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Original Paper

COMPOSITION OF MASTITIS CAUSING MICROORGANISMS AND CYTOKINES IN HEALTHY COW'S MILK: A PILOT STUDY

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The aim of this study was to examine clinically healthy cow's udder milk microbiota and presence of cytokines in different seasons. Milk samples taken from the cows were checked for the presence of Gram-positive and Gram-negative bacteria, and the somatic cell count was detected. Immunohistochemistry methods were performed to detect interleukin (IL) -2, IL-4, IL-8, IL-10, IL-12, IL-17a, β-defensin-3, transforming growth factor (TGF)-β1, interferon-γ and nuclear factor (NF)-κB presence in the milk. S. agalactiae, S. uberis, S. aureus, E. coli, *and* Klebsiella, Enterobacter, Citrobacter *spp. were found in healthy cow's milk. In the first round, the highest prevalence was observed for* S. aureus*. In the second round, the highest mean levels were observed for* S. uberis*, then followed* S. aureus*. IL-4, IL-17a and TGF-β1 demonstrated the highest expression in the milk samples. NF-κB had the lowest expression among all factors. The presence of a rich bacterial microbiome (mostly* S.aureus*,* S.uberis*) in the milk of healthy animals, as well as changing bacterial species between in spring and autumn seasons occur as a result of both the immune state of the animal and many external factors, which consequently affects the amount of expressed cytokines.*

Keywords: *microbiota, interleukins, immunocytochemistry, microscopy, growth factors.*

INTRODUCTION

Dairy products, unfortunately, are an ideal culture medium for pathogenic microorganisms (Delavenne *et al.*, 2012). The most common monitoring methods and indicators of milk quality that are used to detect possible pathology in animals are the evaluation of somatic cell counts (SCC) in milk samples, electrical conductivity of milk, detection clinical signs (temperature, pain etc.) and bacteriological analysis (De Silva *et al.*, 2016; Sørensen *et al.*, 2016; Paudyal *et al.*, 2019).

Milk obtained from a healthy bovine mammary gland usually contains few or no viable bacteria (Ruegg, 2022). In 2022, Steinberg *et al.* reported that milk microbiota is associated with udder health status and breed of animals (Steinberg *et al.*, 2022). After start of milking, the mammary gland becomes a functionally open system with a direct connection to the environment (Taponen *et al.*, 2019). The pathogenic bacteria are part of the environment and are known to be widely distributed in the area of ruminant habitat (Gopal *et al.*, 2015). Bacteria present in expressed milk are likely to come from contamination through bacterial ex-

posure of the udder as well as from endogenous sources via a yet hypothetical enteromammary pathway (Oikonomou *et al.*, 2020). Studies of various aspects and pathways through which pathogenic bacteria can enter the mammary gland of healthy animals are gaining great popularity (Verdier-Metz *et al.*, 2012; Braem *et al.*, 2013). Previous studies also showed the impact of seasonal change on biodiversity of microorganisms in cow's milk (Doyle *et al.*, 2016).

In response to the invasion of pathogenic bacteria, udder tissue is protected by various defence mechanisms, from the teat (providing a physical barrier) and subsequently by innate and adaptive immunity (Riollet *et al.*, 2000; Porcellato *et al.*, 2020).

Interleukin-2 (IL-2) is a cytokine secreted by activated T lymphocytes, which induces the proliferation of activated mononuclear cells and epithelial cells (Forseca *et al.*, 2009; Zecconi *et al.*, 2009). Interleukin-4 (IL-4) produced by Th2 and natural killer T-cells as well as by mast cells and basophils stimulates the formation of B lymphocytes, affects endothelial cells, and participates in haematopoiesis (Luzina *et al.*, 2012). Interleukin-8 (IL-8) is a pro-inflammatory cytokine and a chemoattractant for neutrophils. It is produced by epithelial cells, endothelial cells, and cells of the immune system with toll-like receptors (Ridhowi *et al.*, 2018; Zinicola *et al.*, 2019). Interleukin-10 (IL-10) is an anti-inflammatory cytokine that limits the immune response to pathogens. 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 (Saraiva and O'Garra, 2010). Interleukin-12 (IL-12) promotes the proliferation and activation of cells and provides immune cell differentiation (Comminis *et al.*, 2010). Interleukin-17a (IL-17a) produced by multiple cell types of both the adaptive and the innate immune systems has an important role in host defence against various microbial and non-microbial pathogens (Chen and Kolls, 2017).

Interferon-γ (IFN-γ) is a multifunctional cytokine secreted by different cells; in innate immune response B lymphocytes, natural killer cells are main producers of IFN-γ, while during adaptive immune response – CD4+ T cells and CD8+ T cells (Ivashkiv, 2018). Transforming growth factor-β1 (TGF-β1) is an anti-inflammatory member of the TGF-β superfamily of cytokines and is produced by a variety of cells, further performing regulation of cellular growth, proliferation, differentiation, and apoptosis functions (Tzavlaki and Moustakas, 2020). β-defensins are peptides that have been reported to function against Gram-negative, Gram-positive bacteria, viruses, fungi, and other unicellular parasites (Guaro *et al.*, 2017). Nuclear factor-κB (NF-κB) has an essential role in many biological processes, such inflammation and immunity-related processes (Khan *et al.*, 2020).

Along with the existing methods, obtaining data on the microbiota and cytokines of milk can be used to monitor the health of the cattle's mammary gland. Thus, the aim of this study was to examine mastitis-causing strains in clinically healthy cow's udder milk and presence of cytokines in spring and autumn seasons.

MATERIALS AND METHODS

Animals. Animals selected for this study were kept in a free stall type farm (in Northern Poland) and came from a herd of 79 purebred Polish Holstein Friesian cows (41 adult animals from which 35 were milking cows; 38 non-lactating young cows). Before each milk collecting round, the health status of animals was examined according to the mastitis severity scoring provided by the Polish Society of Animal Production Polskie Towarzystwo Zootechniczne (Jakiel *et al.*, 2011). Before sample collection, udders were cleaned, dried, and disinfected. The foremilk was discarded before taking the sample. Milk was collected manually after removing the milking apparatus from the udder. The milk samples were aseptically collected in sterile containers from each udder quarter of each cow after the standard milking procedure without affecting the cattle's welfare and immediately transported to the laboratory to conduct SCC measurements, prepare sediments and perform microbiological analysis. Milk samples were checked for the presence of Gram-positive and Gram-negative bacteria, and the SCC was detected (examination was made at RIC Pro-Akademia, Poland). LactoScan SCC based on fluorescent image cytometry (ISO 13366-1 IDF 18-1) was used to detect SCC in quarter milk of animals selected for the study (cow number × udder 4 quarters). Each measurement was replicated 16 times with the range of 10,000–10,000,000 cell/ml.

In both, spring (1st round) and autumn (2nd round) milk sample collection rounds, five clinically healthy dairy cattle with low SCC (under 200,000 cells/ml) in milk were selected for the study. Average age of animals was five years, average day of lactation — 182, and average daily yield — 35.31 (kg of milk/day).

Bacteriological examination. Collected milk samples were 10-times diluted in 0.9% saline solution. A series of ten-fold dilutions were made from the first suspension and inoculated directly on the surface of the appropriate medium by an automatic spiral plater (easySpiral®Pro, Interscience, Saint Nom la Bretêche, France). Each sample dilution (0.05 ml) was surface inoculated on Plate Count Agar (Biomaxima, Lublin, Poland) for a total count of aerobic mesophilic bacteria and incubated at 30 °C for 48 h in an incubator according to the ISO 4833-2:2013 standard for total bacteria count. The same amount of sample was plated on prepared Petri dishes with solidified CHROMagar Mastitis Gram-Positive and CHROMagar Mastitis Gram-Positive media. The mentioned plates were placed in an incubator at 37 °C for 24 hours. For each microorganism group, manual counts were made and the characteristic growth was described following the manufacturer's medium manual. Finally, the results were expressed as the decimal logarithm of the count of Colony Forming Units in one millilitre of milk ($\log_{10}$CFU/g).

Milk sample collection. To evaluate the appearance of cytokines, samples of milk were prepared at RIC Pro-Akademia (Poland) two times (at the spring and autumn). Before sample collection, which was performed in the morning, udders were cleaned, dried, and disinfected. The foremilk was discarded before taking the sample. Milk was collected manually after removing the milking apparatus from the udder. The milk samples were aseptically collected in sterile containers from each udder quarter of each cow after the standard milking procedure without affecting the cattle's welfare and immediately transported to the laboratory to conduct SCC measurements, prepare milk sediments and perform microbiological analysis. The samples were analysed within a few hours of sampling. Samples awaiting analysis were kept at 5 °C. Milk samples for cytokine analysis were separated into eppendorfs 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 Institute of Anatomy and Anthropology, Rīga Stradiņš University, Latvia. Then samples were prepared by centrifugation at 2000 rpm/min for 3 min to separate sediment. From each Eppendorf, the precipitate was collected and centrifuged again at 2000 rpm/min for 3 min. The supernatant was repeatedly removed. The remained precipitate was stored in portions at –20 °C until it was used in immunohistochemistry (IHC) staining.

Cytokine analysis. Cow milk samples that were tested by immunohistochemical analysis of different anti-inflammatory proteins were smeared on a microscope slide and fixed by methanol-acetone mixture for 2 h. The IHC method was performed to detect IL-2 (ab92381, 1:250, Abcam, UK), IL-4 (orb10908, 1:100, Biorbyt Ltd., UK), IL-8 (sc264 28343, 1:100, Santa Cruz Biotechnology Inc., USA), IL-10 (sc-8438, 1:100, Santa Cruz, USA), IL-12 (orb10894, 1:100, Biorbyt Ltd., UK), IL-17A (ab79056, 1:200, Abcam, UK), β-defensin-3 (ab19270, 1:200, Abcam, UK), TGF-β1 (cs-130348, 1:100, Santa Cruz, USA), IFN-γ (ab218426, 1:100, Abcam, 268 UK), and NF-κB (ab16502, 1:100, Abcam, UK) in the milk. Microslides with milk samples were evaluated by four independent scientists using a Leica light microscopy at 400 times magnification. The calculation of positively stained cells for each investigated IHC biomarker was performed by semiquantitative counting method, by calculating 100 cells for each smear. Cells in milk smear samples were graded into three groups: 0 – no positive structures; + – weak positive structures; ++ – strong positive structures (Gulbe *et al.*, 2020).

Statistical analysis. 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 of reaction to the factors, data were presented as absolute numbers, whose mean values with standard error were displayed in tables and graphs.

To compare the difference of mean values between positive and negative cells, the paired sample test was applied (Riffenburgh and Gillen, 2020). Significant differences between mean values of positive cells for each sample collecting day and between seasons were tested by one-way ANOVA, LSD (Riffenburgh and Gillen, 2020).

RESULTS

Somatic cell count in cow milk. Animals selected for the study had low SCC (under 200,000 cells/ml) in their milk, which made them relevant for further milk analysis. The mean SCC in the spring sample collection was 25,866 cell/ml in day 1, and then slightly decreased to 21,645 cells/ml and 21,252 cells/ml in days 2 and 3, respectively. In the second sampling round/autumn sample collection mean SCC was 23,638 in day 1 and slightly declined to 20,315 cells/ml and 16,885 cells/ml in days 2 and 3 (Fig. 1). In both the first round and second round, statistical analysis did not reveal any statistical difference in the mean SCC between the days of milk collection. Comparison of all three-day sample mean SCC number between rounds also did not show any statistical difference.

Bacterial examination. A variety of bacteria communities were identified in the healthy cattle milk samples collected in both study rounds. The logarithmic values of the total bacterial count (TBC) were calculated for all quarter milk samples, with consequent identification of pathogenic microorganisms in milk.

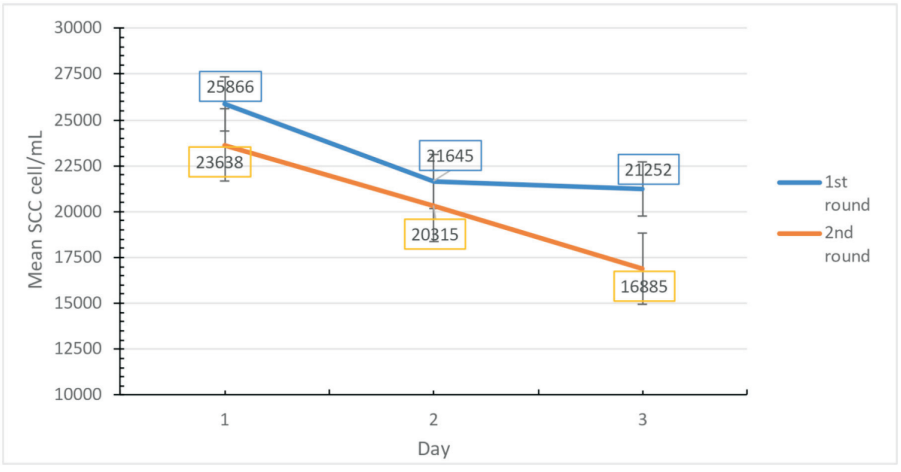

Fig. 1. Somatic cell count (SCC) in the milk of healthy cattle.

In the first sampling round, the TBC was 2.59 ($\log_{10}$CFU/ml) in day 1 and then gradually increased to 3.34 ($\log_{10}$CFU/ml) and 3.69 ($\log_{10}$CFU/ml) in days 2 and 3. There was a weak, but statistically significant difference ($p = 0.049$) of TBC values between days 1 and 3. In the second sampling round the TBC was 2.81 ($\log_{10}$CFU/ml) in day 1, then increased to 3.80 ($\log_{10}$CFU/ml) in day 2, but then decreased to 3.40 ($\log_{10}$CFU/ml) in day 3, not showing any statistical differences (Fig. 2).

S. agalactiae, *S. uberis*, *S. aureus*, *E. coli*, and *Klebsiella*, *Enterobacter*, *Citrobacter* spp. were found in healthy cow's milk from both the first and second rounds, and counts varied by day of sampling.

In the first round, the highest prevalence was observed for *S. aureus*, and the mean value from all three days was 2.72 ($\log_{10}$CFU/ml). The next most common species was *S. uberis* — 2.45 ($\log_{10}$CFU/ml), followed by representatives of *Klebsiella*, *Enterobacter*, *Citrobacter* spp. — 0.94 ($\log_{10}$CFU/ml), *E. coli* — 0.64 ($\log_{10}$CFU/ml), and *S. agalactiae* — 0.39 ($\log_{10}$CFU/ml). In the second round, the highest mean counts were observed for *S. uberis* 2.73 ($\log_{10}$CFU/ml), then followed *S. aureus* with 2.48 ($\log_{10}$CFU/ml). *Candida albicans* was additionally detected in the second round on the third day of milk sampling.

During the first round, among the bacteria found in milk samples, the *S. aureus* count was significantly higher ($p = 0.043$) than for *S. uberis* (day 1), *E. coli*, *S. agalactiae*, and *Klebsiella*, *Enterobacter*, and *Citrobacter* spp. (Fig. 3A). In the second round, in day 1 the *S. aureus* count was significantly higher ($p = 0.043$) than for *S. agalactiae*, *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter* spp., and *C. albicans*. Similar results were found for *S. uberis*. Milk samples from day 2 showed a statistically significant difference ($p = 0.043$) between mean values of *S. aureus* and all other tested microorganisms, while *S. uberis* had statistically higher ($p = 0.043$) mean values than for *S. agalactiae*, *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter* spp., and *C. albicans*. Day 3 milk samples showed slight differences, as *S. aureus* had a significantly higher ($p = 0.043$) mean count than for *S. agalactiae*, *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter* spp. Same results were found for *S. uberis* and *C. albicans*, with $p = 0.043$ and $p = 0.042$, respectively (Fig. 3B).

A significant difference was also found between mean count of *S. uberis*, which was lower in day 1 in comparison with day 2 ($p = 0.047$), and day 3 ($p = 0.028$) in the spring round. In the autumn round, a significant difference was observed in counts of *C. albicans*, which were higher in day 3 in comparison with day 1 ($p = 0.005$), and day 2 ($p =$

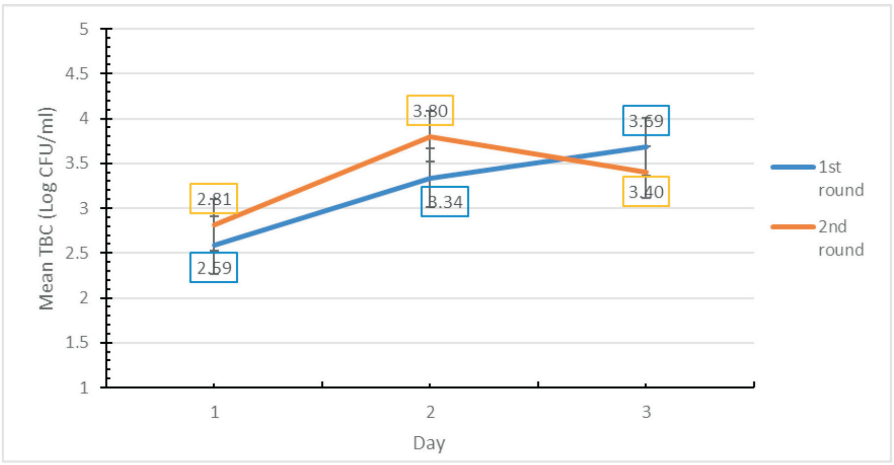

Fig. 2. Mean total bacterial count (TBC) in the milk of healthy cattle.

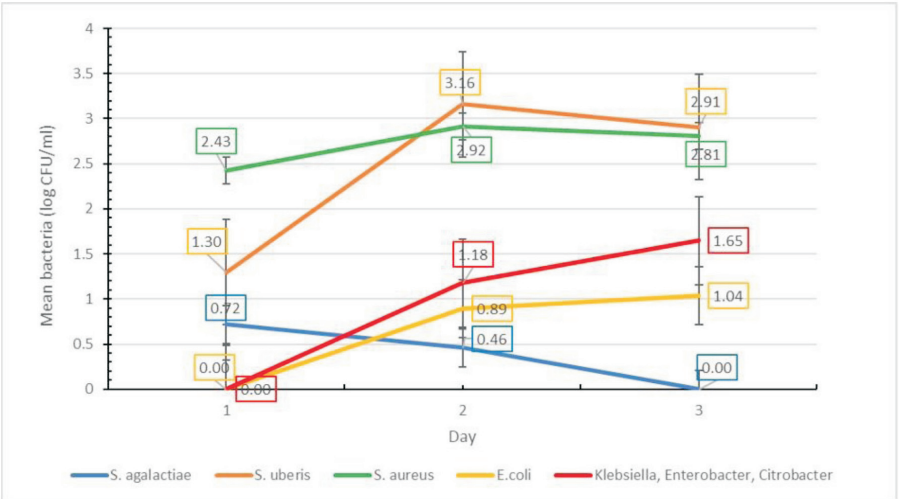

Fig. 3A. Mean total bacterial count in the milk of healthy cattle (1st round).

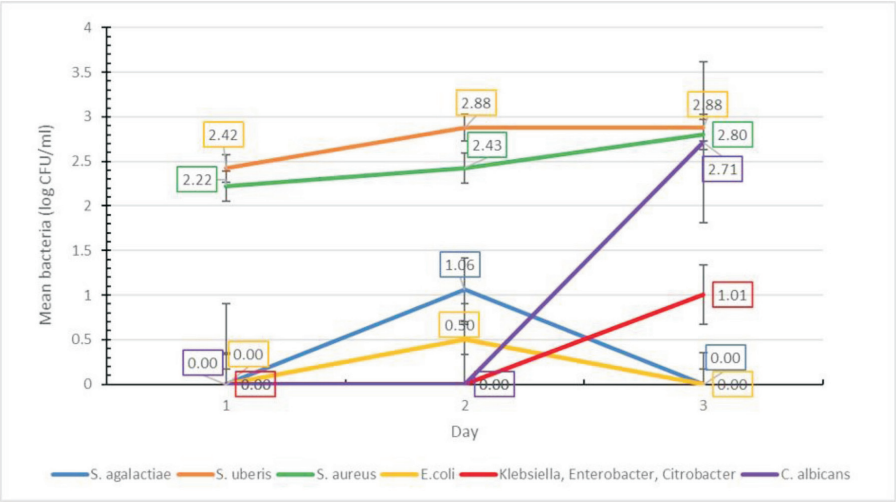

Fig. 3B. Mean total bacterial count in the milk of healthy cattle (2nd round).

0.005). Analysis of all three-day TBC mean values and their comparison between rounds did not show any statistical difference — similar results were obtained from comparison of all evaluated bacteria between rounds.

Cytokine analysis. All of the ten analysed factor positive cells were present in healthy cow milk samples, and the mean counts for each marker did not differ significantly between sample collecting days (Table 1). The only exception was for interleukin-8 (IL-8) counts in the second round; there was a significantly higher (p = 0.009) number of immunopositive cells in day 1 than in day 3 (95.4 and 71 cells from 100 calculated) (Table 2).

Interleukin-4 (IL-4), interleukin-17a (IL-17a) and transforming growth factor-β1 (TGF-β1) demonstrated the highest expression in the milk samples in both rounds (Tables 1 and Table 2; Fig. 4 (B, E, F); Fig. 5 (B, E, F). Interleukin-2 (IL-2) also showed high counts in the first round (Table 1; Fig. 4 (A)), but in the second round the number of IL-2 positive cells slightly decreased, although the changes were not statistically significant (Table 2; Fig. 5 (A)). IL-8 had also high presence in the milk samples, but its expression was observed only in the second round (Table 2; Fig. 5 (J)).

From all examined factors, interleukin-10 (IL-10), interleukin-12 (IL-12), interferon-γ (IFN-γ), β-defensin-3 (β-def-3) showed a difference in expression between milk collecting rounds. IL-10 and IFN-γ showed high expression in the first-round milk samples (p = 0.001) (Table 1; Fig. 4 (C, G)), whereas in the second round IL-10 number decreased almost twice (Table 2; Figure 5 (C)). The number of positive IFN-γ cells not only decreased in the second round, but also fell rapidly with each subsequent day of sampling (Table 2; Fig. 5 (G)). β-def-3 and IL-12 were present in the half of calculated cells in the first round (Table 1; Fig. 4 (D, H)), but had a slight increase in expression in the second round (p = 0.04) (Table 2; Fig. 5 (D, H)). Nuclear factor-κB (NF-κB), and IL-8, was studied only in the second round. This protein complex had the lowest expression among all factors (Table 2; Fig. 5 (I)).

Table 1. Mean number of immunopositive cells in milk of healthy cattle from the first round

Parameter	Day 1					
	++	SD	+	SD	0	SD
IL-2	65	10.16	32	13.88	3	3.71
IL-4	51	8.14	45	2.07	4	6.36
IL-10	81	10.83	10	4.02	9	8.29
IL-12	29	14.98	28	9.12	43	16.66
IL-17a	14	7.97	65	36.16	21	43.96
β-def-3	19	7.20	79	16.07	2	21.72
TGF-β1	19	5.46	33	25.38	48	1.00
IFN-γ	42	22.69	32	28.76	26	20.44

Parameter	Day 2					
	++	SD	+	SD	0	SD
IL-2	77	7.73	23	7.73	0	0.00
IL-4	64	6.88	34	5.03	2	2.12
IL-10	84	6.83	7	6.95	8	4.87
IL-12	28	15.80	26	9.81	46	22.37
IL-17a	21	3.16	74	2.28	5	2.05
β-def-3	17	17.64	81	17.92	2	32.24
TGF-β1	17	1.58	32	2.51	51	1.14
IFN-γ	42	16.96	34	16.76	24	17.42

Parameter	Day 3					
	++	SD	+	SD	0	SD
IL-2	70	13.83	30	13.83	0	0.00
IL-4	54	15.72	43	14.18	3	1.64
IL-10	70	17.82	18	13.28	12	6.83
IL-12	21	11.19	30	11.65	49	19.10
IL-17a	23	4.39	74	3.65	3	1.64
β-def-3	20	5.68	74	18.39	6	21.05
TGF-β1	11	11.25	29	7.23	60	10.06
IFN-γ	35	16.16	34	17.78	31	21.34

IL-2, interleukin-2; IL-4, interleukin-4; IL-10, interleukin-10; IL-12, interleukin-12; IL-17a, interleukin-17a; β-def-3, β-defensin-3; TGF-β1, transforming growth factor-β1; IFN-γ, interferon-γ; ++, strong positive structures; +, weak positive structures; 0, no positive structures.

Table 2. Mean number of immunopositive cells inmilk of healthy cattle from the second round.

Parameter	Day 1					
	++	SD	+	SD	0	SD
IL-2	62	12.60	34	13.86	4	2.97
IL-4	94	3.96	6	3.96	0	0.00
IL-10	19	13.85	22	12.46	59	18.13
IL-12	28	7.22	41	13.13	31	10.09
IL-17a	30	5.68	67	5.18	3	3.08
β-def-3	37	13.87	62	11.43	1	13.05
TGF-β1	40	10.47	23	10.78	37	1.34
IFN-γ	48	37.48	13	12.30	39	33.34
IL-8	53	12.74	42	10.88	5	7.13
NF-κB	0	0.00	16	5.66	84	5.66

Parameter	Day 2					
	++	SD	+	SD	0	SD
IL-2	78	13.07	18	6.07	4	7.87
IL-4	90	17.09	8	13.16	2	4.02
IL-10	17	9.42	23	14.79	60	11.35
IL-12	28	7.16	34	7.65	38	7.54
IL-17a	35	3.65	62	7.09	3	3.83
β-def-3	50	13.13	49	15.17	1	9.24
TGF-β1	37	13.29	26	10.87	37	0.89
IFN-γ	23	27.54	13	6.63	64	25.76
IL-8	39	18.27	41	18.24	20	16.66
NF-κB	6	5.66	24	4.24	70	9.90

Parameter	Day 3					
	++	SD	+	SD	0	SD
IL-2	62	34.94	20	4.00	18	32.42
IL-4	88	4.85	12	4.85	0	0.00
IL-10	22	11.37	26	15.95	52	13.47
IL-12	30	6.14	35	9.24	35	8.96
IL-17a	43	18.93	54	18.12	3	2.12
β-def-3	59	11.15	41	12.10	0	18.59
TGF-β1	36	10.64	27	10.64	37	0.00
IFN-γ	13	16.04	16	9.63	71	24.46
IL-8	32	10.80	39	12.68	29	11.31
NF-κB	3	2.12	23	7.78	74	7.78

For abbreviations see Table 1

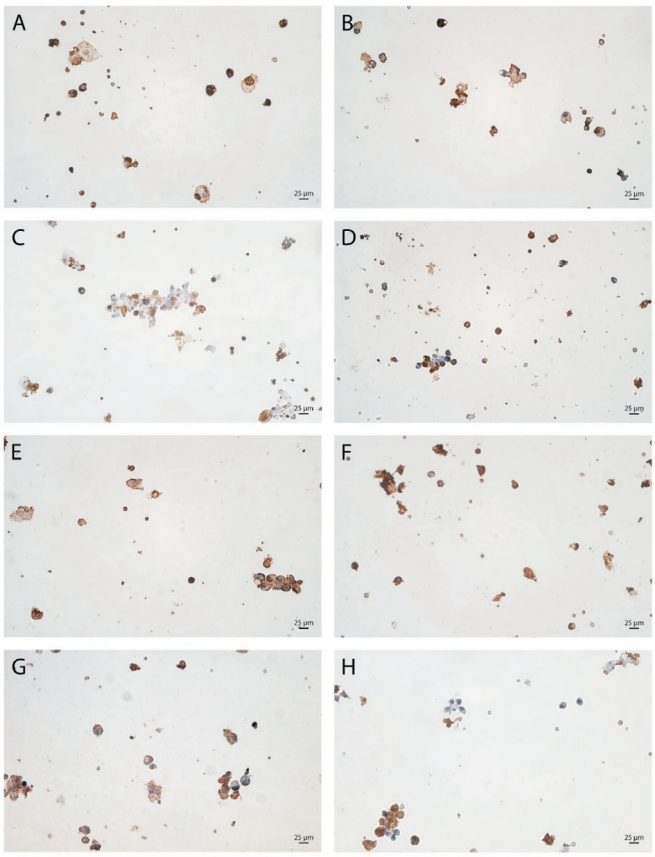

Fig. 4. Immunopositive cells in the milk of the first sample collecting round.

IL-2 (A), IL-4 (B), IL-10 (C), IL-12 (D), IL-17a (E), TGF-β1 (F), IFN-γ (G), and β-def-3 (H) positive cells in the milk of the first sample collecting round (IHC, x400).

DISCUSSION

For a long time, it was believed that milk secreted in the mammary gland alveoli was a sterile substance, but new methods and modern technologies have found opposite results, causing a controversy among scientists (Murphy *et al.*, 2010; Rahimi *et al.*, 2011; Meng *et al.*, 2018). Our study involved animals with no visible signs of disease (e.g., swelling, redness, temperature, or pain of the udder), and the SCC was also within the normal range (d,000 cells/ml). However various bacteria were detected on the plates for the milk samples. Despite the absence of a significantly confirmed difference, in the spring period of milk sampling we observed slightly higher values of each type of bacteria than in the autumn period. In our study, among all the bacteria found, the *S. aureus* and *S. uberis* had significantly higher numbers of colonies. In was previously reported that *E. coli*, *S. uberis* and *S. aureus* are major intramammary pathogens (Keane, 2019). It is interesting that *S. aureus* was dominant in the first round of our study, while *S. uberis* had larger counts in the second round, which may indicate competition between these orrganisms. In a study on milk samples from two farms and in two different seasons, there was a high abundance of two bacterial families, *Corynebacteriaceae* and *Staphylococcaceae*, which accounted for almost 50% of the udder microbiota of healthy cows, and there was a negative correlation between these bacterial families, suggesting competition (Porcellato *et al.*, 2020).

In the second sample collecting round we detected *Candida albicans*. Species of this genus are known to be involved in intramammary infection and can be found on various niches (skin of the udder, milking machines, and other equipment) (Seker, 2010; Dworecka-Kaszak *et al.*, 2012). Many species of *Candida* have also been isolated from cow milk of healthy mammary glands (Zaragoza *et al.*, 2011). Presence of this yeast in our samples might be interpreted as a dynamic community of microbiota or may be fungal ability to overcome the protective mechanisms of the udder due to immunosuppression or damage to the teat.

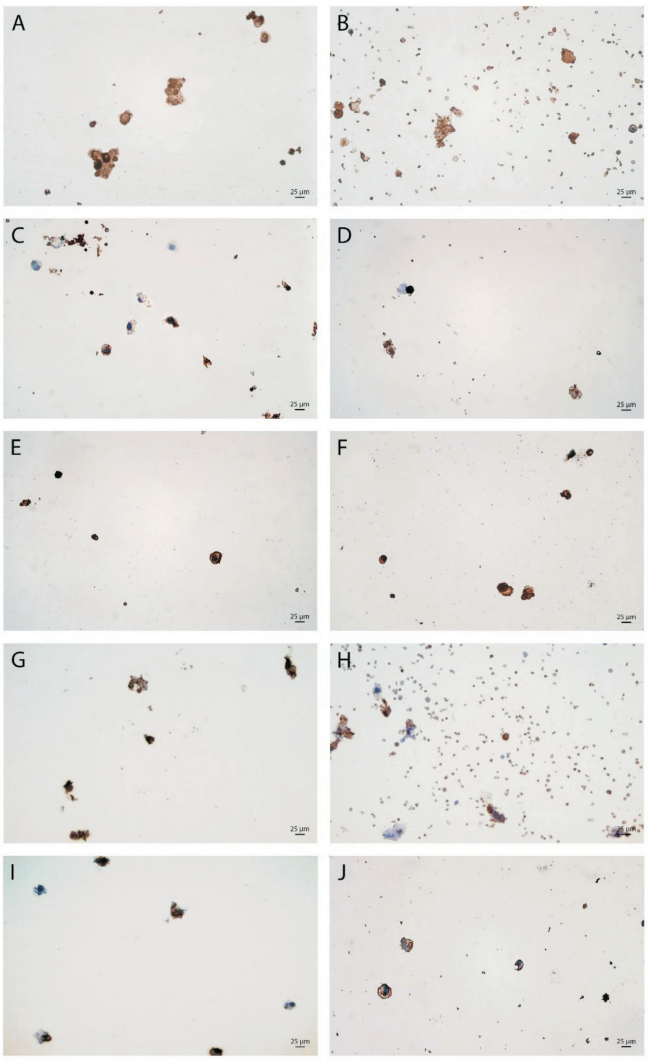

Fig. 5. Immunopositive cells in the milk of the second sample collecting round.

IL-2 (A), IL-4 (B), IL-10 (C), IL-12 (D), IL-17a (E), TGF-β1 (F), IFN-γ (G), β-def-3 (H), NF-κB (I), and IL-8 (J) positive cells in the milk of the second sample collecting round (IHC, ×400).

Cytokine analysis might provide good information on animal health status, as presence, absence or changes can indicate animal immune system defence against pathological bacteria. Proinflammatory (e.g., IL-2, IL-8, IL-12, IL-17a, IFN-γ, NF-κB) and anti-inflammatory (e.g., IL-4, IL-10, TGF-β1, β-def-3) cytokines play important roles in both adaptive and innate immune systems, as they can stimulate or inhibit differentiation, proliferation, degeneration, regeneration, and even cell death (Bannerman, 2009).

Our study showed high numbers of IL-4, IL-17a and TGF-β1 positive cells and stability during all sampling days from both collecting rounds. Other studies also found high levels of IL-4 in serum and in milk of healthy cows in comparison to animals affected by mastitis (Riollet *et al.*, 2000; Fonseca *et al.*, 2009). Cow's milk is a source of growth factors including TGF-β (Panahipour *et al.*, 2018). IL-17a has a protective role and defence properties against micro-organisms (Rainard *et al.*, 2020). Cows suffering from mastitis were found to produce increased levels of IL-17a (Tassi *et al.*, 2013; Roussel *et al.*, 2015).

In the first round we found high levels of IL-2, which decreased non-significantly in the second round. IL-2 stimulates cellular responses against intracellular pathogens and there are studies describing IL-2 treatment for prevention of intramammary infections (Fonseca *et al.*, 2009; Zecconi *et al.*, 2009).

Significant differences between rounds were found for IL-10, IL-12, and IFN-γ positive cell counts. In the spring round, collected milk samples contained more IL-10 and IFN-γ positive cells than in the autumn collecting round. IL-10 can inhibit the activation of T cells, monocytes and macrophages, inhibit the synthesis of proinflammatory cytokines, chemokines, and can also influence hemopoietic cells (Saraiva and O'Garra, 2010). A previous study found that the level of IL-10 was significantly higher in milk of healthy cows than in infected cows (Bochniarz *et al.*, 2017). INF-γ was observed to have low levels in serum and whey taken from healthy cattle, while in mastitic animals in both serum and whey there were significantly higher concentrations (Hisaeda *et al.*, 2001). IFN-γ deficiency is associated with increased susceptibility to infections (Fonseca *et al.*, 2009).

Contrasting results were found for IL-12. We found higher IL-12 positive cells counts in the second collecting round than in the first round. In contrast to IL-10, IL-12 stimulates IFN-γ production and induces natural killer cells to produce other cytokines (Commins *et al.*, 2010). B-defensin-3 also showed a non-significant difference in positive cell values between spring and autumn collecting rounds. Elevated expression of β-defensins in infected udder confirms their key role in the defence of cow mammary glands against mastitis (Kościuczuk *et al.*, 2014). B-defensin increases expression of other cytokines such as IFN-γ, IL-12 and IL-6 (Meade and O'Farrelly, 2014).

Higher IL-8 protein levels were found in mastitis than in healthy udders, although no significant differences were found between the study groups (Kawecka-Grochocka *et al.*, 2021). IL-8 is a potent chemoattractant in recruiting neutrophils and macrophages from the bloodstream to the site of inflammation (Ridhowi *et al.*, 2018; Kawecka-Grochocka *et al.*, 2021). In the current study, we studied IL-8 only in the second round and detected more IL-8 positive cells on the first day.

NF-κB protein complex had the lowest expression among all factors in the current study. Our data are consistent with other studies that did not detect active NF-κB in milk cells from healthy cows (Boulanger *et al.*, 2003; Khan *et al.*, 2020). The activation of NF-κB is highly correlated with granulocyte/macrophage colony-stimulating factor and IL-8 and might play a role in inflammatory processes (Boulanger *et al.*, 2003). Activation of NF-κB is critical for mammary cell differentiation (Zinicola *et al.*, 2019).

CONCLUSION

The presence of a rich bacterial microbiome (*S. aureus*, *S. uberis*) in milk of healthy animals, as well as changing bacterial species between the collecting rounds (in spring and autumn) occurred a result of both the immune state of the animal and many external factors, which consequently affect the amount of expressed cytokines to establish a congruently high expression of IL-2, IL-4, IL-17a and TGF-β1.

ETHICS

The milk collecting procedure was pleasant and in no way harmed the health of the cows, and therefore it did not fall under the Act on the Protection of Animals Used for Scientific or Educational Purposes, adopted in Poland on 15 January 2015 (Journal of Laws/2015/266/1). Thus, no permission from the Local Ethical Commission was required for this study.

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MASTĪTU IEROSINOŠO MIKROORGANISMU UN CITOKĪNU SASTĀVS VESELU GOVJU PIENĀ: PILOTPĒTĪJUMS

Pētījuma mērķis bija izpētīt mikrobiotu un citokīnu klātbūtni pienā, kas iegūts no klīniski veselu govju tesmeņiem dažādos gadalaikos. Tika noteikta Gram-pozitīvo un Gram-negatīvo baktēriju klātbūtne un somatisko šūnu skaits govju piena paraugos. Interleikīna (IL)-2, IL-4, IL-8, IL-10, IL-12, IL-17a, β-defensīna-3, transformējošā augšanas faktora (TGF)-β1, interferona-γ un kodola faktora kappa beta (NF)-κB noteikšanai tika izmantota imūnhistoķīmijas metode. Klīniski veselu govju pienā tika atrastas sekojošas baktērijas — *S. agalactiae*, *S. uberis*, *S. aureus*, *E. coli*. Citi biežāk sastopamie mikroorganismi piederēja *Klebsiella*, *Enterobacter* un *Citrobacter* ģintīm. Pavasara sezonā visaugstāk prevalēja *S. aureus*, savukārt rudens sezonā *S. uberis*, kam sekoja *S. aureus*. Izvērtējot citokīnu sadalījumu piena paraugos, visaugstāko ekspresiju uzrādīja IL-4, IL-17a un TGF-β1, savukārt visretāk tika konstatēta NF-κB klātbūtne. Dzīvnieku imūnais stāvoklis un ārēji faktori nosaka bagātīgo baktēriju mikrobioma (pamatā *S. aureus* un *S. uberis*) klātbūtni un tā mainību atkarībā no sezonas (pavasara un rudens) veselu govju pienā, kas sekojoši ietekmē citokīnu sadalījumu.