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*Original article*

# Concentration of lactoferrin and immunoglobulin G in cows' milk in relation to health status of the udder, lactation and season

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## Abstract

The aim of this study was to analyze an effect of udder health status, somatic cell count (SCC), stage and number of lactations, and different seasons on the concentration of lactoferrin (LF) and immunoglobulin G (IgG) in quarter milk samples (n=120) from crossbreed (Lithuanian Black-and-White & Holstein) dairy cows. Quarter health status was based on SCC and microbiological analysis. The highest mean value of LF and IgG were observed in quarters with subclinical mastitis  $0.1 \pm 0.02$  mg/ml and  $0.41 \pm 0.06$  mg/ml, respectively. Grouping the data according to SCC revealed increased LF ( $0.07 \pm 0.01$  mg/ml as against  $0.06 \pm 0.01$  mg/ml) and IgG values ( $0.27 \pm 0.05$  mg/ml as against  $0.23 \pm 0.02$  mg/ml) in DQ (SCC from 201,000  $\geq$  401,000 cells/ml) compared to HQ (SCC up to 200,000 cells/ml). The milk LF and IgG levels were effected by stage of lactation ( $p < 0.01$  and  $p < 0.05$ , respectively) and season of the year ( $p < 0.001$  and  $p < 0.001$ , respectively). Nevertheless, SCC and subsequent lactation ( $p > 0.05$ ) had no effect on these immunity components.

**Key words:** udder, milk, lactoferrin, immunoglobulin G, mastitis

## Introduction

Among the ailments that affect dairy ruminants, mastitis plays a prominent part. The prevention and treatment of mastitis represent a serious burden to producers and are primary concerns of the dairy industry. Innate immunity is a target of choice for selec-

tion against infectious diseases (Rainard and Riollet 2006). Immune factors in colostrum and milk play an important role in the host defense of the mammary gland itself, protecting it from pathogenic organisms (Sordillo et al. 1997, Oviedo-Boyso et al. 2007). The mammary host defense system has evolved into an extremely well-developed, complex, and highly effec-

tive barrier against pathogens, integrating both the innate and acquired immune response. If the activity of the immunological system is reduced or the virulence of pathogenic microorganisms is high, infection, partly eliminated, may continue for a longer period, and this process becomes apparent as subclinical or chronic mastitis (Ali-Vehmas and Sandholm 1995). Investigations indicate that those humoral immunity components as IgG and antibacterial factor of the udder LF play an important role in the immunological defence response of the cow udder (Korhonen and Kaartinen 1995, Marnila and Korhonen 2002, Kocina et al. 2012).

LF is an iron-binding protein, and because iron is required by some bacteria to grow, LF can reduce the growth of certain bacteria (Lotti et al. 2013). In cattle milk, bovine LF is present in polymorphonuclear lymphocyte secondary granules and is synthesized by cells of the glandular epithelium of the mammary gland (Molenaar et al. 1996). It has been shown that LF concentration increases during mammary involution (Hurley and Rejman 1993) and infection (Harmon et al. 1976, Kawai et al. 1999, Komine et al. 2005). A number of studies described factors that affect the relative LF concentration in milk. Harmon (1994) reported that the LF concentration of milk was significantly associated with SCC, levels of bovine serum albumin, stage of lactation, and milk production. Tsuji et al. (1990) reported that the LF content of multiparous cows was 2 to 3 times higher than that of primiparous cows. After the third lactation, no differences in LF content were observed. Hagiwara et al. (2003) reported that the concentration of milk LF was significantly related to the age of cows but not to the stage of lactation.

Immunoglobulins form specific immunity components and they are translocated by receptors through the blood-udder barrier (Kehrli and Harp 2001, Pyörälä 2002). Antibodies in milk are a reflection of the specific antibodies in the serum of cows. Immunoglobulin concentration increases during colostrumogenesis due to an increase of receptors and accumulation of IgG in the udder. The increase in immunoglobulins during mastitis is mainly caused by a leaky blood-udder barrier. Immunoglobulins are able to prevent the adhesion of microbes, inhibit bacterial metabolism, agglutinate bacteria, augment phagocytosis of bacteria, kill bacteria through activation of complement-mediated bacteriolytic reactions, and neutralize toxins and viruses (Pyörälä 2002, Korhonen 2009).

Gaining more insight in the self-defence mechanism of the udder may help to develop alternative protocols to enhance udder protection and to better identify cows at risk.

In order to understand and evaluate the role of LF and IgG in maintaining udder health, the dynamics of their amount in cow's milk in relation to pathogenic bacteria presence in the udder, SCC, stage and number of lactations during spring, summer and autumn seasons were investigated.

## Materials and Methods

### Selection of animals and collection of milk samples

The dairy herd tested consisted of 30 Lithuanian Black-and-White crossbreed with Holstein dairy cows. In total, 120 milk samples were collected individually from normal lactating dairy cows. Milk samples from individual quarters were collected once during the spring, summer and autumn (10 cows/40 quarter samples/each season). In order to test and estimate the udder health status, in all cows included in the investigation, we evaluated the udder and teats visually and palpated both before milking. All cows were clinically healthy with no signs of udder infection (by checking the presence of redness, swelling, hardness, and pain in the udder, or the presence of clots in milk) at sampling time. Evaluation of milk samples was carried out according to the method described by Sandholm and Pyörälä (1995).

Milk samples were collected for laboratory examination aseptically in accordance with the method recommended by the standard ISO 707:2008. Milk was obtained from udder quarters of the 1st, 2nd and 3rd lactations at early, middle and late stages of lactation. Criteria that included both SCC and a microbial analysis were used to assess the health status of the quarters.

### Microbiological examination of milk samples

Microbiological examination for identification of pathogenic microorganisms was carried out as soon as the milk samples were delivered to the accredited central milk testing laboratory. Standard procedures for identifying pathogenic microorganisms in milk were performed in compliance with the laboratory criteria ISO/IEC 17025:2005 and according to standard operating procedures SOP 5.4.4.B.6:2009. A microbiologically positive quarter was defined as isolation of 1 or 2 bacterial species from a quarter milk sample. A sample was considered contaminated when three or more dissimilar colony types (mixed cultures) were observed with no predomination of a single colony type (Hogan et al. 1999).

### Estimation of SCC

For determination of SCC, milk samples were preserved with bronopol (2-bromo-2-nitropropane-1,3-diol and 2-bromo-2-nitropropanol) in microtubs and analysed with the flow cytometric analysis method using a SomaScope cell counter (Foss, 3400 Hillerzd, Denmark) according to standard EN ISO 13366-1:2008/Cor.1:2009.

### Estimation of LF and IgG concentration

The enzyme-linked immunosorbent assay (ELISA) used to determine LF and IgG concentration in bovine biological fluids is distributed as Biopanda Reagents (United Kingdom). Determinations were carried out in whey obtained by centrifugation of 50 ml of fresh milk for 20 minutes at a temperature of 4°C and a speed of 3,000 rpm (Sobczuk-Szul et al. 2014). The resultant whey was stored frozen at a temperature of -20°C until analysed. The concentration of LF and IgG in quarter milk samples was assayed using ready kits, following the procedure recommended by the manufacturer. For LF analyses the milk samples were diluted at the ratio of 1:1,000 and for IgG 1:2,000. In both analyses, a standard curve was plotted for each plate separately. The test results were estimated by measuring the optical density of samples at wave length  $\lambda=450$  nm using a Thermo Scientific Multiskan EX spectrophotometer (Thermo electron corporation, China, 2005).

### Grouping of the numerical material for statistical analysis

The numerical material was divided into groups according to the health status of the udder quarters, milk SCC, lactation and season of the year.

The health status of the udder quarters was assessed according to microbiological analysis and SCC in milk (Chaneton et al. 2013). Quarters with presence of bacterial growth, nonspecific mastitis, or subclinical mastitis were classified as diseased quarters (Table 1).

On the basis of the milk SCC the data were allocated into four groups: group I – up to 100,000 cells/ml (n=76); group II – from 101,000 to 200,000 cells/ml (n=15); group III – from 201,000 to 400,000 cells/ml (n=9) and group IV – over 401,000 cells/ml (n=20). Udder quarters from group I and group II corresponded to the healthy quarters (n=91) and quarters from group III and group IV to the diseased quarters (n=29).

To test the reliance of milk LF and IgG on subsequent lactation and its stage, the data were classified in three age classes: 1<sup>st</sup> (n=32), 2<sup>nd</sup> (n=44) and 3<sup>rd</sup> (n=44) lactation, as well as three lactation stages: early stage (until 120 day, n=48), middle stage (from 121 up to 200 day, n=36) and late stage (from 201 up to 305 day, n=36).

The data were divided into spring (n=40), summer (n=40) and autumn (n=40) for the analysis of seasonal effect on LF and IgG in milk.

### Statistical analysis

The data obtained were statistically processed by using the SPSS program 20.0 for Windows using a one-way ANOVA procedure. The results are presented as mean and standard error of mean (mean  $\pm$  SEM). The significance of differences between mean values of the evaluated groups was determined with the *post-hoc* Fisher *LSD* criterion. A value of  $p<0.05$  was considered significant.

### Results

The distribution of bacterial findings in 120 quarter milk samples, the proportions of bacterial isolates and the corresponding SCC, LF, IgG are shown in Table 1. Samples with mixed cultures (n=10) were excluded from these figures as these quarters cannot be categorised either as healthy or mastitic. No growth of bacteria was determined in 38.66% of quarter milk samples. Pathogenic staphylococci (two samples were positive for *Staphylococcus aureus*) and CNS were the most common bacterias of the microbiologically positive quarters isolated (35.2 and 20.17%, respectively). Gram-negative bacteria were isolated from 4.2%, and streptococci from 1.68% of the samples. LF and IgG concentrations were higher in quarters where bacteria were isolated compared with quarters where no growth of bacteria was determined.

Under the udder health assessment, 35% of diseased quarters (DQ) corresponded to the category „presence of bacterial growth (BG)”, 13% to subclinical mastitis (SM) and 11% corresponded to nonspecific mastitis (NM) (Table 2). The DQ showed increased concentrations of LF and IgG ( $0.08 \pm 0.01$  mg/ml and  $0.32 \pm 0.03$  mg/ml, respectively) compared with healthy quarters (HQ) ( $0.03 \pm 0.01$  mg/ml and  $0.12 \pm 0.02$  mg/ml,  $p<0.001$ , respectively) in Fig. 1. The highest mean value of LF ( $0.1 \pm 0.02$  mg/ml) and IgG ( $0.41 \pm 0.06$  mg/ml) were observed in quarters with SM. Higher LF and IgG concentrations ( $0.08 \pm 0.01$  and  $0.35 \pm 0.03$  mg/ml, respectively) were

Table 1. Bacterial findings.

Bacterial isolate	No of samples n=119	% of isolates n=73	LF mg/ml	IgG mg/ml	SCC (10 <sup>3</sup> /ml)
Pathogenic staphylococci	42	57.53	0.08 ± 0.01	0.34 ± 0.04	273
CNS	24	32.88	0.05 ± 0.01	0.22 ± 0.03	166
<i>Streptococcus spp.</i>	2	2.74	0.08 ± 0.01	0.52 ± 0.03	113
Gram-negative bacteria	5	6.85	0.07 ± 0.01	0.56 ± 0.04	232
No growth	46	–	0.03 ± 0.01	0.09 ± 0.02	100

CNS: Coagulase Negative Staphylococci. The figure in the first column is the number of all quarter milk samples. The second column represents percentages of bacterial isolates.

Table 2. Grouping of udder quarters according to microbiological analysis and SCC in milk.

Group	Specification	Pathogen isolation (+/-)	SCC (10 <sup>3</sup> /ml)	Health status of the quarters	Number of samples
BG	Presence of bacterial growth	+	< 200	diseased	42
NM	Nonspecific mastitis	–	≥ 200	diseased	13
SM	Subclinical mastitis	+	> 200	diseased	16
HQ	Healthy quarters	–	< 200	healthy	49

(+/-) presence /absence of pathogenic microorganisms in milk sample

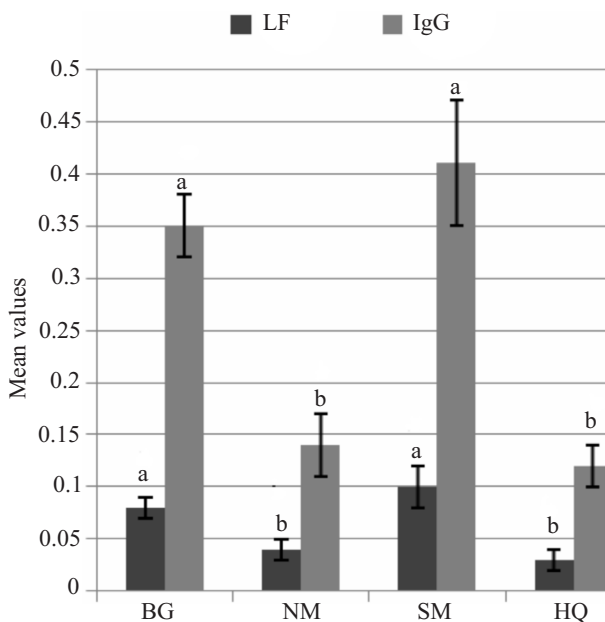


Fig. 1. Lactoferrin and immunoglobulin G concentrations mg/ml (mean ± SEM) in healthy and diseased quarters according to microbiological analysis and SCC groups. BG-positive for presence of bacterial growth, NM-nonspecific mastitis, SM-subclinical mastitis, HQ-healthy quarters. a,b Different letters indicate a significant difference at p<0.05.

detected in quarters with BG compared to HQ (0.03 ± 0.01 and 0.12 ± 0.02 mg/ml, respectively) and quarters with NM (0.04 ± 0.01 and 0.14 ± 0.03 mg/ml, respectively).

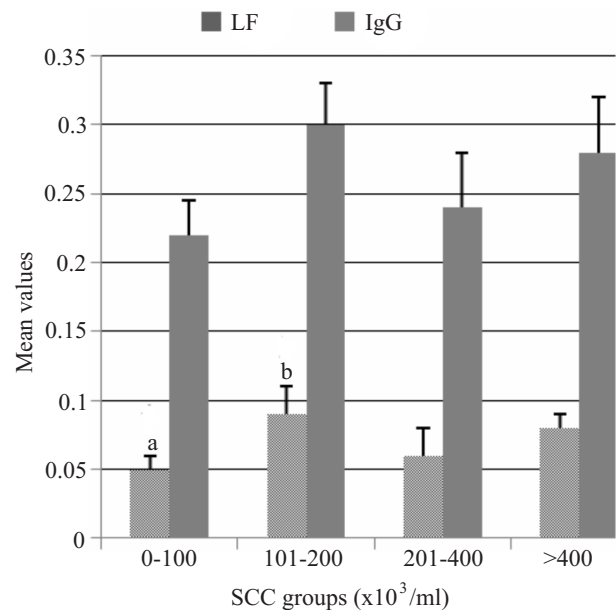


Fig. 2. Lactoferrin and immunoglobulin G concentrations mg/ml (mean ± SEM) in healthy and diseased quarters according to SCC groups. a,b Different letters indicate a significant difference at p<0.05.

Exploration of the SCC effect on LF and IgG revealed increased LF (0.07 ± 0.01 mg/ml as against 0.06 ± 0.01 mg/ml) and IgG values (0.27 ± 0.05 mg/ml as against 0.23 ± 0.02 mg/ml) in DQ (SCC from 201,000 ≥ 401,000 cells/ml) compared to HQ (SCC up to 200,000 cells/ml) (Fig. 2). The highest mean value of LF 0.09 ± 0.02 mg/ml and IgG 0.3 ± 0.03 mg/ml was

Table 3. Effect of subsequent lactation on lactoferrin and immunoglobulin G content (mg/ml) in cows' milk.

Specification	Subsequent lactation			Effect
	1 <sup>th</sup> n=32	2 <sup>nd</sup> n=44	3 <sup>rd</sup> n=44	
LF	0.08 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	2.6% p=0.220
IgG	0.26 ± 0.04	0.30 ± 0.04 <sup>a</sup>	0.18 ± 0.02 <sup>b</sup>	4.7% p=0.060

<sup>a, b</sup> – values (mean ± SEM) in the same row with different superscripts differ significantly (p<0.05).

Table 4. Effect of lactation stage on lactoferrin and immunoglobulin G content (mg/ml) in cows' milk.

Specification	Stage of lactation			Effect
	Early n=48	Middle n=36	Late n=36	
LF	0.08 ± 0.01 <sup>a</sup>	0.05 ± 0.01 <sup>b</sup>	0.04 ± 0.01 <sup>b</sup>	8.5% p=0.005
IgG	0.30 ± 0.03 <sup>a</sup>	0.25 ± 0.04	0.16 ± 0.03 <sup>b</sup>	6.1% p=0.025

<sup>a, b</sup> – values (mean ± SEM) in the same row with different superscripts differ significantly (p<0.05).

Table 5. Effect of season on lactoferrin and immunoglobulin G content (mg/ml) in cows' milk.

Specification	Season			Effect
	Spring n=40	Summer n=40	Autumn n=40	
LF	0.12 ± 0.01 <sup>a</sup>	0.03 ± 0.01 <sup>b</sup>	0.03 ± 0.01 <sup>b</sup>	46.5% p=0.000
IgG	0.54 ± 0.03 <sup>a</sup>	0.08 ± 0.01 <sup>b</sup>	0.11 ± 0.01 <sup>b</sup>	79.6% p=0.000

<sup>a, b</sup> – values (mean ± SEM) in the same row with different superscripts differ significantly (p<0.05).

observed in HQ group II (SCC from 101,000 to 200,000 cells/ml) and significant differences between HQ groups (I and II) of LF mean values were found (p<0.05). The lowest mean value of LF 0.05 ± 0.01 mg/ml and IgG 0.22 ± 0.03 mg/ml was observed in HQ group I (SCC up to 200,000 cells/ml). No significant effects of SCC on milk LF and IgG concentrations were found (p>0.05).

The richest source of LF appeared to be milk obtained from primiparous cows, whereas the highest amount of IgG was at the 2<sup>nd</sup> lactation. At subsequent lactations LF concentrations decreased and lactation numbers had no effect on LF content in milk. The lowest level of IgG was found at the 3<sup>rd</sup> lactation and in the 2<sup>nd</sup> and 3<sup>rd</sup> lactations significant differences in IgG content were determined (p<0.05) (Table 3).

Milk collected during the first months of lactation (≤120 days) had the highest LF and IgG content. Significantly lower LF content was determined in the middle and late stages of lactation (p<0.05). Milk obtained at the late stage of lactation also showed the lowest level of IgG. At the late stage of lactation IgG content was significantly lower compared to IgG mean values at the early stage of lactation (p<0.05). A significant effect of stage of lactation on LF

(p<0.01) and IgG (p<0.05) content was noted (Table 4).

A further seasonal effect on LF and IgG content in milk was evaluated. LF and IgG peak concentrations were clearly observed in the spring season. LF concentration did not change in summer and autumn seasons and was 60% lower than during the spring. In summer, IgG level was the lowest compared to other seasons. A significant effect of season on LF (p<0.001) and IgG (p<0.001) contents was noted (Table 5).

## Discussion

The LF and IgG concentrations in the milk of dairy cows might depend on the pathogenicity of each bacterial species. In the present study the lowest LF and IgG concentrations were found in milk infected with CNS, suggesting that CNS has a low pathogenicity. The present research data are in agreement with the results of Hagiwara et al. (2003) where LF concentration in quarters of cows infected with *S.aureus* and with other streptococci species were higher in comparison to CNS (*coagulase negative staphylococcus*). Investigations by Chaneton et al. (2008) and

Kocina et al. (2012) showed that LF and IgG concentrations are induced by different bacteria, with *Streptococcus uberis* causing the highest increase. Kocina et al. (2012) indicated that in the case of *S.aureus* infection the IgG mean value was lower even than in healthy quarter milk.

The effect of different factors, such as milk SCC, lactation stage, cow's age and season of the year, on milk LF and IgG were evaluated.

SCC is a commonly recognized indicator of bovine udder health, milk quality and its technological usability (Krol et al. 2012). As reported by Hillerton (1999), Hamann (2002) and Cheng et al. (2008), milk containing less than 100,000 somatic cells per 1 ml originates from a healthy udder. The SCC in milk constitutes a good diagnostic tool which allows early detection of either the subclinical or acute form of mastitis (Green et al. 2004, Cheng et al. 2008). According to Krol et al. (2012) a bovine quarter producing milk with a SCC of over 200,000 cells/ml shows the symptoms of subclinical mastitis. In the present research, the concentration of LF and IgG in the cows' milk related to health status of udder quarters was analysed. Two combined variables were used to determine the quarter health status: the SCC value and the isolation of pathogenic bacteria. Similar criteria have been used by other authors (Pyörälä 2002, Chaneton et al. 2013). In the present study 59% corresponded to diseased quarters and 41% to healthy quarters. Chaneton et al. (2013) show that the majority of analyzed milk samples consisted of healthy quarters (86.8%) and of the diseased quarters 62% corresponded to nonspecific mastitis. Most of the analysed milk samples with SCC levels lower than 200,000 were positive for bacterial isolation. This observation means that some specific bacterial species could be associated with weak immune responses (Schwarz et al. 2010) and it is possible that the physiological status of the mammary gland could induce a diminished immune response to bacterial invasion. Chaneton et al. (2013) established that most of the samples with an SCC level higher than 200,000 were negative for bacterial isolation; their explanation for this is that an inflammation process can occur without detectable bacteria in milk. The present research demonstrated higher LF and IgG concentrations in diseased quarters compared to healthy quarters ( $p < 0.001$ ). Significant differences in these immunity components were observed between quarters with BG, NM, SM and HQ ( $p < 0.05$ ). The same tendency of LF was also shown by Chaneton et al. (2013), but no significant differences were observed between quarters. Kocina et al. (2012) reported that IgG mean values in milk samples with pathogenic agents and without pathogens differed slightly and insignificantly.

The data for those indicators in groups formed by SCC showed increased values of LF ( $0.07 \pm 0.01$  mg/ml as against  $0.06 \pm 0.01$  mg/ml) and IgG ( $0.27 \pm 0.05$  mg/ml as against  $0.23 \pm 0.02$  mg/ml) in DQ (SCC from 201,000  $\geq$  401,000 cells/ml) compared to HQ (SCC up to 200,000 cells/ml). Similar results were also obtained by Cheng et al. (2008) where a relationship between milk LF concentration and SCC and LF correlation with SCC were indicated ( $r = 0.375$ ,  $p < 0.001$ ). Krol et al. (2012) calculated high correlation coefficients for LF  $r = 0.65$  and for IgG  $r = 0.79$  and these results confirmed the effect of SCC on LF and IgG. The effect of SCC on the content of IgG was reported also by other authors (Liu et al. 2009). In this research the highest mean value of LF was observed in the group with SCC from 101,000 to 200,000 cells/ml and the lowest mean value of LF in the group with SCC up to 100,000 cells/ml. According to Hamann (2002) the „gold standard” for a cell count to be up to 100,000 somatic cells/ml. Counts that reached above this point provided evidence of disturbed milk secretion that would lead to changes in its chemical composition.

Age of cows, usually referred to as the subsequent lactation, is one of the main physiological factors affecting the productivity and chemical composition of cow milk (Krol et al. 2012). In the present study, the milk LF and IgG concentrations tended to be low in lactating cows of advanced age ( $p > 0.05$ ). These results agree with Hagiwara et al. (2003) where the milk LF concentration in 5-yr-old lactating cows was lower than that in 2-yr-old ( $p \leq 0.01$ ) and 3-yr-old ( $p = 0.05$ ) lactating cows. On the other hand, Krol et al. (2010, 2012) reported reverse results: primiparous cows were shown to produce significantly less LF and IgG as compared to cows at the 2<sup>nd</sup> to 4<sup>th</sup> lactations ( $p \leq 0.05$ ) and older ( $p \leq 0.01$ ). The lowest level of LF and IgG was found in the 1<sup>st</sup> lactation ( $0.89 \pm 0.17$  and  $0.45 \pm 0.16$  mg/ml, respectively) and in subsequent lactations LF and IgG compounds increased gradually (Krol et al. 2012). Tsuji et al. (1990) reported that the LF content of multiparous cows was 2 to 3 times higher than that of primiparous cows. These conflicting results might reflect differences in the sample size and criteria for selecting milk samples.

While some researchers (Cheng et al. 2008, Krol et al. 2012, Sobczuk-Szul et al. 2014) noticed the highest mean concentration of LF in cow's milk obtained from the late lactation period, the present studies showed opposite results. The mean values of LF in milk in the early and middle stages of lactation were higher than in the late stage of lactation. These findings agree with Chaneton et al. (2013), where high levels of LF in early lactation was associated with infected quarters. These results suggest that some specific

quarters are highly susceptible to infection or are chronically infected. Concentrations of IgG during the lactation period were investigated by many other researchers. The significantly higher IgG content was reported in milk obtained in late lactation (Krol et al. 2012, Musayeva et al. 2015). In the present investigation the highest IgG content was observed in the early stage of lactation and significantly differed between stages of lactation ( $p < 0.05$ ). In this research we observed a significant effect of the lactation stage on LF ( $p < 0.01$ ) and IgG ( $p < 0.05$ ) content. Cheng et al. (2008) also found high correlation coefficients between the content of LF and lactation stage ( $r = 0.557$ ).

The present study on the seasonal effect on these immunity components in cow's milk indicated significantly higher LF and IgG values in the spring season ( $p < 0.05$ ). This is in agreement with Conesa et al. (2005), who reported that IgG values from the whole population of analysed samples were found to be significantly higher also in the spring. In another study, the seasonal effect was the only significant variation factor observed, with the highest values in the spring for LF and in the winter for IgG (Konuspayeva et al. 2007). In our research data, a significant effect of season was estimated on LF ( $p < 0.001$ ) and IgG ( $p < 0.001$ ) contents. These results are in agreement with the findings of Korcina et al. (2012), who noted a significant effect of the seasonal storage of cows on the concentration of IgG in cows milk ( $p < 0.001$ ) and Brodziak et al. (2014) whose research results indicated a significant effect of the season on LF content in goats milk ( $p < 0.01$ ).

In terms of mastitis, it can be concluded that LF and IgG concentration in milk were elevated during subclinical mastitis as well as in quarters positive for bacterial growth. It should be assumed that the increased concentrations of LF and IgG in the diseased quarters indicated immune response activity. This suggests that LF and IgG content in milk might be a useful tool in mastitis detection and for evaluation of innate resistance against intramammary infections. Furthermore, the findings showed the high contribution of lactation stage and season of the year on milk LF and IgG concentrations, whereas parity showed no association.

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