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The Relation between Biochemical Parameters, Milk Amyloid A, Somatic Cell Count, and Some Pathogens in Buffalo Milks

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ABSTRACT

Being resistant to hard environmental conditions and diseases makes Buffalo to have its valuable milk. Microbial contamination may occur due to undesirable conditions such as mastitis, environmental contamination, and stress. If microorganisms are not removed from the milk, it causes many production disadvantages including inadequacy of production, failure of fermentation and shortening of the shelf life. This study was conducted to determine the relationship between somatic cells count (SCC), the presence of some pathogens, and milk amyloid A (MAA) in the buffalo milk. In addition, oxidative stress in buffalo milk was evaluated. For this purpose, 70 samples were collected and Enterobacteriaceae, coliform microorganisms, *Escherichia coli*, *Salmonella* spp. analyses were performed. Biochemical parameters [Malondialdehyde (MDA), glutathione (GSH), nitric oxide (NO), catalase, superoxide dismutase (SOD), and total antioxidant capacity (TAC)] and serological parameter (MAA) were measured. The SCC was not statistically different according to Enterobacteriaceae, coliform microorganisms, *E. coli* groups. While MDA, NO, SOD, and TAC values were not statistically different according to the SCC groups, GSH and catalase values were statistically different. MAA values were statistically significant compared to the SCC groups. Moreover, there was a positive correlation between MAA and MDA/SCC. Detection of MAA may prevent the mixing of healthy and mastitic milk. Therefore, more reliable buffalo milk products will be presented to consumption.

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Manda Sütlerindeki Somatik Hücre Sayısının Bazı Patojenler, Biyokimyasal Parametreler ve Süt Amiloid A ile İlişkisi

ÖZET

Mandaların olumsuz çevre koşullarına, hastalıklara karşı dirençli yapısı sütünü de değerli kılmaktadır. Manda sütlerine mastitis, çevresel bulaşma, stres gibi istenmeyen bazı durumlara bağlı olarak mikrobiyel bulaşma olabilir. Bu mikroorganizmaların sütten uzaklaştırılamaması sonucunda yetersiz üretim, fermantasyonun gerçekleşmemesi, ürünlerin raf ömrünün kısalması gibi üretim sorunlarına neden olmaktadır. Bu nedenle, manda sütlerindeki somatik hücre sayısının bazı patojenlerin varlığı ve süt amiloid A ile ilişkisinin belirlenmesi amaçlanmıştır. Buna ilaveten manda sütlerinde meydana gelen oksidatif stres değerlendirilmiştir. Bu amaç doğrultusunda, toplanan manda sütlerinde Enterobacteriaceae, koliform mikroorganizmalar, *Escherichia coli* ve *Salmonella* spp. analizleri yapılmıştır. Biyokimyasal parametrelerden malondialdehit (MDA), glutatyon (GSH), nitrik oksit (NOx), katalaz (KAT), süperoksit dismutaz (SOD), total antioksidan seviye (TAS) ölçülmüştür. Serolojik olarak ise süt amiloid A (SAA) seviyesi belirlenmiştir. Bu analiz sonuçlarına göre Enterobacteriaceae,

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koliform mikroorganizma, *E. coli* gruplarına göre SHS ortalamaları istatistiksel olarak anlamlı farklılık göstermemiştir. MDA, NOx, SOD, TAS değerleri SHS gruplarına göre istatistiksel farklılık göstermezken; GSH ve KAT değerlerinin SHS gruplarına göre istatistiksel olarak farklı olduğu tespit edilmiştir. SAA değeri SHS gruplarına göre istatistiksel olarak anlamlı farklılık göstermiştir. Buna ilaveten SAA ile MDA ve SHS değeri arasında istatistiksel olarak anlamlı, düşük düzeyde, pozitif yönde bir korelasyon tespit edilmiştir. Manda sütlerinde süt amiloid A'nın tespiti ile, hastalıklı hayvan sütlerinin sağlıklı sütlerle karışmasına engel olunarak daha güvenilir manda süt ve süt ürünleri tüketime sunulma olanağı kazandırır.

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INTRODUCTION

The presence of food-borne pathogens in milk may be presented by direct contact of contaminated material or by secretion of animals with mastitis (Oliver et al., 2005). If the microorganisms are removed from the milk effectively, the fermentation will not be realized effectively and the shelf life of products will be reduced (Urech et al., 1999). Mastitis is a serious mammary gland infection that causes economic losses by reducing milk production, also decreasing the nutritional value of milk in herds (Schultz et al., 1978). The somatic cell count (SCC) is an important criterion for determining milk quality (Harmon, 1994). In mastitis cases, superoxide radicals and other oxygen metabolites (Free Radicals, FR) occur due to the number of neutrophils in the mammary gland and increases oxygen utilization in the tissue. Such kinds of FR cause to change chemistry of milk (Mayer et al., 1988). Acute phase proteins are a group of proteins which are secreted in the body during infection or stress (Whelehan et al., 2011; Ceciliani et al., 2012). Some of the acute phase proteins increases during infection and some other decreases (Sevimli et al., 2015). Milk Amyloid A (MAA) is one of the basic acute phase proteins and it is released into milk during infection (Whelehan et al., 2011; Ceciliani et al., 2012). Acute phase proteins are formed by stimulating the acute phase response due to bacterial, chemical, thermal or mechanical damage of the mammary gland (Haghkhah et al., 2010). Therefore, the high amount of acute phase proteins in milk increases the importance as an indicator of infection in recent years (Singh et al., 2015).

Buffalo milk is valuable because buffaloes are resistant to hard environmental conditions and diseases. Even if buffaloes feed the poor quality, it can secrete more quality milk than cows. Furthermore, it has less mastitis risk than cows because long and narrow teat channel prevent the passage of microorganism (Wanasinghe, 1985). On the other hand, having the drooping teats make them to prone mastitis (Badran, 1985; Bansal et al., 1995). In this context, the aim of this study was to detect MAA in buffalo milk and to investigate the relationship of the SCC with some pathogenic microorganisms that should not be presented in milk and to search the biochemical changes caused by these microorganisms for MAA.

MATERIAL and METHODS

Collection of milk samples

The milk samples were collected from Afyonkarahisar province in Turkey on 70 buffaloes, aged 3-10 years, in different stages of lactation, and held in either private farm or public farm. Before the milk samples were collected, the teats had been washed and dried with paper towels. Teats were thoroughly disinfected with 95% alcoholic cotton. The pre-milk was discarded before the milk was taken into the sterile falcon tubes. Milk samples were collected under aseptic conditions and brought to the laboratory under the cold chain.

Preparations for microbiological, biochemical and serological analyses

The SCC was performed according to International Dairy Federation method (Anonymous, 1981; Özenç et al., 2008). Two groups were formed according to the SCC. One of those had SCC of less than 400.000 cells/ml which was named group I (GI). The other had SCC greater than or equal to 400.000 cells/ml and this group was named group II (GII). Enterobacteriaceae, coliform microorganisms, *E. coli* and *Salmonella* spp. were isolated for microbiological analyses (Halkman, 2005; Anonymous, 2017). Malondialdehyde (MDA), glutathione (GSH), nitric oxide (NO), catalase, superoxide dismutase (SOD) and total antioxidant capacity (TAC) measurements were made for biochemical evaluation (Ohkawa et al., 1979; Beutler et al., 1963; Miranda et al., 2001; Luck, 1955; Sun et al., 1988; Erel, 2004). The concentrations of MAA were determined using the commercial ELISA kit (Tridelta Development, Maynooth, Ireland). Optical densities

were read at 450 nm in an automatic plate reader (Model ELx 800; Bio-tekInc, Winooski VT, USA).

Statistical analysis

In addition to descriptive statistics, the normality of data obtained from the study was done with Shapiro Wilk test. It was determined that the data didn't have normality according to the groups ($p<0.05$). According to normality results Mann-Whitney U test was performed for two group comparison and Spearman Correlation analysis was used to analyze the relationship between biochemical analysis and the SCC. Data were analyzed using SPSS 22.0 package program.

RESULTS

The SCC of 12 samples are greater than 400000 cells/ ml (665231.42 ± 357936.69 cells/ ml), while 58 samples are less than 400000 cells/ ml (133495.70 ± 92950.16 cells/ ml). According to the groups, formed as a result of microbiological analysis, the statistical difference of the SCC is shown in Table 1. Reference limits (RL) for microbiological assessment are determined according to the Turkish Food Codex Regulation on Microbiological Criteria (Anonymous, 2011). The SCC were not statistically significant difference according to Enterobacteriaceae, coliform microorganism, and *E. coli* groups ($p>0.05$).

The number of Enterobacteriaceae was not statistically different ($p>0.05$) (Table 2). As a result of biochemical identification, 5 milk samples (7.1%) were confirmed as *E. coli* biotype 1. In addition, 4 milk samples (5.7%) were evaluated as *Salmonella* spp.

While MDA, NO, SOD, and TAC values were not statistically different according to the SCC groups ($p>0.05$); GSH and catalase values were statistically different ($p<0.05$) (Table 3). GI had a high GSH value and low catalase value compared to the GII.

Table 1. Comparison of the SCC according to microbiological measurements
Çizelge 1. Mikrobiyoloji ölçümlerine göre somatik hücre sayısının değerlendirilmesi

Variables	RL	N	Median (Q1-Q3)	*p*
Entorobactericeae (cfu/ml)	$<10^1$	27	105000.0 (47500.0-262499.75)	0.120
	$\geq10^1$	43	155000.0 (80000.0-338676.25)	
Coliform (MPN/ml)	<3	27	160000.0 (78571.0-290000.0)	0.976
	≥3	43	130000.0 (70000.0-300000.0)	
E. coli (MPN/ml)	<3	39	135000.0 (79642.75-293480.25)	0.920
	≥3	31	140000.0 (65416.5-300000.0)	

N: Number of samples; cfu: colony forming unit; MPN: Most Probable Numbers; RL: Reference Limits.

Table 2. Comparison of the Enterobacteriaceae according to SCC groups
Çizelge 2. Enterobactericeae sayısının somatik hücre sayısına göre karşılaştırılması

Variables	SCC (cells/ml)	N	Median (Q1-Q3)	*p*
Enterobactericeae (log cfu/ml)	GI	58	2.0 (1.0-3.01)	0.206
	GII	12	2.80 (1.33-3.23)	

N: Number of samples; cfu: colony forming unit; RL: Reference Limits.

Table 3. Comparison of oxidative stress parameters according to the SCC groups
Çizelge 3. SHS gruplarına göre oksidatif stres parametreleri sonuçlarının karşılaştırılması

Variables	SCC (cells/ml)	N	Median (Q1-Q3)	*p*
MDA (nmol/L)	GI	58	4.98 (4.09-5.49)	0.258
	GII	12	5.30 (4.90-5.78)	
GSH (µmol/L)	GI	58	33.73 (31.10-35.32)	**0.001***
	GII	12	29.11 (21.64-30.87)	
NO (µmol/L)	GI	58	24.90 (19.78-34.22)	0.821
	GII	12	25.00 (21.70-28.97)	
SOD (U/ml)	GI	58	1.12 (0.98-1.35)	0.362
	GII	12	1.08 (0.92-1.25)	
Catalase (U/ml)	GI	58	1.74 (1.38-2.14)	**0.049***
	GII	12	2.06 (1.91-2.39)	
TAC (mmolTroloxEquiv./L)	GI	58	1.53 (1.36-1.65)	0.300
	GII	12	1.60 (1.44-1.72)	

*p<0.05; N: Number of samples.

The correlation analyses result between the oxidative stress and the SCC are shown in Table 4. The relationship between the SCC and GSH/catalase values were statistically significant ($p<0.05$). A low

and negative correlation was found between the SCC and GSH (r=-0.272). Moreover, a low and positive correlation was found between the SCC and catalase (r=0.230).

There was a statistically significant difference in MAA values compared the SCC groups (p<0.05). The MAA value in GII were higher than GI (Table 5). MAA values were not statistically different according to Enterobacteriaceae, coliform microorganisms, and *E. coli* groups (p>0.05) (Table 5).

A low and positive correlation between MAA and MDA was determined. In addition, there was moderate and positive correlation between MAA and SCC (p<0.05) (Table 6).

Table 4. Correlation analysis between the SCC and oxidative stress parameters
Çizelge 4. SHS ile oksidatif stres parametreleri arasındaki korelasyon analizi

Variables *(Değişkenler)*		MDA	GSH	NO	SOD	Catalase	TAC
SCC (cells/ml)	r	0.200	-0.272	-0.046	-0.099	0.230	-0.100
	p	0.096	**0.023***	0.707	0.415	**0.045***	0.412
MDA (nmol/L)	r	1	0.086	0.027	0.140	-0.133	-0.202
	p		0.478	0.826	0.249	0.272	0.093
GSH (µmol/L)	r		1	0.258	0.257	-0.125	0.062
	p			0.055	0.195	0.303	0.610
NO (µmol/L)	r			1	0.020	0.074	0.004
	p				0.867	0.540	0.972
SOD (U/ml)	r				1	0.057	-0.125
	p					0.639	0.303
Catalase (U/ml)	r					1	0.102
	p						0.399

**p<0.05; r: Spearman correlation coefficient.*

Table 5. Comparison of MAA (µg/ml) values according to the SCC and microorganisms
Çizelge 5. Süt amiloid A düzeyinin (µg/ml) SHS ve mikroorganizmalara göre karşılaştırılması

Variables *(Değişkenler)*	RL *(Referans Limit)*	N	Median *(Medyan)* (Q1-Q3)	*p*
SCC (cells/ml)	GI	58	2.23 (1.60-3.94)	**0.044***
	GII	12	3.72 (1.97-6.32)	
Entorobactericeae (cfu/ml)	$<10^1$	27	2.20 (1.71-4.10)	0.685
	$\geq10^1$	43	2.46 (1.80-4.34)	
Coliform (MPN/ml)	<3	27	2.20 (1.54-4.58)	0.656
	≥3	43	2.33 (1.88-4.10)	
E. coli (MPN/ml)	<3	39	2.21 (1.60-4.57)	0.682
	≥3	31	2.46 (1.84-4.02)	

**p<0.05; N: Number of samples; RL: Reference Limits.*

Table 6. Correlation analysis between MAA and biochemical parameters
Çizelge 6. Süt Amiloid A ile biyokimyasal parametreler arasındaki korelasyon analizi

Variables *(Değişkenler)*		MDA	GSH	NO	SOD	Catalase	TAC	SCC
MAA	r	0.253	-0.218	-0.090	0.142	0.157	-0.217	0.432
	p	**0.035***	0.069	0.461	0.242	0.193	0.071	**0.001***
	N	70	70	70	70	70	70	70

**p<0.05; N: Number of samples; r: Spearman correlation coefficient.*

DISCUSSION

Kumar et al. (2014) did not found any differences between the SCC with clinical and subclinical mastitis in buffalo milk. Catozzi et al. (2017) informed that the SCC were greater than 200000 cells/ml in 110 milk which had no clinical signs of mastitis and had negative microbiological results. Harmon (1994) argued that the SCC was an important determinant for the diagnosis of mastitis. Even if the SCC was less than 400000 cells/ml and there was no microbial reproduction in buffalo milk, it may not said that the animals have a healthy mammary gland. At the same time, buffalo milks with the SCC greater than 400000 cells/ml may be suspected of mastitis due to another microorganism.

In mastitis cases, the use of antioxidants increases due to the effects of free radicals during inflammation, therefore, the level of antioxidants reduces. GSH is

effective for protecting tissues from oxidative damage. Moreover, leukocytes utilize GSH for preventing tissues from phagocytosis (Erskine et al. 1987). It was determined that value of GSH changed according to the SCC groups ($p<0.05$) (Table 3). Erişir et al. (2011) found similar results in cow milk. Dimri et al. (2013) determined that the level of glutathione decreased in buffalo milk with mastitis. The decrease in GSH levels and high levels of the SCC can be related to the use of GSH as an antioxidant by leukocytes. It was reported that catalase was an antioxidant enzyme and multiplied 2-4 times in buffalo's milk (Khan et al. 2017). There is a positive and low correlation between the SCC and catalase ($r=0.230$) (Table 4). Dimri et al. (2013) found that catalase level was significantly higher in buffaloes with subclinical mastitis than healthy ones. Silanikove et al. (2009) concluded that catalase played a critical role in redox control of milk and increased continuously during mastitis. Catalase activity was a useful indicator in the diagnosis of mastitis because of the positive correlation with the SCC (Table 4). In addition, it was also reported by some researchers (Silanikove et al., 2009; Andrei, 2010). It is observed that antioxidant enzyme system is stimulated with increased catalase activity in milk with greater SCC and antioxidant enzyme production is increased in order to compensate for increasing oxidative stress.

Inflammatory cells lead to release a number of reactive species in the area of infection (Collins 1999). Therefore, infection and oxidative stress are closely linked and pathophysiological events (Anderson et al. 1994; Flohe et al. 1997). The MAA value was not related to microbial reproduction (Table 6). However, there is a statistically significant difference according to the MAA in the SCC groups (Table 5). Kumar et al. (2014) and Singh et al. (2015) found the MAA value of healthy buffaloes as 0.06 ± 0.03 µg/ml, and 0.03 ± 0.01 µg/ml; in the cases with subclinical mastitis 2.37 ± 0.81 µg/ml, and 1.22 ± 0.44 µg/ml, respectively. In addition to MAA differences in the SCC groups, a positive correlation between the MAA and MDA strengthens the diagnosis of mastitis. It can be concluded that the change in the oxidative stress parameters may be a determinant for the diagnosis of mastitis in buffaloes, such as acute phase proteins.

Cleaning and disinfection of the place can be ignored because of the production of milk and dairy products were mostly done in small scale enterprises. Furthermore, the diagnosis of mastitis may be neglected due to the disease resistant of the buffaloes. Some effective precautions should be taken to prevent the contamination and ensuring the elimination of Enterobacteriaceae, coliform microorganism, *E. coli*, and *Salmonella* spp. from milk. Critical microorganisms were noticed at The Turkish Food Codex and the European Union (Commission Regulation (EC) No: 1441/2007) in the Regulation on Microbiological Criteria. If milk is not stored under suitable conditions, the load of microorganisms will increase until the production. The consumption of unprocessed food, raw milk such as street milk has been increasing due to cultural reasons and consumer's tendency to natural product. The growing demand could increase the microbiological hazard. Therefore, the detection of pathogenic microorganisms is very important in buffalo milk and related products. It is determined that chemical change is not occurred with increasing the SCC. As a result, GSH and catalase which are associated with the SCC can be considered as biomarkers for the detection of mastitis in buffaloes. However, it could not be safe enough to make decisions about mastitis solely by amount of the SCC. Further upcoming studies should be increased about the SCC in buffalo milk especially in the cases of subclinical mastitis. Therefore, establishment of MAA in buffalo milk could be a useful diagnostic tool to detect mastitis and monitoring herd health. Development of biosensors for detection of MAA level in field conditions can ensure to distinguish healthy milk from contaminated ones. Furthermore, facilitating the diagnosis of mastitis may reduce economic losses.

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Conflict of interest statement

There are no conflicts to declare.

Author's Contributions

The contribution of the authors is equal.

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