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Lactoferrin concentrations in bovine milk during involution of the mammary glands, with different bacteriological findings

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ABSTRACT

Lactoferrin is an iron-binding glycoprotein of the transferrin family, present in high concentrations in secretions from the mammary glands during the involution period, and has antimicrobial ability. To determine lactoferrin concentrations in bovine milk with different bacteriological findings, 151 quarter milk samples were collected on a dairy farm of the Holstein-Friesian breed in Vojvodina, Republic of Serbia. Classical microbiological methods were used for bacteria isolation, and ELISA analysis was used for lactoferrin concentration quantification. The most common isolated bacteria in bovine milk samples were *Corynebacterium* spp. (32.45%) and coagulase-negative staphylococci (4.64%) with lactoferrin concentrations of 6.0497 ± 1.6774 mg/mL and 5.2961 ± 1.3633 mg/mL, respectively. The lowest mean value of lactoferrin concentration was observed in uninfected quarters and quarters infected with environmental pathogens, while the highest concentration of lactoferrin was in udder quarters infected with *Streptococcus agalactiae*. One in four milk samples where *Staphylococcus aureus* was isolated had much lower lactoferrin concentrations (1.1736 mg/mL) than the other three samples (6.2089 ± 0.5016 mg/mL), which requires further research.

Key words: bovine lactoferrin, milk, dairy cows, involution period, lactation, mastitis pathogens

Introduction

In the modern dairy industry, mastitis is one of the most significant and complex diseases of highly productive cows that causes severe economic losses and occurs in a high percentage (20-80%) in both developing and developed countries. Mastitis

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is a multicausal disease that results from interaction between a variety of microbial infections, host factors, environment and management practices (BAČIĆ, 2012). The most important major mastitis pathogens are: *Staphylococcus aureus* and *Streptococcus agalactiae* (contagious pathogens), and *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli* and *Enterococcus* (environmental pathogens). Lately, there has been a significant increase in udder infections with minor mastitis pathogens (*Corynebacterium* spp. and coagulase-negative staphylococci), probably because the prevalence of major pathogens is decreasing (PYÖRÄLÄ and TAPONEN, 2009; REYHER et al., 2012; INDRISS et al., 2013). MAĆEŠIĆ et al. (2012) indicated a rise in the importance of environmental pathogens in the epidemiology of bovine mastitis.

Mammary glands are most susceptible to the emergence of new intramammary infections during early involution (first two weeks) and before partus (GAUNT et al., 1980; SMITH et al., 1985; OLIVER and BUSHE, 1987; DINGWELL et al., 2003). The involution period has an important influence on udder health, productivity, overall health, and fertility performance in the next lactation (PETZER et al., 2009). During this period, mammary gland secretion undergoes changes, with a drastic increase in concentrations of natural protective factors, such as phagocytic cells, lactoferrin and immunoglobulins, which are related to the resistance of the udder to intramammary infections (BUSHE and OLIVER, 1987; NICKERSON, 1989; CHENG et al., 2008).

Lactoferrin (lactotransferrin) is an iron-binding antimicrobial glycoprotein present in milk and other external secretions, synthesized by neutrophilic polymorphonuclear leukocytes and glandular epithelial cells (MASSON et al., 1966, and 1969). Concentrations of lactoferrin in milk vary according to the stage of lactation and health status of the udder. Mature bovine milk has relatively low levels of lactoferrin (0.02-0.35 mg/mL), while this concentration dramatically increases in the involution period (20-100 mg/mL) (WELTY et al., 1976).

Inflammatory processes in udders increase lactoferrin content in milk. During inflammation, the epithelial cells of the mammary glands are stimulated and production of lactoferrin is intensified. Additionally, lactoferrin is released from secondary granules of neutrophilic polymorphonuclear leukocytes (LASH et al., 1983; KUTILA et al., 2004). Lactoferrin increases the productivity of inflammatory cytokines and chemokines, and infiltration of leukocytes (KOMINE et al., 2005). Previous reports indicated that major mastitis pathogens (*Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis*) lead to a more significant increase in lactoferrin concentrations in cow's milk than minor mastitis pathogens (*Corynebacterium* spp., coagulase-negative staphylococci) (HARMON et al., 1975; KAWAI et al., 1999; HAGIWARA et al., 2003; NEWMAN et al., 2009).

One of the first functions ascribed to lactoferrin is its ability to bind and sequester environmental iron, which is necessary for the growth of certain bacteria. Beside bacteriostasis, lactoferrin has bactericidal activity which results from the direct interaction between lactoferrin and the bacteria (FARNAUD and EVANS, 2003).

Materials and methods

The experiment was conducted on a dairy farm of the Holstein-Friesian breed in Vojvodina, Republic of Serbia. A total of 151 udder quarters from cows in the involution period were examined with a Draminski mastitis detector, and milk samples were collected for bacteriological examination and lactoferrin concentration determination. The results of the Draminski mastitis detector were interpreted according to the manufacturer's instructions (above 300 - high quality and healthy milk; the incidence of subclinical mastitis is very low; below 300 - a progressively increasing incidence of subclinical infection as the reading decreases).

Quarter milk samples for bacteriological analysis were taken during the morning milking, aseptically from the teat into sterile test tubes. Before sampling, the udder teats were cleaned and disinfected using 70% alcohol. The samples were labeled and submitted to the laboratory at refrigeration temperatures for analysis.

From each sample, 0.1 mL of milk was plated on a Columbia blood agar base (Oxoid, Basingstoke, UK, CM0331) with 5% defibrinated ovine blood, MacConkey agar (Oxoid, CM0007) and Sabouraud dextrose agar (Oxoid, CM0041), and incubated for 24 h-5 days (yeasts, mould) at 37 °C. Bacterial colonies were determined 24, 48 and 72 hours after incubation. Bacterial species were identified according to colony morphology (shape, size and structure) and physiological features (Gram staining, pigment formation, catalase test, CAMP test, coagulase test). Isolated microorganisms were categorized on the basis of the bacteriological findings: major mastitis pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*) and minor mastitis pathogens (coagulase-negative staphylococci, *Corynebacterium* spp.).

Milk samples for lactoferrin concentration determination were skimmed at 1,400 × g for 45 minutes (Eppendorf 5702, Germany). Then, the skimmed milk was drained into test tubes marked with each cow's ID number, and stored at -20 °C until analysis.

Lactoferrin concentrations in bovine milk were determined using a Bovine Lactoferrin ELISA Quantitation Set (Bethyl Laboratories. Inc.) according to the manufacturer's instructions. Microtitre plates were coated with 100 μL of diluted goat anti-bovine lactoferrin coating antibody. Skimmed milk samples were diluted at a ratio of 1:10,000 (NEWMAN et al., 2009) in 50 mM Tris, 0.14 NaCl, 0.05% Tween 20, pH 8.0. Standards were designed through serial dilution using the bovine lactoferrin calibrator. Goat anti-bovine lactoferrin HRP conjugate antibody was used as the detection antibody, at a dilution of 1: 200,000. Plates were read at 450 nm absorbence values by a Labsystems Multiskan plate reader. The obtained concentration of bovine lactoferrin was multiplied by the dilution factor to determine the amount of lactoferrin in the undiluted samples.

Statistical data - mean values, standard deviation, minimum and maximum values were calculated using IBM SPSS Statistics Version 20 (IBM Corp. Armonk, NY, USA).

Results

The study included 151 quarter milk samples from cows in the involution period for bacteriological examination and determination of lactoferrin concentration. Bacteria and mould were not isolated in 76 quarter milk samples (50.33%), while in the other 75 samples bacteria and mould were found (49.67%).

Table 1. Bacteriological findings and Draminski mastitis test

			Draminski mastitis test	
Bacteriological findings	N	%	Below 300	Above 300
Negative	76	50.33	53 (69.74%)	23 (30.26%)
Staphylococcus aureus	4	2.65	2 (50.00%)	2 (50.00%)
Streptococcus agalactiae	3	1.99	3 (100.00%)	0 (0.00%)
Environmental pathogens	3	1.99	3 (100.00%)	0 (0.00%)
Corynebacterium spp.	49	32.45	37 (75.51%)	12 (24.49%)
Coagulase-negative staphylococci	7	4.63	3 (42.86%)	4 (57.14%)
Other*	9	5.96	5 (55.56%)	4 (44.44%)
Total samples	151	100.00	106 (70.20%)	45 (29.80%)

* Other (different types of bacteria and mould)

The most common isolated bacteria were *Corynebacterium* spp. (32.45%) and coagulase-negative staphylococci (4.64%). Contagious mastitis pathogens (*Staphylococcus aureus* and *Streptococcus agalactiae*) were identified in 4.63% of the milk samples. The Draminski mastitis test indicated an increased incidence of subclinical mastitis in 70.20% of the samples.

Lactoferrin concentrations in quarter milk samples with different bacteriological findings are presented in Table 2 and Fig. 1. The lowest mean value of lactoferrin concentration was observed in uninfected quarters and quarters infected with environmental pathogens, while the highest concentration of lactoferrin was in udder quarters infected with *Streptococcus agalactiae*.

Table 2. Lactoferrin concentration in cow milk samples with different bacteriological findings (mg/mL)

Bacteriological findings	N	Lactoferrin concentration		
		min.	max.	mean value ± SD
1. Major mastitis pathogens	10	1.1736	6.8777	5.4549 ± 1.6839
a. Contagious pathogens	7	1.1736	6.8777	5.6069 ± 2.0026
Staphylococcus aureus	4	1.1736	6.6774	4.9501 ± 2.5508
Streptococcus agalactiae	3	5.9099	6.8777	6.4827 ± 0.5078
b. Environmental pathogens	3	4.6818	5.8833	5.1003 ± 0.6786
Streptococcus dysgalactiae	2	4.6818	4.7359	4.7089 ± 0.0383
Enterococcus faecium	1	-	-	5.8833
2. Minor mastitis pathogens	56	2.3868	9.8413	5.9555 ± 1.6497
Corynebacterium spp.	49	2.3868	9.8413	6.0497 ± 1.6774
Coagulase-negative staphylococci	7	4.0753	7.6016	5.2961 ± 1.3633
3. Other*	9	2.2582	8.3376	5.8789 ± 2.3538
4. Negative	76	0.7267	8.8492	5.1237 ± 1.7689

* Other (different types of bacteria and mould)

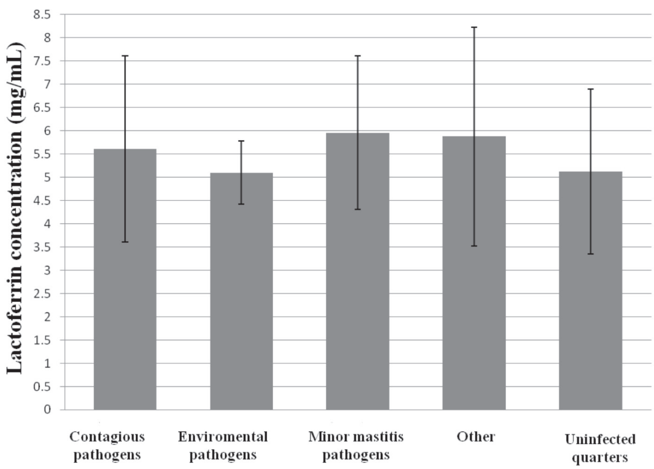

Fig. 1. Lactoferrin concentrations in quarter milk samples with various mastitis pathogens (mean value ± SD)

One in four milk samples where *Staphylococcus aureus* was isolated had a lower lactoferrin concentration (1.1736 mg/mL), while the other three samples had approximately the same concentrations of lactoferrin, with a mean value of 6.2089 ± 0.5016 mg/mL.

The lowest lactoferrin concentration was determined in uninfected quarters (0.7267 mg/mL). The highest concentration of lactoferrin was observed in milk with isolated *Corynebacterium* spp. and it was 9.8413 mg/mL.

Discussion

Over the last decade, minor mastitis pathogens have become the predominant cause of intramammary infections. DE VLIEGHER et al. (2012) and AHMED et al. (2015) reported an increased prevalence of subclinical mastitis caused by coagulase-negative staphylococci. In this study, minor mastitis pathogens were detected in 56 milk samples that indicated an increase in its prevalence. Our results correspond with the conclusions of INDRISS et al. (2013), who reported an increase in intramammary infections with minor mastitis pathogens in 106 out of 390 (27.18%). In our previous study, a similar incidence of udder infection with *Corynebacterium* spp. and coagulase-negative staphylococci was found (GALFI et al., 2015). This study indicates that the Draminski mastitis detector cannot be used alone in detecting intramammary infections which corresponds to the conclusions of GALFI et al. (2015). The Draminski mastitis detector indicated an increased incidence of subclinical infection, but could not detect disorders in 22 of 75 infected quarters.

There are many reports on lactoferrin content in milk from different dairy animals (YANG et al., 2000; HISS et al., 2008; GIACINTI et al., 2013; AHMED et al., 2015). GIACINTI et al. (2013) observed higher lactoferrin concentrations in buffalo milk than that reported in bovine milk, suggesting that buffaloes are more resistant to mastitis than cows, due to the higher lactoferrin content in buffalo milk. Several different methods have been used to quantify lactoferrin concentration in milk, such as capillary electrophoresis (RIECHEL et al., 1998), radial immunodiffusion test (HAGIWARA et al., 2003), ELISA test (NEWMAN et al., 2009) and SDS-polyacrilamide gel electrophoresis (GIACINTI et al., 2013). SOYEURT et al. (2012) confirmed the possibility of computation of an indicator of lactoferrin content in milk by mid-infrared spectrometry.

Lactoferrin has an important role in the host innate defense system with its antimicrobial properties. Several studies have investigated the antibacterial activity of lactoferrin alone or in combination with antibiotics against bovine bacterial pathogens (KAI et al., 2002; DIARRA et al., 2003; KUTILA et al, 2004). A synergistic effect has been reported during intramammary therapy with lactoferrin and antibiotics, suggesting that this may be an effective combination for the treatment of infections caused by bacterial pathogens resistant to antibiotics (LACASSE et al., 2014).

Lactoferrin concentrations in secretions of the bovine mammary gland vary according to the stage of lactation and milk production. Concentrations of lactoferrin are high in colostrum, dry secretion and during intramammary infections. SÁNCHEZ et al. (1988) found the highest lactoferrin concentration in the first milking (0.83 mg/mL), but it decreased sharply during the first days of lactation. A definitive value of lactoferrin concentration in milk was reached during the third week postpartum and amounted to 0.09 mg/mL (SÁNCHEZ et al., 1988). Milk of clinically healthy lactating cows contains a relatively low concentration of lactoferrin, varying from 0.02 to 0.35 mg/mL (WELTY et al., 1976; SCHANBACHER et al., 1993). GAUNT et al. (1980) and CHENG et al. (2008) reported that the mean lactoferrin concentration in bovine milk increases as lactation progresses. Lactoferrin concentrations in the involution period vary between 20 and 30 mg/mL. WELTY et al. (1976) reported the highest physiological concentration of lactoferrin in the whey of dry secretions, which amounted to 118.5 mg/mL. It has also been reported that the concentration of lactoferrin significantly increases in milk from cows with subclinical and clinical mastitis (HARMON et al., 1975; GAUNT et al., 1980; HAGIWARA et al., 2003; CHANETON et al., 2013). During the lactation period, lactoferrin concentrations were detected in mammary glands with chronic mastitis in amounts 7 times higher than in healthy, lactating cows. Concentrations of lactoferrin in milk samples from cows with acute mastitis can be 13 times higher than those of healthy cows (KOMINE et al., 2005).

Major mastitis pathogens, such as *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae,* have been reported to increase lactoferrin concentrations in bovine milk above the levels associated with coagulase-negative staphylococci and *Corynebacterium* spp. (HARMON et al., 1975; HAGIWARA et al., 2003; NEWMAN et al., 2009). HAGIWARA et al. (2003) observed the highest lactoferrin concentrations in milk samples infected with *Streptococcus agalactiae,* which corresponds with our findings. On the other hand, SORDILLO et al. (1987) reported a decrease in lactoferrin concentrations in quarters infected with major mastitis pathogens, which resulted in increased occurences of intramammary infections. In their research, the highest lactoferrin concentrations were measured in milk samples from uninfected quarters.

The lactoferrin concentration in cow's milk might depend on the pathogenicity of each bacterial species (HAGIWARA et al., 2003; KAWAI et al., 1999). KAWAI et al. (1999) assumed that significant differences in lactoferrin concentrations among normal, sublinical and clinical mastitis milk samples may be associated with the severity of the intramammary inflammation. Concentrations of lactoferrin in milk samples where environmental pathogens were isolated, were lower than in negative milk samples (Table 2). On the other hand, our research indicates that samples with different bacteria and mould have high mean lactoferrin concentrations (5.8789 ± 2.3538 mg/mL).

In the present study, quarter milk samples infected with minor mastitis pathogens had higher lactoferrin concentrations than negative milk samples. HAGIWARA et al. (2003) reported the lowest lactoferrin levels in milk infected with *Corynebacterium* spp. suggesting that *Corynebacterium* spp. has low pathogenicity. However, this study showed that milk samples infected with *Corynebacterium* spp. had the highest lactoferrin concentrations (Table 2).

Conclusion

Major and minor mastitis pathogens increase lactoferrin concentrations in bovine milk above the level in uninfected quarters. Indirectly, concentrations of lactoferrin may indicate the existence of intramammary infections. In order to determine whether measuring the concentrations of lactoferrin can be used for identification of intramammary infections and the purpose of diagnostics, further research needs to be done.

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SAŽETAK

Laktoferin, glikoprotein koji veže željezo, pripadnik porodice bjelančevina transferin, prisutan je u visokoj koncentraciji u sekretu mliječne žlijezde tijekom involucije i posjeduje antimikrobnu sposobnost. Radi utvrđivanja koncentracije laktoferina u mlijeku krava s različitim bakteriološkim nalazima, prikupljen je 151 pojedinačni uzorak mlijeka na farmi holštajn-frizijske pasmine na području Autonomne pokrajine Vojvodine Republike Srbije. Za identifikaciju bakterija korištene su klasične mikrobiološke metode i ELISA za određivanje koncentracije laktoferina. Najčešće izdvojene bakterije u uzorcima mlijeka krava bile su *Corynebacterium* spp. (32,45%) s koncentracijom laktoferina 6,0497 ± 1,6774 mg/mL i koagulaza negativni stafilokoki (4,64%) s koncentracijom laktoferina 5,2961 ± 1,3633 mg/mL. Najniža srednja vrijednost koncentracije laktoferina zabilježena je u negativnim četvrtima vimena i četvrtima inficiranim bakterijama iz okoliša, dok je najviša koncentracija laktoferina bila u četvrtima vimena inficiranima vrstom *Streptococcus agalactiae*. Jedan od četiriju uzoraka mlijeka gdje je bio izdvojen *Staphylococcus aureus* imao je znatno nižu vrijednost laktoferina (1,1736 mg/mL) od ostala tri uzorka (6,2089 ± 0,5016 mg/mL), što zahtijeva daljnja istraživanja.

Ključne riječi: laktoferin, mlijeko, visokomliječne krave, razdoblje involucije, laktacija, bakterije, mastitis

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