Polish Journal of Veterinary Sciences Vol. 25, No. 2 (2022), 269–277

DOI 10.24425/pjvs.2022.141811

Original article

Identification of bacterial species in milk by MALDI-TOF and assessment of some oxidant-antioxidant parameters in blood and milk from cows with different health status of the udder

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Abstract

This study aimed to identify bacterial pathogens in milk samples from dairy cows with subclinical and clinical mastitis as well as to assess the concentrations of oxidant-antioxidant parameters [malondialdehyde (MDA), reduced glutathione (GSH), and total GSH levels] in both blood and milk samples. From a total of 200 dairy cows in 8 farms, 800 quarter milk samples obtained from each udder were tested in the laboratory for the presence of udder pathogens. Cultivated bacteria causing intramammary infection from milk samples were identified by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF). In addition, from tested animals 60 cows were selected includıng 20 healthy cows that were CMT negative, 20 cows with subclinical mastitis (SM), and 20 cows with clinical mastitis (CM) for detection of MDA, GSH, and total GSH levels in blood and milk samples. Three hundred and eighty (47.5%; 380/800), 300 (37.5%; 300/800), and 120 (15%; 120/800) of milk samples, respectively were CMT positive or SM and CM, and those positives were cows from different farms. We observed that 87.4% (332/380), 25.3% (76/300), and 34.2% (41/120) of cows with CMT positive, CMT negative, and CM had bacterial growth. The most predominantly identified bacteria were *Staphylococcus chromogenes* (18.7%) obtained mainly from SM and *Staphylococcus aureus* (16.7%) as the most frequent cause of CM. According to our results, dairy cows with CM had the highest MDA levels, the lowest GSH, and total GSH levels in both blood and milk samples however, high MDA levels and low GSH levels in milk samples with SM were observed. Based on our results, lipid oxidant MDA and antioxidant GSH could be excellent biomarkers of cow's milk for developing inflammation of the mammary gland. In addition, there was no link between nutrition and MDA and GSH levels.

Key words: dairy cows, CMT, mastitis, malondialdehyde, glutathione, MALDI-TOF

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Introduction

Despite a plethora of research, bovine mastitis continues to affect the animal health and economic profitability of dairy farms (Zajac et al. 2012, Zigo et al. 2019a). The inflammatory reaction of the mammary gland (i.e. mastitis) generally occurr due to microorganisms and is contemplated as the most costly disease for dairy cows. Both subclinical mastitis (SM) and clinical mastitis (CM) produce great economic losses lowering quality and quantity of the milk produced (Huijps et al. 2008).

Early detection of mastitis is especially important for the successful treatment of affected cows. Several methods or biomarkers have been recently used for detecting mastitis which include: the evaluation of somatic cell count (SCC), California mastitis test (CMT), the measurement of electrical conductivity, pH, levels of proteins, enzymes, peptides, milk constituents, molecular tests, genomics, and proteomic analyses (Viguier et al. 2009, Chakraborty et al. 2019). However, most of these methods are not yet commonly used because of their high cost, difficult use and low availability (Singhal et al. 2015, Sharifi et al. 2018).

New perspective biomarkers reflecting the mammary gland health include malondialdehyde (MDA) and reduced glutathione (GSH). The MDA is one of the lipid peroxidation products and the most commonly used parameter for the evaluation of oxidative stress from blood or milk (Castillo et al. 2006). The GSH is a tripeptide consisting of glutamic acid, cystine, and glycine, and it protects against oxidative damage of cells by reacting with free radicals and peroxides (Turk et al. 2017). In previous studies, it has been demonstrated that a decrease in antioxidant levels may increase the risk of mastitis and antioxidant supplements may be effective in preventing mastitis (Simsek and Aksakal 2006).

Webster (2020) and Taponen et al. (2017) reported that up to 95% of intramammary infections (IMI) that occurred due to bacterial pathogens. The bacteria which are most widely detected in mastitis cases can be divided into two groups: contagious and environmental pathogens.

Contagious pathogens are *Staphylococcus aureus, Streptococcus agalactiae*, or *Streptococcus dysgalactiale*. Among environmental pathogens, *E. coli* is the most common bacteria followed by *Streptococcus uberis*, non-aureus staphylococci (NAS), *Corynebacterium* spp., *Enterobacter* spp., *Listeria* spp., *Leptospira* spp., *Pseudomonas* spp., *Serratia* spp., *Proteus* spp., *Pasteurella* spp., *Yersinia* spp., *Brucella* spp. and *Mycobacterium* spp. (Zigo et al. 2021). Recent studies reported *S. aureus* and NAS as the most predominant bacteria causing mastitis in cows (Garcia and Shalloo 2015, Taponen et al. 2017, Zigo et al. 2019b).

The success of mastitis treatment and prevention programmes on dairy farms is dependent on the rapid identification of the offending bacterial pathogens. Traditional culture methods can take up to six days before an organism can be accurately identified. MALDI-TOF MS is a newly established identification method in routine clinical diagnosis of microorganisms that has been used since 2009. In this method, ribosomal proteins of bacteria or fungi are ionized and blown away with the matrix, the molecular masses are calculated according to the flight time of the proteins, and the protein spectrum of the microorganism is defined. Satisfactory results have been reported about the performance of this method, and MALDI-TOF MS has been accurate at a level of 90-100% for Gram-positive and negative, aerobic and anaerobic bacteria, fungi, and mycobacteria (Kassim et al. 2017).

This study aims to identify the bacterial pathogens at the species level in quarter milk samples from dairy cows in Malatya province of Turkey using MALDI-TOF MS and to assess the concentrations of oxidant-antioxidant parameters (MDA, GSH, and total GSH levels) in both blood and milk.

Materials and Methods

Herds investigation and samples collection

For this study, the clinical examinations of the cows with the collection of milk and blood samples were conducted according to the ethical standards approved by the Firat University Ethics Committee under protocol number: FU-2021/2630.

This study includes 200 dairy cows (120 Simmental, 18 Brown Swiss, 62 Holstein) from 8 private farms in Malatya Province of East Turkey. The cows were housed in semi-open barns. The daily average milk production of cows was 15-25 kg. Nutrition consists of grass silage, corn silage, and hay. In addition, concentrated feed based on factory feed and barley.

From each farm 25 dairy cows were selected at 3-9 years of age in lactating phase 14-100 days after calving which had not received any treatment the last three months. Cows were evaluated by clinical observations, udder secretions of the mammary gland with an assessment of CMT score, and the collection of milk and blood samples. The CMT test was made according to Jackson and Cockcroft (2002) and the score was graded from 0 to 3. CMT score of 0 and trace (±) was accepted as negative whereas CMT scores of +1, +2, and +3 were accepted as indicators of SM.

A total of 800 quarter milk samples from 200 cows

were taken for bacteriological identification according to Zigo et al. (2019c). Additionally, 60 cows divided into three groups (20 cows in each group) based on clinical observation and evaluation of CMT score were chosen for MDA, GSH, and total GSH detection in milk and blood. The first group included 20 healthy cows without clinical signs, negative score of CMT and negative bacterial growth of udder pathogens from cultivated quarter milk samples. The second group included 20 cows with SM determined by the positive score of CMT and bacterial growth without clinical signs, and the third group of 20 cows with clinical signs ranging from mild to severe with positive CMT score, bacteriological cultivation, high level of SCC, changing the consistency of the milk with the presence of flakes, clots or pus and reduction or loss of milk production (Zigo et al. 2019b).

From the selected groups 10 cc milk samples were collected from each udder quarter for detection of MDA, GSH and total GSH. In addition, blood (10 cc) was collected in tubes containing EDTA from these dairy cows. All samples were kept in a cooler containing ice and immediately transported to Inonu University, Department of Microbiology Laboratory for Analysis.

Laboratory analyses

The milk and blood levels of MDA, GSH, and total GSH were measured using the Immuchrom kit (malondialdehyde, LOT, and glutathione LOT IC3401-160114) and high-performance liquid chromatography (Shimadzu-HPLC) according to Ustuner et al. (2017). Samples were subsequently analyzed by an HPLC column on a Mircosorb-MV using an isocratic solvent system (3% methanol and 10 mM potassium phosphate, pH 3.0) and detection at 200 nm (Ustuner et al. 2017). The obtained results were expressed in nmol of MDA, in µmol/L per GSH as well as total GSH from milk and blood samples.

Bacterial Isolation and Identification using MALDI-TOF MS

Milk samples were used to detect udder bacterial pathogens. Qarter milk samples (10 µL) were cultured to detect udder bacterial pathogens on blood agar with 5% of defibrinated blood with parallel cultivation on selective agar Eosin Methylene Blue Agar (Oxoid, Basingstoke, UK), MacConkey agar (Oxoid, Basingstoke, UK) and Edwards Medium (Oxoid, Basingstoke, UK) (Shell et al. 2017). Then, the plates were incubated at 37°C for 24-72 h. We evaluated contamination if the milk samples exhibited three or more different colonies on a culture. After that, single colonies were selected. Suspect colonies were isolated on blood agar and re-incubated at 37°C for 24 h and subsequently identified using MALDI-TOF MS-based VITEK MS (database v3.0) (BioMérieux, France) system identification (Dubois et al. 2012). Controls indicated that the isolates were identified with a high score value (99.9%).

Statistical analysis

The SPSS 22.0 software was utilised for all analyses. Analysis of variance (ANOVA) followed by Duncan's test which can perform multiple comparisons in evaluated anti-oxidative parameters was utilised to define whether there are significant differences between the selected groups of dairy cows. All the data are shown as mean (±) and standard error (SE). The 5% level of significance was used to confirm differences.

Results

Of 800 quarter milk samples examined, 380 (47.5%; 380/800), 300 (37.5%; 300/800), and 120 (15%; 120/800) of milk samples, respectively were CMT positive, CMT negative and CM, and those positive cows were from different farms. In this study, bacterial growth was observed in 56.1% (449/800), following 87.4% (332/380), 25.3% (76/300) and 34.2% (41/120) in CMT positive, CMT negative and CM cows (Table 1). We detected microbial contamination in 98 of 800 (12.3%) milk samples in CMT negative, SM and CM groups. In the remaining samples, bacterial growth was not observed. The present study identified 20 bacterial species by MALDI-TOF. From the 380 CMT positive samples, the most isolated organism was *Staphylococcus chromogenes* (*S. chromogenes*) at 18.7% (71/380), followed by *S. aureus* at 17.4% (66/380), *S. epidermidis* at 10.3% (39/380) and *E. coli* at 7.9% (30/380) as identified by MALDI-TOF. From the 300 CMT negative samples, the most isolated organism were non-aureus staphylococci such (NAS) as *S. epidermidis* at 7% (21/300) and *S. haemolyticus* at 5% (15/300). In milk samples from the 30 cows with clinical mastitis, we detected five bacterial species by MALDI-TOF and the most widely spread species was *S. aureus* (20/120, 16.7%) (Table 1). The MALDI-TOF MS technique identified additionally *Brevibacterium luteolum (B. luteolum), Enterococcus faecalis (E. faecalis), Lactococcus garviae (L. garviae),* and *Pseudomonas fleurescens (P. fleurescens),* which were unidentified or misidentified using biochemical tests from SM samples.

The levels of MDA, GSH, and total GSH measured in the blood are listed in Table 2. According to our results, the highest MDA levels (1.74±0.02 nmol/ml) and the lowest GSH levels (523.3±3.62 µmol/L) and

Table 1. Results of bacterial strains identification in dairy cows from 800 quarter milk samples being CMT-positive, CMT-negative, and from clinical mastitis by MALDI-TOF MS.

Bacteria	CMT positive n (%)	CMT negative n (%)	Clinical mastitis n (%)
Aerococcus viridans	16 (4.2)	7 (2.3)	-
Bacillus circulans	4 (1.1)	-	-
Bacillus licheniformis	10 (2.6)	-	-
Bacillus subtilis	4 (1.1)	2 (0.7)	-
Enterococcus faecalis	6 (1.6)	-	-
Escherichia coli	30 (7.9)	3 (1)	4 (3.3)
Klebsiella pneumoniae	3 (0.8)	-	-
Lactococcus garviae	2 (0.5)	-	-
Pseudomonas fleurescens	3 (0.8)	-	-
Staphylococcus aureus	66 (17.4)	2 (0.7)	20 (16.7)
Staphylococcus chromogenes	71 (18.7)	13 (4.3)	10 (8.3)
Staphylococcus epidermidis	39 (10.3)	21 (7)	-
Staphylococcus haemolyticus	16 (4.2)	15 (5)	5 (4.2)
Staphylococcus hyicus	5 (1.3)	-	-
Staphylococcus saprophyticus	1 (0.3)	-	-
Staphylococcus simulans	8 (2.1)	2 (0.7)	-
Staphylococcus warneri	10 (2.6)	4 (1.3)	-
Staphylococcus xylosus	5 (1.3)	3 (1)	-
Streptococcus dysgalactiae	3 (0.8)	-	-
Streptococcus uberis	5 (1.3)	-	2 (1.7)
S. aureus + E. coli	11 (2.9)	-	-
S. aureus + S. dysgalactiae	5 (1.3)	-	-
S. chromogenes + S. epidermidis	9 (2.4)	4 (1.3)	-
Total	332 (87.4)	76 (25.3)	41 (34.2)

Table 2. Levels of MDA, GSH, and total GSH in blood from dairy cows.

	BLOOD			
	Healthy cows[1]	Subclinical[2]	Clinical[3]	P
MDA (nmol/ml)	1.74 ± 0.02^{c}	2.31 ± 0.01^{a}	1.64 ± 0.02^{b}	***
GSH (µmol/L)	980 ± 2.74^{a}	918 ± 3.81^{a}	523.3 ± 3.62^{b}	***
Total GSH (µmol/L)	1656.57 ± 9.90^{a}	1147.15 ± 12.01^{b}	806 ± 2.11^{c}	***

Note: Healthy cows[1] – healthy cows without clinical signs and a negative score of CMT and negative bacterial growth, Subclinical[2] – cows with subclinical mastitis with the positive score of CMT, and bacterial growth without clinical signs, Clinical[3] – cows with clinical signs and a positive score of CMT and bacterial growth. *** $p<0.001$, Significant, ^{abc} Mean values with different superscripts within a row are significantly different.

total GSH levels (806±2.11 µmol/L) were quantified in cows with CM. Elevated MDA and total GSH levels ($p<0.001$) have been reported in the blood of cows with SM.

The levels of MDA, GSH, and total GSH measured in milk are listed in Table 3. According to our results, the highest MDA levels (2.10±0.08 nmol/ml) and the lowest GSH levels (369.32±7.12 µmol/L) and total

Table 3. Levels of MDA, GSH, and total GSH in milk from dairy cows.

	MILK			
	Healthy cows[1]	Subclinical[2]	Clinical[3]	P
MDA (nmol/ml)	0.12 ± 0.02[a]	1.38 ± 0.03[b]	2.10 ± 0.08[c]	***
GSH (µmol/L)	712.78 ± 11.03[a]	600.45 ± 15.96[a]	369.32 ± 7.12[b]	**
Total GSH (µmol/L)	1047.2 ± 11.16[a]	180.18 ± 8.44[a]	699.22 ± 5.30[b]	***

Note: Healthy cows[1] – healthy cows without clinical signs and a negative score of CMT and negative bacterial growth, Subclinical[2] – cows with subclinical mastitis with the positive score of CMT, and bacterial growth without clinical signs, Clinical[3] – cows with clinical signs and a positive score of CMT and bacterial growth. ** $p<0.01$, *** $p<0.001$, [abc] Mean values with different superscripts within a row are significantly different.

GSH levels (699.22±5.30 µmol/L) were found in cows with CM. In addition, increased levels ($p<0.001$) of MDA in milk samples from cows with SM were observed. There was no associate between nutrition and MDA and GSH levels.

Discussion

There are three forms of mastitis involved in the intramammry infection. These are clinical, subclinical, and chronic mastitis (Taponen et al. 2017). Among these, SM is the main form of this disease in dairy cows that is more predominant worldwide and causes reduced milk production (Zeryehun and Abera 2017, Ndahetuye et al. 2019). The high incidence of SM was also confirmed in our study. Of the 380 quarter milk samples, up to 332 samples (87.4%) showed a positive CMT score with confirmed bacteriological cultivation (Table 1). After laboratory analysis of milk samples were observed relatively high percentage (25.3%) of positive bacteriological results in cows with CMT-negative. These bacteria pathogens sufficiently stimulated inflammatory response but they had not yet colonized the intramammary tissue. There is a high probability in the future that these udder pathogens may cause mastitis and early identification is require to provide diminish the occurrence of intramammary infection (Ozbey et al. 2022).

Multiple pathogenic species in the etiology of mastitis have been reported (Zeryehun and Abera 2017). The major pathogens of mastitis in earlier studies were reported as *Staphylococcus aureus, Streptococcus agalactiae*, and coliforms (Amer et al. 2018). Current studies have indicated *Staphylococcus aureus* as still dominant pathogen however other bacilli such as NAS are becoming major udder pathogens (Mpatswenumugabo et al. 2017, Ndahetuye et al. 2019). NAS are a heterogeneous group of more than 52 species, of which approximately 10 are associated with intramammary infections in dairy cows (Zigo et al. 2022). Among the NAS species that cause mastitis, the most common are; *S. chromogenes*, *S. haemolyticus*, *S. epidermidis*, *S. simulans*, *S. sciuri* and *S. xylosus* (Malinowsky et al. 2006, Sztachańska et al. 2016, Taponen et al. 2017).

In recent years, the MALDI-TOF technology has been used for correct and rapid species determination. Therefore, a great number of bacteria traditionally identified as NAS can be analyzed using MALDI-TOF (Cameron et al. 2018, Dufour and Munoz 2018). The MALDI-TOF MS technique has detected correctly all of the bacteria in comparison with biochemical methods.

The most commonly NAS identified from mastitic quarter milk samples in our study were *S. chromogenes, S. epidermidis*, *S. haemolyticus* and *S. warneri*. Similar to our study, *S. chromogenes* has been detected most frequently in most published researches (Supré et al. 2011, Mørk et al. 2012, Bexiga et al. 2014, Taponen et al. 2017, Kırkan et al. 2018). The cause for different proportions of NAS species obtained from dairy cows in various countries is associated with various environmental conditions and herd management (Vanderhaeghen et al. 2013, De Visscher et al. 2016).

In many cases, persistent or chronic mastitis that has developed from SM caused by *S. chromogenes* and *S. warneri* are the cause of decrease in the antioxidant potential and is related to the accumulation of reactive oxidant species (ROS) and oxidation products (Castillo et al. 2006, Sharma et al. 2011, Zigo et al. 2019d). Our results are consistent with the data of Supré et al. (2011) and Zigo et al. (2019c), who identified *Staphylococcus* spp. as one of the main causes of mastitis in dairy cows followed by *Streptococcus* spp., *E. coli*, and *Klebsiella*. In addition, IMI caused by *S. aureus* and *S. uberis* showed a significant reduction in antioxidant enzyme activity.

Staphylococcus aureus (16.7%, 20/120) in this study was found as the most frequently identified bacteria from CM followed by NAS such as *S. chromogenes* (8.3%, 10/120) and *S. haemolyticus* (4.2%, 5/120) (Table 1).

Contrary to our findings in a study performed in Canadian cows with clinical mastitis, NAS

(*S. chromogenes, S. epidermidis*, and *S. haemolyticus*) were the most frequently isolated bacteria from SM (Condas et al. 2017) and *E. coli* was the main bacterial agent isolated from clinical mastitis study carried out in Wisconsin, USA (Oliveira et al. 2013). Concurrent with our result, the most commonly isolated bacteria were *Staphylococcus* spp. (31.9% and 11.2%, respectively) from SM and CM cases by MALDI-TOF conducted in dairy farms in the western part of Romania (Pascu et al. 2022).

Cows affected by an IMI often have reduced antioxidant capacity due to ongoing inflammation in the mammary gland. Synthesis and accumulation of ROS in the affected organs and tissues are balanced via antioxidant defence systems. There are several mechanisms to prevent oxidative damage including nonenzymatic scavengers such as GSH (Andrei et al. 2011, Celi 2011, Zigo et al. 2019a). A study carried out by Simsek and Aksakal (2005) reported that there was a statistically significant relationship between groups with healthy udder and mastitis, an increase in plasma MDA level, and also a decrease in erythrocyte GSH level. In this study, GSH values in cows with mastitis are in line with the previous study (Simsek and Aksakal 2006) when compared with controls.

Our analysis confirmed that the plasma and milk from the afected cows with SM and CM had higher MDA levels as compared to plasma and milk from the healthy cows without clinical signs of IMI and with negative CMT. There was no relation between nutrition and MDA and GSH levels.

Thus, it could be regarded as a potential indicator in the early detection of IMI. Suriyasathaporn et al. (2012) also found increased MDA levels in mastitic milk samples from cows with SM and CM caused by staphylococci or streptococci.

The high MDA levels and low GSH levels in milk samples with SM and CM were observed and the most frequently identified bacteria were *S. chromogenes* and *S. aureus*, respectively. This may be due to variabilities in an oxidative environment in udders (Zigo et al. 2019c).

Most diseases in dairy cows occur after calving or during first 100 days of lactation which is a period associated with endocrine changes, decreased intake of critical nutrients resulting in immune suppression and increased susceptibility to infections. Among the most important nutrients often deficient in compound feeds, involved in the biological functions and antioxidative activity are selenium (Se) and vitamin E (Vasil' et al. 2022).

There are many biological functions of Se, mainly as the component of various selenoproteins. The most important of these is glutathione peroxidase (GPx) that prevents oxidative damage in living organisms. The biological functions of GPx is complemented by vitamin E, which also functions as a cellular antioxidant.

Earlier studies performed by Andrei et al. (2011) indicated a decrease in the activity of antioxidant enzymes with a consequent increase in MDA levels in cows with SM mainly due to insufficient intake of Se from the feed ration. This may be caused by the tissue damage emerging from mammary inflammation (Carvalho-Sombra et al. 2021). On the contrary, reduction in oxidative stress in affected cows was evident after peroral or parenteral supplementation of vitamin-mineral products (Sharma et al. 2016, Vasil' et al. 2022).

The antioxidant status does not only depend on the Se and vitamin E supply or current status of GPx activity (Vasil' et al. 2022). Glutathione peroxidase action is complemented by other vitamins and redox systems in the elimination of ROS. Consequently, the consumption of one antioxidant may influence the concentration of the others, since the action of antioxidant enzymes also depends on their sparing effect and target tissue (Celi 2011, Vasil' et al. 2022).

The association between enzymatic antioxidants (SOD, GPx) from blood of infected cows and cumulation of MDA in mastitic milk has been reported in previous studies by Mahapatra et al. (2018), Zigo et al. (2019a) and Abdel-Hamied and Mahmoud (2020) that indicate the importance of increasing this lipid peroxidant in cows with mastitis. This might be due to the higher levels of udder defence mechanism (Zigo et al. 2019c). Thus, MDA is an important biomarker to be in dairy cows mastitis (Carvalho-Sombra et al. 2021).

Products of lipid peroxidation are prevented from forming by a defense line against oxidative stress protecting polyunsaturated fatty acids in membrane phospholipids in the early stages of peroxidation by GSH, a powerful endogenous antioxidant (Simsek and Aksakal 2005). The antioxidant defence system has several control mechanisms containing non-enzymatic scavengers such as GSH to prevent the accumulation of ROS and hinder oxidative damage (Andrei et al. 2011, Celi 2011).

The decrease in the GSH levels may occure because of the increased demand for GSH in the cell to compensate for the formation of ROS during mastitis (Kaya et al. 2019). GSH can serve as a nonenzymatic antioxidant with direct interaction with the − SH group of ROS, or join the enzymatic detoxification reaction for ROS as a cofactor or coenzyme (Ma et al. 2011). The primary cause of the reduced GSH levels in cows with mastitis oxidative stress is the inhibition of enzy-

matic activities by ROS. A secondary cause may be ROS binding to proteins and causing changes in their structure, herewith resulting in their oxidation (Kaya et al. 2019).

Oxidative stress seems to be more restricted to the mammary gland in SM with the accumulation of the lipid peroxidation products in milk, however, CM causes systemic oxidative stress with failure of multiple defense mechanisms (Zigo et al. 2019a, Carvalho-Sombra et al. 2021). According to previous study, the milk quality is affected by the accumulation of the oxidation products, which positively correlates with SCC and elevation of CMT score in cows with CM or SM (Zigo et al. 2019a).

Conclusion

Based on our results the levels of MDA were higher in the mastitic milk samples originating from cows with CM and SM compared with the normal milk; therefore, MDA could be regarded as a marker of mastitis. Further investigations are necessary to conduct microbiological analyses to identify the bacterial pathogens causing mastitis in various regions, to search the impact on different biomarkers in the early diagnosis of IMI. This is very important in developing alternative methods to prevent mastitis and economic losses in dairy animals.

Acknowledgements

This research was funded by Professor Barış OTLU from Department of Medical Microbiology, Faculty of Medicine, Inonu University, Malatya, Turkey and Slovak grant KEGA no. 006UVLF-4-2020.

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