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Can intramammary infections change immunoglobulins and acute phase proteins of colostrum and transition milk in dairy goats?

[*A infecção intramamária pode alterar imunoglobulinas e proteínas de fase aguda do colostro e do leite de transição de cabras leiteiras?*]

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ABSTRACT

Proteiongram analysis is useful for the early diagnosis of intramammary infections during the period of colostrogenesis. This study aimed to evaluate the profile of total proteins, immunoglobulins, lactoferrin, and gamma-glutamyl transferase (GGT) in the colostrum of dairy goats with intramammary infections. Animals were divided in groups: GI (n=12) of goats without mammary gland infections, and GII (n=8) of goats with mammary gland infections. Intramammary infections were diagnosed using microbiological isolations and somatic cell counts (SCCs). Total protein was evaluated in the samples using SDS-PAGE shortly after parturition, and 24 and 48 hours after that event. Non-aureus *Staphylococcus* (NAS) were detected in all isolates. At 48 h, GII had high IgG levels and a SCC of 1660.25 × 10³/mL. Levels of total protein were high in this group at 24 and 48 h. Albumin levels were high in goats with mastitis at 24 h. Overall, the IgG, lactoferrin, and albumin levels differed between animals with and without intramammary infections at M0. GGT activity was not influenced by the intramammary infection. The results of this study reinforce the importance of the proteinogram as an auxiliary tool in the diagnosis of mastitis in dairy goats.

Keywords: bacteria, colostrum, immunoglobulins, proteins, transition milk

RESUMO

O proteinograma do colostro/leite pode ser útil para diagnóstico precoce de infecções intramamárias, assim como para a avaliação da intensidade da resposta inflamatória. Este estudo teve como objetivo avaliar o perfil de proteínas, imunoglobulinas, lactoferrina e gamaglutamiltransferase no colostro de cabras leiteiras portadoras de infecções intramamárias. Os animais foram distribuídos em dois grupos, GI (n = 12), composto por cabras sem isolamento microbiológico das glândulas mamárias, e GII (n = 8), composto por cabras com resultados positivos na cultura do leite de pelo menos uma das glândulas mamárias. O diagnóstico de infecção intramamária foi realizado logo após o parto, por meio de isolamento microbiológico e de contagem de células somáticas (CCS). Os níveis de proteína total, imunoglobulina A (IgA), imunoglobulina (G) (IgG), lactoferrina, albumina e atividade da gamaglutamiltransferase (GGT) foram avaliados em amostras de colostro/leite de transição, usando-se SDS-PAGE logo após o parto bem como às 24 e às 48 horas após esse evento. Staphylococcus *não aureus (NAS) foram encontrados em todos os isolamentos. A concentração de IgG foi superior no GII apenas às 48h, ao mesmo tempo em que o CCS foi de 1660,25 x 10³/mL. No entanto, a proteína total foi maior neste grupo às 24 e às 48h. A albumina foi maior nas cabras com mastite às 24h, e a lactoferrina no momento 0h (M0) e às 48h. Em geral, os valores de IgG, lactoferrina e albumina diferiram entre os animais com e sem infecções intramamárias no M0. A atividade da gamaglutamiltransferase não foi influenciada pela infecção intramamária. Os resultados deste estudo reforçam a importância do proteinograma como uma ferramenta auxiliar no diagnóstico da mastite em cabras leiteiras.*

Palavras-chave: bactérias, colostro, imunoglobulinas, proteínas, leite de transição

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INTRODUCTION

The risk of intramammary infections in goats might be higher during the dry period, similar to what happens in dairy cows (Leitner *et al*., 2007). New infections during this period may occur at two time points. At the beginning of the dry period, when the mammary gland begins to shift from a lactating to a non-lactating state (involution), and subsequent changes occur that prevent the risk of new infections. Alternatively, new infections may occur at the end on the dry period, which happens at the same moment with "colostrogenesis," a phase during which the gland undergoes alterations to enable the transition from a non-lactating to lactating state, and during which the protective barriers of the gland disappear (Zobel *et al*., 2015).

The presence of intramammary infections in goats with less than ten days of lactation may represent a pre-existing persistent intramammary infection. The elimination of such bacteria may be less efficient in goats that have been infected during previous lactation or during the dry period because such pathogens are well established in the mammary glands. In addition, the immune system of a goat may be less effective in clearing infections at the beginning of lactation (between calving and the tenth day of lactation) (Gosselin *et al*., 2019).

The immune system uses phagocytosis as a mechanism to eliminate antigen-antibody complexes at the site of infection (Leitner *et al*., 2000). Previous studies have shown that immunoglobulin G1 (IgG1), IgG2, and IgM can act as bacterial opsonin that improve phagocytosis via neutrophils and macrophages. These antibodies can bind the bacteria either directly or with the help of the C3B component of the complement (Howard *et al*., 1980). The inflammatory response against the mammary gland infections during the "colostrogenesis" period may cause alterations in the vascular permeability, extravasation of inflammatory proteins, and diapedesis of polymorphonuclear leukocytes (Leite *et al*., 2017).

The concentrations of IgG, albumin, and lactoferrin were elevated in cows with asymptomatic mastitis at the first hour after calving (Santos *et al*., 2018). *Staphylococcus aureus* infections during the periparturient period can reduce the synthesis and secretion of milk by the epithelial cells of the mammary glands in cows (Sordillo and Nickerson, 1989). Non-aureus *Staphylococcus* (NAS) are the most prevalent pathogens that cause subclinical mammary infections in small ruminants (Gosselin *et al*., 2019). Despite being less pathogenic than *S. aureus*, NAS may cause persistent subclinical mastitis, a significant increase in the somatic cell count (SCC), and clinical mastitis (Supré *et al*., 2011; Deinhofer and Pernthaner, 1995; Contreras *et al*., 1997; Ariznabarreta *et al*., 2002), in addition to producing thermostable enterotoxins (Udo *et al*., 1999).

Although it has been reported that mastitis can persist during the dry period, scientific literature on the alterations of colostrum components during bacterial infections remains scarce. The main role of the colostrum is to provide passive immunity to newborn ruminants against pathogens as it is the main source of IgG in the first month of life (Macías *et al*., 2014; Kessler *et al*., 2019). In this context, the health and survival of goat kids is intimately linked to the ingestion of colostrum (O'Brien and Sherman, 1993).

The colostrum is not only a source of IgGs but also of enzymes, such as gamma- glutamyl transferase (GGT). Although GGT is not associated with the defense of the mammary gland, it can be used to diagnose a failure in the transfer of passive immunity in goats because it is associated with IgG (Yalcin *et al*., 2010).

In cows, the profile of milk components, such as proteins, lipids, carbohydrates are consolidated even during mastitis (Negri Filho *et al*., 2016). However, in goats with higher numbers of NAS infections, the profile of defense milk components, such as IgGs and enzymes remains unclear. Overall, intramammary infections can affect the constitution of colostrum and transition milk. This study aimed to evaluate the profile of total protein (using a proteinogram), IgGs, lactoferrin, and GGT in the colostrum of dairy goats with intramammary infections.

MATERIAL AND METHODS

A total of 20 female *Saanen* and Alpine goats, in the puerperal phase, were obtained from a farm in São José do Rio Preto City, São Paulo State,

Brazil and used in this study. All experimental procedures were approved by the Ethics Committee for the Use of Animals (CEUA) of the São Paulo State University, Araçatuba Campus (approval number 2013-01450).

The animals in this study were allocated to two groups (Fig. 1): GI, composed of 12 goats without any infection in both mammary glands and GII, consisted of eight goats with infections in at least one of the two mammary glands. Animals were maintained in intensive production and fed Tifton grass hay, corn silage, and commercial ration. In addition, they had free access to water and mineral salts throughout the day.

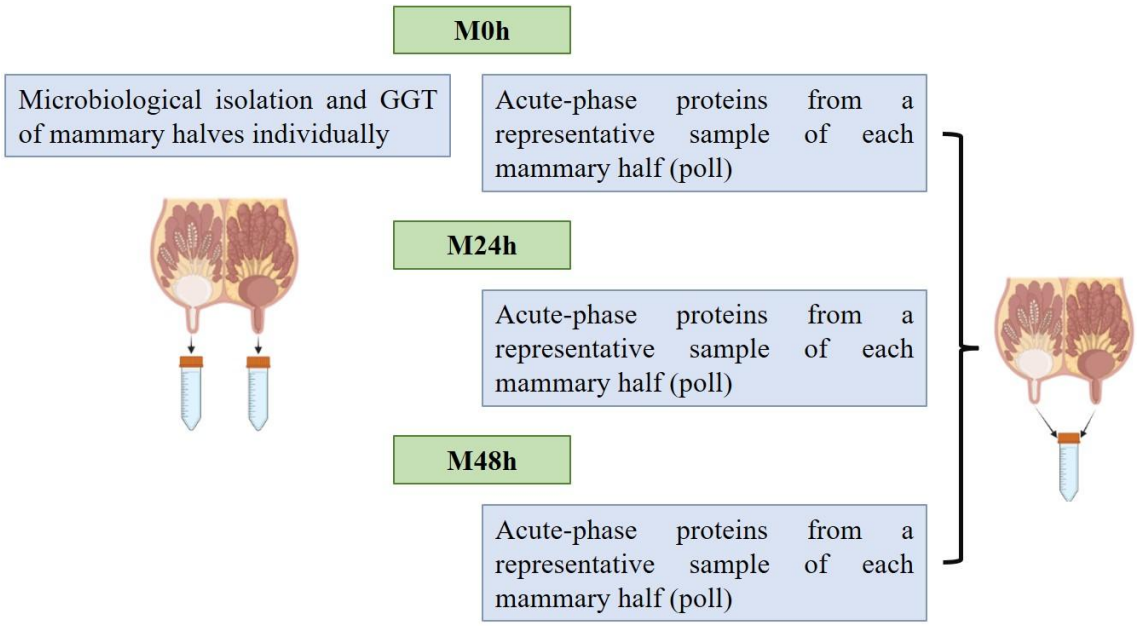

Figure 1. Experimental design of the groups and sample collection. A portion of this image was Created with BioRender.com.

The milking was interrupted (drying off) when the animals had completed approximately 305 days of lactation, which marked the beginning of the 60-day dry period. Drying off was performed abruptly using intramammary antibiotics. All births were attended, and goat kids were separated from their mothers at birth.

Samples of colostrum/transition milk from the mammary halves of puerperal goats were obtained the moment 0h (M0) after birth and at 24 and 48 h. Microbiological analysis and determination of GGT activity were performed from samples taken individually from each mammary half, after parturition. In addition, a representative sample of each mammary half (pool) was obtained shortly after delivery as well. The time point for obtaining the sample was at 24 and 48 hours after this event. This was performed for the determination of acute-phase proteins and somatic cell count (SCC). For obtaining the samples, the first three streams of the colostrum/transition milk were discarded in a strip cup to evaluate the macroscopic characteristics. Next, teat antisepsis was performed using cotton soaked in 70% alcohol. Thereafter, 10mL samples were collected in sterile falcon tubes at an inclination of 90° to prevent contamination.

For the microbiological analyses, samples were gently homogenized, streaked on defibrinated horse blood and MacConkey agars, and incubated under aerobic and microaerophilic conditions at 37ºC in a bacteriological incubator for at least 72 h.

The macroscopic characteristics of colonies were recorded after 24, 48, and 72 h of incubation. Samples that gave rise to at least three identical

colonies in the same medium were considered positive (National Mastitis Council, 2017). Next, colonies from the samples were subjected to Gram staining to investigate the micromorphological characteristics of the microorganisms. Biochemical tests were performed to identify the isolates (Quinn *et al*., 1994).

Samples of fresh milk, without preservatives, were used for SCC. SCC was performed immediately after sample collection using a portable automatic counter (DeLaval cell counter DCC, DeLaval®, Sweden).

Samples of colostrum/transition milk were distributed in 1.5 mL microtubes, and renin solution (Chr. Hansen Brasil Ind. E Com. LTDA, São Paulo, Brazil) was added to 10% of the total volume of colostrum in the tube. The tubes were thereafter maintained in a water bath at 37 °C for 20 min until coagulation. Centrifugation was performed at 143 4,200 × *g* for 20 min at 15°C. Subsequently, fat was removed and the triphasic intermediate solution was obtained and stored in 1.5 mL microtubes at -20°C until further use. Milk serum obtained via this process was used to evaluate the levels of total protein, IgG, IgA, albumin, lactoferrin, and GGT.

The IgG, IgA, and acute phase proteins in colostrum and transition milk were quantified using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS- PAGE). Fractions were measured using Image Quant TL GE Healthcare (Buckinghamshire, UK). A marker solution (Sigma, MO, USA) with different molecular weights of wide and strict spectra was used as reference. Four protein fractions were evaluated: IgG, IgA, lactoferrin, and albumin.

Biochemical analyses were performed using a semi-automatic biochemical analyzer (Celm, São Paulo, Brazil). The total protein in milk serum was determined by the biuret method using commercial reagents (Labtest Diagnóstica, Minas Gerais, Brazil) at an adequate wavelength for the test. The activity of gamma-glutamyl transferase (GGT) was determined using a commercial kit for GGT (Labtest Diagnóstica, Minas Gerais, Brazil) by using the kinetic photometric method as recommended by the International Federation of Clinical Chemistry (IFCC) (Szasz, 1969).

Variables were tested for normality and homoscedasticity using the Kolmogorov–Smirnov and Bartlett tests (when necessary), respectively. Variables for the IgA and GGT test were analyzed using the Friedman test with multiple comparisons. The Dunn test was used to verify the effect of time within the same group. The Mann–Whitney test was used to verify the differences between the groups. The variables for IgG, total protein, albumin, and lactoferrin were analyzed by analysis of variance (ANOVA) with repeated measures and multiple comparisons using the Tukey's test to verify the effect of time within the same group. An unpaired t-test was used to verify the differences between the groups. Correlations were assessed using the Pearson or Spearman tests. All analyses were performed using GraphPad Prism Software Inc. v.6.0, San Diego, CA. $p<0.05$ was considered statistically significant.

RESULTS

Non-aureus *Staphylococcus* (NAS) were present in all microbiological cultures from GII. Among the eight animals from which NAS was isolated, only one animal had infections in both udders. Macroscopic alterations in the milk, as indicated by the lumps in the strip cup test, were observed only in one animal from GII.

In both groups, there was no difference in the SCC over time. The SCC was higher in GII than that in GI (Table 1) at 48 h. The IgG concentrations (Table 2) in milk serum did not differ significantly between groups for samples collected immediately after birth and at 24 h. However, in case of samples collected after 48 h, the concentration of IgG was higher in GII (Tab. 2) than that in GI. In both groups, IgG levels decreased during the study, with minimum values being attained at 48 h.

Table 1. Median and 25th and 75th percentiles (in parentheses) of somatic cell count (SCC x 10^3/mL) in colostrum and milk samples from goats without microbiological isolation (GI, n=12) and goats with microbiological isolation (GII, n= 8), at delivery (0h), at 24 and 48 hours postpartum

Group	SCC ($\overline{X}$ ± S)		
	0h	24h	48h
I	149.5 (39-1033)Aa	587.5 (260.8-1179)Aa	304(154.5-1556)Aa
II	599.5 (460.5-813)Aa	891.5(608-2351)Aa	1287 (802-2637)Ba

Mean values with distinct letters, uppercase in columns and lowercase in rows, presented significant difference ($p<0.05$).

Table 2. Mean ($\overline{X}$) and standard deviation (S) of total protein (g/dL), immunoglobulin A, lactoferrin, albumin, immunoglobulin G levels and GGT in samples from goats without microbiological isolation (GI, n=12) and goats with microbiological isolation (GII, n=8) at birth, 24 and 48h post-partum

Protein fraction	Moment (h)	Groups	
		GI	GII
Total protein (g/dL)	0	9.44±4.24Aa	11.43±3.93Aa
	24	3.71±1.93Ba	5.72±2.30Bb
	48	2.51±0.72Ca	4.46±1.67Cb
Lactoferrin (mg/dL)	0	947.07±439.31Aa	1762.21±741.79Ab
	24	316.83±174.26Ba	502.93±289.97Ba
	48	235.23±80.81Ba	357.95±140.06Bb
Albumin (mg/dL)	0	1774.50±896.58Aa	3345.94±1749.22Ab
	24	534.19±287.15Ba	897.93±501.84Ba
	48	419.89±145.86Ba	619.91±300.16Ca
IgG (mg/dL)	0	4847.52±1950.77Aa	6329.94±1513.98Aa
	24	2612.44±1250.11Ba	3607.03±1920.81Ba
	48	1.623,89±722.71Ca	2901.08±1313.13Bb

Mean values with distinct letters, uppercase in columns and lowercase in rows, presented significant difference ($p<0.05$).

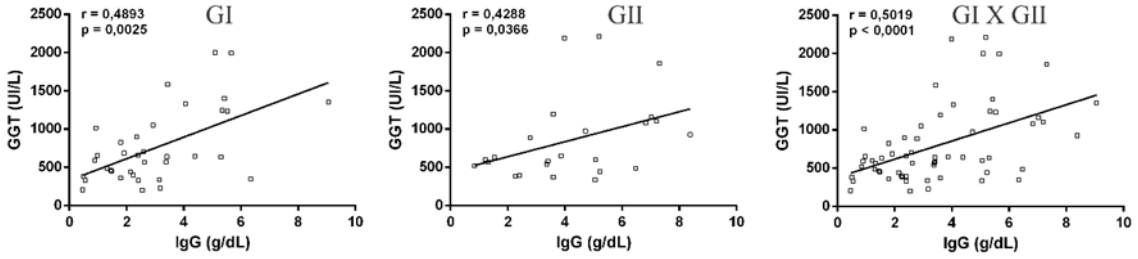

Figure 2. Correlation between the total of immunoglobulin G (IgG) and GGT levels in samples from goats without microbiological isolation (GI, n=12) and goats with microbiological isolation (GII, n=8) at birth.

The concentration of lactoferrin was higher at birth in milk serum from both groups than at 24 and 48 h. The levels of lactoferrin were higher in GII than in GI at birth and 48 h post-partum. Albumin concentration was high in females with intramammary infection at 0 h. A comparison between the different time points for the same group demonstrated that goats without

intramammary infections had higher concentrations of albumin at birth than at 24 and 48 h post-partum. Similarly, the albumin concentrations in goats with intramammary infections were also higher at birth than that at 24 and 48 h post-partum. The IgA concentration in milk serum differed between the groups after birth. Goats without intramammary infections had higher levels of IgA at 0 h than at 48 h. However, in goats with intramammary infection, the IgA levels were higher at 0 h than at 24 h.

In case of GGT activity, significant differences were observed between samples collected at all time points from animals without intramammary infections. Moreover, the GGT levels were higher at 0 h than at other time points (Tab. 2). The animals from GII had higher GGT activity at 0 h than at 24 h. However, the GGT activity did not differ statistically between samples collected at 24 and 48 h. Nonetheless, there was no significant difference between the GI and GII. The mammary halves from goats with intramammary infection were analyzed individually for GGT at birth. No significant difference (p=0.1061) was observed between glands that were positive for NAS and those that were negative.

Table 3. Correlation coefficients between total protein (TP) and immunoglobulin G (IgG) of the samples at birth, 24 and 48h post-partum

Correlations		GI			GII		
		0h	24h	48h	0hr	24h	48h
TP x IgG	p-value	0.0014	<0.0001	0.0001	0.4599	0.0003	<0.0001
	r Pearson	0.8087	0.9254	0.8825	0.3068	0.9507	0.9766

Significant when p<0.05.

Table 4. Median and 25th and 75th percentiles (in parentheses) of immunoglobulin A and GGT in samples from goats without microbiological isolation (GI, n=12) and goats with microbiological isolation (GII, n=8) at birth, 24 and 48h post-partum

Protein fraction	Moment (h)	Groups	
		GI	GII
IgA (mg/dL)	0	76.92(76.92-76.92) Aa	55.81 (55.81-55.81) Ab
	24	27.56 (15.40-44.60) ABa	20.75 (13.73-20.75)Ba
	48	21.45 (11.60-25.14) Ba	32.95 (20.49-33.23)ABa
GGT	0	1288.00 (1025.00-1539.00)Aa	1133(999.80-2104.00) Aa
	24	582.80 (364.90-690.40) Ba	560.00 (415.60-645.00) Ba
	48	423.50(339.50-612.50)Ca	553.5 (399.90-595.0) Ba

As shown in Fig. 2, a positive correlation was observed between the IgG and GGT levels in both groups, which indicated that the two variables (IgG and GGT) were in indirect proportion. The total protein in goats without infection was low at 24 and 48 h post-partum. In addition, the levels of total protein from both groups were high at birth (moment 0) and low at 24 and 48 h. In animals without intramammary infections, a correlation between the total protein and IgG levels was observed for all samples collected at 24 and 48 h post-partum (Table 4).

DISCUSSION

The presence of microorganisms in milk secretions soon after birth is indicative of an infection at the end of lactation or during the dry period. One study showed that 67.70% of intramammary infections caused by NAS persisted in the mammary gland for a period of 61 days (Gosselin *et al*., 2019), indicating that an infection acquired late in lactation and not cured may persist throughout the dry period, which is usually two months.

The environment might play an important role during this period by serving as a source of pathogens that cause intramammary infections. In contrast, the intramammary infections that occur during established lactation have been associated with a *Staphylococcus* genus that was not isolated from an environmental source (Jácome *et al*., 2014). An intramammary infection with macroscopic changes in colostrum was detected in only one animal (1/8), and it demonstrates the low incidence of clinical mastitis in goats immediately post-partum.

Intramammary infections cause an increase in the SCC of milk during lactation and lead to the infiltration of blood leukocytes, which substantially alter the proportion and distribution of leukocytes in milk (Leitner *et al*., 2012). In this study, an increase in SCC was observed in samples collected at 48 h. This can be explained by the fact that the response to an infectious agent is not uniform. Further, factors such as the bacterial species involved and duration of infection also play an important role in defining the extent to which leukocyte levels in milk vary in response to an infectious agent (Leitner *et al*., 2012).

The high concentration of IgG and total protein in the first hour of parturition in both groups coincided with colostrum secretion. This can be explained by the fact that, at birth, ruminants lack gamma globulins in their bloodstream, and the ingestion and absorption of adequate amounts of IgGs in the colostrum is essential for establishing passive immunity (Kessler *et al*., 2019). Some biochemical modifications in milk can be attributed to the defense mechanisms that prevent bacterial infections in the mammary gland. Acute inflammation of the mammary gland hinders selective transport of IgG1, in addition to allowing serum protein extravasation in milk (Turk *et al*., 2021). In cows, it was reported that clinical or subclinical mastitis was responsible for the increase in IgG levels in colostrum (from birth to 48 h after birth), which indicates that the presence of microorganisms in the mammary gland alone can alter some components in the colostrum (Santos *et al*., 2018).

In that study, colostrum with an infectious agent had higher IgG content only at 48 h. That can be explained by the microorganisms that were isolated since NAS can cause inflammatory reactions in the udder, may not adequately stimulate, or even inhibit immune responses in the mammary glands of goats (Ferrer *et al*., 1997; Pengov, 2001; Ezzat Alnakip *et al*., 2014). NAS infections likely induce a milder and delayed inflammatory response, since SCC, as well as IgG concentrations, were higher only at 48 hours.

In cattle, an intramammary infection with *S. simulans* caused a greater increase in the concentration of cytokines and inflammatory indicators in milk than a *S. epidermidis* infection (Simojok *et al*., 2011). An additional factor to be considered is the ongoing inflammatory process. During the recuperation of acute inflammation, metabolic functions of the gland epithelium are compromised, and the synthesis of milk components and selective transfer of IgG to milk decrease (Darton and McDowell, 1980).

A previous study reported that animals with intramammary infections had higher lactoferrin levels (Galfi *et al*., 2016a). During mastitis, the neutrophils that accumulate in milk release lactoferrin in the local microenvironment (Chen *et al*., 2004). Consistent with a previous study, we showed that the milk from goats with mastitis had higher levels of lactoferrin than that from goats without any infection (Galfi *et al*., 2016b).

This increase in lactoferrin levels has been associated with its ability to limit the availability of iron to microbes, one of its crucial amicrobial properties (Kell *et al*., 2020).

In fact, lactoferrin can neutralize bacteria in addition to performing its other immune functions (Kell *et al*., 2020). However, lactoferrin can also support bacterial activities as microorganisms can remove the ferric iron from this protein (Rosa *et al*., 2017; Kell *et al*., 2020). Unlike CCS, lactoferrin was higher in GII right after delivery, proving to be a useful tool in the early diagnosis of mastitis in goats. Lactoferrin has been used for the diagnosis of subclinical mastitis in goats and is more effective in differentiating between infected and uninfected mammary halves when compared to CCS (Barth *et al*., 2010).

The concentration of albumin in milk increases during episodes of clinical and subclinical

mastitis in cows, goats, and sheep (Bannerman *et al*., 2003; Leitner *et al*., 2004a, 2004b). For a long time, it was assumed that this process occurred due to the flow of this protein through the side junctions (tight junction) (Bannerman *et al*., 2003), which form the innate immune system of the organ. However, evidence suggests that albumin is expressed and synthetized by the mammary gland. Mammary gland cells can synthesize and secrete albumin from the precursors of amino acids (Shamay *et al*., 2005).

The presence of an infectious agent in the mammary gland is responsible for antigenic stimulation and migration of immunoglobulins from the blood to the milk (Mehraa *et al*., 2006). IgA does not fix the complement or opsonizes bacteria but agglutinates antigens, neutralizes viruses and bacterial toxins, and affects the adhesion of enteropathogenic bacteria to epithelial cells in the mucosa (Marnila and Korhonen, 2002). The main function of IgA is to passively protect the gastrointestinal tract by binding to pathogens and preventing them from binding to the mucosal epithelial cells (El-Loly, 2019). However, in this study the group with mastitis did not show higher levels compared to the one composed of healthy animals. This can be explained by the significant elevation of IgG causing proportionally lower levels of IgA. In a similar study carried out with cows, colostrum IgA was not interfered with due to the presence of an infectious agent causing clinical or subclinical mastitis (Santos *et al*., 2018).

The GGT level in milk serum can be a good indicator of IgG levels in goat colostrum and may be used as a marker for evaluating colostrum quality in this species (Batmaz *et al*., 2019). We concluded that the presence of intramammary infections does not influence GGT concentrations because in our study, the GGT values did not differ significantly between the two groups. This suggests that GGT levels may serve as indicators for evaluating the quality of colostrum, despite being dependent on infectious agents.

CONCLUSION

The immunological profile of colostrum/ transitional milk changes in response to intramammary infection by non-aureus *Staphylococcus* in goats, however, the magnitude of the inflammatory response seems to be dependent on the etiologic agent. Colostrum/milk GGT activity is not influenced by intramammary infection.

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