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ASSESSMENT OF LIPID PEROXIDATION IN DAIRY COWS WITH SUBCLINICAL AND CLINICAL MASTITIS

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ABSTRACT

Mastitis is still one of the major causes of economic losses in dairy sector. The routine application of bacteriologic examination of milk samples is often insufficient and for this reason, alternative parameters are used to identify trends in the development of the udder health. Therefore, the objectives of this study were to determine the relationship of oxidative product levels, using malondialdehyde (MDA) as a marker on occurrence of mastitis and its causing pathogens. Dairy herd of 223 Slovak spotted cattle were tested for etiology and occurrence of mastitis based on assessment of clinical signs, abnormal udder secretions, Californian Mastitis Test (CMT) with subsequent collecting of milk samples for bacteriological examination. From 892 quarter milk samples were selected for MDA detection 51 subclinical (SM) and 26 clinical mastitis (CM) quarters with positive CMT score and positive bacteriological examination of *Staphylococcus* spp. and *Streptococcus* spp. as well 40 healthy quarters. Results showed that among the current pathogens of the mammary gland belong CNS, *S. aureus, S. sanguinis, S. uberis* and *E. coli*, which were the most frequently isolated from SM and CM. The highest MDA level was observed from clinical cases of mastitis however, increased MDA levels were detectable from subclinical cases. Bacterial isolates from subclinical quarter milk samples are different levels of MDA. In this study, we found that quarter milk samples infected with *S. uberis* were higher compared to other pathogens. In conclusion, differences in both severity of mastitis and mastitic pathogens were associated with differences of oxidative products in infected udders.

Keywords: cows; lactation; mastitis; lipid peroxidation; *S. uberis*; coagulase negative staphylococci

INTRODUCTION

The quality and quantity of milk obtained from the cows is important for the dairy sector. Infection of mammary gland - mastitis is one of the biggest problems of dairy producers causes great losses every year in the livestock economy. The disease is usually local but may become systemic, although rarely, in immunocompromised animals **(Tančin et al., 2006; Shari, Umer and Muhammad, 2009; Andrei et al., 2010; Vršková et al., 2015)**.

Mastitis is characterized by several physical and chemical alterations of the milk and corresponding pathological changes in the mammary tissue depending on the type of the disease. Intramammary infection (IMI) is caused by interaction of various factors associated with the animal, pathogens and the environment, so nature and duration of the disease varies accordingly **(Taponen et al., 2006; Suriyasathaporn et al., 2006; Zajác et al., 2012)**.

More than 140 different microorganisms are recognized to cause mastitis. Infectious agents like bacteria, viruses, fungi and algae are mostly the primary causes of the disease.

Authors **Vasil' et al. (2009)** and **Tenhagen et al. (2006)** indicate that up to 95% IMI is caused by bacterial pathogens. At routine bacteriological examination of milk from suspect cows, or secretion of the cow udder with clinical mastitis we can detect *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, CNS, *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., *Pseudomonas* spp. and others.

During a bacterial infection the number of somatic cells in milk increases, especially that of polymorphonuclear cells (PMN). 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 **(Castillo et al., 2006; Sharma et al., 2011)**.

Oxidative stress in veterinary medicine and particularly in ruminant health is a relatively young field of research. Early identification of udder health problems is essential for dairy farmers and veterinarians to ensure not only the animal well-being but also the milk quality and dairying productivity. Economic aspects interfere with the routine application of bacteriologic examination of quarter milk samples. For this reason, alternative indicators of oxidative stress are used to identify trends in the development of the udder health in a dairy herd. Although these parameters belong detection of lipid peroxidation reactive aldehydes such as malondialdehyde (MDA) **(Suriyasathaporn et al., 2012; Sharma et al., 2016)**.

MDA is one of the several low molecular weight end products formed during the radical induced decomposition of polyunsaturated fatty acid. MDA modifies the physical structures of cell membranes and is indirectly involved in the synthesis of protein, DNA, and RNA. In addition, it has mutagenic and carcinogenic properties **(Turk et al., 2017; Kapusta et al., 2018)**.

Scientific hypothesis

Lipid peroxidation reactive aldehydes have been identified as a key factor in numerous pathologies, including udder inflammation. Detection of MDA may be helpful in the health management of cows. Therefore, the goal of this study was to evaluate the etiology of mastitis and to assess of lipid peroxidation by measuring milk malondialdehyde level in raw cows' milk.

MATERIAL AND METHODOLOGY

Animals and milking

The experiment was carried out in dairy herd of 270 Slovak Pied cattle in north of the Slovakia. Dairy cows from monitored herd were kept in a free housing system with a separate calving barn and equipped with individual boxes with bedding and were allowed *ad libitum* access to water. Their diet was formulated according to international standards **(NRC, 2001)** to meet the nutritional requirements of a 600 kg cow, yielding 15 - 25 kg of milk per day. The cows were milked twice a day at 4:30 a.m. and 4:30 p.m. in the fishing-milking parlour (FarmTec) 2x10. Milking and pulsation vacuum were set at 42 kPa. Pulsation ratio was 60:40 at a rate of 52 c.min^{-1}.

Examination of health status

The examination of health status included clinical examination of the mammary gland, examination fore-strip of milk, with CMT reaction, subsequent collecting of milk samples for bacteriological examination, subsequent cultivation and identification of pathogenic bacteria. Clinical examination consisted of heart rate, body temperature, respiratory rate, udder health including the mammary gland palpation, evaluation of macroscopic changes in milk, and evaluation of somatic cell count in milk using the CMT (Indirect diagnostic test, Krause, Denmark). CMT was performed from the 223 milked cows on 892 quarter milk samples and the score was evaluated (Table 1) according to **Jackson and Cockcroft (2002)**. Following washing and drying the mammary teats, 70% ethanol was sprayed, and a few streams of milk were discarded. Afterwards, quarter milk samples of the secretion (10 mL) were then collected with aseptic techniques in accordance with **National Mastitis Council guidelines (2001)**. The samples were cooled and immediately transported to the laboratory.

Laboratory analyses

Bacteriological examinations were performed according to commonly accepted rules **Malinowski et al. (2006)**. Milk samples (10 μL) were cultured at the respective veterinary practice according to their routine procedures, usually employing Columbia Blood Agar Base with 5% of defibrinated blood, Staphylococcal medium N° 110, Baird-Parker agar, Edwards Medium, Mac Conkey Agar (Oxoid, (OXOID Ltd., Basingstoke, Hants, UK) incubated at 37 °C for 24 h (Figure 1).

Figure 1 Bacteriology analysis cultured on selectives medium. Note: *S. aureus* (1A - 1B), *S. warneri* (2A - 2B), *S. uberis* (3A - 3B), *E. coli* (4), *A. viridans* (5).

Beside evaluation of bacterial growth characteristics another assays were used to bacterial species determination: pigment and coagulase production, catalase activity, haemolysis, Gram staining and other virulence factors. Bacteria *Staphylococcus* spp. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, SR). Suspect colonies *Staphylococcus* spp., *Streptococcus* spp. and *Enterobacteriaceae* spp. were isolated on blood agar and cultivated at 37 °C for 24 h and detailed identified biochemically using the Staphy test, Strepto test, resp. Entero test and identification by software TNW Pro 7.0 (Erba-Lachema, CZ) according to the manufacturer's instructions. Identification of typical *Trueperella pyogenes* colonies derived from pure or mixed culture were distinguished by colony morphology, post incubation haemolysis, Gram staining, catalase test. Specific colonies were plated on defibrinated sheep blood agar (5%) and incubated aerobically at 5% CO_2 atmosphere at 37 °C for 72 hours. Colonies morphologically compatible with *T. pyogenes* were subjected to a conventional phenotypic assay API Coryne strips (BioMe'rieux, France).

MDA determination from quarter milk samples

For milk MDA detection were selected three groups from all monitored cows. The first group of 10 healthy cows (40 quarters milk samples) without clinical signs, negative score of CMT and negative bacteriological examination. From the 29 cows (51 quarters milk samples) on the basis of positive CMT score, without clinical signs and positive bacteriological examination of *Staphylococcus* spp. and *Streptococcus* spp. were selected second group with subclinical mastitis.

The third group of 11 cows (26 quarters milk samples) was selected on the basis of clinical signs, positive CMT score and positive bacteriological examination of *Staphylococcus* spp. and *Streptococcus* spp.

Milk MDA level from selected milk samples was measured by the photometric method based on a reaction with thiobarbituric acid (TBA) described by **Andrei et al. (2010)**.

Briefly, one hundred microliters of milk sample were properly mixed with 1 mL of trichloroacetic acid with a vortex mixer. Afterwards 400 mL of TBA was added. The mixture was boiled for 30 min and subsequently cooled down by tap water. The solution was duplicate analysed by UV spectrophotometry at 532 nm against its blank reaction mixture. The results were expressed in $nmol.mL^{-1}$ milk.

Statistical analysis

The data of milk MDA level from selected groups of cows and selected mastitis pathogens are presented as the mean (M) ±standard error of the mean (SEM). Difference between groups and pathogens causing subclinical mastitis were analysed by using analysis of variance (ANOVA) followed by Tukey comparison test and minimum criteria for statistical significance was set at $p \leq 0.05$ for all. Approximate probabilities were evaluated with Post Hoc Test for the statistical analysis of MDA level between selected pathogens.

RESULTS AND DISCUSSION

Table 1 shows the health status udder of 223 lactating dairy cows. From (892 quarter milk samples were recorded in 161 positive samples (18.1%) with CMT score trace or 1 - 4. Negative CMT score were recorded in 731 quarter milk samples (81.1%). A total of 127 infected quarter milk samples from 67 mastitis cows were the most commonly diagnosed form of SM 8.7% from all investigated quarters. At quarter levels, 16 (1.8%), 34 (3.8%) quarters were classified as latent and clinical mastitis, respectively. Numbers and percentages of isolates separated for their severity of mastitis are shown in Table 2. CNS gave the highest percentages (37%) representation on the etiology of mastitis in the monitored herd. SM were represented by up to 61.4% from all mastitis samples. The most common pathogens in CM were *S. aureus*, *S. uberis* and *S. sanguinis*. Other bacteria and *S. intermedius* had very few data and were excluded from analyses.

Table 1 Evaluation of CMT in monitored herd.

All exanimated quarters*		Healthy quarters	Quarters with positive CMT score*	Infected quarters	Evaluation of CMT score				
					CMT score	n	%	SCC x 10*	Interpretation*
n	892	731	161	127	0 (negative)	731	81.1	0 - 200	Healthy quarters
%	100	81.1	18.1	14.2	T (trace)	29	3.3	200 - 400 (±50)	Healthy or LM[1]
					1	61	6.8	400 - 650 (±150)	SM[2]
					2	40	4.4	850 - 1,200 (±200)	SM or CM[3]
					3	23	2.6	1,500 - 5,000 (±300)	CM
					4	8	0.9	Over 5,500	CM

Note: n - number of tested quarters, All exanimated quarters* - quarters with milk secretion, 8 quarters were rejected, CMT score* - quarters with positive evaluation of Californian Mastitis Test with score trace, 1, 2, 3 or 4, SCC*- Somatic cell count and interpretation of severity mastitis according to **Jackson and Cockcroft (2002)**.

LM[1] - Latent mastitis are characteristic only with the presence of bacterial pathogens in samples of milk without changing its consistency and SCC. SM[2] - Subclinical mastitis are characteristic with positive CMT score, bacteriological cultivation, increased SCC, reduced milk yield without clinical signs. CM[3] - Clinical mastitsare characteristic with positive CMT score, bacteriological cultivation, high level of SCC, changing the consistency of the milk, reduced or loss of milk production with clinical signs.

Table 2 Isolated microorganisms from infected quarters in monitored herd.

Isolated microorganisms	n	%	Latent		Subclinical		Clinical	
			n	%	n	%	n	%
***Staphylococcus* spp.**								
S. aureus	16	1.8	-	-	7	0.8	9	1.0
S. haemolyticus	18	-	4	0.4	12	-	2	0.2
S. chromogenes	15	1.7	-	-	8	8.9	7	0.8
S. warneri	11	1.2	2	0.2	9	1.0	-	-
S. xylosus	9	1.0	3	0.3	6	0.7	-	-
S. intermedius	5	0.6	1	0.1	2	-	2	0.2
***Streptococcus* spp.**								
Str. sanguinis	9	1.0	-	-	4	0.4	5	0.6
Str. uberis	8	0.8	-	-	5	0.6	3	0.3
Str. spp.	6	0.7	2	0.2	4	0.4	-	-
Other bacteria								
E. coli	7	0.8	1	0.1	5	0.7	1	0.1
Aerococcus viridans	7	0.8	-	-	7	0.8	-	-
Trueperella pyogenes	5	0.6	1	0.1	4	0.4	-	-
Mixed infection*	11	1.2	2	0.2	4	0.4	5	0.6
Total	**127**	**14.2**	**16**	**1.8**	**78**	**8.7**	**34**	**3.8**

Note: n - number of isolated bacteria from exanimated quarters; Mixed infection* - mixed infection caused two or more bacteria.

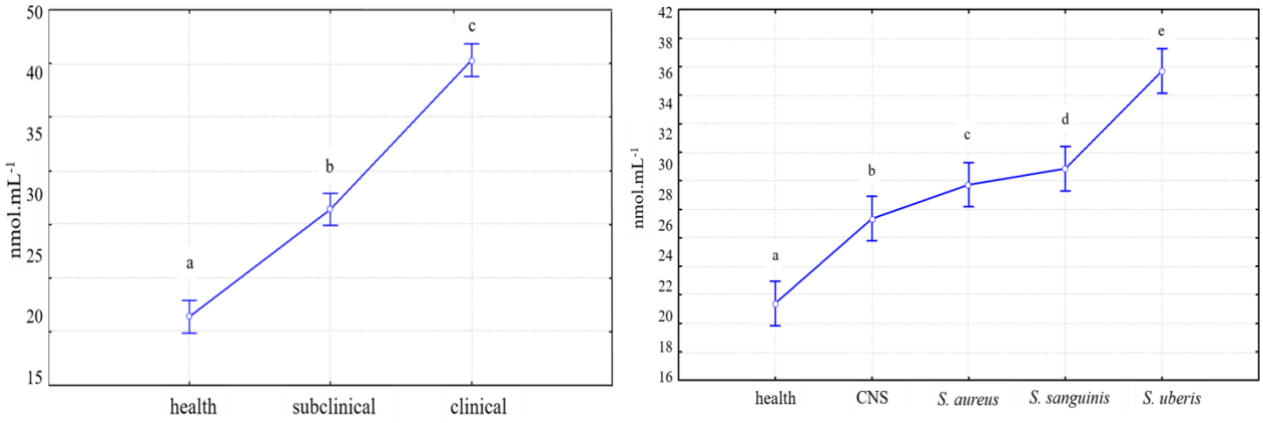

Figure 2 Milk malondialdehyde level (MDA) in raw cows' milk. Note: (A) MDA concentration separated by healthy quarters (n = 40), quarters with subclinical mastitis (n = 51) and quarters with clinical mastitis (n = 26); (B) MDA concentrations separated by isolates (n = 51) from quarters with subclinical mastitis. [a-e]Overall MDA values without common superscript differ significantly (p <0.05).

Table 3 MDA concentrations separated by isolates (n = 51) from quarters with subclinical mastitis.

	Groups	Normal	CNS	*S. aureus*	*Str. sanguinis*	*Str. uberis*
		21.40	27.34	29.72	30.85	37.71
1	Normal		**0.000159**	**0.000134**	**0.000134**	**0.000134**
2	CNS	**0.000159**		0.206953	**0.019734**	**0.000134**
3	*S. aureus*	**0.000134**	0.206953		0.838401	**0.000134**
4	*Str. sanguinis*	**0.000134**	**0.019734**	0.838401		**0.000134**
5	*Str. uberis*	**0.000134**	**0.000134**	**0.000134**	**0.000134**	

Note: Tukey HSD test; variable MDA approximate probabilities for Post Hoc Tests Error: Between MS = 5.9733, df = 45.000.

Averages of milk MDA for the selected groups and for the selected pathogens causing SM were shown in Figures 2(A) and (B), respectively. Averages, values of milk MDA in this study from health, subclinical and clinical group were 19.6, 28.8, and 41.3 nmol.mL^{-1}, respectively. From univariate analyses, results show that differences between selected groups and pathogens causing SM were related to MDA concentrations (p <0.001). Milk from SM and CM quarters had highest MDA concentrations and were statistically different (p <0.001) compared to healthy quarters (Figure 2(A)). MDA from milk samples with *S. aureus*, CNS, *Str. sanguinis* and *Str. uberis* was

significantly higher compared to MDA from health milk samples (Figure 2(B)). Results from Post Hoc probabilities showed, that MDA from milk samples infected with *Str. uberis* were statistically higher (p <0.001) compared to other pathogens (Table 3).

Bacteria are the most common cause of mastitis and hence bacteriological culture is routinely used in the laboratory diagnosis. The bacterial pathogens isolated from 127 affected quarters were CNS (41.7%), followed by CPS (16.5%), *Str. sanguinis* (7.0%), *Str. uberis* (6.3%) and other bacteria with mixed infections (8.6%) (Table 2).

The present findings are in accordance with the findings of **Kivaria and Noordhuizen (2007)** isolated *Staphylococcus* spp. followed by *Streptococcus* spp., *E. coli* and *Klebsiella*.

Tenhagen et al. (2006) isolated *Staphylococcus* spp. followed by *E. coli*, streptococci and *Pseudomonas*.

In many countries, CNS have been acknowledged more and more frequently as a cause of intramammary infections in dairy cattle **(Pyörälä and Taponen, 2009; Lange et al., 2015)**.

In the case of CNS, there has been not only an increasing prevalence of such infections but also an expanding list of species reported to be involved in the process. Results of studies from different countries and continents revealed that more than 20 CNS species have been isolated from milk samples of mastitic cows, the most common being *S. chromogenes, S. haemolyticus, S. epidermidis, S. simulans* and S. *xylosus* **(Zadoks et al., 2011; Lange et al., 2015; Khazandia et al., 2018)**.

In our study the most common CNS species such as *S. haemolyticus, S. chromogenes, S. warneri* and *S. xylosus* have been isolated from infected quarter milk samples of cows.

According to **Vasil' et al. (2016)** staphylococci and streptococci are the main etiological agents of ruminant IMI. *Staphylococcus aureus* with CNS are the most frequent isolates from subclinical and clinical cases of IMI.

Monday and Bohach (1999) present in their study that CNS are the most prevalent pathogens causing subclinical mastitis in dairy ruminants. Although less pathogenic than *S. aureus*, CNS can also produce persistent SM as well as producing thermostable enterotoxins. Nevertheless, despite the accepted role of these bacteria as major IMI causing pathogens in dairy ruminants, the pathogenicity of the different CNS species varies widely.

In our study, CNS, *S. aureus* and streptococci were most common isolated from SSM what is generally seen as an increase in the SCC in milk of the infected quarter with positive CMT score.

Increase in SCC during the inflammatory process in mammary glands indicates increased neutrophils in milk which result in oxidation reactions and increase MDA levels. In cows with mastitis, serum lipid peroxidation levels were increased, and the level of blood glutathione peroxidase was decreased compared to the levels in healthy cows **(Atroshi et al., 1996)**.

According to our previous study **Zigo et al. (2014)** infected lactating cows are more sensitive to oxidative stress than cows without IMI. An imbalance between increased production of ROS and the availability of antioxidant defenses needed to reduce ROS accumulation during the infection and may expose cows to increased oxidative stress.

Lipid peroxidation is one of the important consequences of oxidative stress. MDA is generated as a consequence of lipid peroxidation and, as such, is assayed as a biomarker of oxidative stress. The significant higher changes in milk MDA concentration of cows with subclinical and clinical mastitis in the present study (Figure 2(A)) are in accordance with the study **Suriyasathaporn et al. (2012)** that showed a significant increase of milk MDA levels in cows with SM and CM.

According to our expectation, CM quarters had highest MDA concentrations. This might be caused by the higher levels of udder defence mechanism. With the perspective of early diagnosis have shown increased MDA levels in SM quarters. This study showed that MDA level was different among pathogens causing subclinical mastitis (Figure 2(B)). Characteristics of pathogens may be the reason for differences in oxidative environment in udders but MDA from milk samples wit *Str. uberis* were statistically higher compared to other pathogens (Table 3).

CONCLUSION

In the study, we confirmed that the current pathogens mammary gland includes CNS, *S. aureus, S. sanguinis* and *S. uberis*, which were most often isolated from SM and CM. The highest MDA level was observed from clinical cases of mastitis however, elevated levels of MDA were detectable from SM quarters. Bacterial isolates from subclinical quarter milk samples are different levels of MDA. In this study, we found that *S. uberis* was statistically higher compared to other pathogens.

Subclinical mastitis is difficult to detect because of a lack of clinical signs that can be easily identified by visual inspection and palpation of the udder. As can be seen from our study, one of the additional methods for detecting SM can be measurement of MDA level in raw cows' milk.

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