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Lactoferrin and Immunoglobulin G Concentration in Bovine Milk from Cows with Subclinical Mastitis during the Late Lactation Period*

Annamaria Galfi[1], Miodrag Radinović[1], Dubravka Milanov[2], Sara Savić[2], Stanko Boboš[1] & Marija Pajić[1]

ABSTRACT

***Background*:** Lactoferrin and immunoglobulin G in milk have an important role in udder resistance to infection in the involution period. Both proteins express antimicrobial activity- lactoferrin by the binding and sequestration of iron ion; and immunoglobulin G by complement activation, bacterial opsonization and agglutination. Many factors affect lactoferrin and immunoglobulin G concentrations in bovine milk, such as the stage of lactation, milk production, and intramammary infections. The aim of this study was to determine concentrations of lactoferrin and immunoglobulin G in milk from healthy cows and subclinical mastitic cows during the late lactation period, and to evaluate the relationship between them.
***Materials, Methods & Results*:** A total of 150 quarter milk samples from 41 cows (Holstein-Friesian breed) in late lactation period were reviewed in this study. Milk samples were collected during morning milking, using aseptic techniques in sterile test tubes. From each sample, 0.1 mL of milk was plated on Columbia blood agar base with 5% defibrinated ovine blood, MacConkey agar and Sabouraud dextrose agar and incubated for 24 h - 48 h (bacteria) and 5 days (yeasts, mould) at 37oC. Milk samples for detection lactoferrin and immunoglobulin G concentration were skimmed at 1,400 *g* for 45 min and stored at -20°C until analysis. Lactoferrin concentration in bovine milk was determined using the Bovine Lactoferrin ELISA Quantitation Set. Milk samples were diluted at a ratio of 1:10,000. Plates were read at 450 nm absorbence values. Immunoglobulin G concentration was determined by the immunodiffusion method using radial immunodiffusion (RID) plates. Milk samples were diluted in a ratio of 1:30. Reading of results was done after incubation for 48 h by measuring the diameter of the precipitation ring. The highest mean lactoferrin concentration was observed in udder quarters infected with contagious pathogens (*Streptococcus agalactiae* and *Staphylococcus aureus*), while the highest mean immunoglobulin G concentration was detected in milk samples where minor mastitis pathogens (coagulase-negative staphylococci and *Corynebacterium* spp.) were isolated. Milk samples where *Staphylococcus aureus* was isolated had the lowest immunoglobulin G concentration, and the lowest lactoferrin concentration was observed in samples infected with enviromental pathogens (*Streptococcus dysgalactiae*).
***Discussion*:** This study showed that lactoferrin and immunoglobulin G concentrations are higher in milk samples from subclinical mastitic cows than in milk from normal lactating cows. Lactoferrin concentrations in milk samples from udder quarters infected with major mastitis pathogens were significantly higher than in milk infected with minor mastitis pathogens. The lowest concentration of immunoglobulin G was detected in milk samples where *Staphylococcus aureus* was isolated, while the highest immunoglobulin G concentration was observed in milk samples from quarters infected with minor mastitis pathogens. Lactoferrin and immunoglobulin G concentrations were significantly and positively correlated in all milk samples. This means that cows with high lactoferrin concentrations have high immunoglobulin G concentrations. In quarter milk samples infected with *Staphylococcus aureus*, lactoferrin and immunoglobulin G concentrations were negatively correlated. The cause of these findings could be the suppression of local immune response of mammary gland.

Keywords: lactoferrin, immunoglobulin G, milk, dairy cows.

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[1]Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Novi Sad, Serbia. [2]Scientific Veterinary Institute "Novi Sad", Novi Sad. CORRESPONDENCE: M. Radinović [radinovic@polj.uns.ac.rs - Tel: +381(21)4853464]. Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovica 8. 21000 Novi Sad, Serbia.

INTRODUCTION

The risk of new intramammary infection is the highest during the early involution period (first two - three weeks) and before partus [3,6,17,20]. Secretions of the mammary gland change with the increase in concentration of natural protective factors which play an important role in the immunology of the mammary gland. Bovine lactoferrin and immunoglobulin G are major proteins in whey from involuted udders [2,19,21].

Lactoferrin is an iron-binding glycoprotein that belongs to the transferrin family present in milk and other external secretions synthesized by neutrophilic polymorphonuclear leukocytes and glandular epithelial cells [14,15]. This protein exhibits its antimicrobial activity by sequestrating and binding free enviromental iron ions, which are necessary for the growth of certain bacteria, such as *Escherichia coli*. Beside bacteriostasis, lactoferrin has bactericidal activity [4]. Lactoferrin concentrations in bovine milk increases during the dry period and intramammary infections.

Beside lactoferrin, milk contains other antimicrobial proteins such as immunoglobulins. Immunoglobulin G is the predominant immunoglobulin in bovine milk and has following activities: complement activation, bacterial opsonization and agglutination. Concentrations of immunoglobulin G are low in healthy cow milk during the middle lactation period, but it increases during the late lactation period and udder inflamation [11,12].

The aim of this study was to determine milk lactoferrin and immunoglobulin G concentrations in healthy cows and subclinical mastitic cows during the late lactation period, and to evaluate the relationship between them.

MATERIALS AND METHODS

Milk samples

The experiment was conducted on a dairy farm of the Holstein-Friesian breed in the Autonomous Province of Vojvodina, Republic of Serbia. The farm is characterized by a closed housing system. General conditions and udder status were evaluated by clinical examinations of the animals. Udders were examined visually and by palpating for the presence of any udder changes (redness, swelling, hardness, pain, heat) in a total of 50 animals. Also, milk samples from each of the quarters were examined for the presence of flakes and clots. Animals with visible signs of udder inflammation were not included in the study. A total of 150 quarter milk samples from 41 cows in the late lactation period were conducted in this study.

Milk samples were collected during morning milking, using aseptic techniques in sterile test tubes. Before sampling, cleaning and disinfection of the udder teats was done using 70% alcohol. The samples were labeled with the cow's ID number and the teat from which sample was collected, and submitted to the laboratory for microbiological examination at refrigerator temperature. Milk samples for lactoferrin and immunoglobulin G concentration detection were skimmed at 1,400 x *g* for 45 min. Then, milk serum was drained into test tubes with each cow's ID number noted, and stored at -20°C until analysis.

Microbiological examination

From each sample, 0.1 mL of milk was plated on Columbia blood agar base with 5% defibrinated ovine blood, MacConkey agar and Sabouraud dextrose agar[1]. Plates were incubated for 24 h - 48 h (bacteria) and 5 days (yeasts, mould) at 37°C under aerobic conditions, and microbial growth was menitored daily. The isolates were identified by their cultural characteristics (shape, size and structure), microscopic appearance in Gram stained preparations, catalase reaction, coagulase test with rabbit plasma, and CAMP test.

Lactoferrin quantification

Lactoferrin concentration in bovine milk was determined using the Bovine Lactoferrin ELISA Quantitation Set[2] 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 in 50 mM Tris, 0.14 NaCl, 0.05% Tween 20, pH 8.0 [16]. Standards were designed through serial dilution using the bovine lactoferrin calibrator. Goat anti-bovine lactoferrin horseradish peroxidase (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[3]. Obtained concentration of bovine lactoferrin was multiplied by the dilution factor to determine the amount of lactoferrin in samples.

Determination of immunoglobulin G concentration

Immunoglobulin G concentration was determined by the immunodiffusion method using radial immunodiffusion (RID) plates[4] according to the manufacturer's instruction. Milk samples were diluted with a physiological solution in a ratio of 1:30, and 5 μL of the milk samples were poured in wells of RID plate. Reading of the results was done after incubation for 48 h at room temperature by measuring the diameter of the precipitation ring with the RID meter. The following formula calculates the value obtained in the concentration of immunoglobulin G. The formula is: $C = (R^2 - b) / a) \times 30$, where "R" is the radius of the precipitation ring, "b" is a constant whose value is 8.69, "a" is a constant with a value of 47.48, and 30 is the dilution ratio.

Data analysis

The criteria for selection of normal milk samples were the absence of bacteria and no clinical sign of udder inflammation. The criteria for selection of milk samples from subclinical mastitic cows were the presence of bacterial growth and no sign of clinical mastitis in cows. Isolated microorganisms were categorized based on the bacteriological findings: major mastitis pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*) and minor mastitis pathogens (coagulase-negative staphylococci, *Corynebacterium* spp.).

Statistical data - mean values, standard deviation, minimum and maximum values, and correlation test were calculated using SPSS, version 20.0, software package for Microsoft Windows[5].

RESULTS

The study included 150 quarter milk samples from cows in the late lactation period for determining lactoferrin and immunoglobulin G concentrations. No bacteria growth was detected in 50.67% (76/150) quarter milk samples, while in the other 49.33% (74/150) samples bacteria and mould were found. Lactoferrin concentrations in milk from normal lactating cows and subclinical mastitic cows ranged from 0.73 to 8.85 mg/mL (n = 76) and 2.26 to 9.84 mg/mL (n = 74), respectively, while immunoglobulin G concentration in normal lactating cows and subclinical mastitic cows ranged from 4.78 to 162.38 g/L (n = 76) and 4.62 to 152.24 g/L (n = 74), respectively (Table 1).

Table 1. The lowest and the highest lactoferrin and immunoglobulin G concentration in milk from normal lactating cows and subclinical mastitic cows.

Findings	Lactoferrin (mg/mL)		Immunoglobulin G (g/L)	
	Minimum	Maximum	Minimum	Maximum
Normal milk	0.73	8.85	4.78	162.38
Subclinical mastitis	2.26	9.84	4.62	152.24

Lactoferrin concentrations in milk from normal lactating cows and subclinical mastitic cows are shown in Table 2 and Table 3. The mean concentration of lactoferrin in quarter milk samples from normal lactating cows was 5.12 ± 1.77 mg/mL, while in milk from subclinical mastitic cows mean lactoferrin concentration was higher and amounted to 5.94 ± 1.65 mg/mL.

The highest mean lactoferrin concentration was observed in udder quarters infected with contagious mastitis pathogens (*Streptococcus agalactiae* and *Staphylococcus aureus*), while the lowest concentration was in quarters infected with environmental pathogens *(Streptococcus dysgalactiae).*

The mean concentration of immunoglobulin G in quarter milk samples from normal lactating cows was 24.64 ± 23.56 g/L, while in milk from subclinical mastitic cows mean lactoferrin concentration was higher and amounted to 29.35 ± 24.38 g/L (Tables 2 & 3).

The highest mean concentration of immunoglobulin G was detected in samples where minor mastitis pathogens (coagulase-negative staphylococci and *Corynebacterium* spp.) were isolated (Table 3). In quarter milk samples where *Staphylococcus aureus* was isolated had the lowest concentration of immunoglobulin G (12.21 ± 8.08 g/L).

Table 2. Lactoferrin and immunoglobulin G concentration in quarter milk from normal lactating cows.

Milk Samples	No	Lactoferrin (mg/mL) mean value ± sd	Immunoglobulin G (g/L) mean value ± sd
Total	76	5.12 ± 1.77	24.64 ± 23.56

Table 3. Lactoferrin and immunoglobulin G concentration in quarter milk from subclinical mastitic cows.

	Milk Samples	No	Lactoferrin (mg/mL) mean value ± sd	Immunoglobulin G (g/L) mean value ± sd
	Total	74	5.94 ± 1.65	29.35 ± 24.38
Bacterial species	*Staphylococcus aureus*	3	6.21 ± 0.50	12.21 ± 8.08
	Streptococcus agalactiae	3	6.48 ± 0.51	22.51 ± 15.18
	Streptococcus dysgalactiae	2	4.71 ± 0.38	28.81 ± 15.88
	Enterococcus faecium	1	5.88	23.19
	Corynebacterium spp.	49	6.05 ± 1.68	31.15 ± 27.30
	coagulase-negative staphyloccoci	7	5.30 ± 1.36	34.11 ± 21.42
	Others*	9	5.88 ± 2.35	24.68 ± 17.51

*Different types of bacteria and mould.

The concentration of lactoferrin and immunoglobulin G in all milk samples (n = 150) were positively correlated (r = 0.34), but in samples where *Staphylococcus aureus* was isolated (*n* = 3) correlation test was negative (r = -0.99).

DISCUSSION

During the first three weeks of the involution period, the risk of occurance of new intramammary infection is the highest [3,6,17,20]. Lactoferrin and immunoglobulins have an important role in udder protection. Many factors affect lactoferrin and immunoglobulin G concentrations in bovine milk, such as the stage of lactation, milk production, and intramammary infections [8].

Lactoferrin concentration is relatively low in milk from clinically healthy lactating cows and it may vary between 0.02 and 0.35 mg/mL. Concentration of lactoferrin in milk increases as lactation progresses, and the maximum lactoferrin concentration can been observed after 3 to 4 week of involution when it amounted to 20 - 30 mg/mL [18,22]. Additionally, higher concentrations of lactoferrin can been detected in milk from subclinical and clinical mastitic cows.

This study showed that lactoferrin concentrations are higher in milk from subclinical mastitic cows than in milk from normal lactating cows (Table 1). The lowest concentration of lactoferrin was detected in milk samples from normal lactating cows (0.73 mg/mL), while the minimal lactoferrin concentration in milk from subclinical mastitic cows was significantly higher (2.26 mg/mL). Some authors indicated that the pathogenicity of bacteria might affect lactoferrin concentrations in milk [7,9]. Lactoferrin concentrations in milk samples from quarters infected with *Staphylococcus aureus* and *Streptococcus agalactiae* were significantly higher than in milk infected with minor mastitis pathogens (Table3). These results correspond to the findings of other authors [7,8,16].

Concentrations of immunoglobulin G in milk changes during lactation. In the early and the middle stage of lactation, immunoglobulin G content is significantly lower than in the late lactation period [1,12,13]. Other authors have also reported that immunoglobulin concentrations increase during mammary gland inflammation [11]. Immunoglobulin G concentrations were higher in milk from subclinical mastitic cows than in milk from normal lactating cows. This study showed that immunoglobulin G concentrations were the highest in milk samples from quarters infected with minor mastitis pathogens, while in milk samples where *Staphylococcus aureus* was isolated had the lowest immunoglobulin G concentrations (Table 3). These results correspond with the findings of Kociņa *et al*. [10].

Lactoferrin and immunoglobulin G concentrations were positively correlated in all milk samples, which correspond to the findings of other authors [5]. This means that cows with high lactoferrin concentrations have high immunoglobulin G concentrations. However, in quarter milk samples infected with *Staphylococcus aureus*, lactoferrin and immunoglobulin G concentrations were negatively correlated. The cause of these findings can be the suppression of a local mammary gland immune response.

CONCLUSION

Lactoferrin and immunoglobulin G concentrations were higher in milk samples from subclinical mastitic cows than in samples from normal lactating cows, which indicates that intramammary infection increases their concentration. This may suggest that the determination of lactoferrin and immunoglobulin G concentrations in milk can be useful in detecting the presence of intramammary infections.

Lactoferrin and immunoglobulin G concentrations in all milk samples were positively correlated, but the concentration of immunoglobulin G was the lowest in samples with *Staphylococcus aureus*, while lactoferrin concentration was high. Further studies are required to elucidate the relationship between lactoferrin and immunoglobulin G concentrations and the occurrence of intramammary infection.

MANUFACTURERS

[1]Oxoid Limited. Basingstoke, Hampshire, UK.

[2]Bethyl Laboratories. Montgomery, TX, USA.

[3]Thermo Electron Corporation. Vantaa, Finland.

[4]INEP, Institute for the Application of Nuclear Energy. Belgrade, Republic of Serbia.

[5]IBM Corporation. Armonk, NY, USA.

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