Original scientific paper
UDC 637.12.045

IMPACT OF MAMMARY GLAND INFECTIVE STATUS ON THE CONCENTRATION OF LACTOFERRIN IN COW'S MILK

Biljana Trajkovska[1*], Ljupche Kochoski[1], Kiro Petrovski[2], Gordana Dimitrovska[1], Zehra Hajrulai-Musliu[3], Risto Uzunov[3], Vladimir Petkov[3]

[1]Faculty of Biotechnical Sciences, University "St. Kliment Ohridski", Partizanska b.b., 7000 Bitola, Macedonia
[2]School of Animal and Veterinary Science, University of Adelaide, Mudla Wirra Rd, 5371 Roseworthy SA, Australia
[3]Faculty of Veterinary Medicine, University "Ss. Cyril and Methodius", Lazar Pop Trajkov 5, 1000 Skopje, Macedonia

[*]e-mail: biljana.trajkovska@uklo.edu.mk

Abstract

Lactoferrin (LF) is glycoprotein, which is normally excreted in cow's milk, but as a result of inflammatory processes in the mammary gland, its concentration is being increased. The aim of this research was to determinate whether the LF concentration in milk is influenced by increased number of milk somatic cell count.

In this research we used milk from (n = 345) Holstein-Friesian cows in Pelagonia region, or more precisely milk from healthy (n = 112) and infected quarters (n = 233). A quarter was classified as healthy when no visible signs of mastitis were detected or the California Mastitis Test (CMT) was negative. A quarter was classified as subclinical when no visible signs of mastitis were detected but CMT was positive. A quarter was classified as clinical mastitis when there were visible signs of mastitis (cow, udder or milk appearance). The milk samples were analysed instrumentally for physico - chemical properties (Lactoscope C4+), somatic cell count (SCC) by Bentley Somacount CC 150, total number of bacteria (CFU) (by Bentley Bactocount IBC), and by ELISA for concentrations of lactoferrin. We used SPSS 15.0 for analysis of gained data.

Significant differences were detected in concentrations of lactoferrin in healthy quarters (915.94 μg/mL) and in quarters with sub-clinical (2,095.87 μg/mL) and clinical mastitis (3,528.99 μg/mL) ($p < 0.001$). A correlation between lactoferrin and lactose ($r = -0.21$, $p < 0.01$), lactoferrin and CFU ($r = 0.28$, $p < 0.01$) as well as between lactoferrin and somatic cells ($r = 0.32$, $p < 0.01$) was observed.

These results initiate that lactoferrin could be used as complementary test to somatic cell count, and potential to be a primary diagnostic test for sub-clinical and clinical mastitis in dairy cows. Furthermore, it could be used as natural antibiotic in treatment of mastitis in dairy cows, in order to decrease the presence of antibiotic residuals in milk, thereby to improve hygiene of raw milk.

Key words: *Lactoferrin, Cow's milk, Somatic cells, Mastitis.*

1. Introduction

Lactoferrin (LF) is an iron-binding glycoprotein in the mammary gland, which is mainly excreted by secretory epithelium and, to a lesser degree, by polymorphonuclear neutrophil (PMN) leukocytes. It plays a significant role in the defensive system of the mammary gland [1]. Lactoferrin contributes to the resistance of the mammary gland in several ways, mainly through its bacteriostatic effect, competing with bacteria for free iron [2, 3] or by binding to the bacterial surface [4]. The ability of LF to destroy the outer membrane of wide spectrum of Gram-negative bacteria by interaction with the lipid A portion and binding proteins to the outer membrane (porins) have been demonstrated. Lactoferrin changes the integrity and permeability of cell membrane occur and by releases lipopolysaccharides, whereof the sensibility of the cell to antimicrobials is increased [5]. Lipopolysaccharides are carriers of specific 0-antigen which is responsible for pathogenicity of certain bacteria [6].

Cow's milk contains between 200 - 485 μg/mL, depending of period of lactation and milk yield [1, 7]. On the other hand, cow colostrum contains very high concentration of LF and then gradually decreases [8]. Also, high LF concentration in milk may indicate on mastitis inflammation [9]. Mastitis is one of the most significant diseases in dairy cows which cause significant economic losses [10]. During mastitis, as a result of inflammatory processes the concentration of LF in milk can be increased up to 30 times, depending on the level of infection [7, 11, and 12], as well as on the cause of inflammation. Different pathogens cause distinctive excretion of LF from mammary epithelial cells [12]. The increase in concentrations of LF occurs during acute mastitis is dramatic [13].

On the other hand, the somatic cells in milk are significant indicator of the mammary gland health and the milk hygiene, and they represent a good diagnostic tool which enables early detection of sub-clinical or acute forms of mastitis, and they are significant components of any monitoring program. Somatic cells count - SCC is related to the cellular immunological response of the mammary gland after the inflammatory process [14]. The increase of somatic cells leads to decrease of milk yield and changes in milk's composition, which could have impact on the quality of dairy processing, e.g. increased time of coagulation with rennin, decrease of cheese moisture, delayed growth of starter cultures, decrease of the stability of the curdles and cheese yield [15, 16].

The aim of this research was to determinate whether the LF concentration in milk is influenced by increased number of milk somatic cell count, and to determinate normal milk LF concentration. LF could be used as a diagnostic test to detect the presence of mastitis on the farm.

2. Materials and Methods

2.1 Materials

2.1.1 Cows

The research was conducted on two dairy farms in Pelagonia region, Macedonia, with Holstein-Friesian (HF) cows. A Total of 345 quarter milk samples were taken by random choice. Before each sampling, the clinical status of the cows and the udder was recorded, based on general demeanor and behavior of the cow, udder condition and the appearance of the milk. Based on detected clinical findings samples were categorized into three groups: quarter foremilk samples with systemic clinical signs of mastitis ('severe clinical mastitis'), quarter foremilk samples with local signs of mastitis ('moderate clinical mastitis') and quarter foremilk samples with an altered appearance of the milk ('mild clinical mastitis'). If there were no visible changes on the udder or in the milk appearance, the next step was taking milk samples from each quarter for California mastitis test as an on farm screening test to detect the existence of sub-clinical infection ('subclinical mastitis'). Milk samples from quarters with moderate or mild clinical mastitis were categorized as clinical. The milk sample in such case was taken before administration of antimicrobial. After milking, around 10 mL of milk was collected from each quarter into plastic tubes.

2.1.2 Milk samples

Milk samples were classified on the basis of somatic cell count as: Group I (NL) - up to 200,000 cells/mL, Group II (SCC1) - between 200,001 and to 400,000 cells/mL, Group III (SCC2) - between 400,001 and 600000 cells/mL and Group IV (SCC3) - above 600,000 cells/mL. Data were also categorized into three groups, depending on the clinical status of the quarter: Group healthy (NL) when no visible signs of mastitis were detected and CMT was negative, Group subclinical (SUB) when no visible signs of mastitis were detected but CMT was positive and a Group clinical (CL) when quarter showed mild, moderate or severe mastitis. A total of 112 quarter milk samples were selected as normal and 233 samples were from infected quarters.

2.2 Methods

The determination of chemical properties of the milk (milk fat, fat-free dry matter and lactose) was performed with precise mid-infrared analyzer Lactoscope C4+ (Delta Instruments B.V, Drachten, the Netherlands), according to IDF 9622:1999 standard. Samples intended for examination of somatic cells were conserved with Broad Spectrum Microtabs II (BSM) (Advanced Instruments INC, Norwood, U.S.A.). Determination of the somatic cell count (SCC) was made by fluoro-optical electronic counter Somacount CC 150 (Bentley Instruments, Inc., Chaska, Minnesota, U.S.A.). Milk-enumeration of SCC was in accordance with MK EN ISO 13366-52:2010 standard. The total number of bacteria was examined by means of Bactocount IBC (Bentley Instruments, Inc., Chaska, Minnesota, U.S.A.), MKC EN ISO 21187:2011.

Quantitative determination of LF was made with commercial immunoenzyme test Bovine Lactoferrin ELISA kit (Biopanda Reagents, Belfast, UK). Milk samples were previously skimmed by centrifugation on 2 - 8 ^{0}C at 3,000 rpm in period of 20 minutes (MPW - 352 R). Results from the testing were obtained by measurement of optical density of the sample on wavelength $\lambda = 450$ nm using microplate reader model 680 (BioRad, Hercules, California, USA). All procedures were performed according to manufacturer's instructions. Milk samples were diluted in proportion 1/10,000 and a standard curve was generated for each set of samples.

2.2.1 Statistical analysis

The statistical software SPSS 15.0 for Windows (Chicago, SPSS Inc.) was used for statistical analysis of the obtained data. Results are presented as mean values and standard deviation of mean (mean ± SD). Significance of differences between mean values of the classified groups was determined with the t - test. A value of when $p < 0.05$ were considered significant. The correlation of the parameters was evaluated according to Pearson correlation coefficients (r). The results are considered to be statistically reliable when $p < 0.001$, $p < 0.01$, $p < 0.05$.

3. Results and Discussion

Somatic cells in milk are a significant indicator of the mammary gland health status as well as the milk hygiene, and represent a good diagnostic tool which enables early detection of a sub-clinical or acute form of mastitis [17]. They are also a significant component of any monitoring program [18]. Factors, such as late lactation, age of the cow and environmental stress can cause a slight increase in somatic cells, but such increases are usually insignificant. The only significant and prolonged increase occurs as a result of infection [19].

During this study, in order to determine the health status of the udder, SCC and the total number of bacteria were used. Data presented in Table 1 showed that the increase of SCC resulted in an increase in concentrations of LF. Therefore, the milk with the SCC (Group IV - SCC3) had the highest concentration of LF 2,812.93 µg/mL, compared to normal milk (NL) with 915.94 µg/mL ($p < 0.001$) (Table 1).

Similar criteria have been previously reported [20, 21]. According to Król *et al.*, [22] quarters which produce milk that contains more than 200,000 somatic cells/mL show signs of subclinical mastitis. Other have indicated that normal milk could only be the one with SCC less than 200,000 cells/mL and each increase above these values indicates inflammation of the udder [23]. The results of our study showed that 68% of the examined samples referred to infected quarters, while 32% referred to healthy quarters. Moreover, there was some correlation between the SCC and concentrations of LF ($r = 0.32$; $p < 0.01$). These findings confirmed the impact of SCC on the concentrations of LF. Hence, results from our study indicated that LF could be used as the complementary test to SCC but it may also be a potential diagnostic test for detection of sub-clinical mastitis in dairy cows [1].

In our research, we observed a negative correlation between LF and lactose ($r = -0.21$, $p < 0.01$), LF and proteins ($r = -0.20$, $p < 0.01$), LF and fat-free dry matter ($r = -0.20$, $p < 0.01$), LF and milk production ($r = -0.24$, $p < 0.01$), and lactoferrin and CFU ($r = 0.28$, $p < 0.01$). Similar results have obtained by other authors [1] were correlation between LF and lactose in their study is ($r = -0.183$, $p = 0.049$), LF and daily milk production ($r = -0.472$, $p < 0.001$). However, they observed a positive correlation between LF and proteins ($r = 0.482$ $p < 0.001$). Correlation between LF and SCC might have resulted due to increased SCC which in turn, decreased the synthetic activity of the milk secretory tissue. This has been previously confirmed by other authors [24, 25] wherein the increase of SCC is usually related to decreases in lactose. In our study, we have examined only the total number of bacterial colony-forming units (CFU)/mL, but not the type of bacteria. Only milk samples with more than 600,000 SCC/mL (Table 1) showed significant increase in CFU number (1,212,820 CFU/mL), while other groups had normal values (11,450 CFU/mL; 29,100 CFU/mL, and 8,200 CFU/mL, respectively for groups I, II and III). Most common bacteria that cause mastitis in dairy cows in the Macedonia are coagulase-negative staphylococci (CNS) (40%), *Streptococcus agalactiae* (25%), *Escherichia coli* (16.6%), *Proteus* spp. (11.7%) and *Staphylococcus aureus* (6.7%) [26].

The average values of concentration of LF in quarters with SUB was 2,095.87 µg/mL and CL 3,528.99 µg/mL, were significantly higher than those from normal milk (NL) with 915.94 µg/mL ($p < 0.001$; Table 2). Significant changes in concentration of LF were obtained between milk from quarters with sub-clinical and clinical mastitis ($p < 0.001$). Similar results have obtained by other authors [7, 27].

Table 1. Impact of somatic cells on concentration of lactoferrin (µg/mL) and chemical parameters in milk

Milk parameters		Categories of somatic cells			
		NL (N = 112)	SCC1 (N = 68)	SCC2 (N = 48)	SCC3 (N = 117)
Milk fat (%)	$\bar{x}$	2.10	3.35	3.36	3.42
Lactose (%)		4.85^{a}	$4.65^{b,a}$	$4.56^{c,b}$	$4.28^{d,c}$
Proteins (%)		3.22	3.10	3.03	2.84
Fat free dry matter (%)		8.82	8.47	8.30	7.78
CFU/mL (*000)		$11,45^{a}$	$29,10^{b}$	$8,20^{a,b,c}$	$1,212.82^{c}$
LF (µg/mL)		915.94^{a}	$1,905.37^{b}$	$1,915.80^{c,b}$	$2,812.93^{d}$
Daily milk production (L)		24.22	21.75	21.33	23.22

Legend: SCC - somatic cells count; NL - healthy group; * The differences in values with different superscripts in same row are statistically significant at level: a:b; a:c; a:d; b:d; b:c ($p < 0.05$).

Table 2. Concentration of lactoferrin (µg/mL) and chemical parameters of milk in samples that originate from healthy and diseased quarters

Chemical parameters of milk	NL (N = 112)		SUB (N = 180)		CL (N = 53)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Milk fat (%)	2.10	1.63	3.68	2.74	2.28	2.08
Proteins (%)	3.22	0.29	2.96	0.46	2.73	0.43
Lactose (%)	4.85	0.44	4.46	0.68	4.10	0.65
Fat free dry matter (%)	8.82	0.80	8.11	1.24	7.46	1.18
CFU/mL (*000)	11.45 [a]	12.65	284.88 [b]	599.93	3,418.00 [c]	1,773.42
LF (µg/mL)	915.94 [a]	822.09	2,095.87 [b]	1,516.46	3,528.99 [c]	1,413.98

Legend: NL - healthy group; SUB - subclinical group; CL - clinical group; *The differences in values with different superscripts in same row are statistically significant at level: a:b; a:c; b:c ($p < 0.05$).

Concentrations of LF in milk are promptly increased when the udder is infected [1, 27], i.e. during mastitis. Concentrations of LF could be increased up to 2,000 µg/mL, depending on cause and degree of inflammation [11, 12, 28]. In clinical mastitis, the average value of LF in dairy cows is between 300 and 3,200 µg/mL, and it is significantly higher, compared to normal milk [7, 11, and 29]. The analysis of variance between the groups of cows with normal milk, sub-clinical and clinical mastitis (Table 2) showed that there was a statistical difference in concentrations of LF in all three categories of milk ($p < 0.001$). Number of studies indicates that the concentration of excreted LF is different, depending on the cause of infection. The difference between concentrations of LF in milk from different quarters of cows, depending on the cause of mastitis, which could refer to the intensity of inflammation, or to point out differences regarding the response of the mammary gland, resulting in different excretion of LF from each quarter [1].

However, according to report by Musayeva *et al.*, [21], most of the analyzed samples where SCC are lower than 200,000/mL are positive, i.e. pathogenic bacteria were isolated. This implies that some specific types of bacteria could be related to weak immunological response [21], i.e. depending on the bacterial infection, the mammary gland could respond with a different degree of immunological response. Also, samples with SCC higher than 200,000 cells/mL may be negative for pathogenic bacteria, i.e. there are no isolated bacteria, and their explanation is that inflammatory process can occur without bacteria being present in the milk [20]. However, the appearance of higher concentrations of LF in infected quarters is characteristic, compared to healthy ones ($p < 0.001$). Musayeva *et al.*, [21], have obtained the same results, wherein statistical significance at the level of $p < 0.05$ is detected. The same tendency is noticed in of the report by [20], but statistical significance between the quarters was not noticed.

4. Conclusions

- Results from our study indicate that most of examined samples originate from inflamed quarters (68%), which confirm the high prevalence of mastitis on the examined farms. The increase of SCC resulted in an increase of the concentrations of LF, which was significantly higher compared to normal milk ($p < 0.001$).
- These results indicate that lactoferrin could be used as a complementary test to SCC, but potentially can be used as a sole diagnostic test for detection of clinical and sub-clinical mastitis in dairy cows. This was supported by the positive correlation of LF with SCC ($p < 0.01$). Highest concentrations of LF were obtained in cows with clinical followed by sub-clinical mastitis. As the increased concentration of LF was related to mastitis only confirms the physiological role of lactoferrin in the unspecific defense of the mammary gland against pathogen insult.

5. References

[1] Cheng J., Wang J., Bu D., Liu G., Zhang C., Wei H., Zhou L., Wang J. (2008). *Factors affecting the lactoferrin concentration in bovine milk*. Journal of dairy science, 91, (3), pp. 970-976.
[2] Clare D., Swaisgood H. (2000). *Bioactive milk peptides: A prospectus*. Journal of dairy science, 83, (6), pp. 1187-1195.
[3] Ward P. P., Uribe-Luna S., Conneely O. M. (2002). *Lactoferrin and host defense*. Biochemistry and Cell Biology, 80, (1), pp. 95-102.
[4] Diarra M., Petitclerc D., Lacasse P. (2002). *Effect of lactoferrin in combination with penicillin on the morphology and the physiology of Staphylococcus aureus isolated from bovine mastitis*. Journal of dairy science, 85, (5), pp. 1141-1149.
[5] Ellison III R. T., Luo Q., Reller L. B. (1990). *Enhancement of the activity of cefotaxime by iron-binding proteins*. Journal of Antimicrobial Chemotherapy, 25, (3), pp. 479-481.
[6] Dimitrovski A. (2000). *Microbiology with Food microbiology* (2nd Ed.). Faculty of Technology and

Metallurgy, University "Ss. Cyril and Methodius" Skopje, Macedonia.
[7] Hagiwara S., Kawai K., Anri A., Nagahata H. (2003). *Lactoferrin concentrations in milk from normal and subclinical mastitic cows*. Journal of Veterinary Medical Science, 65, (3), pp. 319-323.
[8] Shimazaki K., Kawai K.(2017). *Advances in lactoferrin research concerning bovine mastitis*. Biochemistry and Cell Biology, 95, (1), pp. 69-75.
[9] Kutila T., Suojala L., Lehtolainen T., Saloniemi H., Kaartinen L., Tähti M., Seppälä K., Pyörälä S. (2004). *The efficacy of bovine lactoferrin in the treatment of cows with experimentally induced Escherichia coli mastitis*. Journal of veterinary pharmacology and therapeutics, 27, (4), pp. 197-202.
[10] Petrovski K., Buneski G., Trajcev M. (2006). *A review of the factors affecting the costs of bovine mastitis*. Journal of the South African Veterinary Association, 77, (2), pp. 52-60.
[11] Kawai K., Hagiwara S., Anri A., Nagahata H. (1999). *Lactoferrin concentration in milk of bovine clinical mastitis*. Veterinary Research Communications, 23, (7), pp. 391-398.
[12] Chaneton L., Tirante L., Maito J., Chaves J., Bussmann L. (2008). *Relationship between milk lactoferrin and etiological agent in the mastitic bovine mammary gland*. Journal of dairy science, 91, (5), pp. 1865-1873.
[13] Nuijens J. H., van Berkel P. H., Schanbacher F. L.(1996). *Structure and biological actions of lactoferrin*. Journal of mammary gland biology and neoplasia, 1, (3), pp. 285-295.
[14] Vangroenweghe F., Dosogne H., Burvenich C. (2002). *Composition and milk cell characteristics in quarter milk fractions of dairy cows with low cell count*. The Veterinary Journal, 164, (3), pp. 254-260.
[15] Lindmark-Månsson H., Svensson U., Paulsson M., Aldén G., Frank B., Johnsson G. (2000). *Influence of milk components, somatic cells and supplemental zinc on milk processability*. International Dairy Journal, 10, (7), pp. 423-433.
[16] Trajkovska B., Hristova V,, Kalevska T. (2011). *Influence of somatic cells in raw milk on yild of dairy products*. Conference of agronomy students with international participation Proceedings, Cacak, Serbia, 7, (7), pp. 142-148.
[17] Green M., Green L., Schukken Y., Bradley A., Peeler E., Barkema H., De Haas Y., Collis V., Medley G. (2004). *Somatic cell count distributions during lactation predict clinical mastitis*. Journal of dairy science, 87, (5), pp. 1256-1264.
[18] Schukken Y. H., Wilson D. J., Welcome F., Garrison-Tikofsky L., Gonzalez R. N. (2003). *Monitoring udder health and milk quality using somatic cell counts*. Veterinary research, 34, (5), pp. 579-596.
[19] Harmon R. (1994). *Physiology of mastitis and factors affecting somatic cell counts*. Journal of dairy science, 77, (7), pp. 2103-2112.
[20] Chaneton L., Bonta M., Pol M., Tirante L., Bussmann L. (2013). *Milk lactoferrin in heifers: Influence of health status and stage of lactation*. Journal of dairy science, 96, (8), pp. 4977-4982.
[21] Musayeva K., Sederevičius A., Želvytė R., Monkevičienė I., Beliavska-Aleksiejūnė D., Kerzienė S. (2016). *Concentration of lactoferrin and immunoglobulin G in cows' milk in relation to health status of the udder, lactation and season*. Polish journal of veterinary sciences, 19, 4, pp. 737-744.
[22] Król J., Brodziak A., Litwinczuk Z., Barlowska J. (2012). *Selected factors determining the content of lactoferrin, lysozyme and immunoglobulins G in bovine milk*. Polish Journal of Veterinary Sciences, 13, 2, pp. 357-361.
[23] Hillerton J. (1999). *Redefining mastitis based on somatic cell count*. International Dairy Federation Bulletin, 345, pp. 4-6.
[24] Sobczuk-Szul M., Wielgosz-Groth Z., Wronski M., Rzemieniewski A. (2013). *Changes in the bioactive protein concentrations in the bovine colostrum of Jersey and Polish Holstein-Friesian cows*. Turkish Journal of Veterinary and Animal Sciences, 37, (1), pp. 43-49.
[25] Korhonen H. J., Kaartinen L. (1995). *Changes in the composition of milk induced by mastitis*. The Bovine Udder and Mastitis. In: Honkanen-Buzalski T., Kaartinen L., Sandholm M., Pyörälä S. (Eds.), University of Helsinki, Faculty of Veterinary Medicine, pp. 76-82.
[26] Branko A., Ljupco M., Ljupco A., Martin N., Marija R., Dean J., Kiro P., Toni D. (2012). *Implementation of strategies for mastitis control in dairy herds in Macedonia: A case report*. Macedonian Veterinary Review, 35, (2), pp. 87-90.
[27] Harmon R., Schanbacher F., Ferguson L., Smith K. (1976). *Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and alpha-lactalbumin during acute experimental and natural coliform mastitis in cows*. Infection and Immunity, 13, (2), pp. 533-542.
[28] Hyvönen P. (2010). *Lactoferrin in bovine intramammary infection*. PhD thesis, University of Helsinki, Faculty of Veterinary Medicine, Helsinki, Finland.
[29] Asfour H., Yassin M., Gomaa A. (2010). *Antibacterial Activity of Bovine Milk Lactoferrin against Some Mastitis Causative Pathogens with Special Regards to Mycoplasmas*. International Journal of Microbiological Research, 1, (3), pp. 97-105.