

# Interrelationships between the content of oxidative markers, antioxidative status, and somatic cell count in cow's milk

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**ABSTRACT:** Bovine mastitis is a major disease affecting dairy cattle worldwide. The milk collected from cows with different type of inflammation, including mastitis, is characterized by an increased number of somatic cells (SCC), especially polymorphonuclear cells (PMN). It was proved that the antibacterial activity of PMN generates reactive oxygen species and nitric oxide-derived metabolites. The accumulation of the reactive species correlating with a decrease in the antioxidant activity specific for milk can lead to oxidative and nitrosative stress. The aim of this study was to evaluate the antioxidant/oxidant status in normal cow's milk and in subclinical mastitis milk using three parameters: total antioxidant status (TAS); levels of malondialdehyde (MDA) – a degradation product of lipid peroxidation; and levels of proteins oxidation expressed as carbonylated proteins analyzed in the reaction with dinitrophenylhydrazine (DNPH). Subclinical mastitis was diagnosed using an electrical conductivity method and by determining the number of SCC in milk. Comparative analysis of TAS in normal and mastitis milk revealed extremely significant differences ( $P = 0.0005$ ), the average of this parameter was lower for mastitis milk samples. The results describing the antioxidant status were correlated with those on lipid and protein oxidative degradation. The average level of MDA in mastitis milk was higher compared with normal milk, the mean difference was 40.69 nmol/ml. A similar variation was observed for DNPH, the mean difference was 191.24  $\mu$ mol/ml. The levels of SCC, MDA, and DNPH were significantly higher in subclinical mastitis milk compared to milk from healthy cows due to the occurrence of oxidative stress state in subclinically inflamed mammary gland.

**Keywords:** milk; mastitis; total antioxidant status; malondialdehyde; protein oxidation

## INTRODUCTION

All milk types contain a certain amount of somatic cells represented by polymorphonuclear cells (PMN), lymphocytes, and macrophages. During a bacterial infection the number of somatic cells in

milk increases, especially that of PMN (Prin-Mathieu et al. 2002). The somatic cell count (SCC) is used as an indicator for the milk hygienic quality; an increase of SCC in bulk tank milk indicates that a significant proportion of milk originates from mastitis cows (Hamed et al. 2008; Andrei et al. 2011).

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Pathological changes occurring in mammary gland tissue during the mastitis infections can lead to undesirable changes in milk composition, dependent on numerous factors such as: severity of infection (which may range from minor changes to complete inhibition of secretion of milk, depending on the type and virulence of the pathogen); size of the infected area; disruption of metabolic activity of milk secreting cells by reducing the synthesis of milk (due to a decrease in the level of galactopoietic hormones and the increased concentration of inhibitor hormones); degradation of some biochemical constituents of milk (under the action of enzymes synthesized by pathogenic microorganisms) (Petrovski 2006).

The antibacterial activity of PMN cells is partially mediated through reactive oxygen species (ROS); an excess of these species correlating with the absence of optimal amounts of antioxidants can lead to oxidative stress. It was shown that the occurrence of oxidative stress in cattle may contribute to some periparturient disorders (retained fetal membranes, udder edema, mastitis) or metabolic diseases (Pintea et al. 2006; Rinaldi et al. 2007; Spears and Weiss 2008).

Subclinical and clinical mastitis in cows was found to be associated not only with oxidative stress, but also with nitrosative stress. In various bacterial infections of the mammary gland an increased concentration of nitric oxide (NO<sub>x</sub>)-derived metabolites, nitrite, nitrate, and oxidatively modified organic components was detected. NO<sub>x</sub> is produced by mammary epithelial cells and milk somatic cells. The increase in released NO<sub>x</sub> is reflected in the accumulation of nitrite and nitrate in milk and it may be associated with nitrosative stress and impairment of milk oxidative stability (Silanikove et al. 2014a, b).

The effects of NO<sub>x</sub> at the cellular level are influenced by many factors such as the level of production and the diffusion of NO; concentration of the potential reactants (superoxide radical anion and hydrogen peroxide); activity of antioxidant enzymes such as catalase, superoxide dismutase, lactoperoxidase; and concentration of non-enzymatic antioxidants (e.g. glutathione, vitamin C, etc.) (Andrei et al. 2014). One of the main functions of catalase present in milk is the conversion of nitrite to nitrate. Thus catalase is one of the key enzymes in the prevention of excessive nitrosative stress in milk. The main bactericidal effect of NO<sub>x</sub> in milk

may be related to the conversion of nitrite into nitrate in a hydrogen peroxide-dependent manner by lactoperoxidase (Silanikove et al. 2014a).

The antioxidant activity of milk is a result of antioxidant enzymes and various non-enzymatic molecules like vitamin antioxidants, glutathione, various polypeptides, and proteins. The enzymatic antioxidant activity of milk is due to the presence of catalase, glutathione peroxidase, superoxide dismutase (SOD), and lactoperoxidase (Fox and Kelly 2006; Silanikove et al. 2014a). Catalase plays a critical role in controlling the redox system of milk (Silanikove et al. 2005, 2009, 2012) which explains an earlier finding that during subclinical mastitis the activity of catalase increases and may be used as an indicator of bacterial infection (Hamed et al. 2008). Lactoperoxidase and glutathione peroxidase activities are increased in mastitis milk and are correlated with SCC (Seifu et al. 2007; Andrei et al. 2011). However, lactoperoxidase increases oxidative stress and antibacterial resistance in milk (Silanikove et al. 2014a) whereas glutathione peroxidase contributes to resolution of oxidative stress. The SOD activity in cow's milk is not influenced by factors such as lactation stage or animal age (Lindmark-Mansson and Akesson 2000) and is not correlated with SCC of milk (Andrei et al. 2010).

Milk contains also non-enzymatic antioxidants such as retinol and carotenoids, tocopherols, and ascorbic acid (Lindmark-Mansson and Akesson 2000; Matei et al. 2011). Concentration of these antioxidants in milk may decrease in the case of different pathological conditions (e.g. mammary gland infection) or due to heat treatment or exposure to ultraviolet light and oxygen during milk processing. Previous studies have shown that in milk with a high SCC the concentration of antioxidant vitamins is lower compared to milk with a low SCC (Andrei et al. 2009). A pronounced decrease in the concentration of vitamin C has been shown for subclinical mastitis caused by infections with various pathogens (Lykkesfeldt and Svendsen 2007). This is because vitamin C (ascorbic acid) is reductively consumed while excreting its antioxidative effect (Silanikove et al. 2014b).

In the state of oxidative stress the modified antioxidant enzymatic activity and decreased concentrations of non-enzymatic antioxidants correlate with the increase in the concentration of products resulting from oxidative degradation of lipids,

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protein, and nucleic acids (Suriyasathaporn et al. 2006; Kizil et al. 2007; Andrei et al., 2009).

The aim of this study was to evaluate the antioxidant/oxidant status in milk of cows with subclinical mastitis using three parameters: total antioxidant status (TAS), levels of malondialdehyde (MDA) – a degradation product of lipids peroxidation, and levels of proteins oxidation.

## MATERIAL AND METHODS

The research was conducted over the period of 3 years in a dairy farm from Cluj County, Romania, on a total of 120 cows. The cows were a mixed race of Austrian Bălțata with Red Holstein and Red Holstein metis. Totally 55 milk samples from cows with bacterial infection and 30 milk samples from healthy cows were examined (Table 1).

**Mastitis detection and milk sampling.** Mastitis was detected with the aid of a Waikato mastitis indicator (MAS D TEC Electronic Mastitis Detector, Hamby Dairy Supply, USA) determining the quality of milk by measuring the conductivity. Milk samples were collected from each quarter of the lactating cows, before morning milking. After teats end cleaning (with 70% ethanol), first streams of foremilk were discarded and then milk was collected aseptically from each teat into sterile vials. Milk samples were stored in a refrigerator, at 4°C, until analysis (for about 2 h). Determination of SCC was performed by an automated counter MT04 (Agro Legato Kft., Budapest, Hungary).

**Biochemical analysis.** The analysis of biochemical parameters in milk samples was performed after a preliminary dissociation of casein micelles (clarifying of milk). Clarification can be defined as a result of the combined action of solvents, detergents, and a base, i.e. the chemical compounds which produce a complete dissolution of casein micelles responsible for milk turbidity (Humbert et al. 2006). The first step in this analysis consisted

in removing lipids from milk (centrifuging the milk at 5000 rpm, for 1 h, at 4°C). Skimmed milks were then clarified by the addition of 2 volumes of 0.1M bis-tris buffer, pH 8.0, containing 8M urea, 1.3% trisodium citrate, and 0.3% dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, USA). It permits to alleviate the protein–protein interactions and thus to dismantle the micellar organization of caseins (Miranda et al. 2004).

**Total antioxidant status determination.** The TAS was estimated spectrophotometrically using a commercial kit (Randox Laboratories Ltd., Crumlin, UK) and a semiautomatic biochemistry analyzer MasterPlus Screen (Hospitex Diagnostics, Florence, Italy). In brief, ABTS (2,20-azino-di-[3-ethylbenzthiazoline sulfonate]) was incubated with methmyoglobin and hydrogen peroxide to produce ABTS<sup>+</sup>, a radical cation with a relatively stable blue-green colour that can be measured at 600 nm. Antioxidants in the sample suppressed the production of this coloured compound, in proportion to their concentration. The results were expressed in mol/ml.

**Determination of lipid peroxidation level.** The lipid peroxidation level was determined by a photometric method based on a reaction with thiobarbituric acid (TBA). It reacts in acid medium, with malondialdehyde resulting from peroxidation processes, forming a red coloured compound which was dosed photometrically at 535 nm using an UV-Vis spectrophotometer Jenway 6315 (Bibby Scientific Ltd., Stone, UK). For calculation we used the MDA standard curve obtained by the acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) (Andrei et al. 2010a). The results were expressed in nmol/ml milk.

**Determination of carbonylated protein.** Carbonylated protein (frequently used as an indicator of proteins oxidation) was determined using the method proposed by Fenaille et al. (2006). The principle is based on the determination of carbonylated proteins by the reaction with dinitrophenylhydrazine (DNPH) to form the corresponding hydrazone, dosed photometrically at 370 nm (using an UV-Vis spectrophotometer Jenway 6315, Bibby Scientific Ltd.). The level of proteins oxidation was calculated using an extinction coefficient of 22 000 M<sup>-1</sup> cm<sup>-1</sup>. The results were expressed in mmol/ml milk.

**Statistical analysis.** Comparative interpretation of the results was performed using modified Welch's *t*-test (ANOVA) with the expression of the

Table 1. Experimental periods and cattle groups considered for the study

| Year | Experimental periods | Subclinical mastitis cattle <i>n</i> | Healthy cattle <i>n</i> |
|------|----------------------|--------------------------------------|-------------------------|
| 1    | May–July             | 18                                   | 10                      |
| 2    | June–August          | 21                                   | 10                      |
| 3    | April–July           | 16                                   | 10                      |

Table 2. Somatic cell count (SCC) values in normal and subclinical mastitis cow's milk

|                          | Low SCC          |         | High SCC                       |          |
|--------------------------|------------------|---------|--------------------------------|----------|
|                          | average $\pm$ SD | range   | average $\pm$ SD               | range    |
| SCC $\times 10^{-3}$ /ml | 143 $\pm$ 34.205 | 120–200 | 750 $\pm$ 205.287 <sup>a</sup> | 500–1000 |

<sup>a</sup> $P \leq 0.001$  (extremely significant)

probability index ( $P$ ). Statistical validity of data obtained by Welch's  $t$ -test was performed by applying tests of normality, using Kolmogorov-Smirnov index calculation. For all calculated and compared variants, the results have passed tests of normality and are therefore deemed correct and the database was used with sufficient variables. The obtained data were correlated using the Pearson's correlation coefficient ( $r$ ) (ANOVA). Interpretation was done according to the  $R^2$  value and the probability index  $P$ .

## RESULTS

The determination of SCC in milk proved that in many of the samples the maximum admitted limit was exceeded, the differences being highly significant ( $P < 0.0001$ ). Healthy animals presented values 120–200 SCC  $\times 10^{-3}$ /ml (low SSC), while samples from cattle with subclinical mastitis showed values 500–1000 SCC  $\times 10^{-3}$ /ml (high SSC) (Table 2).

Total antioxidant status showed different variations in milk samples (Table 3). Comparative analysis of TAS in low SCC and high SCC milk samples revealed extremely significant differences ( $P = 0.0005$ ), the average of this parameter being lower for mastitis than for normal milks, with a mean difference of 8.461 nmol/ml.

The average level of MDA in mastitis milk was higher, with an average of 70.465  $\pm$  2.060 nmol/ml compared with normal milk (29.774  $\pm$  7.765 nmol/ml), mean difference being 40.691 nmol/ml (extremely significant –  $P < 0.00001$ ). A similar variation was observed for protein oxidation levels (DNPH), the mean difference being 191.24  $\mu$ mol/ml (mean of subclinical mastitis minus mean of normal) (extremely significant –  $P = 0.0007$ ) (Table 3).

Milk samples with high SCC were in direct correlation with TAS ( $r = 0.087$ ), MDA ( $r = 0.5046$ ), and DNPH ( $r = 0.7715$ ). On the other hand, TAS was directly correlated with MDA ( $r = 0.5777$ ). The

Table 3. Total antioxidant status (TAS), lipids (MDA), and proteins (DNPH) oxidation in normal and subclinical mastitis cow's milk

| Parameters           | Low somatic cell count           |               | High somatic cell count          |               |
|----------------------|----------------------------------|---------------|----------------------------------|---------------|
|                      | average $\pm$ SD                 | range         | average $\pm$ SD                 | range         |
| TAS ( $\mu$ mol/ml)  | 15.809 $\pm$ 2.435 <sup>a</sup>  | 11.595–17.564 | 7.348 $\pm$ 1.343 <sup>a</sup>   | 6.004–9.368   |
| MDA (nmol/ml)        | 29.774 $\pm$ 7.765 <sup>b</sup>  | 20.20–38.44   | 70.465 $\pm$ 2.060 <sup>b</sup>  | 64.330–80.220 |
| DNPH ( $\mu$ mol/ml) | 366.43 $\pm$ 32.272 <sup>c</sup> | 340.01–421.21 | 557.67 $\pm$ 38.295 <sup>c</sup> | 449.82–744.39 |

<sup>a</sup> $P = 0.0005$ , <sup>b</sup> $P < 0.0001$ , <sup>c</sup> $P = 0.0007$  – all extremely significant

Table 4. Correlation between SCC, TAS, MDA, and DNPH in subclinical mastitis milk

| Correlation              |                      | Pearson's $r$ | Probability index $P$ | $R^2$   |
|--------------------------|----------------------|---------------|-----------------------|---------|
| SCC $\times 10^{-3}$ /ml | TAS ( $\mu$ mol/ml)  | 0.08700       | 0.8529                | 0.00757 |
|                          | MDA (nmol/ml)        | 0.5046        | 0.2022                | 0.2546  |
|                          | DNPH ( $\mu$ mol/ml) | 0.7715        | 0.0250                | 0.5951  |
| TAS ( $\mu$ mol/ml)      | MDA (nmol/ml)        | 0.5777        | 0.3077                | 0.3337  |
|                          | DNPH ( $\mu$ mol/ml) | –0.03528      | 0.9551                | 0.00124 |

SCC = somatic cell count, TAS = total antioxidant status, MDA = lipid oxidation, DNPH = proteins oxidation

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correlation coefficient between TAS and DNPH in high SCC samples ( $r = -0.0.3528$ ) showed an indirect trend (Table 4).

## DISCUSSION

The International Dairy Federation recommended the classification of cow's milk as subclinical mastitis or non-mastitis (normal) using a SCC threshold of 500 000 cells/ml (Asadpour et al. 2008). In Europe, the Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 stated that milk with SCC over  $400 \text{ SCC} \times 10^{-3}/\text{ml}$  cannot be used for human consumption. Based on this recommendation, our milk samples were divided into two categories, normal milk with low SCC values (below  $400 \text{ SCC} \times 10^{-3}/\text{ml}$ ) and subclinical mastitis milk with high SCC values (above the limit of  $400 \text{ SCC} \times 10^{-3}/\text{ml}$ ).

Total antioxidant activity of milk can be determined using various methods, the most common being the spectrophotometric methods: the ABTS method, the ORAC (oxygen radical absorbance capacity) method, and the FRAP (ferric reducing antioxidant power) method (Zulueta et al. 2009). The results of the antioxidant capacity assay of milk depend on the method employed and the conditions of the assay. The ABTS method monitors the antioxidant capacity of both whey and total milk, and is more sensitive to caseins. The FRAP method is suitable only for the whey fraction, and less suitable for the assay of protein antioxidant capacity (Chen et al. 2003). However, the use of DTT for pre-treated samples does not allow comparisons with non-treated milks because of the oxidative properties of DTT.

In our study we determined the total antioxidant status in milk samples using the ABTS method. The results show that the total antioxidant activity of milk presents significant changes, being lower in mastitis milk samples compared with normal. The data obtained were correlated with those presented by Atakisi et al. (2010) in cows and Silanikove et al. (2014b) in goats. According to them, milk from cows with subclinical mastitis has a low antioxidant activity. Moreover, the studies performed noted a direct correlation between the antioxidant status and severity of infection (reflected by the number of somatic cells present in milk).

The results obtained in the determination of antioxidant status also correlate with those ob-

tained in the determination of lipids and proteins oxidative degradation. MDA, one of the compounds formed by peroxidation of unsaturated fatty acids, is frequently used as a marker of peroxidation (Del Rio et al. 2005). It was noted that the average concentration of MDA was higher in milk samples from cows with subclinical mastitis than in milk samples from healthy cows.

Because the most important substrate for peroxidation is represented by polyunsaturated fatty acids, monitoring of lipid peroxidation can be achieved by determining the polyunsaturated/saturated fatty acids (PUFA/SFA) ratio which decreases with the increase of peroxidation. In a previous study published by Andrei et al. (2010b), in normal milk, the PUFA/SFA ratio had an average value of 0.174, while in milk from cows with subclinical mastitis this value was lower (0.123 on average). Also, by comparing percentages of fatty acids with SCC, it was observed that PUFA decreased with increasing SCC.

Changes induced by ROS in proteins structure resulted in inactivation of enzymes, increased susceptibility to precipitation of proteins and proteolysis (Shacter 2000). Oxidative modification of proteins is initiated mainly by the reaction with hydroxyl radicals but it is still dependent on the superoxide radical anion concentrations and hydrogen peroxide. These ROS react with amino acid residues of polypeptide chains leading to their fragmentation. According to data published by Requena et al. (2001), about 60% of total carbonylic compounds are represented by amino adipic semialdehyde and glutamic semialdehyde.

There is a close relationship between the level of lipid peroxidation and protein oxidation (Silanikove et al. 2005, 2014a). In cellular systems, protein oxidation can take place under the action of radicals or carbonylic products resulting from lipid peroxidation and similar responses were found in milk under oxidative stress (Silanikove et al. 2009, 2012). Thus, an increased oxidation of PUFA elevates the protein carbonyl levels. PUFA oxidation leads to the formation of unsaturated aldehydes such as MDA and 4-hydroxynonenal, which interacts with amino acid residues with the formation of Schiff base type compounds. Also alcoxyl radicals, formed by decomposition of lipid hydroperoxides, may interact with different amino acids (Refsgaard et al. 2000). In the case of milk samples analyzed in this study these correlations

were observed. In all samples of milk collected from cows with subclinical mastitis, increased lipid peroxidation processes led to elevated protein carbonyl levels, so to an increase in oxidative degradation of proteins. In general, the results in this study are consistent with the model described in Silanikove et al. (2014a).

## CONCLUSION

The total antioxidant activity showed significant changes in mastitis milk compared to normal milk samples. The milk from cows with subclinical mastitis showed a decreased antioxidant activity, which led to the accumulation of reactive oxygen species and oxidative stress installation, and a direct correlation between the antioxidant status and severity of infection (determined by the number of somatic cells present in milk) was observed. Oxidative stress occurred in the infected mammary gland and milk from cows with subclinical mastitis exhibited a higher concentration of compounds resulting from oxidative degradation of lipids and proteins.

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