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Analysis of Inflammatory and Regulatory Cytokines in the Milk of Dairy Cows with Mastitis: A Comparative Study with Healthy Animals

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ABSTRACT

Bovine mastitis remains a major problem in the global dairy cattle industry. The acute invasion of udder by pathogens induces innate immune response as the first defence mechanism in subclinical and clinical mastitis. The aim of the study was to determine inflammatory and regulatory cytokines IL-2, IL-4, TGF-β1, IL-17A, beta-defensin 3 and IL-10 and their potential changes in milk of dairy cows with subclinical and clinical mastitis, and to compare the findings with healthy animals. Milk samples from 15 holstein Friesian breed cows were used in the study. Cows were divided into three groups based on their health status (5 healthy, 5 subclinical and 5 clinical animals). All samples were tested using immunohistochemistry to evaluate IL-2, IL-4, IL-10, IL17A, TGF-β1 and β-Def 3 proteins. Expression of all proteins was detected in all milk samples. High expression of IL-2, IL-4, IL17A, TGF-β1 was detected in healthy cows' milk and in milk of cows with subclinical and clinical mastitis. However, expression of IL-10 and β-Def 3 in milk samples of healthy cows was significantly higher compared to the milk of cows with subclinical and clinical mastitis ($p < .001$). IL-10 and β-Def 3 can be considered as informative biomarkers in diagnosis of subclinical and clinical mastitis.

KEYWORDS
cytokines; inflammatory markers; mastitis; bovine milk; inflammation

Introduction

Bovine mastitis is an inflammatory disease of the mammary gland which remains a major problem in the global dairy cattle industry that is accompanied with economical loss (Bradley, 2002; Roussel et al., 2015; Verschoor et al., 2009). Economic losses due to bovine mastitis include health care costs (diagnosis, veterinary service, medications), decrease in milk production (discarded milk, future milk production loss), and increase in culling and death rates (Hogeveen et al., 2011; Melchior et al., 2006; Rollin et al., 2015; Santos et al., 2004).

Fungi, yeast, algae, Chlamydia, and viruses have been studied in causing mastitis, with bacteria remaining as the principle causative agents (Ezzat Alnakip et al., 2014). Infection

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can be caused by Gram-positive pathogens (*Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus agalactiae, coagulase-negative staphylococci*) and by Gram-negative pathogens (*Escherichia coli*), but the most common pathogen isolated from mammary glands in several dairy farms of the infected cows remains *S. aureus* (Oliveira et al., 2007; Östensson et al., 2013; Saidi et al., 2013; Verschoor et al., 2009). However, despite *S. aureus* and *S. uberis* significant role in the udder infection development, these bacterial species are a part of the normal microbiota of the mammary gland and predominantly are opportunistic environmental pathogens (Oikonomou et al., 2014; Rainard, 2017; Zadoks et al., 2005). Mastitis then develops when pathogen intrudes the mammary gland through the teat canal (Sordillo et al., 1997). The teat canal of bovine mammary gland is the first mechanism of defense against pathogens, as it provides a physical barrier and antimicrobial substances. The second line of defense is immune cell participation in bacteria elimination, like leukocytes ingesting pathogens (Nickerson, 1985). Intramammary infections caused by pathogens lead to inflammatory reactions. Antimicrobial peptides, immunoglobulins, lysozyme, lactoferrin, and oligosaccharides are some of the immunoregulatory components of milk (Derakhshani et al., 2018). Further effects of infection caused inflammation rely on the host immune responses in the early stages of the disease, including the mechanisms achieved by secreted cytokines (Alluwaimi, 2004; Murphy et al., 2019; Shah et al., 2018).

Interleukin-2 plays a key role in regulation of the adaptive immune response, as well as stimulates T cells to express other cytokines. It is associated with expansion of CD4+ and CD8+ T cells' antigen-specific clones via both proliferation and anti-apoptotic mechanisms and stimulate CD8+ cells proliferation and development to B cells (Abbas et al., 2018; Gaffen & Liu, 2004). Also, IL-2 is an important factor in modulation of T-cells differentiation promoting naive CD4+ T-cell differentiation into T helper-1 and T helper-2 cells (Abbas et al., 2018; Gaffen & Liu, 2004). Barron et al. (2010) reported that IL-2 promotes the expansion of regulatory T cells (Treg) and in the absence of IL-2, Treg numbers significantly decrease in the peripheral lymphatic organs (Barron et al., 2010). Secretion of the IL-2 has been detected in normal bovine mammary glands with enhanced level of the cytokine at the end of the lactation period (Alluwaimi, 2000; Taylor et al., 1997). Alluwaimi et al. in 2003 and in 2004 observed that IL-2 mRNA levels significantly dropped between 24- and 32-h post infection in the milk of *S. aureus* challenged bovine (Alluwaimi, 2004; Alluwaimi et al., 2003). Upon stimulation with IL-2, lymphoid cells that are isolated from bovine mammary gland with natural killer (NK)-like activity demonstrated an increased ability to kill *S. aureus* in a nonspecific manner. Importantly, IL-2 inhibits the early differentiation of bovine Th17 cells, one of the main IL-17A producers (Cunha et al., 2019). Altogether, IL-2 regulates bovine mammary gland adaptive immune system by enhancing B lymphocyte proliferation, improving cytotoxic and bactericidal effects of T lymphocytes, increasing plasma cell numbers, and activating NK cells (Ezzat Alnakip et al., 2014).

IL-4 was detected as T derivate cytokine and later studies have shown dominant expression of IL-4 by T helper 2 lymphocytes (Celik et al., 2020; Howard et al., 1982; Li-Weber et al., 1997). Although, IL-4 main source is Th2 cells, the number of other cells (not only immune) have demonstrated involvement in IL-4 expression: NK, mast cells, eosinophils, basophils, macrophages, fibroblasts, and endothelial cells (Arai et al., 1990; Bochniarz et al., 2017; Paul, 1991). When IL-4 is secreted by T cells, it also in turn provides survival, growth, proliferation, and differentiation of B cells into active plasma cells or memory cells (C.

Riollet et al., 2000). IL-4 is one of the most important B cell activation cytokines what means it is one of the major humoral immunity regulation cytokines (Bochniarz et al., 2017; Goenka & Kaplan, 2011). IL-4 is a cytokine which forms a positive feedback loop by stimulating the differentiation of Th2 cells (Ho & Miaw, 2016). Increased level of IL-4 additionally stimulates endothelial cells, inducing the expression of very late antigen-4 which is involved in migration of eosinophils, monocytes, and T cells to tissues (Li-Weber et al., 1997). IL-4 is involved in activation of macrophages in tissue through alternative activation pathway what results in formation of alternatively activated macrophages which mainly produce anti-inflammatory cytokines like IL-10 (Celik et al., 2020; Stein et al., 1992).

IL-10 is a member of a big cytokine family with mainly anti-inflammatory function (Bochniarz et al., 2017; Ouyang et al., 2011). At first, IL-10 was defined as Th2 cell produced cytokine, but later studies have reported numbers of other cells that are involved in synthesis and expression of IL-10 (Lutfalla et al., 1993). As an anti-inflammatory cytokine, IL-10 is involved in activity suppression of different immune cells including CD4+ and CD8 + T cells, B cells, antigen presenting cells, NK cells, macrophages, and pro-inflammatory cytokines (IL-6, IL-8, TNF-α) and inflammatory chemokines (macrophage inflammatory protein-1α) (Li & Flavell, 2008; Mills, 2004; Sakaguchi et al., 2010; Sato et al., 1999). Expression of IL-10 by different cells was detected in bovine milk and serum (Verschoor et.al., 2009). The increase in IL-10 concentration was noted during an intramammary infection caused by various species of pathogens including *E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa*, and *Streptococcus spp* (Bannerman, 2009). Interesting, the earlier expression of IL-10 was observed in response to gram-negative bacteria than gram-positive bacteria (Bannerman, 2009). These studies demonstrate the central role of IL-10 as a protective and regenerative mucosal cytokine.

IL-17A is a member of the big pro-inflammatory mediator family and it is important in host defense at mucosal surfaces. The main source of IL-17 was detected to be CD4+ T helpers which became known as "Th17 cells" (Amatya et al., 2017). Several types of immune cells actively produce IL-17A, including CD4+, CD8+ T lymphocytes, macrophages, and neutrophils (Rainard et al., 2013). In healthy udder, epithelial cells are the key contributors to sensing bacteria. In relevant studies, production of IL-17A is induced during clinical and subclinical mastitis by *E. coli, S. uberis* or *S. aureus* (Bruno et al., 2010; Roussel et al., 2015; Tao & Mallard, 2007; Whelehan et al., 2011). This further leads to the initiation of antigen-specific mammary inflammation via innate immunity mechanisms. IL-17A is implicated in activation of epithelial cells,' fibroblasts' and endothelial cells' receptors which lead to production of chemokines, antimicrobial peptides, matrix metalloproteinases, cytokines and mucins attracting and stimulating neutrophils, as well as favoring granulopoiesis (Amatya et al., 2017; Brembilla et al., 2018; Y. Chen et al., 2003; Lorè et al., 2016). Bovine IL-17 has been associated with the induction of IL-6 and IL-8 production as a part of intramammary immune defense system (D. C. Riollet et al., 2006).

Beta-defensin 3 is a small cationic antimicrobial peptide which shows antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, parasites, and viruses (Dhople et al., 2006; McGlasson et al., 2017; PBJr & Bentley, 1997). Unfortunately, data about beta-defensin 3 in bovine udder defense system is quite limited, but it remains that primary source of beta-defensin 3 is epithelial cells dominantly in respiratory tract and genitourinary tract (Harder et al., 2001). Furthermore, HBD-3 expression was detected in non-epithelial tissues like heart muscles, leukocytes, and skeletal muscles (Schneider et al., 2005). TNF-α, INF-γ, IL-1, LPS connection with specific receptors (TLR2, TLR5 and TLR9), stimulate synthesis of the beta-defensin mRNA through NFkB pathway (Albanesi et al., 2007; Scharf et al., 2010; Wang et al., 2017). Furthermore, beta-defensin 3 has chemoattractant activity and can mobilize dendritic cells, memory T cells and monocytes (Jin et al., 2010; Kulkarni et al., 2016). Interestingly, beta-defensin 3 increases secretion of IL-6, IL-10, IL-8 (Niyonsaba et al., 2007; Wang et al., 2017).

Lastly, transforming growth factor-beta 1 (TGF-β1) has been studied for its wide pleiotropic functions. TGFβ-1 plays a crucial role as an anti-inflammatory cytokine mainly operates at mucosal surface through regulatory T cells (Bingisser & Holt, 2001; Lehner, 2008; MacDermoit, 1996). Like elsewhere, TGF-β1 mediates extracellular matrix (ECM) synthesis and protects the ECM from degradation. TGFβ-1 stimulates production of different ECM components (collagen type I, laminin, vimentin, fibronectin, α-smooth muscle actin) by epithelial and mesenchymal cells (fibroblasts) (Q. Chen et al., 2017; Ignotz & Massague, 1986; Sugiyama et al., 2013). Overall, increased stromal development and fibrosis is associated with an overexpression of TGF-β1. Similar like IL-10, the inclusion of TGFβ-1 in the group of anti-inflammatory cytokines is associated with the number of certain properties: 1) inhibition of macrophages and with them associated activities like production of chemokines, pro-inflammatory cytokines; 2) inhibition of different pro-inflammatory factors activity – limitation of IFN-γ secretion and increase expression of IL-1 receptor antagonists; 3) suppression of other immune cells – CD4+ and CD8+ T cells, B cells, antigen presenting cells, NK cells (Ashcroft, 1999; Letterio & Roberts, 1998; Li & Flavell, 2008; Mills, 2004; Sakaguchi et al., 2010; Sato et al., 1999). TGFβ-1 is possible to detect in the milk of healthy milky cows and in the milk of infected cows. Together, *S. aureus* and *E. coli*-associated mastitis has induced the highest concentration of TGF-β1 (Safak & Risvanli, 2021).

Our research, as the previous studies (Šerstņova et al., 2022; Vitenberga-Verza et al., 2022), was performed as a part of the ICRAD project which was targeted on evaluation of different inflammatory and regulatory cytokine expression changes in milk of healthy dairy cows and in milk of dairy cows with subclinical and clinical mastitis. Previously, we have detected pronounced numbers of these factors in milk cells where expression of 2 factors was significantly different among clinical groups (Šerstņova et al., 2022; Vitenberga-Verza et al., 2022). In our study, we evaluated changes of factors expression in wider period of time to confirm significance of these factors as subclinical and clinical mastitis diagnostic markers.

Hereby, the aim of the study was to determine inflammatory and regulatory cytokines IL-2, IL-4, TGF-β1, IL-17A, beta-defensin 3 and IL-10 and their potential changes in milk of dairy cows with subclinical and clinical mastitis, and to compare the findings with healthy animals.

Materials and methods

Ethics and consent of participation

This is the final study of the 3 years long ICRAD project. The project and the study aim to perform an analysis of cow's milk to determine the significance of different inflammatory and regulatory cytokines in diagnosis of mastitis. Farm owners gave their informed consent to perform the research and have assisted us in collecting milk samples and performing our research.

According to the national regulations of the partner in charge of validation (RIC Pro-Akademia, Poland), the study was performed in accordance with the norms defined in the Law of January 15 2015 on the protection of animals used for scientific or educational purposes (Journal of Laws of 2015 pos. 266 with further amendments) (Rakoczy, 2015). In the sense of the above mentioned, the study did not involve experiments on animals. Animal studies were carried out humanely according to national and international Animal Care and Use Committee protocols. The study involved obtaining milk samples by standard milking procedure without affecting the cow's routine. The milk samples collection causes no pain, suffering, distress, or damage to the participating animals. Therefore, no Ethical Approval was necessary to conduct this study.

Animals and study design

The research was carried out on a group of 41 holstein Friesian cows in production with an average milk yield of 31.7 kg/cow/day. The animals are confined and feed with silage, corn silage, and grain by-products. A preliminary diagnosis of subclinical (somatic cells count) and clinical (clinical signs) mastitis was made at farm. Animals were evaluated for presence or absence of mastitis into three groups: healthy (control group; $n = 5$; average parity = 2.2; average DIM = 244.4; history of mastitis = 0/5), cows with subclinical mastitis (SCM group; $n = 5$; average parity = 2.6; average DIM = 113.8; history of mastitis = 4/5) and cows with clinical mastitis (CM group; $n = 5$; average parity = 2.0; average DIM = 186.2; history of mastitis = 4/5). The diagnosis was made based on 3-day observations (days 1–3) and measurements of the number of somatic cells in milk collected from each quarter of the udder from all 41 cows.

Milk sample collection

After 3 days of observation, collecting milk from each quarter of 41 cows and determining the number of somatic cells, 15 cows were selected for further tests with selected specific quarters of the udder from which milk was collected during further tests. All milk samples were collected during morning milking. Milk samples for detailed analyses were collected on days 4, 7, 10, 12 and 14 from selected 15 cows for the further somatic cell count (SCC) monitoring, bacteriological examination, and milk sediment preparation. Samples were collected into sterile containers and immediately transported to the laboratory. Before sample collection, udders were cleaned, dried, and disinfected. During the period of milk sampling, no veterinary procedures were performed on selected dairy cows, unless antibiotics were necessary; this was done after the first 3 days (diagnosis period). The foremilk was discarded before taking the sample. Samples analysis and smears preparation was conducted on the same day, immediately after collecting the milk samples.

The following analytical procedures of milk samples were performed: differential counting of somatic cells, bacteriological examination, and immunocytochemical analysis.

SCC in cattle milk and cow selection criteria

Rapid increase of SCC was observed after udders were triggered by mastitis associated antigens in a number of different studies (Rainard et al., 2018; Wellnitz et al., 2010). Changes of SCC in milk during mammary infection shows its essential role as a marker in detection of inflammatory processes of bovine udders (Rainard et al., 2018). SCC in the selected dairy cows' milk was detected by LactoScan SCC based on fluorescent image cytometry (ISO 13,366–1 IDF 18–1). Further, the selected 15 dairy cows were assigned to the three study groups using the following criteria: (1) the average number of somatic cells in milk; (2) observation of udders, in line with the mastitis severity scoring provided by the scientific annals of the Polish Society of Animal Production (Polskie Towarzystwo Zootechniczne, PZH) (Jakiel et al., 2011). The healthy group included cows with SCC under 200,000 cells/mL milk and udders do not showing redness, swelling, or pain equivalent to PZH severity score of I or II. The subclinical mastitis group included cows with an SCC of more than 200,000 cells/mL of milk and with no signs of udder inflammation equivalent to PZH severity score of III or IV. Clinical mastitis group included cows with SCC above 500,000 cells/mL and clinical signs of udders inflammation like mammary gland redness, swelling, or pain equivalent to PZH severity score higher than IV. No exclusion criteria for each group were applied.

Bacteriological examination

Microbiological analysis was performed on days 4, 7, 10, 12 and 14 using dedicated CHROMagar Mastitis product to determine presence of strains connected with mastitis (Chromagar Mastitis Gram positive and Chromagar Mastitis Gram negative). Milk samples were collected at day 4, 7, 10, 12 and 14 from each selected cow (1 selected quarter per cow). Milk were 10 times diluted in 0.9% saline solution. Plating was performed using automatic Easy Spiral (Interscience, Saint-Nom-la -Bretèche, France). Petri dishes were incubated in aerobic conditions at 37°C for 24 h. *S. aureus, S. uberis, Streptococcus agalactiae, Klebsiella, Enterobacter, Citrobacter, E. coli* were detected during bacteriological milk analysis.

Anti- and pro-inflammatory cytokine analysis

To perform qualitative evaluation of the expressed cytokines in a dairy cows' milk, the five consecutive repetitions (day 4, day 7, day 10, day 12 and day 14) of the milk sample collection were made in Poland. In total, the obtaining of 100 mL of milk from the 15 cows of a selected quarter of each were performed during every milk sample collection. Milk samples of 100 mL were obtained directly after the milking. The centrifugation of milk samples at 2000 RPM/min for 3 min was performed to separate the sediment. The supernatant was carefully removed and discarded from each Eppendorf. Samples were again centrifuged at 2000 RPM/min for 3 min and the sediment was repeatedly removed. Fifteen Eppendorf tubes of 5 mL (with 2 mL of milk sample (sediment) and 2 mL of Tyrode's solution buffer) were prepared from each day of sample collection round. In total, 75 Eppendorf tubes were prepared. Samples were stored at −20°C and shipped in boxes with dried ice to Latvia, Laboratory of Morphology, Institute of Anatomy and Anthropology, Riga Stradins University where preparation and staining of milk smears were performed.

Bovine milk samples immunohistochemical staining for protein identification was done by biotin-streptavidin method (Gulbe et al., 2020; Hsu et al., 1981; Junga et al., 2018). The following antibodies were used for the immunocytochemical staining of milk smears: IL-2 (ab92381, 1:250, Abcam, UK), IL-4 (code orb10908, dilution 1:100, rabbit, Biorbyt Ltd., Cambridge, UK), IL-10 (sc-8438, 1:100, Santa Cruz, USA), IL-17A (code ab79056, dilution 1:200, rabbit, Abcam, Cambridge, UK), TGFβ-1 (cs -130,348, 1:100, Santa Cruz, USA) and β-defensin 3 (ab19270, 1:200, Abcam, UK).

The stained slides were analyzed by using bright-field microscopy with a Leica DC 300F camera microscope (Leica DM500RB, Leica Biosystems Richmond, Buffalo Grove, IL, USA) nonparametric evaluation. The results were evaluated by grading the appearance of the positively stained cells in the visual field (Gulbe et al., 2020): 0 – no positive immunoreactive cells in the visual field; + – a few positive immunoreactive cells in visual field; ++ – a moderate number of positive immunoreactive cells in visual field. For visual illustration, a Leica DM500RB digital camera and Microsoft Photo editor (version 19,051.162100) were used.

Data statistical analysis

The statistical data processing was performed with IBM SPSS (Statistical Package for the Social Sciences) version 26.0. After counting the cells and their distribution into groups depending on the presence and immunoreactivity of the factors, data were presented as absolute numbers.

In this study, milk samples from 15 cattle with distinct health conditions were analyzed within five sampling days. Nonparametric statistical tests were chosen to perform after Shapiro–Wilk test of normality was run. To compare the difference between positive and negative cells, the Wilcoxon test was used. Distinction between rank values of positive cells for each animal group and between sample collecting days was performed using Kruskal–Wallis H test. Contrast of the rank values of all days immunopositive cell count between animal groups was analyzed using Friedman test. In Kruskal–Wallis H test and Friedman test, a Bonferroni adjustment for multiple comparison were run for post-hoc test. p value < 0.05 was considered statistically significant.

Results

Evaluation of milk quality

The mean SCC in healthy cows was 15 755 cells/ml on day 1 and increased till 70 054 cells/ml on day 7. The highest SCC in healthy cows was detected on day 10 when it reached 108 856 cell/ml. Mean SCC decreased on day 12 till 41 974 cells/ml and slightly increased on day 14 (55 322 cells/ml).

Here, 728 465 cells/ml was observed on the 4th day of subclinical cows. The mean SCC decreased on day 7 till 687 162 cell/ml and on day 10 till 305 259 cells/ml. The mean SCC on day 12 was 527 676 cells/ml and on day 14–648 933 cell/ml.

The highest mean SCC in clinical cows was detected on day 4 (2 596 147 cells/ml) with further decrease in the mean SCC. The mean SCC was 985 676 cells/ml on day 7 and 795

330 cells/ml on day 10. The mean SCC slightly increased on day 12 till 851 853 cell/ml. 757 178 cells/ml was observed on day 14 in clinical cows.

Bacteriological examination

Different types of bacteria were detected in milk samples of all groups. Almost all milk samples were *S. aureus and S. uberis* positive. The most common species of bacteria was *S. uberis*, which prevalence was almost similar between all three group milk samples (Table 2). Althought, *S. aureus* incidence was like *S. uberis*, the average number of *S. uberis* was significantly higher than *S. aureus* (data is not shown).

The highest prevalence of *S. agalactiae* was detected in clinical cows with an increase on day 4 and day 10. Only in some specimen, *Klebsiella, Enterobacter, Citrobacter spp and E. coli* were detected without any variation between days (Table 1).

Identification of anti- and pro-inflammatory proteins producing cells in milk of dairy cows

In healthy cows, mean (%) IL-2 immunoreactive cell count was fluctuating through time. On day 4 the mean immunoreactive cell count was 62.80, which increased to 81.60 on day 7 (Table 2). However, immunoreactive cells significant decrease was noticed on day 10 when mean (%) value of IL-2 immunoreactive cell count decreased by two times (Figure 1(a), Table 2). Despite this decrease, mean (%) immunoreactive cell count on day 12 reached 78.20 (Table 2). Immunoreactivity of IL-4 was stable through all days. Moreover, the increase of weakly stained (+) cells increased while intensively stained (++) cell count decreased through time (Table 2). Positive cells increased of IL-4 mean number (%) was detected from day 4 to day 7, with following insignificant decrease on day 10 (Figure 1(d), Table 2). Overall, immunoreactivity has increased again reaching maximum of mean (%) cell count on day 14 (Table 2). The number of IL-4 negative stained (0) cells slightly decreased from day 4 to day 14 (Table 2). Immunoreactivity of IL-10 in healthy cow milk samples was stable. In addition, mainly weakly stained (+) immunoreactive cells were observed on day 4, day 7 and day 10, meanwhile, intensively (++) and weakly (+) stained cell ratio was 1:1 on day 12 and day 14 (Figure 2(a), Table 2). A mean (%) IL-17A immunoreactive cell count was stable through time (Figure 2(d), Table 2). β-Def 3 stained

Table 1. Number of cows' positive for monitored microorganisms.

	Healthy					Subclinical					Clinical				
	Day 4	Day 7	Day 10	Day 12	Day 14	Day 4	Day 7	Day 10	Day 12	Day 14	Day 4	Day 7	Day 10	Day 12	Day 14
S. aureus	5	5	4	3	5	5	5	5	4	5	5	5	4	5	3
S. uberis	5	5	5	2	5	5	5	5	3	5	5	5	5	5	2
S. agalactiae	5	2	4	0	0	4	0	4	0	2	4	1	4	3	3
Klebsiella, Enterobacter, Citrobacter spp.	0	1	3	1	0	0	1	3	0	1	1	0	2	0	1
E. coli	0	0	3	0	0	0	0	2	0	0	0	0	2	0	0

Table 2. The appearance and mean numbers of IL-2, IL-4, IL-10 and IL-17A in healthy cows and cows with subclinical and clinical mastitis.

	IL-2															IL-4														
	Day 4			Day 7			Day 10			Day 12			Day 14			Day 4			Day 7			Day 10			Day 12			Day 14		
	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0
Healthy cows																														
Mean (%)	29	34	37	58	24	18	23	24	53	53	25	22	49	25	26	51	30	19	53	39	8	39	48	13	35	54	11	41	58	1
±		63/37*			82/18*			47/53			78/22*			74/26*			81/19*			92/8*			87/13*			89/11*			99/1*	
Subclinical mastitis																														
Mean (%)	57	28	15	48	45	7	58	30	12	53	34	13	59	38	3	70	30	0	39	61	0	20	70	10	29	68	3	21	77	2
±		85/15*			93/7*			88/12*			87/13*			97/3*			100/0*			100/0*			90/10*			97/3*			98/2*	
Clinical mastitis																														
Mean (%)	59	30	11	48	51	1	41	54	5	37	58	5	50	33	17	23	75	2	22	76	2	14	66	20	7	79	14	25	68	7
±		89/11*			99/1*			95/5*			95/5*			83/17*			98/2*			98/2*			80/20*			86/14*			93/7*	
	IL-10															IL-17A														
	Day 4			Day 7			Day 10			Day 12			Day 14			Day 4			Day 7			Day 10			Day 12			Day 14		
	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0
Healthy cows																														
Mean (%)	14	67	19	2	83	15	5	74	21	44	42	14	41	34	25	45	53	2	40	57	3	31	66	3	43	56	1	42	57	1
±		81/19*			85/15			79/21*			86/14*			75/25*			98/2*			97/3*			97/3*			99/1*			99/1*	
Subclinical mastitis																														
Mean (%)	3	14	83	0	17	83	2	13	85	0	13	87	3	12	85	27	61	12	29	59	12	28	58	14	29	65	6	36	61	3
±		17/83*			17/83*			15/85*			13/87*			15/85*			88/12*			88/12*			86/14*			94/6*			97/3*	
Clinical mastitis																														
Mean (%)	0	15	85	0	13	87	5	14	81	0	5	95	10	2	88	25	59	16	25	63	12	25	60	15	33	57	10	33	58	9
±		15/85*			13/87*			19/81*			5/95*			12/88*			84/16*			88/12*			85/15*			90/10*			91/9*	

Mean – mean value (%) of intensively (++), weakly (+) and negatively (0) stained immunoreactive cells, numbers are indicated with zero decimals; ±—immunoreactive cells (both values of intensively (++) and weakly (+) stained immunoreactive cells) (+) vs. negative cells (no immunoreactivity) (–).

Abbreviations in table: *—statistically significant difference, Wilcoxon test ($p < .05$); IL-2, IL-4, IL-10, IL-17A – interleukins (IL)-2, –4, –10, –17A.

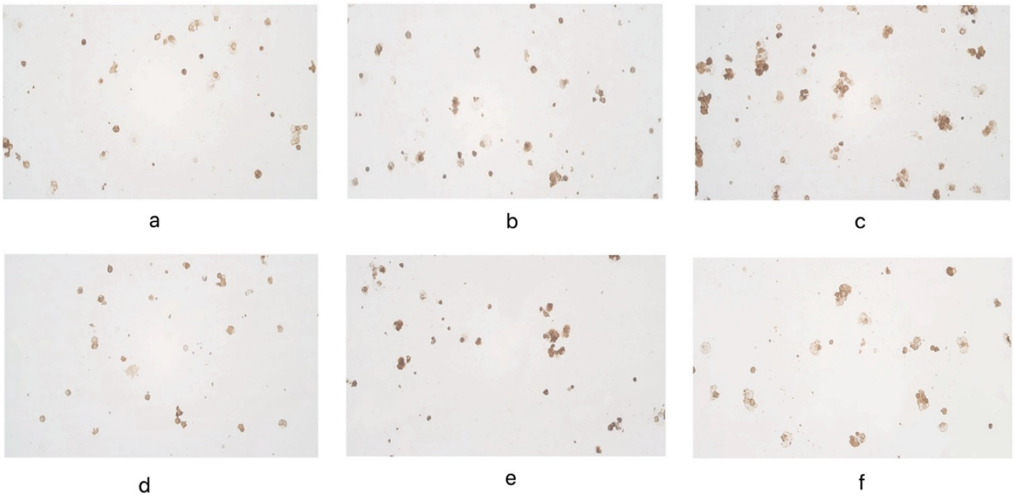

Figure 1. IL-2 immunoreactive cells in the milk. (a) A moderate number of positive cells in the milk of a healthy cow on day 10, ×400; (b) Numerous positive cells in the milk of a cow with subclinical mastitis on day 12, ×400; (c) A moderate number of positive cells in the milk of a cow with clinical mastitis on day 14, ×400. IL-4 immunoreactive cells in the milk. (d) A moderate number of immunoreactive cells in the milk of a healthy cow on day 4, ×400; (e) A moderate number of immunoreactive cells in the milk of a cow with subclinical mastitis on day 14, ×400; (f) A moderate number of positive cells in the milk of a cow with clinical mastitis on day 10, ×400.

milk samples showed that ration of positive (intensively (++) and weakly (+) stained cells) and negative (0) cells mean value is almost 1:1. Immunoreactivity of β-Def 3 has started to decrease from the 4th day with slight increase of mean numbers on day 12. However, a sharp drop in mean numbers of cells was observed on day 14 (Figure 3(d), Table 3). Finally, the mean (%) value of TGFβ-1 immunoreactive cells was stable from day 4 to 14 (Figure 3(a), Table 3).

In summary, mean (%) values of IL-4, IL-10, IL-17A and TGFβ-1 immunoreactive cells were stable through time. The ratio of intensively (++) and weakly (+) stained cells was approximately 1:1 through all days or only on last two days (for IL-10). However, mean (%) values of IL-2 and β-Def 3 immunoreactive cells showed a tendency to decrease on day 10. The highest positive immunoreactive cell count was detected in healthy cows' milk samples stained with TGFβ-1, IL-17A and IL-4.

In subclinical cows, immunoreactivity of IL-2 was stable. A mean (%) cells count increased from day 4 to day 14 (Figure 1(b), Table 2). Similarly, IL-4 positive cells have stayed stable through time, but positive cells mainly consisted of weakly (+) stained cells. Mean (%) value of IL-4 immunoreactive cells showed stability from the 4th day to the 14th day (Figure 1(d), Table 2). Further, a mean (%) of IL-10 positive cell count was very low and decreased through time in subclinical mastitis affected cows. Mainly negative (0) cells were detected in milk samples with IL-10 staining. Although, immunoreactivity of IL-10 was low, it decreased even more from the 4th day to the 14th day (Figure 2(b), Table 2). Similarly to IL-10 activity, immunoreactivity of β-Def 3 also remained the decreased during the research only with slight increase on the 7th day (Figure 3(e), Table 4). IL-17A positive cells mean value started to increase from the 4th day, reaching a mean value of 97.20 cells on the 14th

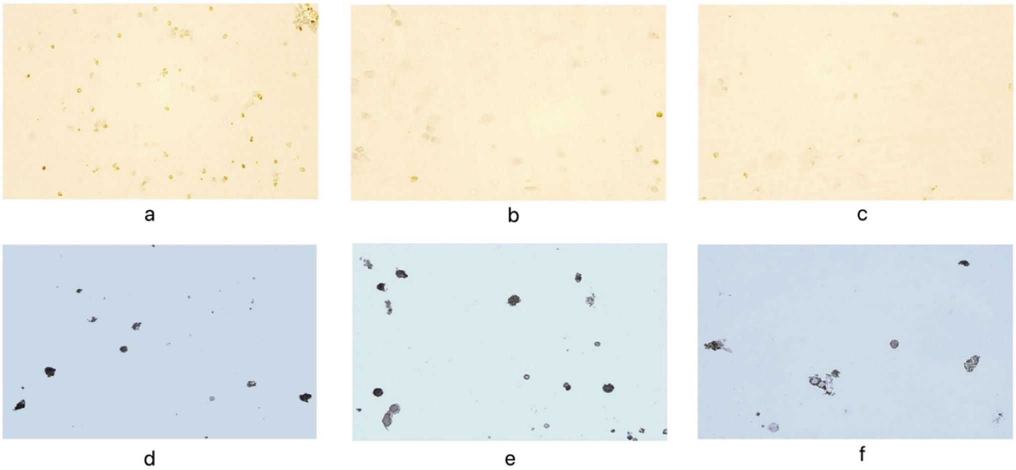

Figure 2. IL-10 immunoreactive cells in the milk. (a) Numerous immunoreactive cells in the milk of a healthy cow on day 4, ×400; (b) A few positive cells in the milk of a cow with subclinical mastitis on day 10, ×400; (c) A few immunoreactive cells in the milk of a cow with clinical mastitis on day 12, ×400. IL-17A immunoreactive cells in the milk. (d) A few immunoreactive cells in the milk of a healthy cow on day 12, ×400; (e) A few positive cells in the milk of a cow with subclinical mastitis on day 7, ×400; (f) A few immunoreactive cells in the milk of a cow with clinical mastitis on day 4, ×400.

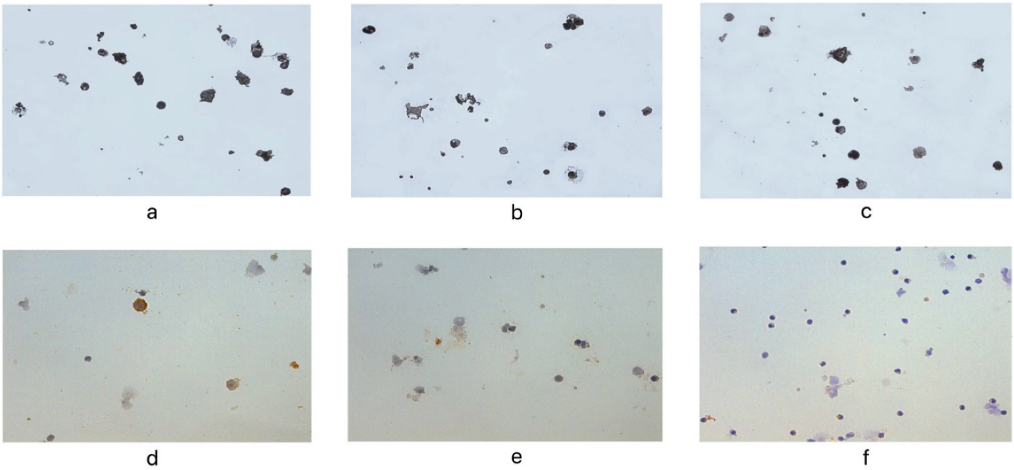

Figure 3. TGFβ-1 immunoreactive cells in the milk. (a) A moderate number of immunoreactive cells in the milk of a healthy cow on day 4, ×400; (b) A moderate number of immunoreactive cells in the milk of a cow with subclinical mastitis on day 7, ×400; (c) A moderate number of immunoreactive cells in the milk of a cow with clinical mastitis on day 12, ×400. β-def 3 immunoreactive cells in the milk of: (d) An occasional to a few immunoreactive cells in the milk of a healthy cow on day 7, ×400; (e) An occasional number of immunoreactive cells in the milk of a cow with subclinical mastitis on day 10, ×400; (f) An occasional number of immunoreactive cells in the milk of a cow with clinical mastitis on day 10, ×400.

Table 3. The appearance and mean numbers of β-def 3 and TGFβ-1 in healthy cows and cows with subclinical and clinical mastitis.

	TGF-β1															β-Def3														
	Day 4			Day 7			Day 10			Day 12			Day 14			Day 4			Day 7			Day 10			Day 12			Day 14		
	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0
Healthy cows																														
Mean (%)	43	57	0	48	51	1	38	61	1	48	52	0	46	54	0	25	33	42	24	32	44	13	30	57	24	36	40	12	29	59
±		100/0*			99/1*			99/1*			100/0*			100/0*			58/42			56/44			43/57			60/40			41/59	
Subclinical mastitis																														
Mean (%)	18	82	0	29	70	1	29	69	2	24	75	1	27	72	1	5	9	86	5	15	80	5	8	87	4	9	87	2	10	88
±		100/0*			99/1*			98/2*			99/1*			99/1*			14/86*			20/80*			13/87*			13/87*			12/88*	
Clinical mastitis																														
Mean (%)	20	75	5	32	65	3	37	62	1	27	73	0	32	67	1	0	13	87	2	4	94	3	6	91	3	7	90	4	9	87
±		95/5*			97/3*			99/1*			100/0*			99/1*			13/87			6/94*			9/91*			10/90*			13/87*	

Mean – mean value (%) of intensively (++), weakly (+) and negatively (0) stained immunoreactive cells, numbers are indicated with zero decimals; ±—immunoreactive cells (both values of intensively (++) and weakly (+) stained immunoreactive cells) (+) vs. negative cells (no immunoreactivity) (–).

Abbreviations in table: *—statistically significant difference, Wilcoxon test ($p < .05$); transforming growth factor-beta 1 - TGFβ-1; Beta-defensin 3 - β-Def3.

day of the study. (Figure 2(e), Table 2). Finally, mainly a weakly (+) stained TGFβ-1 immunoreactive cell count remained stable over time of the study (Figure 3(b), Table 3).

Overall, the highest mean (%) values of immunoreactive cell count were detected in subclinical cows' milk samples stained with IL-4 and TGFβ-1. Expression of IL-10 and β-Def 3 were significantly decreased in subclinical cows and mainly negative (0) cells were observed. Mean (%) values of IL-2 and IL-17A immunoreactive cells slightly increased from day 4 to day 14.

In clinical mastitis-associated cows, mean (%) values of IL-2 immunoreactive cell count showed increase from day 4 to day 12 reaching a mean value of cells 95.20 (Table 2). However, a sharp drop was observed on the 14th day of study, showing a mean cell value of 83.40 (Figure 1(c), Table 2). Further, mainly a weakly (+) stained IL-4 immunoreactive cell count fluctuated over the time. Although mean cells values remained above 93 cells, a significant decrease was observed on the 10th and 12th day. (Figure 1(f), Table 2). Immunoreactivity of IL-17A positive cells slightly increased from day 4 to day 14 and mainly remained stable (Figure 2(f), Table 2). TGFβ-1 immunoreactive cell count stayed stable over time (Figure 3(c), Table 3). Immunoreactivity of both IL-10, and β-Def 3, remained nearly absent. Mean (%) values of IL-10 immunoreactive cells did not increase over 20 positive cells and mainly fluctuated from 5 to 15 positive cells in field of vision (Figure 2(c), Table 2). Finally, similar behaviour was observed in β-Def 3 immunoreactive cells (Figure 3(f), Table 3).

In summary, high expression of IL-2, IL-4, IL-17A and TGFβ-1 was observed in clinical mastitis associated cows. Meanwhile, the expressions of IL-10 and β-Def 3 were barely detectable. The highest expression of immunoreactive cells mean (%) values was detected in milk samples stained with TGFβ-1.

Graphical illustration of IL-2, IL-4, IL-10, IL-17A, TGFβ-1 and β-Def 3 mean value (%) distribution over time is shown in Figure 4.

Table 4. Wilcoxon test revealing statistical interaction between mean values of positive and negative cell counts.

Health status	Factor	Mean value of negative cell count	Mean value of positive cell count	*p*-value
Healthy	IL-2	31.36	68.64	.043
	IL-4	10.40	89.60	.043
	IL-10	19.00	81.00	.043
	IL-17A	1.88	98.12	.043
	β-Def 3	48.52	51.48	.686
	TGF-β1	0.20	99.80	.034
Subclinical	IL-2	9.92	90.08	.043
	IL-4	3.08	96.92	.043
	IL-10	84.88	15.12	.043
	IL-17A	9.44	90.56	.043
	β-Def 3	85.88	14.12	.043
	TGF-β1	0.60	99.40	.041
Clinical	IL-2	7.76	92.24	.043
	IL-4	8.88	91.12	.042
	IL-10	87.00	13.00	.043
	IL-17A	13.84	87.36	.043
	β-Def 3	89.76	10.24	.043
	TGF-β1	1.92	98.08	.043

IL-2, IL-4, IL-10, IL-17A – interleukins (IL)-2, −4, −10, −17A; TGFβ-1 – transforming growth factor-beta 1; β-Def 3 – Beta-defensin 3.

Statistical analysis

Statistically significant difference between ranks of positive and negative cells was determined after Wilcoxon test was performed. A statistically significant difference of positive and negative cell count was detected in IL-2, IL-4, IL-10 IL-17A and TGFβ-1-stained samples of healthy, subclinical, and clinical cows in all days ($p < .05$) (Table 4).

A Kruskal–Wallis H test determined that rank values of IL-2 ($p = .017$) and IL-4 ($p = .001$) immunoreactive cells indicated statistically significant difference of cell count over time (between days 4 to 12). Pairwise comparison of days with post-hoc Bonferroni adjustment revealed no statistically significant difference between days of IL-2 immunoreactive cells. Meanwhile, rank values of IL-4 immunoreactive cells showed that cell count was statistically higher on day 14 vs. day 4 ($p < .001$) and on day 14 vs. day 10 ($p = .032$).

For dairy cows of subclinical group, a Kruskal–Wallis H test determined that rank values of IL-4 ($p = .011$) and IL-17A ($p = .012$) immunoreactive cell count showed statistically significant difference between days. A statistically higher IL-17A immunoreactive cell count was observed on day 14 vs. day 10 ($p = .032$) and on day 14 vs. day 4 ($p = .045$) after Bonferroni adjustment was performed. Furthermore, post-hoc Bonferroni adjustment revealed statistically higher rank values of IL-4 immunoreactive cells on day 4 vs. day 10 ($p = .016$) and on day 7 vs. day 10 ($p = .024$).

A Kruskal–Wallis H test determined that rank values of IL-2 ($p = .004$) and IL-4 ($p = .012$) immunoreactive cells indicated statistically significant difference of cell count over time. Pairwise comparison of days with post-hoc Bonferroni adjustment revealed no statistically significant difference between days of IL-4 immunoreactive cells. Meanwhile, rank values of IL-2 immunoreactive cells showed that cell count was statistically higher on day 7 vs. day 14 ($p = .003$) after Bonferroni adjustment was performed.

On day 10, IL-2 immunoreactive cell count was almost two times lower in the healthy group compared to the subclinical and clinical groups. Friedman tests demonstrated statistically significant influence of status and time on IL-2 immunoreactive cell count ($p < .001$). The rank values of IL-2 immunoreactive cells in clinical cows was statistically significantly greater than in healthy cows on day 4 ($p = .034$), day 7 ($p = .013$) and day 10 ($p = .013$). Furthermore, rank values of IL-2 immunoreactive cells in subclinical cows was statistically significantly higher than in healthy cows on day 14 ($p = .013$) (Table 5).

Although high level of IL-4 immunoreactive cells was detected in all groups and days, Friedman test demonstrated statistically significant difference of IL-4 immunoreactive cells between status and time ($p = .001$). The rank values of IL-4 immunoreactive cells in subclinical cows was statistically significantly greater than in healthy cows on day 4 ($p = .013$) and day 7 ($p = .022$). Moreover, a statistically significant greater immunoreactive cell count was detected in subclinical cows than in clinical cows on day 12 ($p = .034$) (Table 5).

Friedman test revealed statistically significant difference of IL-10 immunoreactive cell rank values between status and time ($p < .001$). A statistically significant greater expression of IL-10 was detected in healthy cows than in clinical cows on day 4 ($p = .034$), day 7 ($p = .013$) and day 12 ($p = .005$) (Table 5).

Table 5. Statistically significant interaction of status and time on ranks of IL-2, IL-4, IL-10, IL-17A, TGFβ-1 and β-def 3 immunoreactive cells in healthy cows and in cows with subclinical and clinical mastitis.

	p-value			
	Time			
	Healthy cows	Subclinical mastitis	Clinical mastitis	Status vs. time
IL-2	.017	.109	.004	<.001
IL-4	.001	.011	.012	.001
IL-10	.193	.838	.132	<.001
IL-17A	.709	.012	.347	<.001
β-Def 3	.124	.650	.542	<.001
TGFβ-1	.536	.243	.108	.054

IL-2, IL-4, IL-10, IL-17A – interleukins (IL)-2, -4, -10, -17A; TGFβ-1 – transforming growth factor-beta 1; β-Def 3 – Beta-defensin 3.

Expression of IL-17A was slightly decreased in subclinical and clinical cows. Rank values of IL-17A immunoreactive cell count indicated statistically significant greater number of positive cells in healthy cows than in clinical cows on day 4 ($p = .034$), day 12 ($p = .034$) and day 14 ($p = .022$) (Table 5).

Decrease of β-Def 3 immunoreactive cell count was detected in clinical and subclinical cows compared to healthy cows in all studied days. Friedman's test determined a statistically significant interaction of status and time on β-Def 3 immunoreactive cells ($p < .001$). Rank values of β-Def 3 immunoreactive cell count were a statistically significant greater in healthy cows than in clinical cows on day 7 ($p = .008$), day 10 ($p = .034$) and day 12 ($p = .034$) (Table 5).

Rank values showed no statistically significant difference of TGFβ-1 immunoreactive cell count in status and time ($p = .054$) (Table 5).

Discussion

SCC, mainly lymphocyte count, has predictive role in subclinical and clinical mastitis manifestation what is widely used in the diagnosis of mastitis (Bannerman, 2009). The acute invasion of udder by pathogens induces innate immune response as the first defense mechanism in subclinical and clinical mastitis (Benoit et al., 2008). Particularly, neutrophils, macrophages, lymphocytes, and epithelial cells are involved in dairy cows' udder protection (C. Riollet et al., 2000). Firstly, lymphocytes and epithelial cells are activated by bacteria released toxins, resulting in secretion of cytokines (IL-2, IL-4, IL-10, IL-17A, TGFβ-1, β-Def 3) and polymorphonuclear cell mobilization and migration (Amatya et al., 2017; Bannerman, 2009; Lorè et al., 2016; Paape et al., 2003). Overall, SCC remains as a prognostic factor for mastitis progression, but detection of factors, like cytokines, defensins and other inflammatory proteins, can give additional information about mastitis progression.

In our study, *S. aureus and S. uberus* was detected almost in all cows through the time and expression of IL-2 was high in all studied groups. Upon mentioned, *S. aureus* and *S. uberus* are a part of the normal microbiota of the mammary gland and predominantly are opportunistic environmental pathogens (Oikonomou et al., 2014; Rainard, 2017; Zadoks et al., 2005). A statistically higher IL-2 immunoreactive cell count was detected in clinical groups compared to healthy cows in the first 3 days (day 4, day 7, day 10) and in subclinical cows compared to healthy cows on day 14. Overall, a statistically lower expression of IL-2 compared to clinical and subclinical groups, suggests its important role in dairy cow udder immunity. However, the number of IL-2 positive cells in healthy cows also was high, which makes it difficult to use IL-2 as a marker to determine cows with clinical and subclinical mastitis. IL-2 is a pro-inflammatory cytokine which is mainly produced by antigen-stimulated CD4+ and CD8+ T lymphocytes (Malek, 2008). As mentioned earlier, IL-2 is a main protein in the regulation of adaptive immune responses (Abbas et al., 2018). Relevant studies have shown significant immunopotentiation in the infected bovine after the infusion of IL-2 what resulted in recruitment of somatic cells, lymphocytes, neutrophils, macrophages, and eosinophils (Nickerson et al., 1989; Quiroga et al., 1993; Reddy et al., 1992). Also, there was detected IL-2 changes in the milk of the infected bovine. Alluwaimi et al. in 2003 and in 2004 and Ferens et al. in 1998 observed IL-2 mRNA increase in the milk of cows infected by *S. aureus*, however, level of IL-2 significantly dropped between 24 and 32 h post infection (Alluwaimi, 2004; Alluwaimi et al., 2003). According to authors thoughts, this drop was provoked by *S. aureus* enterotoxin induced production of IL-4 and IL-10 mRNA (Ferens et al., 1998).

The level of IL-4 positive cells in clinical and subclinical groups has dropped to the healthy cows' level on day 10. Since then, immunoreactive cell count stayed similar in all three groups. Park et al. in 2006 stimulated bovine udders with staphylococcal enterotoxin C and observed increase of Th-1 cytokines (IL-12, IL-2, INF-γ) and Th-2 cytokines (IL-4, IL-13) with following decrease 24 h after stimulation (Gieseck et al., 2018). Interestingly, the second peak of IL-4 was observed on the 3rd day (Park et al., 2006). These findings show that IL-4 expression mainly is observed in the first days of the infection development and is higher in clinical mastitis compared with subclinical. IL-4 in bovine mammary glands is produced by CD4+ Th2 cells, CD8+/T-suppressors, and B cells mainly providing the differentiation of T cell, favoring Th2 subset development, and inhibiting the production of IFN-γ (Ezzat Alnakip et al., 2014; C. Riollet et al., 2000). Sipka et al. (2013) measured IL-4 expression in the milk of the infected untreated and infected treated with cefapirin or cefapirin and prednisolone dairy cows (Sipka et al., 2013). Increase of IL-4 was observed in all groups 24 h post-infection, meanwhile, the highest level of IL-4 was observed in the infected untreated cows (Sipka et al., 2013). In our study, IL-4 immunoreactive cell count was higher in clinical and subclinical groups compared to healthy cows. Moreover, a statistically greater amount of IL-4 was observed in clinical mastitis associated dairy cows' milk compared with healthy cows' milk on day 4 and day 7. IL-4 is a negative regulator of pro-inflammatory cytokines and decreases inflammation processes. IL-4 initiate activation of Stat6, induces expression of GATA3 what leads to Th1 and Th17 differentiation inhibition (Gieseck et al., 2018; Ho & Miaw, 2016). As a result, decreases production of such pro-inflammatory cytokines like IL-17, IL-1, IL-6, IL-12, and others (Gieseck et al., 2018; Hahn & Ghoreschi, 2017).

In our study the highest expression of IL-17A was detected in healthy cows, despite IL-17A pro-inflammatory activity. However, IL-17A immunoreactive cell count in subclinical and clinical mastitis cows did not show any statistically significant difference with healthy cows and remained high through all study days. Decreased expression of IL-17A in mastitis cows can be associated with increased activity of anti-inflammatory cytokines. In healthy bovine udder, IL-17A is produced mainly by Th 17 cells. It influences stromal cells such as fibroblasts and epithelial cells, inducing the secretion of chemokines attracting and stimulating neutrophils, as well as favoring granulopoiesis (Roussel et al., 2015). Furthermore, IL-17A has been studied at the protein level in the milk of the challenged mammary glands. Several types of immune cells actively produce IL-17A, including CD4+, CD8+ T lymphocytes, macrophages, and neutrophils (Rainard et al., 2013). Tassi et al. (2013) challenged 24 healthy Holstein cows with *S. uberis* (Tassi et al., 2013). The data have shown increase of IL-17A in the time gap from 51 h to 144-h post infection with the peak expression at 81-h post infection (Chockalingam et al., 2005). Roussel et al. in 2015 challenged 5 healthy Holstein cows with *E. coli* and observed signs of clinical mastitis 12 h post infection (Roussel et al., 2015). Animals were slaughtered 24 h post infection but, unfortunately, there was not detected expression of IL-17A (Roussel et al., 2015). However, additional analysis was done using real time polymerase chain reaction (RT-PCR) and there was detected induction of IL-17A genes 24 h post infection (Roussel et al., 2015). Overall, bacterial induced IL-17A expression in the udder of dairy cows increased very rapidly and reached the highest level approximately 12–24 h post infection.

TGFβ-1 plays a crucial role as an anti-inflammatory cytokine and mainly operates at mucosal surface through regulatory T cells (Bingisser & Holt, 2001; Lehner, 2008; MacDermoit, 1996). However, TGFβ-1 is not associated only with pathogen caused inflammation processes in the organism. The TGFβ-1 primary function is to regulate production of ECM and provide wound healing (Sugiyama et al., 2013). Prolonged intramammary infection can stimulate TGFβ-1 induced excessive growth of connective tissue and the formation of sclerosis, scars, and strictures (Min et al., 2016). Obviously, these changes influence dairy cows' ability to produce milk. Relevant studies have demonstrated an increase of TGFβ-1 level in challenged cows. Chockalingam et al. (2005) challenged healthy Holstein cows with *E. coli* and observed TGFβ-1 peak concentration 48 h post infection (Chockalingam et al., 2005). Bannerman et al. (2008) challenged two breeds of cows with *S. aureus* (Gieseck et al., 2018). While in the Holstein cows the first concentration peak of TGFβ-1 was observed at 36 hpi and the second one at 60 hpi, Jersey cows' TGFβ-1 level fluctuated with peaks at 6, 18, 42 and 60 hpi (Chockalingam et al., 2005). However, in our study high expression of TGFβ-1 was detected in all studied groups. Moreover, a statistically significant difference was not detected nor by status neither by time. Overall, other authors detected TGFβ-1expression peaks in the first hours after cows' udders were challenged by pathogens, meanwhile in our study expression of inflammatory proteins was observed during prolonged acute phase up to 14th day.

Although, expression of IL-2, IL-4, IL-17A and TGFβ-1 was high in subclinical and clinical mastitis from day 4 to day 14, the level of these cytokines in healthy cows was almost the same. Similar data was observed in earlier studies of IL-2 and TGFβ-1 from day 1 to day 3, and of IL-4 and IL-17A from day 4 to day 6 (Šerstņova et al., 2022; Vitenberga-Verza et al., 2022). Comparing these data with other author data, expression changes of these cytokines should be detected hourly to observe the difference of secretion levels in infected and healthy cows.

Upon above mentioned, IL-10 is an anti-inflammatory cytokine what is produced by different cells (Lutfalla et al., 1993; Ouyang et al., 2011). Expression of IL-10 in mammary glands can be triggered by different pathogens like *E. coli, Kl. pneumonia, P. aeruginosa*, and *Streptococcus spp* (Bannerman, 2009). As a result, IL-10 level increases not only in the milk of the infected cow, but also in the serum [44]. Varzandian et al. (2017) detected expression of IL-10 in the milk and serum of the mastitis cow and observed increase of the cytokine in both substances which were statistically significant compared with healthy cows (Varzandian et al., 2017). Bannerman et al. (2005) reported increase of IL-10 level in the milk of *P. aeruginosa* infected cows within 24 h post infection and return to pre-challenge level within 48 h post infection (Bannerman et al., 2005). Meanwhile, Tassi et al. (2013) found increased expression of IL-10 in the milk of *S. uberis* challenged cows with the peak of the expression at 48 h post infection (Tassi et al., 2013). In our study, greater expression of IL-10 immunoreactive cells was detected in healthy cows compared with clinical and subclinical dairy cows. A statistically higher numbers of IL-10 positive cells were detected in healthy dairy cows compared with clinical cows on day 4, day 7 and day 12, and with subclinical cows on day 10. We assumed that decreased expression of IL-10 in subclinical and clinical mastitis can be caused by prolonged acute inflammatory phase when IL-10 expression starts rapidly decrease. However, in previous study expression of IL-10 in healthy cows remained higher than in clinical and subclinical mastitis associated cows from day 1 to 3, despite increased production of IL-10 in subclinical mastitis on day 2 (Šerstņova et al., 2022). Overall, other suppressive factors, which should be detected, influence IL-10 secretion during inflammation and can be used as diagnostic factors.

Defensins are antimicrobial peptides which can be found in a variety of animals (Merriman et al., 2015). Unfortunately, there is limited amount of data showing β-Def 3 and other defensin family members expression in the infected bovine udder. However, Merriman et al. in 2018 evaluated effects of vitamin D on immunity of cows challenged with *E. coli* endotoxin (Merriman et al., 2018). The results of the study showed significantly increased expression of β-Def 3 genes in milk after stimulation with lipopolysaccharides (Merriman et al., 2018). The main expression of β-Def 3 was detected by macrophages and neutrophils (Merriman et al., 2018). In a previous study decrease of β-Def 3 level was observed in between groups (Šerstņova et al., 2022). The highest expression was in healthy cows, then in subclinical mastitis and the lowest expression was observed in clinical mastitis from day 1 to day 3 (Šerstņova et al., 2022). In our study, the highest expression of β-Def 3 was observed in healthy cows, meanwhile, almost no immunoreactive cells were detected in the cows with subclinical and clinical mastitis. This finding suggests another factor increasing influence on β-Def 3 expression in infected cows. Moreover, this influence increases in prolonged acute phase of inflammation what lets β-Def 3 use as mastitis diagnostic marker. However, the main problem to evaluate changes of β-Def 3 expression remains deficiency in knowledge about defensin family expression in dairy cows' milk.

Overall, IL-10 and β-Def 3 expressions significantly decrease in infected cows what can be helpful in diagnosis of mastitis. However, limited knowledge about other factors suppressive influence on these factors' expression should be evaluated in the future.

Impact of different factors is associated with relevant changes in expression of anti- and pro-inflammatory proteins in the milk of healthy and mastitis affected cows. Meanwhile the majority of studies are based on experimentally induced mastitis, in our study cows naturally accruing subclinical and clinical mastitis were accepted for research. Expression of immune defense proteins can differ because of pathogens involved in inflammatory process, for example, contagious or environmental pathogens or even combination of different pathogen organisms. Changes in cytokines and other protein production amount and type can be influenced by other trigger factors like environmental (e.g., season, temperature, humidity), animal factors (e.g., age, cattle breed, dietary management) (Bagath et al., 2019; Bannerman et al., 2008). In summary, our data has differed from other author data as a result of the above-mentioned factor combination.

The limitations of our study are associated with the relatively small sample number. To get more accurate results it is necessary to increase the number of subjects in each group. However, our study still corresponds to the standards for experiments using morphological methods for diagnosis. In this study, only immunohistochemistry method was used, but it could be valuable to compare the results applying other methods like ELISA, PCR, Western Blot. Performing ELISA and mRNA of certain proteins can also be used as diagnostic tools in the future.

Conclusion

The stable expression of IL-2 IL-4, IL-17A and TGFβ-1 from day 4 to day 14 in the milk of subclinical and clinical mastitis affected animals and in the healthy cows' milk indicates possible insignificant role of these cytokines in inflammatory processes and suggests not to use these cytokines as biomarkers for diagnostic purpose of mastitis.

Decreased expression of IL-10 and β-Def 3 in the milk of subclinical and clinical animals indicates significant changes in factors expression. This lets us assume IL-10 and β-Def 3 as possible diagnostic biomarkers of inflammatory acute phase in subclinical and clinical mastitis. However, further studies are necessary to evaluate the nature of β-Def 3 expression indication and suppression.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Institutional review board statement

The study aims to perform an analysis of cow's milk. The research involving animal use has the clear scientific purpose of increasing their welfare, which justifies their engagement in the research project. The research involved techniques administered in a way that did not cause pain, suffering, distress, or damage to the body of the participating animals. In this sense and in view of the national regulations of the partner in charge of validation (RIC Pro – Akademia, Poland), the study did not involve experiments on animals as defined in the Law of January 15 2015 on the protection of animals used for scientific or educational purposes (Journal of Laws of 2015 pos. 266 with further amendments). Animal studies were carried out humanely according to national and international Animal Care and

Use Committee protocols. The study involved obtaining milk samples by standard milking procedure without affecting the cow's routine. Therefore, no ethical committee's consent was required.

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