Characterization of blood redox status of early and mid-late lactating dairy cows

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ABSTRACT: The effect of the stage of lactation on blood redox homeostasis of bovine and buffalo cows was evaluated. The investigation was carried out on early lactating and mid-late lactating cows, reared in a farm located in Campania (southern Italy). Plasma concentration of α-tocopherol and ascorbate, the total antioxidant capacity (TAC), glutathione peroxidase (GPx), and superoxide dismutase activities were higher ($P < 0.01$) in mid-late lactating cows, thus suggesting a higher consumption of antioxidants during early lactation. Plasma concentration of protein-bound carbonyls (PC) and nitrotyrosine (N-Tyr), and the level of lipid hydroperoxides (LPO) were higher ($P < 0.01$) in early lactating cows, thus suggesting that lipid peroxidation and peroxynitrite production are crucial in determining oxidative modifications in plasma. TAC was positively correlated with ascorbate concentration ($P < 0.03$), and negatively correlated with PC concentration ($P < 0.002$), and ascorbate was negatively correlated with PC ($P < 0.03$) in mid-late lactating group. These findings demonstrate that circulating ascorbate plays a major role in preventing protein modifications induced by carbonyls, and that ascorbate scavenging effect is impaired during early lactation. We calculated a protein oxidative stress index as the ratio (PC + N-Tyr)/TAC multiplied by 100, and we found that this parameter was higher ($P < 0.0001$) in early lactating cows. Therefore, it could be useful for assessing the extent of protein oxidative damage in relation to the whole antioxidant status. Further, we suggest that the LPO/GPx ratio multiplied by 100 might be used as lipid oxidative stress index in lactating cows. This index was higher ($P < 0.0001$) in early lactating cows, and might represent a standard parameter for evaluating the lipid damage depending on a deficiency of the enzymatic antioxidant defence. These parameters are proposed for a possible effective description of physiological changes associated with lactation.

Keywords: lactational status; antioxidant defence; oxidative damage; biomarkers; Mediterranean Italian buffalo; Italian Holstein Friesian bovine

Lactation is associated with a physiologically increased rate of metabolic processes, and is characterized by a high energy requirement, especially in the early stage, when milk yield is higher. Cows mobilize body tissues to satisfy the increased energy requirement for milk production, and preferentially use lipids as energetic substrate (Contreras and Sordillo, 2011; Wathes et al., 2013). The enhanced lipid mobilization disrupts several inflammatory and immune functions (Lacetera et al., 2004; Scalia et al., 2006; Contreras and Sordillo, 2011) and promotes free radicals production by leukocytes and endothelial cells (Valko et al., 2007; Schönfeld and Wojtczak, 2008). In physiological conditions, the

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antioxidant defence system, provided by enzymes and antioxidants, scavenges reactive oxygen species (ROS), thus limiting or preventing oxidative damage (Miller et al., 1993a; Halliwell and Gutteridge, 2000). An increased production of free radicals or deficiencies of antioxidants may lead to oxidative stress (Cadenas and Davies, 2000; Halliwell and Gutteridge, 2000), which impairs physiological functions, thus contributing to health disorders in lactating animals (Ranjan et al., 2005; Lykkesfeldt and Svendsen, 2007; Zhao and Lacasse, 2008; Salman et al., 2009). As a matter of the fact, oxidative stress can increase the susceptibility of dairy cattle to several diseases and metabolic disorders, particularly during the transition period (Gröhn et al., 1989; Lomba, 1996; Allison and Laven, 2000; Bernabucci et al., 2002, 2005; Castillo et al., 2005; Sordillo, 2005; Wilde, 2006), thus affecting health and zootechnical or reproductive performance of dairy cows (Miller et al., 1993a; Davies, 2000). Further, mitochondrial damage and apoptotic mechanisms triggered by ROS were reported to damage oocytes and the endometrium, thus contributing to the reduction of fertility in dairy cows (Wathes et al., 2013). Therefore, the evaluation of blood redox homeostasis has increasingly contributed to the knowledge of the processes involved in reproductive and metabolic disorders (Miller et al., 1993a; Campbell and Miller, 1998; Kankofer, 2002; Sordillo and Aitken, 2009), and it has become important as a complementary tool for the evaluation of health and metabolic status of dairy cows (Castillo et al., 2003, 2005, 2006; Bernabucci et al., 2005), and feedlot calves (Castillo et al., 2012). In addition, it has recently been reported that redox balance of colostrum strongly affects oxidative status of new-born calves, and that the assessment of the oxidative/antioxidative profile of colostrum might represent a good index of colostrum quality (Abuelo et al., 2013b). In this context, it is worth mentioning that although it is well recognized that oxidative stress is implicated in the initiation or progression of numerous diseases, discrepancies between the different models and methodologies used for the evaluation of oxidative status make it very difficult to compare results of the different studies carried out in veterinary medicine (Celi, 2011). Therefore, it has recently been proposed to develop oxidative stress indices for accurate describing redox balance, and for evaluating the association between oxidative status and disease onset (Celi, 2011; Abuelo et al., 2013a).

The main objective of this study was to characterize the blood redox status of early and mid-late lactating bovine and buffalo cows, in order to define the preferential targets of oxidative damage in blood, and to identify biomarkers of physiological changes of oxidative status associated with lactation.

In this study, plasma concentrations of non-enzymatic antioxidants (ascorbic acid, retinol, and α-tocopherol), and the activities of enzymatic antioxidants (superoxide dismutase and glutathione peroxidase) were measured, and used as markers of the antioxidant defence system. The TAC was also assessed, as it well reflects the overall antioxidative potential of the whole organism (Kankofer et al., 2010), and effectively describes the dynamic equilibrium between pro-oxidants and antioxidants in plasma (Ghiselli et al., 2000). Oxidative modifications were monitored by measuring plasma concentrations of nitrotyrosine (N-Tyr) and protein-bound carbonyls (PC), for evaluating the extent of oxidative damage to protein, and plasma level of lipid hydroperoxides (LPO) for assessing the extent of lipid peroxidation, induced by the interaction of free radicals with polyunsaturated fatty acids.

MATERIAL AND METHODS

Ethic statement. The care and use of animals in this study complies with Italian animal welfare laws, guidelines, and policies (Legislative Decree No. 116 of January 27, 1992; Authorization No. 169/94-A, issued December 19, 1994 by the Ministry of Health), and conforms to the Guide for the care and use of agricultural animals in research and teaching. Animals were managed according to the local farm-production practices. Animal housing was carried out without altering natural environmental conditions, and respected all standards of dairy cattle welfare (thermal comfort, physical comfort, disease control, and freedom from fear). Blood sampling was carried out kindly, and always by the same veterinarian, for avoiding animal suffering and stress.

Farm selection and animals. The study was carried out in a conventional dairy farm localized in Campania (southern Italy). The farm was selected in an attempt to obtain no dietary variability between bovine and buffalo cows during the study, in order to avoid a possible effect of diet on blood parameters evaluated. Farm management

Table 1. Experimental design of blood sampling

	Early lactating		Mid-late lactating	
	bovine	buffalo	bovine	buffalo
December	1, 2, 3	1, 2, 3	no sample	8
January	1, 2, 3, 4, 5	1, 2, 3, 4, 5	8, 10	8, 9, 10
February	4, 5, 6, 7	4, 5, 6, 7	1, 2, 3, 9	1, 2, 3, 8, 9, 10
March	no sample	no sample	1, 2, 3, 4, 5, 6, 7, 8, 10	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
April	no sample	no sample	4, 5, 9	4, 5, 6, 7, 9, 10
Samples	12	12	18	26

Each cow was indicated with a number (1, 2, etc.) during the experimental period, and was assigned to early-lactating or mid-late lactating group on the basis of days in milk

was not changed through the experiment. Ten bovine cows (Italian Holstein Friesian breed) and ten Mediterranean Italian buffalo cows (*Bubalus bubalis*), homogeneous in parity (= 3), body condition score, previous milk production, and health condition, were included in the study.

Cows from 35 up to 80 days in milk (DIM) were grouped as early lactating cows. Cows from 85 up to 160 DIM were grouped as mid-late lactating. These classification criteria ensured that all cows, in each category, were analyzed at least twice during the experimental period (December 2011–April 2012). In detail, the study included ten bovine and ten buffalo cows, seven of which were analyzed both as "early" and "mid-late" lactating, while three cows were assigned only to "mid-late" group on the basis of DIM. Therefore, the distribution of bovine cows in the two categories was (number of analyzed samples in parenthesis) seven early lactating cows (12) and ten mid-late lactating cows (18), while the distribution of buffalo cows in the two categories was seven early lactating cows (12) and ten mid-late lactating cows (26). The experimental design of blood sampling is reported in Table 1. Climatic conditions during the experimental period were constant both in winter and in spring, with temperatures never falling below 4°C or exceeding 20°C. In particular, average temperature and humidity recorded during winter (December–February) were 6 ± 1°C and 52 ± 1.7%, respectively, while those recorded during spring (March–April) were 11 ± 1.5°C and 65.3 ± 6.4%, respectively.

Ingredients and chemical composition of diets of early and mid-late lactating cows are shown in Table 2. In particular, the diet of early lactating cows was adjusted to maintain the requirements for milk output, by increasing (10% dry matter basis) the amount of concentrate, and, accordingly, that of corn silage. Daily feed intake was 18–20 kg dry matter. Chemical analysis of the diet was carried out according to published procedures (Van Soest et al., 1991) and the energy value was calculated by validated methods (Jarrige, 1988).

Blood samples were collected early in the morning, on the same day and under the same environmental conditions, into heparinized tubes. Plasma was obtained by centrifugation (500 *g*, 4°C, 15 min), and processed (by the same operator) for titration of ascorbic acid (Asc), retinol (Ret), α-tocopherol (Toc), superoxide dismutase

Table 2. Chemical composition (expressed as % of DM) and net energy (expressed as Milk Forage Units) of diets

	Lactation	
	early	mid-late
Ingredients (% DM)		
Corn silage	38	34
Straw	10	9
Polyphyte hay	24	21
Concentrate	30	28.3
Nutrient		
Crude protein	14.4	15.5
Crude fibre	28.2	26.3
Ashes	9.8	10.0
Neutral detergent fibre	37.5	40.2
Acid detergent fibre	23	24
ADL	3.9	4.5
UFL/kg DM	0.85	0.90

DM = dry matter, ADL = acid detergent lignin, UFL = milk forage unit, Ret = retinol, Toc = α-tocopherol
Diets were supplemented with Vitasol complex (Vitasol, Brescia, Italy) (4000 IU Ret/kg of concentrate (about 1.2 mg Ret/kg DM), and 89 IU Toc/kg of concentrate (about 26.2 mg Toc/kg DM))

(SOD), and glutathione peroxidase (GPx) activity, total antioxidant capacity (TAC), nitrotyrosine, protein-bound carbonyls, and lipid hydroperoxides.

Determination of antioxidants and lipid hydroperoxides. Plasma samples were processed for determination of Asc concentration as previously described (Spagnuolo et al., 2011), and analyzed by high performance liquid chromatography (HPLC) using an anion exchange column (Nucleosil 100-NH_2, 5 µm, 250 × 4.6 mm i.d.). Ret and Toc levels were measured according to a published procedure (Spagnuolo et al., 2003), and analyzed by HPLC using a reverse phase C18 column (Nova-PAK C18, 4 µm, 125 × 2 mm i.d.). TAC was measured by the Trolox equivalent antioxidant capacity assay, according to Miller et al. (1993b). Plasma samples were reacted with the radical 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), and the antioxidant capacity was measured as the decrease of the absorbance at 734 nm and expressed as µM concentration of Trolox equivalents (Miller et al., 1993b; Spagnuolo et al., 2001).

LPO concentration was measured by a colourimetric quantitative assay, using the Lipid Hydroperoxide Assay Kit (Cayman Chemical, Ann Arbor, USA), according to the manufacturer's instructions.

Determination of nitrotyrosine. Nitrated protein levels in plasma samples were measured by ELISA, as previously reported (Spagnuolo et al., 2001). Plasma samples were diluted (1 : 500, 1 : 1000, 1 : 3000, 1 : 9000, and 1 : 15 000) with coating buffer (7mM Na_2CO_3, 17mM $NaHCO_3$, 1.5mM NaN_3, pH 9.6), and incubated in the wells of a microtitre plate at 4°C overnight. Standard curves were obtained with serial dilutions of nitrated bovine serum albumin (BSA). N-Tyr was detected by incubation with Rabbit anti-N-Tyr antibody (1 : 1000 dilution in 130mM NaCl, 20mM Tris-HCl, 0.05% Tween 20, pH 7.3, supplemented with 0.25% BSA, 37°C, 1 h), followed by Goat anti Rabbit IgG-horseradish peroxidase linked (GAR-HRP) diluted 1 : 2000 as the primary antibody. Colour development was monitored at 492 nm, as previously described (Spagnuolo et al., 2003). Data were reported as nmol of N-Tyr per mg of protein.

Determination of protein-bound carbonyls. PC concentration in plasma samples was titrated by ELISA according to Buss et al. (1997). Protein derivatization was carried out with a dinitrophenylhydrazine (DNP) solution (10mM in 6M guanidine hydrochloride, 0.5M potassium phosphate buffer, pH 2.5), to a final protein concentration of 3 mg/ml. Samples were incubated at room temperature for 45 min, vortexing every 10–15 min. Each sample was then diluted (1 : 1000–1 : 20 000) with10mM sodium phosphate buffer, pH 7.0, containing 140mM NaCl, and incubated (overnight at 4°C) in the wells of a microtitre plate. PC were detected by incubation (1 h, 37°C) with Rabbit anti-DNP antibody diluted 1 : 1500 with PBS supplemented with 0.2% gelatine and 0.05% Tween 20, followed by GAR-HRP antibody (diluted 1 : 3000 as the primary antibody). Colour development was monitored at 492 nm, as previously described (Spagnuolo et al., 2003). A six-point standard curve of oxidized BSA was included in each plate. A blank for DNP reagent in PBS without protein was subtracted from each absorbance. Data were reported as nmol of carbonyls per mg of proteins.

Evaluation of plasma activity of glutathione peroxidase and superoxide dismutase. GPx activity was measured indirectly by a coupled reaction with glutathione reductase (GR), using the glutathione peroxidase assay kit (Cayman Chemical), according to the manufacturer's instructions. GPx activity was expressed as nmol of NADPH oxidized per min/ml of sample.

SOD activity was measured with the superoxide dismutase assay kit (Cayman Chemical), according to the manufacturer's instructions. SOD activity was expressed in Unit/ml. One unit of SOD is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical.

Material. BSA, chemicals of the highest purity, GAR-HRP IgG, Rabbit anti-DNP IgG, and standards for HPLC were purchased from Sigma-Aldrich (Milan, Italy). The Nucleosil 100-NH_2 column and the Nova-PAK C18 column were obtained from Macherey-Nagel (distributed by Delchimica Scientific, Milan, Italy). Organic solvents were purchased from Romil (distributed by Delchimica Scientific). Polystyrene 96-wells plates were purchased from Nunc (distributed by VWR International, Milan, Italy). Nitrated BSA, rabbit anti-Nitrotyrosine IgG of Covalab, as well as the kits for titration of lipoperoxide and for measurement of SOD and GPx activities of Cayman Chemical were purchased from Vincibiochem (Vinci, Italy).

Statistical analysis. The samples for measurement of SOD and GPx activities, PC, N-Tyr, or LPO concentration were processed in triplicate. The titration of Toc, Ret, and Asc was carried out on duplicates. Values were reported in tables as mean ± SEM. Normal distribution of data was

assessed by the D'Agostino and Pearson' tests, and the analysis of statistical difference between groups was carried out by using the unpaired *t*-test, according to the "Prism guide to interpreting statistical results" suggestion (www.graphpad.com; GraphPad Software Inc., San Diego, USA). Differences were considered statistically significant when the two-sided *P*-value was less than 0.05. The GraphPad Prism 5.01 program was also used to compare the variances by *F* test, and to calculate Pearson's correlation coefficient (*r*). Coefficient of variation (CV), *P* and *F* values are shown in each table.

RESULTS

Analysis of antioxidants and total antioxidant capacity. Plasma concentrations of Ret, Toc, and Asc, the TAC, and the activities of GPx and SOD are shown in Table 3 (bovine cows) and Table 4 (buffalo cows). Plasma concentration of Ret was positively correlated with Toc concentration both in bovine cows (early lactating, $r = 0.80$, $P < 0.05$; mid-late lactating, $r = 0.63$, $P < 0.01$; data not shown) and buffalo cows (early lactating, $r = 0.92$, $P < 0.0001$; mid-late lactating, $r = 0.56$, $P < 0.01$; data not shown) as expected since they are both dietary liposoluble antioxidants. Plasma concentration of Ret did not differ between early and mid-late lactating cows, both in bovine and buffalo cows, while the concentration of Toc was significantly lower in plasma of early lactating than in plasma of mid-late lactating bovine ($P < 0.02$) or buffalo ($P < 0.0001$) cows. Plasma concentration of Asc and TAC was higher in mid-late lactating than in early lactating bovine cows ($P < 0.02$ and $P < 0.001$, respectively) or buffalo cows ($P < 0.0001$). In addition, TAC was found positively correlated with plasma concentration of Asc in mid-late lactating cows (bovine, $r = 0.82$, $P < 0.0001$; buffalo, $r = 0.44$, $P < 0.05$; data not shown), but not with the concentration of liposoluble antioxidants. SOD and GPx activities were lower in early lactating than in mid-late lactating bovine ($P = 0.0012$ and $P < 0.02$, respectively) and buffalo ($P < 0.0001$) cows. In addition, SOD activity was found positively correlated with plasma Asc concentration both in mid-late lactating bovine ($r = 0.84$, $P \leq 0.0001$; data not shown) and buffalo ($r = 0.67$, $P < 0.001$; data not shown) cows.

Analysis of protein-bound carbonyls, nitrotyrosine, and lipid hydroperoxides. The extent of oxidative damage to proteins and lipids was evaluated by measuring plasma concentrations

Table 3. Markers of the antioxidant defence system and markers of oxidative stress in plasma of early lactating and mid-late lactating bovine cows

	Early lactating ($n = 12$)		Mid-late lactating ($n = 18$)		P (<)	F
	mean	CV (%)	mean	CV (%)		
Retinol (μg/ml)	0.81 ± 0.03	10.3	0.80 ± 0.02	8.0	ns	1.7
α-Tocopherol (μg/ml)	1.99 ± 0.12	15.2	2.38 ± 0.08	14.8	0.03	1.3
Ascorbate (μM)	11.44 ± 0.11	2.5	13.02 ± 0.37	12.2	0.03	32.0**
GPx (nmol/min/ml)	170.5 ± 22.4	34.7	221.3 ± 8.77	16.8	0.03	2.5
SOD (U/ml)	1.57 ± 0.09	14.7	1.99 ± 0.06	13.4	0.01	1.3
PC (nmol/mg protein)	8.63 ± 0.28	8.7	7.67 ± 0.14	7.7	0.01	1.6
N-Tyr (nmol/mg protein)	7.09 ± 0.20	7.4	6.02 ± 0.11	7.4	0.001	1.2
LPO (μM)	4.28 ± 0.19	11.5	3.35 ± 0.08	9.9	0.001	2.2
TAC (μM)	167.4 ± 1.77	2.8	199.4 ± 3.25	6.9	0.001	8.7*
p-OS (arbitrary unit)	9.4 ± 0.3	7.3	6.9 ± 0.2	12.4	0.001	1.6
l-OS (arbitrary unit)	2.9 ± 0.5	48.1	1.6 ± 0.1	31.8	0.01	7.5**
Milk yield (kg)	24.86 ± 0.83	7.3	17.3 ± 0.96	5.4	0.01	3.5

PC = protein-bound carbonyls (nmol per mg of protein), N-Tyr = nitrotyrosine (nmol per mg of protein), LPO = lipid hydroperoxides (μM), TAC = total antioxidant capacity (μM concentration of Trolox equivalents), GPx = glutathione peroxidase activity (expressed as nmol of NADPH oxidized per min per ml), SOD = superoxide dismutase (U/ml), p-OS = protein Oxidative stress index, l-OS = lipid Oxidative stress index, ns = not significant, CV = coefficient of variation, P = significance of differences between the means calculated by *t*-test, *F*-value calculated by *F* test

*$P < 0.01$, **$P < 0.001$

Table 4. Markers of the antioxidant defence system and markers of oxidative stress in plasma of early lactating and mid-late lactating buffalo cows

	Early lactating (n = 12)		Mid-late lactating (n = 26)		P (<)	F
	mean	CV (%)	mean	CV (%)		
Retinol (μg/ml)	0.90 ± 0.03	11.8	0.95 ± 0.01	6.8	ns	2.7*
α-Tocopherol (μg/ml)	2.57 ± 0.05	7.2	3.02 ± 0.06	10.4	0.001	2.9
Ascorbate (μM)	13.4 ± 0.25	6.4	16.4 ± 0.29	9.0	0.001	2.9
GPx (nmol/min/ml)	211.3 ± 3.51	5.8	259.6 ± 3.74	7.4	0.001	2.5
SOD (U/ml)	2.11 ± 0.03	5.3	2.44 ± 0.05	9.6	0.001	4.4*
PC (nmol/mg protein)	6.99 ± 0.21	10.6	5.74 ± 0.17	14.7	0.001	1.3
N-Tyr (nmol/mg protein)	3.59 ± 0.05	4.8	3.19 ± 0.05	8.2	0.001	2.3
LPO (μM)	3.21 ± 0.09	9.6	2.36 ± 0.06	12.7	0.001	1.1
TAC (μM)	193.3 ± 4.28	7.7	234.6 ± 5.67	12.3	0.001	3.8*
p-OS (arbitrary unit)	5.5 ± 0.2	11.9	3.9 ± 0.1	18.7	0.001	1.3
l-OS (arbitrary unit)	1.5 ± 0.07	15.6	0.9 ± 0.03	17.4	0.001	2.2
Milk yield (kg)	13.4 ± 0.67	4.2	9.48 ± 0.42	8.4	0.01	1.2

PC = protein-bound carbonyls (nmol per mg of protein), N-Tyr = nitrotyrosine (nmol per mg of protein), LPO = lipid hydroperoxides (μM), TAC = total antioxidant capacity (μM concentration of Trolox equivalents), GPx = glutathione peroxidase activity (expressed as nmol of NADPH oxidized per min per ml), SOD = superoxide dismutase (U/ml), p-OS = protein Oxidative stress index, l-OS = lipid Oxidative stress index, ns = not significant, CV = coefficient of variation, P = significance of differences between the means calculated by t-test, F-value calculated by F test

*$P < 0.05$

of PC, N-Tyr, and LPO. Plasma concentrations of N-Tyr and PC were significantly lower in mid-late lactating than in early lactating bovine ($P < 0.0001$ and $P < 0.01$, respectively; Table 3) and buffalo ($P < 0.0001$; Table 4) cows. Also, we found a higher level of LPO in the plasma from animals of early lactating group ($P < 0.0001$) than in that of mid-late lactating group, both in bovine and buffalo.

Plasma level of PC was negatively correlated with plasma titre of Asc in mid-late lactating cows, both in bovine ($r = –0.82$, $P < 0.0001$), and buffalo ($r= –0.44$, $P < 0.05$) (Figure 1, panel A). A negative correlation between plasma PC and the total antioxidant capacity was observed in mid-late lactating bovine ($r = –0.69$, $P < 0.01$) and buffalo ($r = –0.65$, $P < 0.001$) cows (Figure 1, panel B). We also found a negative correlation between plasma level of N-Tyr and Asc in mid lactating bovine ($r = –0.58$, $P < 0.02$; data not shown), and buffalo ($r = –0.57$, $P < 0.01$; data not shown) cows, according with the recognized role of Asc as scavenger of peroxynitrite (Halliwell, 1997). Plasma concentration of N-Tyr was negatively correlated with total antioxidant capacity in mid-late lactating bovine ($r = –0.72$, $P < 0.001$) and buffalo ($r = – 0.64$, $P < 0.001$) cows (Figure 2, panel A). A negative correlation between SOD activity and plasma concentration of N-Tyr was found in mid-late lactating bovine ($r = –0.62$, $P < 0.01$) and buffalo ($r = –0.59$, $P < 0.01$) cows (Figure 2, panel B). Further, GPx activity was negatively correlated with LPO concentration both in early lactating (bovine, $r = –0.84$, $P < 0.02$; buffalo, $r = –0.91$, $P < 0.0001$) and mid-late lactating (bovine, $r = –0.85$, $P < 0.0001$; buffalo, $r = –0.47$, $P = 0.015$) cows, in agreement with the key role of this enzymatic antioxidant in protecting against lipid peroxidation.

As TAC was positively correlated with Asc concentration and negatively correlated with both PC and N-Tyr level, we calculated the value of the ratio (PC + N-Tyr)/TAC multiplied by 100, and we used it as a protein Oxidative stress index (p-OS). This parameter was significantly higher in early lactating than in mid-late lactating cows (bovine 9.4 ± 0.3 vs. 6.9 ± 0.2, $P < 0.0001$; buffalo 5.5 ± 0.2 vs. 3.9 ± 0.1, $P < 0.0001$; Tables 3 and 4).

Further, as LPO concentration was negatively correlated with GPx activity but not with the other markers of the antioxidant defence system here evaluated, we suggest that the LPO/GPx ratio, multiplied by 100, might be useful as lipid Oxidative stress index (l-OS) in lactating cows. This index was found significantly higher in early lactating than in mid-late lactating cows (bovine 2.9 ± 0.5

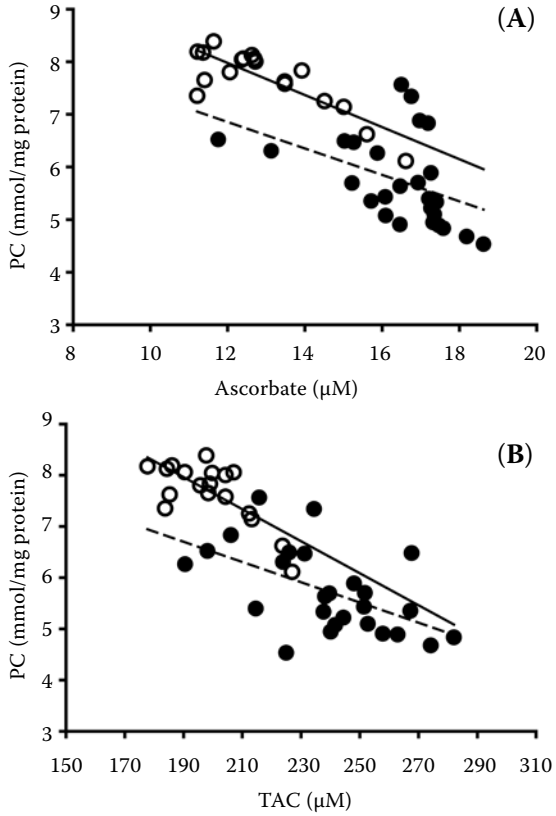

Figure 1. Correlation between the concentration of protein-bound carbonyls (PC) and the concentration of ascorbate or the total antioxidant capacity (TAC) in the plasma of dairy cows

(**A**) Levels of ascorbic acid (Asc) and PC were measured in plasma samples from mid-late lactating bovine cows (open circles), and in plasma from mid-late lactating buffalo cows (full circles). Asc was isolated from deproteinized plasma samples by HPLC, and detected by a UV-spectrophotometer. PC level was measured by ELISA and expressed as nmol/mg of protein. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over 5% from the mean were not found. The statistical program GraphPad Prism 5.01 performed the regression analysis and the calculation of *P* (bovine: $r = -0.82$, full line; $P < 0.0001$; buffalo: $r = -0.44$, dotted line; $P < 0.03$)

(**B**) TAC was measured by the Trolox equivalent antioxidant capacity assay in plasma samples from mid-late lactating bovine cows (open circles), and in plasma from mid-late lactating buffalo cows (full circles). The antioxidant capacity was measured as a decrease of the absorbance at 734 nm, and expressed as μM concentration of Trolox equivalents. PC level was measured by ELISA and expressed as nmol/mg of protein. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over 5% from the mean were not found. The statistical program GraphPad Prism 5.01 performed the regression analysis and the calculation of *P* (bovine: $r = -0.69$, full line; $P < 0.01$; buffalo: $r = -0.65$, dotted line; $P < 0.001$)

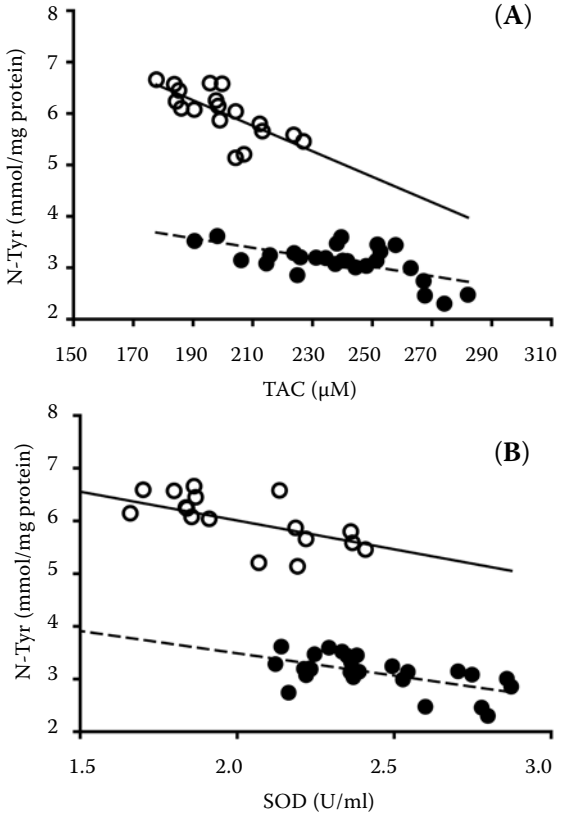

Figure 2. Correlation between the nitrotyrosine (N-Tyr) concentration and the total antioxidant capacity (TAC) or the superoxide dismutase (SOD) activity in the plasma of dairy cows

(**A**) Concentrations of N-Tyr and TAC were measured in plasma samples from mid-late lactating bovine cows (open circles), and in plasma from mid-late lactating buffalo cows (full circles). N-Tyr level was measured by ELISA and expressed as nmol/mg of protein. TAC was measured by the Trolox equivalent antioxidant capacity assay, and expressed as μM concentration of Trolox equivalents. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over 5% from the mean were not found. The statistical program GraphPad Prism 5.01 performed the regression analysis and the calculation of *P* (bovine: $r = -0.72$, full line; $P < 0.001$; buffalo: $r = -0.64$, dotted line; $P < 0.001$)

(**B**) SOD activity was measured in plasma samples from mid-late lactating bovine cows (open circles), and in plasma from mid-late lactating buffalo cows (full circles), and expressed as U/ml. N-Tyr concentration was measured by ELISA and expressed as nmol/mg of protein. Each sample was analyzed in triplicate, and the average value was calculated. Deviations over 5% from the mean were not found. The statistical program GraphPad Prism 5.01 performed the regression analysis and the calculation of *P* (bovine: $r = -0.62$, full line; $P < 0.01$; buffalo: $r = -0.59$, dotted line; $P < 0.01$)

vs. 1.6 ± 0.1, $P = 0.002$; buffalo 1.5 ± 0.07 vs. 0.9 ± 0.03, $P < 0.0001$).

DISCUSSION

The periparturient and lactation periods are particularly critical for dairy cows' health, productivity, and fertility (Goff and Horst, 1997; Castillo et al., 2005, 2006**;** Kowalska and Jankowiak, 2009; Roche et al., 2009). In these periods the energy requirement significantly increases, essentially to support milk production, and the physiological adaptation to this condition is represented by the implementation of metabolic activities and by an extensive mobilization of body fat reserves, particularly in early lactation. The accumulation of triglycerides in the liver and the increase of lipid metabolites concentration in the blood result in the enhancement of ROS production, decrease of paraoxonase activity, and onset of oxidative stress (Turk et al., 2008; Morris et al., 2009; Pilarczyk et al., 2012), which in turn leads to a higher vulnerability of dairy cows to bacterial infections (Kehrli et al., 1989; Mallard et al., 1998; Keane et al., 2006; van Knegsel et al., 2007), thus causing metabolic disorders and diseases (LeBlanc et al., 2004; Rizzo et al., 2007, 2009; LeBlanc, 2008; Sharma et al., 2011; Politis et al., 2012; Sordillo and Raphael, 2013; Wathes et al., 2013).

In this paper we report data about oxidative status of early lactating and mid-late lactating bovine and buffalo cows. It was previously reported that total antioxidant status and the level of malondialdehyde, a lipid peroxidation marker, did not differ between late pregnant and lactating cows, as well as between early and late-lactating cows (Castillo et al., 2005, 2006). In our investigation blood redox homeostasis was characterized by evaluating different specific markers of the antioxidant defence system, and of oxidative damage to protein and lipid. This detailed analysis revealed that plasma concentrations of Toc and Asc, as well as the total antioxidant capacity, and the activities of both SOD and GPx were significantly higher in mid-late lactating cows, both in bovine and buffalo. These results suggest that Toc and Asc play a key role in the protection against oxidative damage during lactation, and that early lactation is associated with a higher consumption of enzymatic and non-enzymatic antioxidants, likely due to the increased metabolic activity needed for milk production. The lower antioxidant capacity of early lactating cows was associated with significant differences in the extent of oxidative modifications to plasma proteins and lipids in the two analyzed groups. As a matter of fact, the concentration of PC, N-Tyr, and LPO was significantly higher in plasma of early lactating cows, both in bovine and buffalo. It is worth mentioning that N-Tyr level represents the footprint of protein oxidative damage induced by peroxynitrite (Halliwell, 1997), while PC may be introduced into proteins by direct oxidative attack to proteins themselves (Kristal and Yu, 1992), or by reactions with aldehydes originated during lipid peroxidation processes (Uchida and Stadtman, 1993). Therefore, our results suggest that lipid peroxidation and its intermediates, as well as peroxynitrite production, are crucial in determining oxidative modifications of proteins and lipids. In addition, we observed that all parameters used as markers of the antioxidant defence system were higher in buffalo than in bovine cows, while the markers of the oxidative modifications were higher in bovine than in buffalo cows. Although a direct correlation between milk yield and the markers of redox homeostasis here selected was not observed, we suggest that the apparent lower efficiency of the antioxidant defence system of bovine might depend on the higher productive effort associated with the higher milk production of lactating bovine cows. Indeed, as milk production of bovine cows both in early and mid-late phase (24.86 ± 0.83 and 17.33 ± 0.96 kg, respectively) (Table 3) was higher ($P < 0.001$) than that of buffalo cows (13.42 ± 0.67 and 9.48 ± 0.42, respectively) (Table 4), it is likely that the antioxidant defence system might come out less effective in bovine, in providing protection against oxidants, because of the increased production of these species.

The positive correlation between Asc concentration and SOD activity was observed only in mid-late lactating cows. This suggests that Asc plays a key role in preserving SOD integrity and activity, and that this scavenging role might be impaired by the perturbation of redox homeostasis associated with early lactation. Also, TAC was positively correlated with Asc concentration, and negatively correlated with PC and N-Tyr concentration, only in mid-late lactating bovine and buffalo cows. Furthermore, a negative correlation between plasma concentration of Asc and PC was observed in this group. These findings suggest that in this lactation phase a significant part of the plasma antioxidant capacity might be accounted for by Asc, and that

circulating Asc plays a major role in preventing protein oxidative modifications induced by carbonyls production, as previously reported for humans (Krajčovičová-Kudláčková et al., 2006). As no correlation between Asc concentration and PC level was observed in early lactating cows, we hypothesize that Asc scavenging effect is impaired in the early lactating phase, likely due to the increased ROS production. Asc is a well known scavenger of peroxynitrite, and SOD catalyzes the dismutation of the superoxide radical anion to hydrogen peroxide (Beckman and Koppenol, 1996; Halliwell and Gutteridge, 2000). The protective role of Asc and SOD against N-Tyr production, expressed by the negative correlation between Asc and N-Tyr concentration, and between SOD activity and N-Tyr concentration, was observed only in mid-late lactating cows. These findings strongly suggest that the increased metabolic activity, associated with early lactation, negatively affects blood redox homeostasis, and interferes with the protective role of SOD and Asc against peroxynitrite production and damage to protein, both in bovine and buffalo cows. On the basis of our results we suggest that changes of plasma levels of these antioxidant defence system markers might be correlated with the extent of metabolic activity, and that the alteration of blood redox balance effectively reflects physiological changes associated with lactation. The increased interest in the role of oxidative stress in physiological and pathological processes has amplified, in the recent years, the need of obtaining specific and reliable indices of oxidative status essentially for comparing results of the different studies carried out in veterinary medicine (Celi, 2011**;** Abuelo et al., 2013 a, b; Po et al., 2013). As TAC was positively correlated with Asc concentration and negatively correlated with both PC and N-Tyr level, we calculated the (PC + N-Tyr)/TAC ratio multiplied by 100, and we used this value as a protein Oxidative stress index (p-OS). This parameter was significantly higher in early lactating cows, thus demonstrating that the higher amount of protein oxidative modifications, caused by peroxynitrite production or by peroxidative processes, found in plasma of early lactating cows, depends on the lower blood antioxidant capacity during this lactation phase. On the basis of our results we suggest p-OS index as possibly useful for the combined quantification of the extent of different kinds of protein oxidative damage taking into account the whole antioxidant status, as described by plasma TAC and Asc concentration. Further, as LPO concentration was negatively correlated with GPx activity, but not with the other markers of the antioxidant defence system, in both bovine and buffalo, we calculated the LPO/GPx ratio, multiplied by 100, and we used it as lipid Oxidative stress index (l-OS). This index was significantly higher in early lactating than in mid-late lactating cows, thus suggesting that the higher amount of lipid hydroperoxides found in plasma of early lactating cows is essentially due to reduced levels and/or activity of glutathione peroxidase, which, in turn, reflects the impairment of the enzymatic antioxidant defence system during early lactation. Therefore, we propose l-OS as a possible standard parameter for evaluating the extent of lipid oxidative damage taking into account the status of enzymatic antioxidant defences. Although further studies are required to validate the use of these indices also for describing physiological changes depending on housing and/or management practices in dairy farms, we believe that p-OS and l-OS might be used as objective indices of the physiological oxidative status in early and mid-late lactation.

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