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Glutathione peroxidase activity and its relationship with somatic cell count, number of colony forming units and protein content in subclinical mastitis cows milk

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Abstract

This study was conducted to determine the relationship between milk and blood glutathione peroxidase (GPx) activity, casein concentration, somatic cell count (SCC) and colony forming units (CFU) in cows with subclinical mastitis. Following these tests carried out on 84 lactating cows, 10 of them were classified as cows with subclinical mastitis (SCC values above the limit of 400,000 cells mL^{-1}) and 74 as normal. After microbiological processing of the subclinical mastitis milk samples, nine microorganism species have been isolated, represented by seven bacteria and two types of fungi. Comparative analysis of GPx activity in milk revealed significant differences ($p = 0.0013$), the average of this parameter is higher for mastitis than normal milk. GPx activity in blood shows no significant changes in cows with subclinical mastitis compared to healthy ones. The average quantity of caseins in mastitis milks was lower compared with regular milks, the difference being significant ($p = 0.0007$). Mastitis milk GPx activity was directly correlated with the total SCC ($r = 0.019$) and CFU ($r = 0.1785$). Correlation coefficient between GPx activity and caseins in mastitis milk showed indirect trend ($r = -0.2606$). The positive correlation between SCC and GPx activity suggests that this enzyme may have potential to detect subclinical mastitis in dairy cows.

Keywords: subclinical mastitis, glutathione peroxidase, milk, blood, cows

Abbreviations: CAT, catalase; CFU, colony forming units; GPx, glutathione peroxidase; GSH, glutathione; LPx, lactoperoxidase; PMN, polymorphonuclear cells ; ROS, reactive oxygen species; SCC, somatic cell count; SOD, superoxide dismutase

Introduction

All around the world, mastitis is one of the most important diseases in dairy production sector. Since it is a disease caused by multiple factors (multiple pathogens), mastitis is difficult to control. Mastitis affects not only the health of dairy animals, with direct consequences for farm profitability, but also affects animal welfare. Most of the time, mastitis negatively influences milk quality having consequences on milk production industry [1]).

All milks contain a certain amount of somatic cells represented by polymorphonuclear cells (PMN), lymphocytes and macrophages. In bacterial infection and other inflammation processes affecting the mammary tissue, the number of somatic cells in milk increases, especially the PMN level. During mastitis, PMN cells migrate from the peripheral blood into milk, through the mammary epithelium [2]). In many countries, somatic cell count (SCC) is used as an indicator for the hygienic milk quality. An increased SCC in a bulk tank milk indicates that a significant proportion of milk originates from mastitis cows.

More than 140 different microorganisms are recognized to cause mastitis. They are classified into four different groups: contagious, environmental, opportunistic and others.

Most mammary gland infections are caused by only a few types of bacteria, including streptococci (*Streptococcus agalactiae*), staphylococci (*Staphylococcus aureus*) and coliforms (*Corynebacterium bovis* [3, 4, 5].

Subclinical infections are characterized by a reduction in milk secretion and composition changes. A few hours after udder infection with pathogens, milk somatic cell count increase in response to activation of inflammatory processes. Mammary epithelium ability to synthesize and secrete the major components of milk is reduced while the secretion of some proteins (such as lactoferrin) is increased. Casein concentration in milk decreases, both due to reduced secretion and to the passage of plasmin from blood into milk, which determines the appearance of hydrolysis products. Simultaneously, the concentration of molecules that have a blood origin, such as serum albumin, sodium and chloride in milk increased after infection [6, 7, 8]. Increasing the concentration of blood proteins during mastitis results in increased levels of soluble proteins in milk. The percentage of □-lactoglobulin decreases, with increasing number of somatic cells while increasing serum albumin and immunoglobulin [9]. Increasing the proportion of proteins associated with inflammatory response in the udder (lactoferrin, serum albumin) compensates significantly lower proportions of casein. Total protein content and ratio of casein and soluble proteins change significantly even in the early sub clinical mastitis. In case of moderate subclinical mastitis, some peptides produced by hydrolysis of milk proteins can be highlighted [10, 11].

Antibacterial activity of neutrophils is mediated, in part, through reactive oxygen species (ROS) [12]. Various infectious diseases of farm animals, such as pneumonia, enteritis, mastitis, are associated with oxidative stress [13, 14, 15]. Although essential for the body, an excess of oxidative reactions of the anti-bacterial processes may cause tissue damage. An excess of ROS and the absence of optimal amounts of antioxidants are leading to oxidative stress. Many cells are susceptible to this oxidative stress, which can cause necrosis or apoptosis. The term oxidative stress is used to characterize the strength imbalance occurring between ROS and antioxidant systems [16].

Antioxidant activity of milk is due to the presence of antioxidant enzymes such as catalase - CAT, glutathione peroxidase - GPx, lactoperoxidase – LPx, xanthine oxidase and superoxid dismutase - SOD, or vitamins and provitamins such as vitamin A and carotenoids, vitamin E, vitamin C. The activity of milk antioxidant enzymes such as xanthine oxidase, catalase and lactoperoxidase increases when SCC increases. Measurement of the activity of some enzymes such as catalase, in milk has been used to monitor udder health in dairy cows. Activity of milk lactoperoxidase has been also used to detect mastitis [17, 18, 19, 20].

Glutathione-peroxidase (GPx - EC 1.11.1.9) is widespread in the cytoplasm of animal cells. The function of this enzyme is to protect cells against the damaging effects of peroxides, as part of an antioxidant enzymatic system. Milk contains low levels of GPx, more than 90% being represented by extra cellular form. The function of this enzyme in milk is not yet fully known, it is the only known enzyme that fixes 30% of total selenium (Se), an important element of diet. It is also known that milk GPx varies according to species and diet [21, 22].

To our knowledge, the correlation between milk glutathione peroxidase activity and cellular counting has not yet been fully considered in cows. Therefore, the aim of the present study was to examine possible relationships between milk somatic cells, the number of colony forming units (markers of the hygienic conditions) and glutathione peroxidase activity (in blood and milk) and casein concentration in cows with subclinical mastitis.

Materials and methods

Animals: The research was conducted in a dairy farm from the Apahida village, Cluj County. On a total of 120 cows (mixed race of Austrian Bălţat with Red Holstein and Red Holstein metis) 84 were lactating cows.

Milk sampling: Samples of milk were collected from each quarter of the 84 lactating cows, before morning milking. No cow had any evidence of clinical mastitis at the time of sampling. After teat end cleaning (with ethanol 70%), first streams of foremilk were discarded and then 10 mL of 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 hours).

Blood Samples were taken by jugular venipuncture, from all the animals, into heparinized tubes.

Determination of SCC was performed by automated counter (MT04, AgroLegato, Hungary).

Microbiological analyses: Samples with subclinical mastitis were processed with the aim to isolate the microbial species from milk and to determine the number of colony forming units (CFU mL^{-1}). To isolate bacteria, from each intense homogenized sample, one microbiological loop (0.01 mL) was sowed on blood agar medium incubated at 37˚C for 24 hours. Identification of bacteria was made after examination of cultural features on blood agar and morphological features using Gram stain and API multitest Biomerio systems [23]. For fungi isolation, Sabouraud medium incubated at 28˚C for 7 days was used, following the appreciation of morphological and cultural features [24]. Determination of the number of colony forming units for bacteria and fungi was made by sowing technique on solid medium [25].

Total protein and casein assay: The first step in this analysis consisted in removing lipids from milk (centrifuging the milk at 5000 rpm, for 1 h, at 4°C) followed by casein dissociation. Total protein content was determined using the Bradford method [26]. In the second step, caseins were precipitated by treating milk with 1M HCl to pH 4.6, after which samples were centrifuged for 20 minutes at 5,000 rpm. Whey, representing the upper aqueous phase, was taken and protein concentration was determined using the same photometric method. By difference between total protein and soluble protein (from whey) the concentration of caseins was calculated.

Enzymatic assays: Glutathione peroxidase activity in milk samples was performed on skimmed milk, using a commercial kit (Ransel, Randox Laboratories) and semiautomatic biochemistry analyzer MasterPlus Screen. The final results were reported in units per ml of milk (U.mL^{-1} milk).

Erythrocytes glutathione peroxidase activity was determined photometrically, on heparinized whole blood, using a commercial kit (Ransel, Randox Laboratories) and semiautomatic biochemistry analyzer MasterPlus Screen. The final results were reported in units per gram of hemoglobin (U.g^{-1} Hb). Hemoglobin was determined photometrically, by the method of hemoglobin cyanide, using a commercial kit (Hospitex Diagnostics).

Statistical analyses: Comparative interpretation of variables (normal milk vs. mastitis milk) was performed using modified Welch T test (ANOVA) with the expression index of probability ("p"). Statistical validity of data obtained by Welch T test was performed by applying tests of normality, using Kolmogorov-Smirnov index calculation [27]. For all calculated and compared variants, the results have passed tests of normality and are therefore deemed correct and the database used with sufficient variables. Correlating expression data was done by measuring the correlation coefficient "r – Pearson" (ANOVA) and 95% confidence interval for it. Interpretation was done according to the R^2 value and the index of probability "p". Correlations were determined between GPx activity and SCC, CFU and caseins in mastitis milk.

Results

Few hours after the infection of the udder with pathogenic microorganisms, the number of somatic cell (SCC) in milk increases in response to activation of inflammatory processes. Macrophages play an important role in overseeing infected gland. The International Dairy Federation recommended classification of cow milk as subclinical mastitis or non mastitic (normal) using a SCC threshold of 500,000 cells.mL^{-1} (ASADPOUR et al., [17]). In Europe, the ECC directive 92/46 (1992) stated that milk with SCC over 400,000 cells per mL cannot be used for human consumption, while limits are 750,000 in the USA and 500,000 in Canada [28]. Starting from this recommendation we have classified milk samples in two categories, normal (values below 400,000 cells.mL^{-1}) and subclinical mastitis (values above the limit of 400,000 cells.mL^{-1}).

Following tests carried out on the 84 lactating cows, 10 of them were cows with subclinical mastitis, representing 12% of the total lactating cows. For these 10 cows, an increased number of somatic cells was observed. Thus, the obtained values were between 500,000 and 1,5 million cells mL^{-1}. In healthy cows, somatic cell count did not exceed the value of 270,000 cells mL^{-1} (Table 1). Somatic cell count was higher in mastitis milks, the average difference (885,455 SCC x 10^{-3} mL^{-1}) being statistically assured (p = 0.0001).

Table 1. Somatic cell count in normal and subclinical mastitis cows milk

Parameter	Normal milk		Subclinical mastitis milk	
	Average±SD	Range	Average±SD	Range
SCCx10^{-3} mL^{-1}	191.81 ± 57.81	120 - 270	1077.27 ± 401.91	500 - 1500

After microbiological processing of the subclinical mastitis milk samples, 9 species of microorganisms have been isolated, represented by seven bacteria and two types of fungi (Table 2). Results show a predominance of staphylococci isolated from subclinical mastitis samples, *Staphylococcus intermedius* being isolated in 45.5% of samples. *Streptococcus agalactiae,* isolated in 31.1% of samples, was also present, followed by *Bacillus cereus* with 27.3%. The two types of isolated fungi, *Mucor spp.* and *Aspergillus spp.*, were isolated in a low rate representing 4.5% and 9.1%, respectively.

Table. 2. Microorganisms isolated from subclinical mastitis milk

Microorganisms	No. of strains	Percentage %	CFU mL^{-1}
Staphylococcus intermedius	10	45.45	2289-890
Staphylococcus chromogenes	3	13.63	617-140
Staphylococcus caprae	2	9.09	4577, 2556
Micrococcus luteus	2	9.09	3560,2225
Streptococcus agalactiae	7	31.81	7629-6103
Bacillus cereus	6	27.27	2130-150
Escherichia coli	2	18.18	3560, 2254
Mucor spp.	1	4.54	7
Aspergillus spp.	2	9.09	16

Regarding the number of germs, it was observed that the most CFU mL^{-1} were obtained from samples from which *Streptococcus agalactiae* was isolated, with the number of microorganisms ranging between 7629 and 6103 CFU mL^{-1}. The lowest number of microorganisms in samples was observed for fungi (7 CFU mL^{-1} for *Mucor* and 16 CFU mL^{-1} as far as Aspergillus is concerned).

The microbiological examinations isolated several microorganisms from the same sample, allowing to observe association between some species of bacteria and between bacteria and fungi. The most frequent associations were between *Staphylococcus intermedius* and *Streptococcus agalactiae* (Table 3). Making a comparison between the number of microorganisms and combinations of them, it is evident that samples having a high value of CFU mL^{-1} had no association between microorganisms, since in these samples only *Streptococcus agalactiae* was isolated.

Table 3. Associations between microorganisms isolated from samples

Association type	Number of associations
Micrococcus luteus *Bacillus cereus* *Escherichia coli*	1
Staphylococcus intermedius *Aspergillus spp.*	2
Staphylococcus caprae *Mucor spp.*	1
Staphylococcus intermedius *Streptococcus agalactiae*	3
Staphylococcus chromogenes *Bacillus cereus*	2

Glutathione peroxidase activity shows different variations in blood and milk samples (Table 4). Thus, in blood samples collected from healthy cows, the activity has an average of 104.96 ± 16,433 $U.g^{-1}Hb$ while in samples taken from cows diagnosed with subclinical mastitis value was 104.26 ± 10.28 (p = 0,838). Comparative analysis of GPx activity in milk revealed significant differences (p = 0.0013), the average of this parameter being higher for mastitis than for normal milks, with 37.37 $U.mL^{-1}$.

Table 4. Milk and blood GPx activity, milk protein concentration and casein concentration in normal and subclinical mastitic cows

Parameter	Normal		Subclinical mastitis	
	Average±SD	Range	Average±SD	Range
Milk GPx U mL^{-1}	23,009±6,562	14 – 32,6	60,379±23,462	33,8 - 92,9
Blood GPx U g^{-1} Hb	104,96±16,433	79,6 - 129	104,26±10,281	90,4 - 121,4
Total milk proteins mg mL^{-1}	29,64±1,832	28,11 - 31,76	29,58±1,149	28,25 - 31,37
Caseins mg mL^{-1}	19,18±1,926	17,71 - 21,53	13,97±2,367	10,31 - 17,29

The average quantity of caseins in mastitis was lower, with an average of 13.97±2.37 $mg.mL^{-1}$, compared with regular milk (19.18 ± 1.93 $mg.mL^{-1}$) the difference being significant (p = 0.0007). In terms of total milk protein content, significant changes were not observed between mastitis and normal milks (Table 4).

Table 5. Correlation between milk GPx activity, somatic cell counts, number of colony forming units and casein concentration in subclinical mastitis milk

CORRELATION		Pearson "r"	IC 95%	"p"	R^2
GPx activity $U.mL^{-1}$	$SCC.mL^{-1}$	0,0191	-0,6534 – 0,6748	0,9610	0,00036
	$CFU.mL^{-1}$	0,1785	-0,5511 -0,7534	0,6459	0,03187
	Caseins $mg.mL^{-1}$	-0,2606	-0.7884 - 0.4881	0,4983	0,06790

Mastitis milk GPx activity was in direct correlation with the total SCC (r = 0.019) and CFU (r = 0.1785). Correlation coefficient between GPx activity and caseins in mastitis milk (r =- 0.2606) showed indirect trend (Table 5).

Discussion

When bacteria invade and colonize the mammary gland, macrophages respond by initiating the inflammatory response, attracting polymorphonuclear (PMN) cells in milk to kill bacteria. More than 90% of somatic cells found in infected glands are neutrophils (PMN). Antibacterial activity of neutrophils is mediated via reactive oxygen species (ROS) (RINALDI et al., [12]). Although essential for the body, an excess of oxidative reactions due to bacterial infection may cause damage to tissues. An excess of ROS and the absence of optimal amounts of antioxidants result in oxidative stress development (ANDREI et al., [29]).

Glutathione peroxidase is an antioxidant enzymes in milk, it catalyses the reduction of different peroxides aided by glutathione or other reducing substrates. Two different classes of GPx - selenium-dependent (EC 1.11.1.9) and selenium-independent (EC 2.5.1.18), are known. Both utilize glutathione for reducing hydroperoxides, but selenium-dependent enzymes are also capable of reducing hydrogen peroxide (H_2O_2). In our study, the average value for GPx activity in normal milk was 23 $U.mL^{-1}$. Our results are in line with those of LINDMARK-MANSSON and AKESSON [20], who reported that GPx activity in cow's milk has values ranging between 12 and 32 $U.mL^{-1}$ whereas its activity is correlated significantly with selenium concentration. The obtained value (33.02 $U.mL^{-1}$) was lower than that reported by HAMED et al. [2].

In milk from cows diagnosed with subclinical mastitis, a significant increase in glutathione peroxidase activity was observed as compared to normal milk. We issued three hypotheses to explain this variation: (1) increased activity in milk is due to increasing enzyme concentration / activity in blood; (2) in milk, the enzyme is associated with caseins, an intensive hydrolysis process of casein can release the enzyme, and thus increase its activity; (3) peroxidases can be produced in milk by pathogens.

In stating the first hypothesis, we started from the origin of GPx enzyme present in milk. Milk contains two different forms of the enzyme: plasma selenodependent enzyme (p-GPx) and cellular enzyme (c-GPx). The latter may be secreted by the liver and transported into milk via blood stream (TORRES et al., [30]). In general, oxidative stress induces increased activity of antioxidant enzymes in blood, which can influence enzyme levels in milk. The first hypothesis was not confirmed by the results obtained in this study. The blood enzyme activity shows no significant changes in blood taken from cows with subclinical mastitis compared to healthy cows. Moreover, data obtained are consistent with those reported in other studies, according to which there is a negative correlation between blood GPx activity and incidence of mastitis in different species [31, 32].

Enzymes in milk occur in various states: as unassociated forms in solution; associated or integral part of membrane fractions (such as the milk fat globule membrane or skim milk membrane vesicles, both of which are derived from the plasma membrane of the secretory cell); associated with casein micelles; and as part of microsomal particles [33].) The GPx activity in human milk is at the same level as in bovine milk, 31 - 39 $U.mL^{-1}$. Most of the glutathione peroxidase activity has been found in fractions corresponding to 150-170 and 92-96 kDa in milk from all species so far examined. These results suggest that a substantial portion of the GPx in milk exists in a complex form attached to high-molecular-weight proteins within the casein fraction [34]). Milk from clinically and subclinically affected

mastitic cows had very high increases in the activity of proteolytic enzymes. The main caseinolytic enzyme in milk, plasmin, is able to rapidly cleave caseins in polypeptide fragments (proteose peptones) which then diffuse into whey [6, 10, 11, 35, 36, 37]. Hydrolysis of casein (demonstrated by their reduced concentration in mastitis milk) can cause release of enzyme and thus its activity in mastitis milk will be higher. In our study, the correlation coefficient between milk GPx activity and caseins in mastitis milk showed indirect trend (r = - 0.2606).

The third hypothesis is based on the action of pathogens responsible for mastitis and how they can adapt to oxidative stress. Increased GPx activity in milk may occur as a result of adaptation mechanisms of pathogens. Moreover, there is a direct correlation between enzyme activity and CFU (r = 0.1785). During phagocytosis, phagocytic cells generate superoxide and other reactive oxygen species, which are involved in antibacterial activity. On the other hand, many pathogens possess antioxidant defenses that may explain their survival. These defenses include antioxidant enzymes such as superoxide dismutase and catalase [38, 39]. In bacteria involved in mastitis infections, such as *Streptococcus agalactiae*, various studies have shown that the glutathione can be synthesized. Glutathione (GSH, □-glutamyl-cysteinyl-glycine) is the predominant non-protein thiol compound in living organisms. In bacteria it plays various important roles in many metabolic processes, such as protection against oxidative stress. The resistance of bacteria to hydrogen peroxides was found to be dependent on the accumulation of glutathione, which can activate glutathione- glutathione peroxidase - glutathione reductase system in cells [40, 41, 42].

Based on the findings of the present study it can be concluded that in samples of milk from cows with subclinical mastitis, a significant increase in glutathione peroxidase activity is recorded. This increase is statistically correlated with milk SCC and CFU. Increased enzyme activity can be explained, first by hydrolysis of casein-enzyme complex, leading to enzyme release and, on the other hand, by the antioxidant defense mechanisms specific for pathogens. The positive correlation between SCC and GPx activity suggests that this enzyme may have potential to detect subclinical mastitis in dairy caws.

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