Polish Journal of Veterinary Sciences Vol. 25, No. 2 (2022), 237–248

DOI 10.24425/pjvs.2022.141808

Original article

Expression of anti-inflammatory markers IL-2, IL-10, TGF-β1, βDEF-2, βDEF-3 and Cathelicidin LL37 in dairy cattle milk with different health status of the udder

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Abstract

Great economic losses to the dairy industry are associated with bovine mastitis, which results in poor milk quality and high treatment costs. Anti-inflammatory proteins play an important role in the suppression of the immune response against invading pathogenic microorganisms and are therefore being studied for possible use in the early diagnosis of mastitis. In our study, we used milk samples from 15 cows of Holstein Friesian breed with different health status (5 healthy, 5 subclinical, and 5 clinical animals), and tested them using immunohistochemical (IHC) analysis to evaluate the presence of IL-2, IL-10, TGF-β1, βDEF-2, DEF-3, and Cathelicidin LL37 proteins. The calculation of positively and negatively stained cells for each biomarker was performed using the semiquantitative counting method. We found the presence of all factors with the exception of Cathelicidin LL37, which was almost absent in milk samples of all animal groups. The significant decrease of IL-10, β-def2, and β-def3 expression levels within the 3 days of sampling, found in the milk of animals with sub- and clinical mastitis, indicates the loss of anti-inflammatory protection of the affected cow's udder. In contrast, the stable increase of IL-2 and TGF-β1 positive cells observed in the milk of mastitis-affected cows, and the similar expression of these factors in the milk of healthy animals, indicate the possible lack of involvement of these cytokines at an early stage of udder inflammation.

Key words: cytokines, interleukins, mastitis, bovine milk

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Introduction

Mastitis is defined as the inflammatory response in domestic dairy animal udder tissue against a variety of microorganisms, most commonly environmental pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus uberis*, resulting in the udder and mammary gland infection (Alnakip et al. 2014). Bovine mastitis is the most common pathology of dairy cattle and is characterized by complexity and high cost of treatment, and as such causes harm to the welfare of animals, leading to reduced milk yields and poor milk quality, and consequently brings economic losses to farmers and impacts the dairy industry worldwide (Azooz et al. 2020). To maintain animal welfare and health, antibiotics are used as the most common antimicrobial treatment for mastitis. However, overuse and misuse of antibiotics lead to antimicrobial resistance (AMR) shown by bacterial populations in treated cattle. Therefore, many experimental studies are aimed at finding a method for predicting and controlling pre-clinical bovine mastitis in order to decrease antimicrobial usage in the dairy industry and thus reduce AMR (Krömker et al. 2017).

Innate immunity of the animal will be the first to encounter the invasion of pathogens through the teat canal of the udder and to protect the tissues of the mammary gland, after which the adaptive immune response takes place. The innate immune system plays a role in the control of adaptive immunity's reactions via intracellular signal transduction cascades that affect acute regulation of different immune cells and molecules (e.g., leukocytes, cytokines, factors, antimicrobial peptides and proteins) (Stelwagen et al. 2009). At the initial phase of inflammation, protection of the organism starts with the ability of the surface receptors of the immune and epithelial cells to recognize microbial molecules, and further to cause an immune reaction by producing pro-inflammatory mediators, which detect and eliminate pathogens, thus preventing their further distribution and damage to the udder tissue (Bannerman 2009, Schukken et al. 2011). Additionally, there are anti-inflammatory cytokines and specific cytokine inhibitors that control proinflammatory cytokine activity, limiting it to reducing the potentially injurious effects of long-term inflammatory reactions (Opal and DePalo 2000). These immune system defense components can be found in both mammary gland tissue and milk and are studied in healthy animals and subclinical or clinical cases. One example of a such component is cytokines, which can induce or suppress the immune response. They are produced by multiple cell types and are also found in healthy udders. Cytokines include interleukins (IL), tumor necrosis factors (TNF), and interferons (IFN) (Alluwaimi and Cullor 2002).

Macrophages are the predominant cell type in the healthy lactating mammary gland, whereas during the inflammation process, neutrophils are prevalent, as they are recruited after bacterial invasion into the udder. In the milk of healthy animals, the predominant lymphocyte phenotype is cytotoxic (CD8+) T lymphocytes, but during the inflammatory reaction, T helper (CD4+) cells become the predominant phenotype (Riollet et al. 2000).

Interleukins (e.g. IL-2 and IL-10) are naturally occurring proteins that mediate communication between cells. IL-2 belongs to gamma chain (γc) cytokines that affect the T cell life cycle through the γ chain or common receptor complex (Jiang et al. 2005, Dooms 2013). IL-2 is a pleotropic cytokine with a complex role in CD4+ cell expansion and survival, CD4+ T helper subset generation, and regulatory T cell development (Cheng et al. 2011)IL-2R signaling also plays an important part in Treg cell growth, survival, and effector differentiation. However, Treg cells somewhat distinctively assimilate IL-2R signaling. In particular, Treg cells require essentially only IL-2-dependent receptor proximal signal transducer and activator of transcription 5 (Stat5. IL-2 regulates immune system reactions by enhancing B and T lymphocytes, as well as by increasing plasma cell counts and activating natural killer (NK) cells (Alnakip et al. 2014).

Another interleukin, IL-10 is known as an anti-inflammatory cytokine that limits and ultimately terminates the immune response to pathogens, thus preventing damage to the host organism. IL-10 is a multifunctional cytokine as it can inhibit the activation and effector function of T cells, monocytes and macrophages, can inhibit the synthesis of proinflammatory cytokines, chemokines, and can also influence hemopoietic cells in various ways. In addition, IL-10 can regulate the growth and differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells. In different immune and other cell types, IL-10 expression passes through different pathways, usually in response to an activation stimulus (Moore et al. 2001, Saraiva and O'Garra 2010). IL-10 inhibits IFN-γ and IL-2 via Th1 lymphocytes; IL-4 and IL-5 via Th2 lymphocytes; IL-1, IL-6, IL-8, IL-12 and TNF-α via macrophages; and IFN-γ and TNF-α via NK cells (Taylor et al. 2007, Commins et al. 2008).

Transforming growth factor TGF-β can regulate numerous cellular functions, e.g it can activate cell proliferation and/or differentiation processes, and apoptosis, but can also inhibit proliferation of macrophages and lymphocytes by binding to the cell surface specific receptors (Gauthier et al. 2006, Dallas et al. 2008). For TGF β1 and its family members (TGF-β2 and

TGF-β3), activation goes through the same signaling system, when growth hormone binds to cell plasma membrane-bound receptors in the sequence TGF βR3, TGF βR2, and TGF βR1. Such downstream signaling activates several cytoplasmic proteins and protein kinases, causing in responding cells biological activity. Similar to IL-10, TGF-*β* is found to be an anti-inflammatory cytokine for its ability to suppress immune response during inflammation (Anton and Glod 2017, Tzavlaki and Moustakas 2020). Both, IL-10 and TGF-β1 inhibit T cell proliferation and differentiation to varying degrees and affect the formation of IL-2 and IFN-γ (Moore et al. 2001, Gorelik and Flavell 2002).

Defensins are small, cationic, amphiphilic peptides with microbicidal activity against both bacteria and fungi. Mammalian defensins can be subdivided into three main classes according to their structural differences: alpha-defensins, beta-defensins, and recently described theta-defensins. Mammalian alpha-defensins are predominantly found in neutrophils and in small intestinal Paneth cells, whereas mammalian beta-defensins have been isolated from both leukocytes and epithelial cells. Recently, two novel human beta-defensins, human beta-defensin-3 (HBD-3), and human beta-defensin-4 (HBD-4) have been discovered (Pazgier et al. 2006). Like HBD-1 and HBD-2, HBD-3 also has microbicidal activity towards Gram-negative bacteria (*Pseudomonas aeruginosa, E. coli*) and the yeasts (*Candida albicans, Malassezia furfur)*. In contrast to HBD-1 and HBD-2, significant expression of HBD-3 has been demonstrated in non-epithelial tissues, such as leukocytes, and heart and skeletal muscle. HBD-4 is expressed in certain epithelia and in neutrophils. Its bactericidal activity against *P. aeruginosa* is stronger than that of the other known beta-defensins (Schneider et al. 2005).

In addition to defensins, other antimicrobial molecules have been found in milk, for example cathelicidins (CATHL), but total milk gene sequencing has revealed about 15 local immune factor genes that provide local protection (Wheeler et al. 2012). In total, 268 proteins in human milk and 269 proteins in bovine milk have been identified. Among them, 44 human milk proteins and 51 bovine milk proteins are related to the host defense system, and 33 of them have been found in both species, but in significantly different quantities. More abundant antimicrobial proteins (5 cathelicidins) are known to be abundant in bovine milk (Hettinga et al. 2011). Bioinformatic analysis of the bovine genome revealed seven protein-coding cathelicidin genes, CATHL1-7, including two identical copies of CATHL4, as well as three additional putative cathelicidin genes, all clustered on the long arm of chromosome 22. Six of the seven protein-coding genes were expressed in leukocytes extracted from the milk of high somatic cell count (SCC) cows (Whelehan et al. 2014). Cathelicidins in significant amounts are present in mastitic milk, and neutrophils are believed to be the main producers of these antimicrobial peptides, while the role of mammary epithelial cells in their production and release is still unclear (Cubeddu et al. 2017).

According to the symptoms and changes of SCC in the animal's milk samples, bovine mastitis is known to be classified as subclinical or clinical. The clinical case is easily recognizable by changes in animal behavior, deterioration of milk quality and its decreased yields, and the visual changes in infected mammary quarters. Diagnosis of the subclinical case is more complicated because of the absence of visual signs of pathology in udder teats. As a result, the main indicators of subclinical mastitis remain a decrease in milk production and an increase in the SCC count (Khan and Khan 2006, Alhussien and Dang 2020). An in-depth understanding of the role of immune components in the different health status of various bovine species, as well as their count change in response to specific pathogens, can help to develop effective methods for early diagnosis of bovine mastitis and udder health control.

The aim of this study was to detect various anti-inflammatory cytokines and factors in cattle milk from an agricultural farm, and examine the change in the levels of these biomarkers between healthy cows, and animals with subclinical and clinical mastitis.

Materials and Methods

Animals and ethics statement

A herd of 79 purebred Polish Holstein Friesian cows (41 adult animals, ofwhich 35 were milking cows, and 38 non-lactating young cows) was kept in a free stall type farm, located in Northern Poland. The animals were fed with silage, corn silage, and grain by-products. In line with the mastitis severity scoring provided by the Polish Society of Animal Production (Polskie Towarzystwo Zootechniczne) PZH, udder quarters were examined and the somatic cell count (SCC) in animal milk was monitored to determine the animals' suitability for the study (Jakiel et al. 2011). Based on the health status of each animal fifteen heads of dairy cattle were selected and divided into three groups (five animals in each). At the beginning of the study, five cows were clinically healthy with low SCC (under 200 000 cells/mL) in udder quarter milk, five animals with medium SCC (200 000 - 500 000 cells/mL) were categorized as having mastitis, and the last five with high SCC (above 500 000 cells/mL) had symptoms

of clinical mastitis. The average age of the healthy cows was 5 years, the average day of lactation was 182, and the average daily yield was 35.31 (kg of milk/day). For cows with subclinical mastitis, the average age was 5 years, the day of lactation was 150, the milk yield was 36.44 (kg of milk/day). For cows with clinical mastitis the average age was 6 years, the day of lactation was 237, the milk yield was 41.87 (kg of milk/day). All milk samples taken from the cows were also examined for the presence of Gram-positive and Gram-negative pathogens. Examination was carried out at RIC Pro--Akademia, Poland.

The milk samples were collected through a standard milking procedure without affecting the cattle's welfare. This method is pleasant and effective, and in no way harms the health of the animals participating in the study, and so it does not fall under the Act on the Protection of Animals Used for Scientific or Educational Purposes, adopted in Poland on January 15th, 2015 (Journal of Laws of 2015, item 266.) Thus, the realization of this study did not require ethical approval.

Somatic cell count in cattle milk

LactoScan SCC based on fluorescent image cytometry (ISO 13366-1 IDF 18-1) was used to detect SCC in bulk milk and then in the animals selected for the experiment (cow number x udder 4 quarters). Each measurement replicates 16 times witha range of 10,000-10,000,000 cell/ml.

Bacteriological examination

Following to the ISO 4833:2013 standard the total bacteria count on Plate Count Agar (PCA), Chromagar Mastitis Gram positive (G+) and Chromagar Mastitis Gram negative (G-) were used to detect the characteristic strains of mastitis in cow milk samples. The results were represented in logarithmic (log) CFU/mL. Before plating, milk samples from 15 cows x 1 selected quarters were 10-times diluted in 0.9% saline solution. Plating was carried out using automatic plater (Easy Spiral; Interscience). Bacteriological milk analysis showed the presence of *S. agalactiae, S. uberis, S. aureus, E. coli, Klebsiella, Enterobacter, Citrobacter*.

Milk sample collection

To evaluate the expression of cytokines, milk samples were collected in Poland from cow udder quarters during the 3 repetitions (following days) x 100 ml of milk from 15 selected animals and then separated into 45 eppendorfs of 5 ml (with 2 ml of sample sediment and 2 ml of Tyrode buffer). Samples were stored at -20°C and shipped in boxes with dry ice to Latvia, Riga, Rīga Stradiņš University, Institute of Anatomy and Anthropology, Department of Morphology. The milk samples were then centrifuged at 2 000 rpm/min for 3 min to separate the sediment. From each eppendorf the supernatant was removed and the precipitate was collected and centrifuged again at 2 000 r/min for 3 min. The supernatant was repeatedly removed. The remaining precipitate was harvested and stored in portions at -20°C until use in immunohistochemistry (IHC) staining.

Anti-inflammatory protein analysis

Bovine milk samples that were tested by immunohistochemical analysis of different anti-inflammatory proteins were smeared on a microscope slide and fixed using a methanol-acetone mixture for 2h. The IHC method was used to detect IL-2 (ab92381, 1:250, Abcam, UK), IL-10 (sc-8438, 1:100, Santa Cruz, USA), β-defensin-2 (ab63982, 1:500, Abcam, UK), β-defensin-3 (ab19270, 1:200, Abcam, UK), TGF-β1 (cs-130348, 1:100, Santa Cruz, USA), Cathelicidin LL37 (cs-166770, 1:100, Santa Cruz, USA) cytokine and factor presence in the milk. Microslides with milk samples were evaluated using a Leica light microscop at 400 times magnification. The calculation of positively and negatively stained cells for each investigated IHC biomarker was performed using the semiquantitative counting method, by calculating 100 cells for each smear, with evaluation of their belonging to the particular cell group. Cells in milk smear samples were graded into three groups: 0 – negative structures; + - weak positive structures; ++ - strong positive structures (Gulbe et al. 2020).

Statistical analyzes

SPSS version 23.0 (IBM Corp., Armonk, NY, USA) was used for statistical data analysis of the identified biomarkers. After counting the cells and their distribution into groups ("0", "+", and "++") depending on the presence and degree of reaction to the factors, data were presented as absolute numbers, whose mean values are displayed in tables and graphs.

In this study, milk samples from 15 cattle with distinct health conditions (5 cows for each group - healthy animals, and animals with subclinical or clinical mastitis) were analyzed within three sampling days. To compare the difference of mean values between positive and negative cells, the Paired - Sample test was used. Distinction between mean values of positive cells for each animal group and between sample collecting days was performed using One-way ANOVA, LSD. Contrast of the total mean values of immunopositive cells (average of three sampling days) between animal groups was analyzed using MANOVA, LSD. P value < 0.05 was considered statistically significant.

Table 1. Numbers of cow's positive for the monitored microorganisms.

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

Results

Bacterial examination and SCC

Different species of bacteria were identified in the milk samples collected from all animal groups. The only exception was *E. coli*, which was not found in the milk from animals with clinical mastitis. We also observed that in naturally occurring bovine mastitis, communities of pathogenic bacteria vary by day of sampling and animal health status. The highest prevalence was observed for *S. aureus*, as it was found in the milkfrom almost every cow in all animal groups. The next most common species was *S. uberis*. It was detected in all bovine milk samples affected by mastitis, its occurrence varied between four and five animals in the subclinical group, and in the healthy group increased from three to five on day 2 and remained the same on day 3. Representatives of *Klebsiella, Enterobacter, Citrobacter spp.* were observed on days 2 and 3, while *S. agalactiae* was present in several animals on days 1 and 2. Lastly, *E. coli* was the least present, but the majority of positive samples were found in the healthy group (Table 1).

The mean SCC in healthy cows was 46 789 cells/ml in day 1, and then slightly decreased to 31 178 cells/ml and 29 113 cells/ml on days 2 and 3, respectively. In the subclinical group, the mean SCC was 312 238 cells/ml on day 1, then increased to 417 256 cells/ml on day 2, but then decreased to 232 791 cells/ml on day 3. In the clinical mastitis group, mean SCC was 2 747 677 cells/ml on day 1, then rose to 3 021 388 cells/ml on day 2, and decreased to 2 406 977 cells/ml on day 3.

IL-2, IL-10, β-Def2, β-Def3, CLL37 and TGF-β1 immunopositive cell presence in cattle milk.

Different amounts of all analyzed factors were present in healthy, subclinical, and clinical cattle milk samples, and the mean amounts for each group did not differ significantly between sample collecting days (Table 2).

IL-2 and TGF-β1 demonstrated the highest expression in the milk samples of all animal groups. In contrast, β-Def2 factor was observed in quite a low number of immunopositive cells, and CLL37 was found to be practically absent. Of all the examined factors, only IL-10 and β-Def3 showed a difference in expression across the animal groups. IL-10 showed high expression in milk from healthy cows, with the amounts subsequently declining in the milk of cows with subclinical and clinical mastitis. β-Def3, in turn, was less expressed than IL-10, but the amounts still differed between animal groups (Fig. 1).

There were significantly more IL-2 (Fig. 2) positive cells ($p<0.05$) number than negative cells in all animal groups, regardless of the day of sampling (Table 2A). Analyzing each of the three sampling days separately, compared to the healthy group, where the high numbers (97;100;100) of all counted cells were positive to IL-2, a slight, but not statistically significant, decline of the mean number of IL-2 positive cells was observed in the subclinical (93;89;86) and clinical (90;99;99) groups (Fig. 1A). However, the total mean of three sampling days showed a significantly higher ($p=0.03$) number of positive cells in healthy than in subclinical animal milk (Table 3A).

Analysis of IL-10 (Fig. 3) presence on each day within each animal group showed a significant difference ($p<0.05$) between IL-10 negative and IL-10 positive cells in all cases, except the 2nd and 3rd day for the subclinical group (Table 2B). The mean amounts of IL-10 positive cells were checked separately each day, and the results showed a significant difference between animal groups. On the 1st, 2nd, and 3rd day healthy animal milk samples contained close to maximal amounts (91;92;88) of IL-10 positive cells from all counted cells, and it was significantly higher ($p<0.001$) than in other animal groups (21;46;24 in subclinical and 3;5;8 in clinical). A significant difference was also found between subclinical and clinical groups ($p<0.05$), but it became insignificant in the 3rd day (Fig. 1B). IL-10 showed a significantly higher ($p<0.001$) total expression in healthy cattle milk among animal groups over all three days, as the number of immunopositive cells sharply decreased in subclinical and clinical

Table 2. Mean number of IL-2, IL-10, β-Def2, β-Def3, CLL37, and TGF-β1 immunopositive cells in the milk of healthy cattle and cows with subclinical and clinical mastitis, recorded in the 1st, 2nd, and 3rd day of sampling.

Animal group	IL-2 A									IL-10 B								
	Day1			Day2			Day3			Day1			Day2			Day3		
	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0
Healthy																		
Mean	65	32	3	77	23	0	70	30	0	81	10	9	84	8	8	70	17	12
+/0	97*/3			100*/0			100*/0			91*/9			92*/8			88*/12		
Subclincal																		
Mean	20	73	7	30	59	11	32	54	14	12	9	79	14	32	54	15	9	76
+/0	93*/7			89*/11			86*/14			21/79*			46/54			24/76		
Clinical																		
Mean	53	37	10	73	26	1	72	27	1	1	2	97	2	3	95	1	7	92
+/0	90*/10			99*/1			99*/1			3/97*			5/95*			8/92*		

Animal group	β-Def2 C									β-Def3 D								
	Day1			Day2			Day3			Day1			Day2			Day3		
	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0
Healthy																		
Mean	18	17	65	6	20	74	9	15	76	20	32	48	20	34	46	10	30	60
+/0	35/65			26/74			24/75			52/48			54/46			40/60		
Subclincal																		
Mean	3	4	93	5	6	89	8	4	88	10	15	75	5	30	65	1	23	76
+/0	7/93*			11/89*			12/88*			25/75			35/65			24/76		
Clinical																		
Mean	0	1	99	0	2	98	0	2	98	0	3	97	6	94		1	3	96
+/0	1/99*			2/98*			2/98*			3/97*			6/94*			4/96*		

Animal group	CLL37 E									TGF-β1 F								
	Day1			Day2			Day3			Day1			Day2			Day3		
	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0	++	+	0
Healthy																		
Mean	0	3	97	0	2	98	0	0	100	18	80	2	17	81	2	20	75	5
+/0	3/97*			2/98*			0/100*			98*/2			98*/2			95*/5		
Subclincal																		
Mean	0	0	100	0	3	97	0	2	98	9	79	12	11	75	15	17	72	11
+/0	0/100*			3/97*			2/98*			88*/12			85*/15			89*/11		
Clinical																		
Mean	0	0	100	0	0	100	0	0	100	11	69	20	12	75	13	13	74	13
+/0	0/100*			0/100*			0/100*			80*/20			87*/13			87*/13		

* – statistically significant difference ($p < 0.05$)

groups. A significant difference ($p<0.001$) was also found between subclinical and clinical animals (Table 3B).

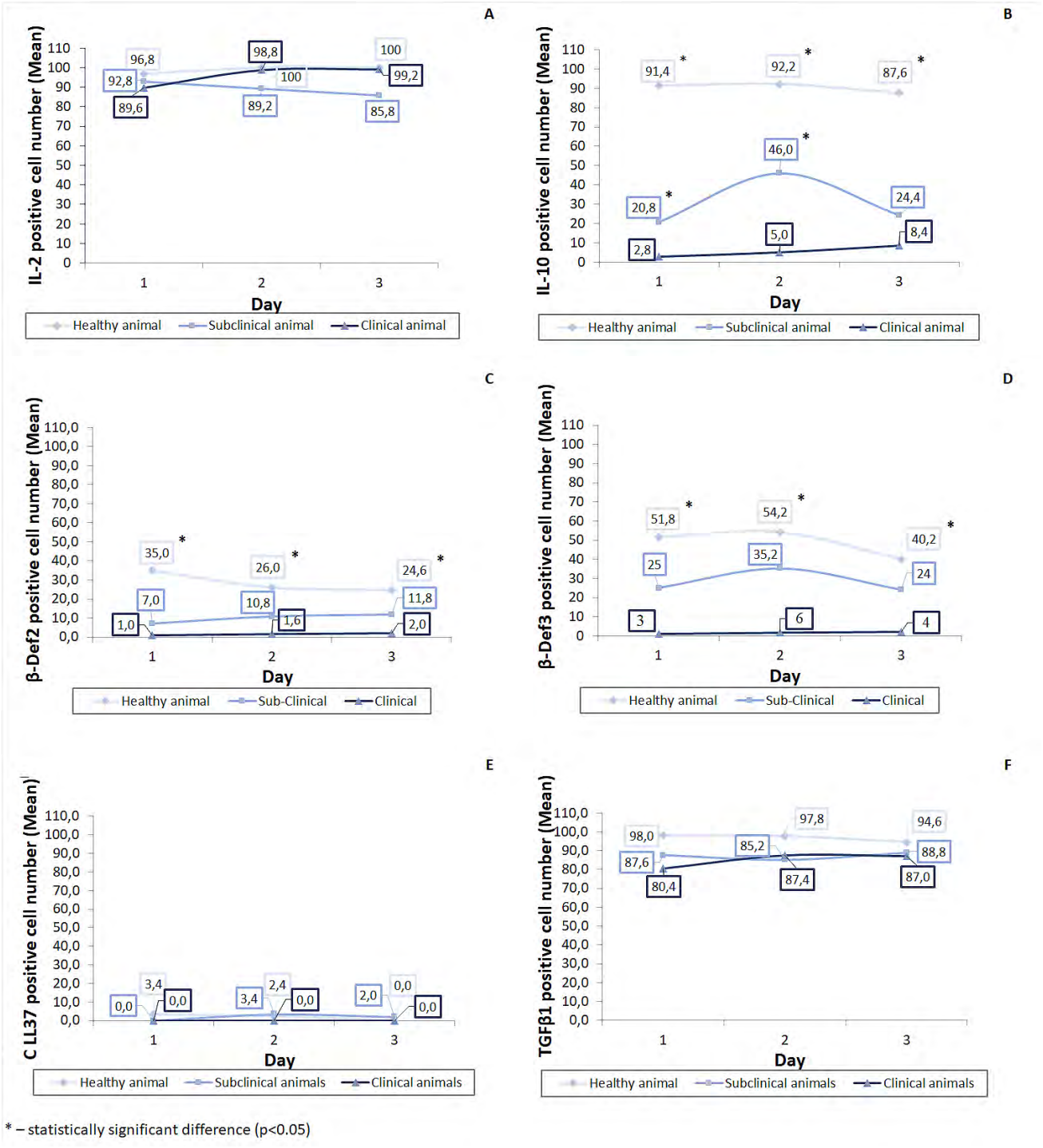
Mean values of β-Def2 (Fig. 4) and β-Def3 (Fig. 5) positive and negative cells did not significantly differ in healthy cattle milk during all three days, while for the subclinical and the clinical groups the difference was significant ($p<0.001$). One exception was the β-Def3 readings in the subclinical group on the 1st, 2nd and the 3rd sampling day, which showed no significant difference (Table 2C and D). Meanwhile, expression of both β-Def2 and β-Def3 on the 1st day of sampling was significantly higher ($p<0.001$) in milk from healthy cattle than in the subclinical or clinical group. On the 2nd and 3rd days such a difference ($p<0.05$) remained only between healthy and clinical cattle milk samples (Fig. 1C and D). Tne total results of all three days demonstrate that β-Def2 positive cells (29% of all counted cells) predominated in healthy cattle milk and their number decreased markedly in subclinical (10%) and clinical (2%) groups, which was also confirmed by statistical analysis giving $p<0,001$ (Table 3C). In the case of β-Def3 factor, it was also predominant in milk from healthy cattle (49%) and differed significantly from both subclinical (28%) and clinical (4%) group. Among the last two, there was also observed a statistically significant ($p=0.012$) difference (Table 3D).

Results for the CLL37 (Fig. 6) and TGF-β1 (Fig. 7) immunoreactive cells showed statistically significant difference ($p<0.001$) between the negative and the positive cells among all animal groups and sample collecting days (Table 2E and F). As for CLL37, this factor was observed in a very low number of cells (2-3% of all cell counted) in healthy and subclinical cattle milk, and was completely absent in the clinical group (Fig.1E). There was also no statistical difference in the mean values of CLL37 positive cells for all checked parameters or for the sum of mean values calculated over the three sampling days (Table 3E). On the other hand, TGF-β1 was detected in a very high number of cells (85-97% of all cells counted), and that did not change significantly between animal groups and between sampling days (Fig. 1F). Similarly, there was no difference between the sum of mean values of TGF-β1 positive cells for all three days between all animal groups (Table 3F).

Discussion

This study aimed at analyzing the presence and change in the levels of anti-inflammatory cytokines IL-2, IL-10, β-Def2, β-Def3, CLL37, and TGF-β1 in the milk of dairy cattle in relation to the health condition of the animals, as they could serve as signs or biomarkers indicating an early stage of mastitis.

Analysis conducted for the study showed that the levels of IL-2 and TGF- β1 were high and quite stable



* – statistically significant difference (p<0.05)

Fig. 1. Graphic illustration of mean number of IL-2, IL-10, β-Def2, β-Def3, CLL37, and TGF-β1 immunopositive cells in the milk of healthy animals and cows with subclinical and clinical mastitis, recorded on the 1st, 2nd, and 3rd day of sampling.

across the sampling days in the milk of dairy cows both with and without mastitis. In contrast, Alluwaimi (2000) demonstrated that IL-2 expression in the late stage of the lactation period in milk cells was low. Meanwhile, Britti et al. (2005) showed that during mid- and late-lactation the T-helper lymphocytes predominantly act as Th1 (cell-mediated immunity) and synthesize IFN-γ and IL-2. Furthermore, in the bovine mammary gland experimentally infected with *S. aureus*, the IL-2 level indicated a significantly continuous decrease during the early inflammation (Alluwaimi et al. 2003). As for TGF-β1, several authors reported that milk samples from udder quarters infected by *E. coli* (Chockalingam et al. 2005) and *S. aureus* (Bannerman et al. 2006) had increased levels of TGF-β1, and these elevations were sustained throughout the study. In a later study, Bannerman et al. (2008), checked the levels of different cytokines (IFN-y, IL-12, TGF-a, and TGF-β1) in milk

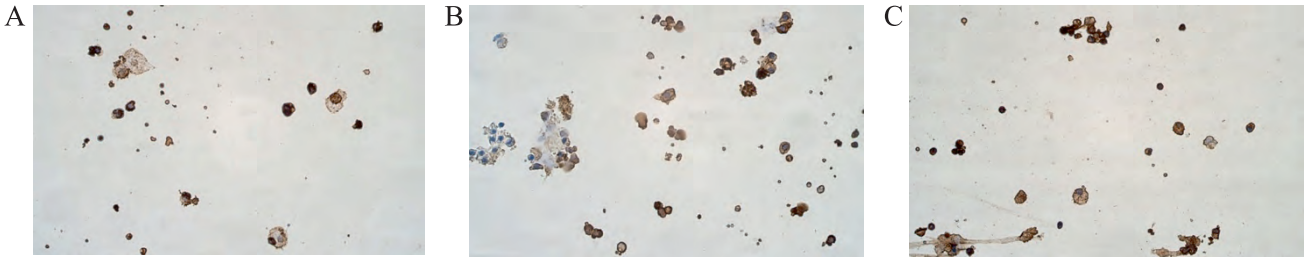


Fig. 2. Interleukin-2 positive cells in the milk of: (A) a healthy cow (sample ID: 2784C), (B) one with subclinical mastitis (sample ID: 2790C), and (C) one with clinical mastitis (sample ID: 4446D) (immunohistochemistry, IHC, x400).

Table 3. Mean immunoreactive cell number of all analyzed factors found in the milk of healthy cattle and cows with subclinical and clinical mastitis.

A

Animal group	IL-2 positive cells		
	Mean†	±	SEM
Healthy	97,47*	±	4,31
Subclinical	89,27	±	4,31
Clinical	95,87	±	4,31

B

Animal group	IL-10 positive cells		
	Mean†	±	SEM
Healthy	90,40*	±	5,40
Subclinical	30,40*	±	5,40
Clinical	5,4	±	5,40

C

Animal group	β-Def-2 positive cells		
	Mean†	±	SEM
Healthy	28,53*	±	4,51
Subclinical	9,87	±	4,51
Clinical	1,53	±	4,51

D

Animal group	β-Def-3 positive cells		
	Mean†	±	SEM
Healthy	48,73*	±	8,99
Subclinical	28,07*	±	8,99
Clinical	4,2	±	8,99

E

Animal group	CLL37 positive cells		
	Mean†	±	SEM
Healthy	1,93	±	1,56
Subclinical	1,80	±	1,56
Clinical	0,00	±	1,56

F

Animal group	TGF-β1 positive cells		
	Mean†	±	SEM
Healthy	96,80	±	6,42
Subclinical	87,20	±	6,42
Clinical	84,93	±	6,42

SPSS 23, MANOVA, LSD, * – statistically significant difference ($p<0.05$)
† mean values of immunoreactive cells from three milk collecting days

samples of Holstein and Jersey cows at various time points after intramammary infusion of *S. aureus* and observed that concentrations of TGF-β1 showed an increase within 6 hours post-infection.

We also recorded high levels of expression of IL-10, β-Def2, β-Def3 in milk samples of healthy animals, and a significant decrease in readings for the milk of animals with subclinical and clinical mastitis. Bannerman (2009) observed that mastitis caused by various species of pathogens, both experimentally and naturally induced, results on the increase in IL-10 and other cytokine (e.g. Il-1, IL-6, IL-8, IL-12) concentration and an increase in the somatic cell count. However, bovine milk with high and persistent concentrations of bacteria shows absent or postponed IL-10 secretion. Bochniarz (2017) showed lower levels of IL-10 in the serum and milk of cows with subclinical mastitis induced by Coagulase-negative staphylococci in comparison with healthy cows.

Concerning defensins, they are little studied in terms of intramammary infections. Petzl et al. (2008) indicates the selective influence of *E. coli* and *S. aureus* on the level of defensins in milk, and presents IHC identified mammary epithelial cells as sites for the upregulated beta-defensin expression. The are some data about the lingual antimicrobial peptide (LAP), belonging to the beta-defensin family and which is localized in the epithelial cells of alveoli in mammary glands and in milk of cattle (Isobe et al. 2009). LAP concentrations increase in milk from mastitic udders and show a positive correlation with the somatic cell count (SCC). The concentration of LAP in milk infected with S. aureus, *Streptococcus bovis*, *Streptococcus dysgalactiae*, and *E. coli* was found to be significantly higher than that in uninfected milk (Kawai et al. 2013). Kitano et al. (2020) states that the concentration patterns of LAP secreted in milk from the udders of healthy lactating cows follow the baseline data. These distinct concentration patterns might indicate various protective responses.

Interestingly, CLL37 positive cells were practically absent in all milk samples collected both from healthy

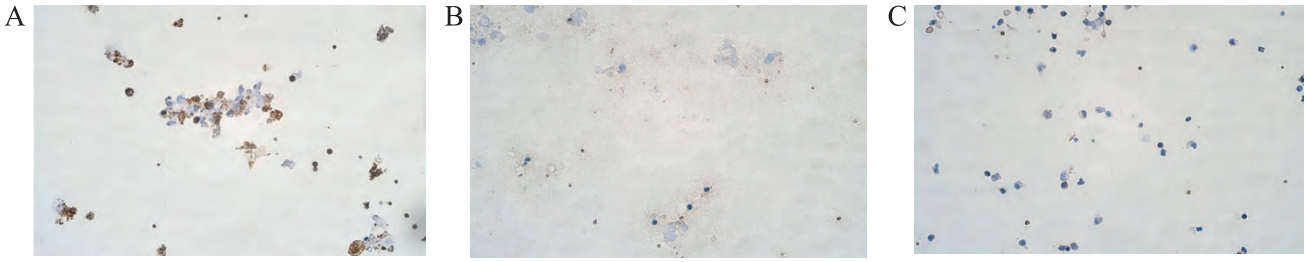


Fig. 3. Interleukin-10 positive cells in the milk of: (A) a healthy cow (sample ID: 2780A), (B) a cow with subclinical mastitis (sample ID: 1518D), (C) a cow with clinical mastitis (sample ID: 4446D) (IHC, x400).

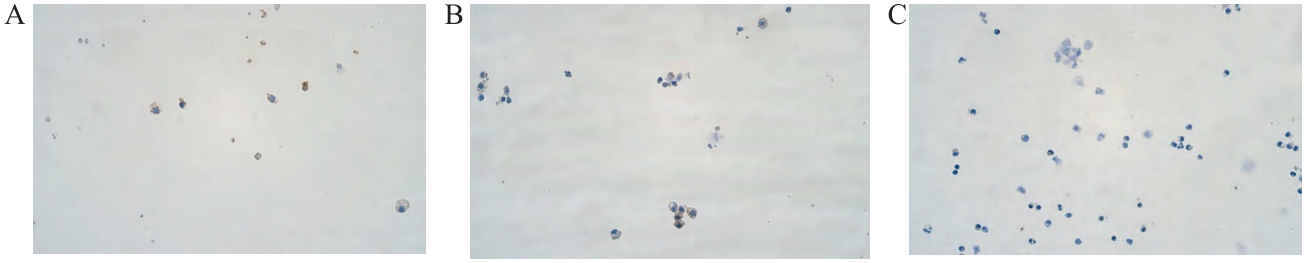


Fig. 4. β-Defensin 2 positive cells in the milk of: (A) a healthy cow (sample ID: 2784C), (B) a cow with subclinical mastitis (sample ID: 2790C), (C) a cow with clinical mastitis (sample ID: 4446D) (IHC, x400).

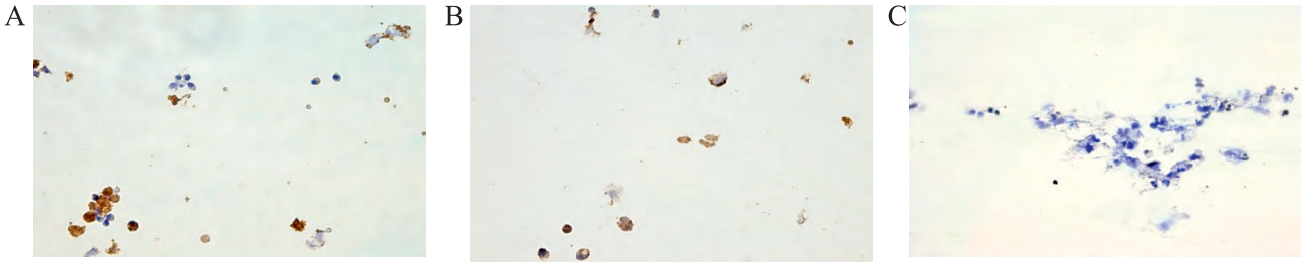


Fig. 5. β-Defensin3 positive cells in the milk of: (A) a healthy cow (sample ID: 2784C), (B) a cow with subclinical mastitis (sample ID: 2790C), (C) a cow with clinical mastitis (sample ID: 2753C) (IHC, x400).

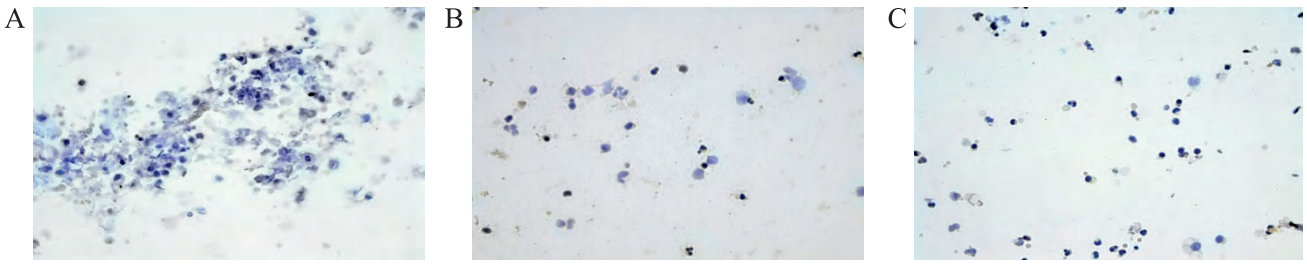


Fig. 6. Cathelicidin LL37 positive cells in the milk of: (A) a healthy cow (sample ID: 1523C), (B) a cow with subclinical mastitis (sample ID: 2790C), (C) a cow with clinical mastitis (sample ID: 4446D) (IHC, x400).

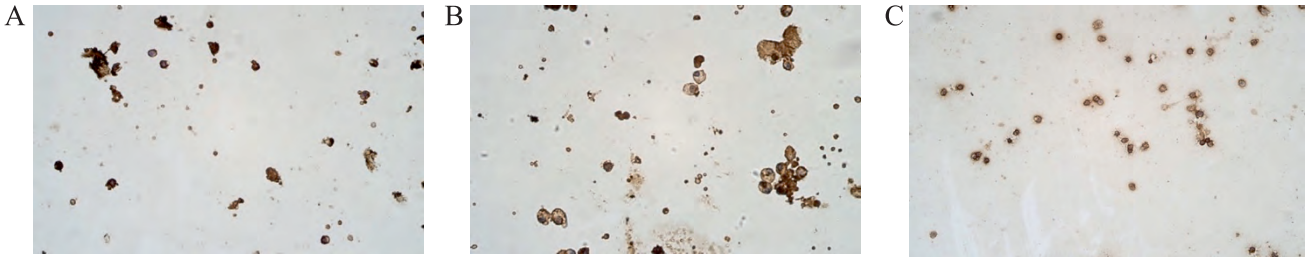


Fig. 7. Transforming growth factor-β1 positive cells in the milk of: (A) a healthy cow (sample ID: 1523C), (B) a cow with subclinical mastitis (sample ID: 2790C), (C) a cow with clinical mastitis (sample ID: 1512D) (IHC, x400).

cows and those with subclinical and clinical mastitis. Meanwhile, other authors reported that during natural inflammatory infections with S*treptococcus agalactiae* (Pongthaisong et al. 2016), *S. uberis* (Eckersall 2019), and *Serratia marcescens* (Addis et al. 2016) cathelicidin levels increase, and even suggested that this increase might serve as a biomarker for determining the stage of infection in dairy cows (Addis et al. 2017). Wollowski et al. (2021) measured cathelicidin in the milk samples from healthy cows and those with subclinical mastitis and showed that cathelicidin was found only in a half of cows with mastitis. Difference in cathelicidin levels in cattle milk might be explained by the influence of different bacteria on the udder tissue.

The mechanism of action of anti-inflammatory factors during the inflammation and with the increase of SCC count may have not only an individual effect, but also a combined effect on the immune response inside the udder. However, the synergistic effects between factors in bovine milk are little studied (Bartee and McFadden 2013, Komai et al. 2018). Thus, in the cow's milk with mastitis, leukocytes are the main producers of IL-2 (by T helper-1 cells) (Alluwaimi 2000), IL-10 (by T helper-2 cells) (Moore et al. 2001, Saraiva and O'Garra 2010), defensins (also by epithelial cells) (Schneider et al. 2005), and cathelicidins (by neutrophils) (Cubeddu et al. 2017). At the same time, TGF-β1 is synthesized by various cells (Dallas et al. 2008). All of them can activate or suppress other cells, e.g. some T cells, macrophages, and epithelial cells. Unfortunately, there is little detailed information regarding the proportions of specific leukocytes in the milk of cows with different pathological stages of bovine mastitis. Paudyal et al. (2008) observed changes in the proportions of leukocytes in milk, that were associated with categories of total leukocyte counts, levels of milk yield, and mastitis-causing pathogen groups, but the deviations were found to be small.

Our data might differ from the results presented by other authors since there are different mechanisms underlining the development of mastitis. Some of the impacting factors, for example, are whether the infection of the bovine mammary gland tissue was caused by contagious or environmental pathogens, or whether it was natural or experimentally induced. The cattle immune system can be triggered differently in these varying conditions, and with that the release of anti-inflammatory factors can also be different. Contradictory data in comparison with other studies may also arise due to various animal factors (e.g. cattle breed, age, udder structure, and nutritional stress) and environment factors (e.g. season, temperature, and humidity), which can also affect the number of different cytokines released by leukocytes and epithelial cells of the mammary gland tissue in dairy cows (De Vliegher et al. 2012, Cheng and Han 2020).

The number of animals involved in our study was small, but still corresponds to the standards for experiments using morphological methods for diagnosis. These included the detection of the expression of various diagnostic cytokines in milk samples. However, in future studies we will consider using larger number of animals for the diagnostic purpose. Another limitation for this study is the use of only the IHC method, and we assume that ELISA and mRNA expression will also be used for diagnostic purposes in further parts of this international project.

Conclusions

The stable increase of IL-2 and TGF-β1 expression within the 3 days of mastitis-affected cows and similar expression of these factors in the milk of healthy animals might indicate the possible lack of such cytokine involvement in the course of early udder inflammation.

The significant decrease of IL-10, β-def2, and β-def3 levels in milk samples of animals with subclinical and clinical mastitis possibly indicates reduced anti-inflammatory protection of the affected udder. With caution we suggest that these molecules can be considered as potential biomarkers for early detection of subclinical and clinical mastitis. Only few CLL37 positive cells detected in the milk of healthy cows and those with mastitis remove this cathelicidin from the significant diagnostic biomarkers in the case of cow bovine mastitis.

Acknowledgements

We would like to express our gratitude to the farm owners, Mirosław and Janina Gontar, who take care of the animals and help to collect milk samples for performing our research, and as well as to our Laboratory Assistant Natālija Moroza, who prepared and processed milk smear samples for further analysis.

The project is implemented under ERA-NET ICRAD (International Coordination of Research on Infectious Diseases) and co-financing by the Spanish State Research Agency (AEI/10.13039/501100011033; PCI2020-120693-2), Hungarian National Food Chain Safety Office, Polish National Centre for Research and Development, and Latvian State Education Development Agency. This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement no. 862605.

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