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Comparative analysis of total protein, casein, lactose, and fat content in milk of cows suffering from subclinical and clinical mastitis caused by *Streptococcus* spp.

Mariola Bochniarz[1]✉, Przemysław Błaszczyk[2], Marek Szczubiał[1], Iosif Vasiu[3], Łukasz Adaszek[4], Katarzyna Michalak[4], Dorota Pietras-Ożga[4], Marco Wochnik[1], Roman Dąbrowski[1]

[1]Department and Clinic of Animal Reproduction, Faculty of Veterinary Medicine, University of Life Sciences, 20-612 Lublin, Poland
[2]Prevent – Veterinary Practice, 09-304 Lubowidz, Poland
[3]University of Agricultural Sciences and Veterinary Medicine, 400372 Cluj-Napoca, Romania
[4]Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences, 20-612 Lublin, Poland
mariolabochniarz@interia.pl

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Abstract

Introduction: The aim of the study was to analyse the total protein (TP), casein (CAS), lactose (LAC), and fat content of milk from cows with subclinical (SCM) and clinical mastitis (CM) caused by *Streptococcus* spp. **Material and Methods:** A total of 60 milk samples from diseased cows and 30 milk samples from healthy cows were included in the study. Milk samples were taken from Holstein-Friesian cows from four dairy farms in Lublin Province. The bacteriological examination of the milk was performed and the somatic cells count in 1 mL of milk was determined using a SomaCount FC automatic cell counter. Determination of TP, CAS, LAC, FAT and FA levels in milk was carried out using a DairySpec FT automated Fourier transform infrared spectrometer. **Results:** Total protein in milk from HE was significantly higher than in milk from cows with mastitis (4.04% *vs* 3.57% in milk from SCM cows and 3.7% in milk from CM cows , P = 0.001). The CAS level was 2.73% in milk from CM cows and 2.92% in milk from SCM cows *vs* 3.30% in milk from HE cows, P = 0.001. The changes in CAS and TP in milk resulted in a significant difference in the CAS/TP ratio (81.7% in milk from HE cows *vs* 73.8% in milk from CM cows). A decrease in levels was also recorded for LAC (4.8% in milk from HE cows *vs* 4.51% in milk from SCM cows and 4.01% in milk from CM cows, P = 0.001). The fat level was significantly higher in milk from healthy cows than in milk from cows with mastitis (4.0% *vs* 2.3% in milk from SCM cows and 1.64% in milk from CM cows, P = 0.001). **Conclusion:** It should be emphasised that the decrease in the levels of TP, LAC and FAT was significant not only in milk from CM cows but also in milk from SCM cows. This is very unfavourable, because the reduction in the main milk components results in poor quality dairy products and impairs line processes.

Keywords: cows, milk components, mastitis, *Streptococcus* spp.

Introduction

Milk is a secretion of the bovine mammary gland with a particularly complex composition. The major part of milk consists of components produced in the lactating cells: casein (CAS), part of whey protein (non-casein protein), lactose (LAC) and fat (12, 23). These are produced from components supplied with blood to the udder: amino acids, glycerol, fatty acids, glucose and acetates. Other milk constituents directly transfer into the milk from the blood: minerals, vitamins, enzymes, free amino acids and non-protein nitrogenous compounds (15).

Particularly important components of milk are proteins because of their nutritional value for humans. Furthermore, a high total protein (TP) content in milk improves the quality of dairy products (12, 14). Nineteen amino acids have been found in milk proteins, all of

which are exogenous. Among the milk proteins, 80% are casein while 20% are whey proteins (α-lactalbumin, β-lactoglobulin, immunoglobulin, serum albumin, lactoferrin, lysozyme and enzymes) (12). The proteins of milk have antibacterial, antiviral, antifungal, and antioxidant properties (14).

Lactose is the primary milk sugar for most mammals (9). It is produced in the milk-producing cells of the udder from glucose supplied there with blood. The synthesis and concentration of LAC in milk depends primarily on the health of the udder and the energy balance and metabolism of the cow (2, 9). Lactose is widely used in the food and pharmaceutical industries, with cow's milk being the main source (2).

Dairy fat remains the most important and cheapest source of fat in the human diet (23). It consists of several groups of organic compounds that are insoluble in water but soluble in organic solvents. The most important lipid fraction of milk fat is acylglycerols, which account for 96–99%. These are predominantly triglycerides comprising saturated (SFA) and unsaturated (UFA) fatty acids (FA). More than 400 FA have been identified in cow's milk, of which only 14 are present in amounts above 1% (23). Saturated fatty acids (long-medium and short-chain) at 65-75% of total milk fat make up by far the largest proportion. A unique property of milk fat is the presence of short-chain FA (accounting for about 7–10%). The smallest proportion of milk fat is polyunsaturated fatty acids (PUFA) (only 4–5%), which have a very important biological function in the human body, *e.g.* as part of lipid metabolism and a source of tissue hormones, regulation of insulin secretion (7, 11, 23, 25).

Milk and dairy products are key components of the human diet (12). For this reason, the appropriate composition of raw milk is extremely important, the nutritional value not being the only aspect determined by composition, however: the physical properties of milk and dairy products are also functions of the composition of the raw milk from which they were made (11, 34). Milk secretion consists of two processes: cellular metabolism within the milk-producing follicles and the seepage of components from the blood plasma (3, 35). In mastitis, the processes of producing milk components are disrupted, resulting in a decrease in both the quantity and quality of milk and milk products (10, 13, 16).

Mastitis is caused by a wide variety of pathogens (bacteria, fungi, algae, and viruses). Bacteria, of which *Streptococcus* are predominant in dairy farming contamination, are still the main aetiological agents of mastitis (19). *Streptococcus* is a genus of Gram-positive bacteria in the family Streptococcaceae, belonging to the order Lactobacillales (lactic acid bacteria) in the phylum Bacillota. Cell division in streptococci occurs along a single axis, so as they grow they tend to form pairs or chains that may appear bent or twisted. Most streptococci are oxidase-negative and catalase-negative, and many are facultative anaerobes (capable of growing under both aerobic and anaerobic conditions). The proportion of streptococci as the aetiological agent of mastitis varies from a few to several per cent (2–15%). Streptococcal infections occur via the galactogenic route, *i.e.* through the teat canal. Infections can be subclinical and clinical, and an acute or chronic course is possible. The most common *Streptococcus* species found in animals are *S. agalactiae*, *S. dysgalactiae* and *S. uberis* (5, 18, 19). Infectious pathogens such as *S. agalactiae* and *S. dysgalactiae* occur inside the cow's udder and spread from infected to uninfected mammary glands during milking (18). *Streptococcus agalactiae* is able to infect both heifers and older cows and is considered one of the main causes of economic losses in dairy herds without a control programme (18). In contrast, environmental pathogens such as *S. uberis* easily colonise, grow in and multiply in the environment. They are found in many places such as faeces, water, and soil. Significant numbers of bacterial cells in the environment contribute to a higher incidence of mastitis (19).

The aim of the study was to comparatively analyse TP, CAS, LAC, fat and FA content of milk from cows in the course of clinical and subclinical mastitis caused by *Streptococcus* spp.

Material and Methods

Study designs and data collection. All procedures for collecting material for animal testing, carried out within the project, were recognised by the Local Ethical Committee for Animal Experiments in Lublin as routine veterinary services for dairy cows. Therefore, the study was conducted in accordance with European Union regulations contained of the Directive 2010/63/EU on the protection of animals used for scientific purposes.

Milk samples were taken from Holstein-Friesian cows from four dairy farms in the Lublin Province. The herds numbered 68, 326, 480, and 650 cows. Milk samples were taken between September and November 2021. The cows were kept in free-stall housing, without access to paddocks, and fed on the total mixed ration system (a full-dose, year-round system of animal nutrition). The average milk yield was 6,760 kg per cow per year. Milk was sampled during morning milking. As a routine, milk was collected for the California Mastitis Test (CMT) (Mastirapid; Vetoquinol, Lure, France) from each cow before being milked. Scoring of CMT results was according to Quinn *et al.* (28).

In case of a positive CMT result, udders and teats were cleaned using warm water with detergent and next disinfected with disposable wipes soaked in alcohol solution. From each quarter for which the CMT result was positive, three aliquots of milk of approximately 10 mL (for bacteriological testing), 30 mL (for somatic cell count (SCC) determination), and 40 mL (for milk composition determination) were taken into separate labelled sterile tubes. In addition, a clinical examination of the mammary gland and of the cow's health was carried out (appetite, thirst, temperature measurement,

and blood collection for haematological tests). All the information obtained from the medical interview with the veterinarian overseeing the herd and the results of the clinical examination were carefully recorded.

The samples were placed in a transporter at 4°C and delivered to the laboratory of the Department of Animal Reproduction at the University of Life Sciences in Lublin.

The determination of the somatic cell count in 1 mL of milk. One sample of the milk (approximately 30 mL) was used for SCC determination using a SomaCount FC automatic cell counter (Bentley Instruments, Chaska, MN, USA).

Bacteriological examination of milk. Agar medium (BTL, Lodz, Poland) with sterile defibrinated sheep's blood (5% volume of agar solution) was used for microbial culture. The incubation was carried out under aerobic conditions at 37°C for 24 h. Preliminary identification of bacteria based on characteristic morphological features of the colonies was made. A catalase test was performed and Gram-stained microscope slides were prepared. Gram-positive and catalase-negative cocci (*Streptococcus* and *Enterococcus* genera) were qualified for further diagnosis by first using differentiation media. Esculin Blood Agar (Oxoid, Basingstoke, UK) allowed differentiation of bacteria into aesculin-positive (*S. uberis* and *Enterococcus* spp.) and aesculin-negative species (*S. agalactiae* and *S. dysgalactiae*). Kanamycin Esculin Azide Agar (Oxoid) was used to distinguish streptococci from enterococci. To confirm the presence of β-haemolysis, the CAMP test (Christie-Atkins-Munch-Petersen) was performed. The species identification of *Streptococcus* spp. was confirmed by a matrix-assisted laser desorption/ ionisation–time of flight mass spectometry (MALDI-TOF) system (Bruker Daltonics, Bremen, Germany).

Determination of TP, CAS, LAC, FAT, and FA levels in milk. All the analyses were performed using DairySpec FT automated Fourier transform infrared spectrometer (Bentley Instruments), which complies with the International Dairy Federation 141C:2000 guidelines for mid-infrared spectrometric analysis of milk and the requirements of organisations such as the International Committee for Animal Recording and AOAC for testing the composition of milk and dairy products. The analysis achieved accuracy at a coefficient of variation < 1% for all components, typically <0.5%. It also had good repeatability, which was borne out by a coefficient of variation of < 0.5% for all components, typically <0.25%.

Study groups. A total of 426 milk samples showing a positive CMT test with or without macroscopic changes in milk, symptoms of inflammation in the mammary gland and/or systemic signs were taken during the prescribed study period. Cows which in addition to a positive CMT result in milk were suffering from associated metabolic or other diseases were not included in the study. The diseased cows were divided into two groups according to the type of mastitis. Cows with subclinical mastitis (SCM) were included in the first group. In the milk of these cows, the presence of microorganisms was found despite there being no visible changes in the milk nor local or general mastitis symptoms in the animal. The SCC was >200,000/mL in the milk samples from this group. Cows with clinical mastitis (CM) comprised the second group. Abnormalities in these cows' milk samples were visible in the form of clots and flakes, and the animals had visible symptoms of mastitis (swelling, redness or pain of the udder) and occasionally occurring general symptoms. An elevated SCC > 200,000/mL was also taken from these milk samples.

Sixty milk samples from cows with streptococcal mastitis (30 milk samples from SCM cows and 30 milk samples from CM cows) were included in the study. The SCC in the milk of these cows ranged from 238,000 to 2,540,000 cells/mL. Milk samples were also taken from 30 healthy cows to create a control group. The criteria for inclusion of cows in the control group were CMTnegative status, freedom from clinical signs of mastitis, a negative outcome of bacteriological examination, and an SCC level of <100,000 cells/mL. For all animals (of the control group and of the groups with mastitis), a haematological examination (including red and white blood cell counts, and measurement of haemoglobin and haematocrit concentrations were performed using Scil Vet ABC Plus+ Animal Haematology Analyzer (Horiba, Kyoto, Japan) as previously described (1). The results of the study are provided in Table 1.

Table 1. Blood cell counts in cows with clinical and subclinical mastitis caused by *Streptococcus* spp. and in healthy cows

	Clinical mastitis n = 30	Subclinical mastitis n = 30	Healthy cows n = 30
Blood WBC (× 10^3/mm^3)*	8.10	7.10	7.86
lymphocytes (× 10^3/mm^3)*	2.45	3.22	3.65
neutrophils (× 10^3/mm^3)*	3.65	2.05	2.33
eosinophils (× 10^3/mm^3)*	0.51	0.48	0.52
Blood RBC (× 10^6/mm^3)*	6.2	6.8	7.00
HGB (g/dL)*	11.6	12.2	13.40
HCT (%)*	32	34	34

* – average counts for all animals from each group; WBC – white blood cell count; RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit

Statistical analysis. Data were expressed as median, minimum and maximum values and compared using the Mann–Whitney U test. A P-value <0.05 was regarded as statistically significant. The calculations were conducted using the SPSS Statistics 24 package (IBM, Armonk, NY, USA).

Results

The study was conducted on 60 samples of milk from cows with subclinical and clinical mastitis and 30 samples of milk from healthy cows. The *Streptococcus* species found in cows were *Streptococcus dysgalactiae* (17 isolates from SCM and 14 isolates from CM cows' milk), *Streptococcus uberis* (10 isolates from SCM and 12 isolates from CM cows' milk), *Streptococcus agalactiae* (4 isolates from CM cows' milk) and *Streptococcus canis* (3 isolates from SCM cows' milk). One milk sample from each cow was qualified for evaluation of TP, CAS, LAC, fat and FA levels.

Total protein levels in milk from healthy cows ranged from 3.64% to 5.28% (median 4.04%) and were significantly higher than in milk from cows suffering from mastitis caused by *Streptococcus* spp. (3.57% in SCM cows' milk, P = 0.001, and 3.7% in CM cows' milk, P = 0.001). A change in CAS content was also noted. The CAS levels in the milk of diseased cows were 2.73% in CM and 2.92% in SCM cows' milk and were markedly lower compared to those in milk from healthy cows (3.30%; P = 0.001). There was no statistical difference in TP and CAS levels associated with the form of mastitis. However, changes in the CAS/TP ratio were noted. The CAS in milk from healthy cows was 81.7% of TP, while in milk of cows with CM the CAS/TP ratio decreased to 73.8%. It should be noted that in SCM cows' milk the CAS/TP ratio was similar to that in milk of healthy cows (81.8%). The LAC levels in milk from healthy cows ranged from 2.64% to 5.19% (median 4.8%) and were significantly higher compared to milk from cows suffering from SCM (4.51%, P = 0.001) and those suffering from CM (4.01%, P = 0.001). There was no statistical difference between LAC levels in milk from SCM and CM cows. The data for these milk components is summarised in Table 2.

The highest variability in levels was found for fat. In milk of healthy cows, fat levels ranged from 2.98% to 5.92% (median 4.0%) and were markedly higher than those in milk from cows suffering from streptococcal mastitis (2.3% in SCM cows' milk, P = 0.001, and 1.64% in CM cows' milk, P = 0.001). The study also found lower FA levels in the milk of diseased cows than in the milk of healthy cows. In both CM and SCM cows' milk, SFA and UFA levels were lower than in healthy cows' milk (1.07 mmol/L and 1.49 mmol/L *vs* 2.59 mmol/L and 0.27 mmol/L and 0.37 mmol/L *vs* 0.81 mmol/L, respectively, P = 0.001). However, there was no statistical difference in fat and FA levels between the samples of milk from cows with different forms of mastitis. Only the PUFA level was lower in the milk of cows with CM than in the milk of cows with SCM (0.02 mmol/L *vs* 0.03 mmol/L, P = 0.001). Contents of fat and the three FA categories in the investigated milk samples are shown in Table 3.

Table 2. The concentration of total protein, casein and lactose in milk from cows with subclinical and clinical mastitis caused by *Streptococcus* spp. and in milk from healthy cows

Symbol	Sample	n	TP median (range) (%)	CAS median (range) (%)	LAC median (range) (%)
A	Healthy cows' milk	30	$4.04^{B,C}$ (3.64–5.28)	$3.30^{B,C}$ (2.84–4.19)	$4.8^{B,C}$ (2.64–5.19)
B	SCM cows' milk	30	3.57^{A} (2.84–4.29)	2.92^{A} (2.11–3.25)	4.51^{A} (2.2–4.94)
C	CM cows' milk	30	3.7^{A} (2.6–4.32)	2.73^{A} (1.87–3.13)	4.01^{A} (1.61–4.82)

TP – total protein; CAS – casein; LAC – lactose; SCM – subclinical mastitis; CM – clinical mastitis; n – number of samples
Data are presented as median, minimum and maximum values
Statistical analysis was performed using the Mann–Whitney U test. Uppercase superscript letters indicate significant difference (P < 0.05) between this parameter and its counterpart in the group indicated by matching symbol (A–C)

Table 3. The concentration of fat and fatty acids in milk from cows with subclinical and clinical mastitis caused by *Streptococcus* spp. and in milk from healthy cows

Symbol	Sample	n	Fat median (range) (%)	SFA median (range) (mmol/L)	UFA median (range) (mmol/L)	PUFA median (range) (mmol/L)
A	Healthy cows' milk	30	$4.0^{B,C}$ (2.98–5.92)	$2.59^{B,C}$ (1.92–4.21)	$0.81^{B,C}$ (0.49–1.38)	0.06^{C} (0.01–0.12)
B	SCM cows' milk	30	2.3^{A} (0.87–3.74)	1.49^{A} (0.59–2.39)	0.37^{A} (0.04–1.11)	$0.03^{A,C}$ (0.01–0.11)
C	CM cows' milk	30	1.64^{A} (0.86–3.13)	1.07^{A} (0.59–1.83)	0.27^{A} (0.01–0.79)	$0.02^{A,B}$ (0.01–0.06)

SFA – saturated fatty acids; UFA – unsaturated fatty acids; PUFA – polyunsaturated fatty acids; SCM – subclinical mastitis; CM – clinical mastitis; n – number of samples
Data are presented as median, minimum and maximum values. Statistical analysis was performed using the Mann–Whitney U test. Uppercase superscript letters indicate significant difference (P < 0.05) between this parameter and its counterpart in the group indicated by matching symbol (A–C)

Discussion

This study investigated the content of protein, casein, lactose, fat and fatty acids in milk from cows with clinical and subclinical mastitis caused by *Streptococcus* spp.

In our study, a significant difference in TP, CAS, LAC and fat levels was recorded in the milk of cows suffering from streptococcal mastitis compared to the milk of healthy cows. Total protein levels in the samples from diseased cows were 11.6% lower in SCM cows' milk and 8.4% lower in CM cows' milk than in healthy cows' milk. However, there was no marked difference in TP levels between the samples of milk from cows with one mastitis and the samples of milk from cows with the other form, although it should be noted that TP levels in milk from CM cows were slightly higher than they were in milk from SCM cows, with a simultaneous decrease occurring in CAS level. Analogous results were obtained by other authors (17, 29, 33, 34) who found a reduction in TP contents in mastitic milk. In contrast, in a study by Ogola *et al.* (27), the level of TP did not differ between healthy and infected quarters, but a clearly elevated content of the non-casein fraction was noted. The authors suggest that the reason for the lower CAS/TP ratio may be endogenous proteolysis in infected quarters due to intensified enzyme activity (27). One of the most important proteolytic enzymes present in milk from both healthy and infected cows is plasmin. In the udder, the activity of plasmin and other proteases is progessively stimulated with the severity of mastitis, and the result is the degradation of casein (34). Simultaneously in mastitis, activation of the immune response causes an inflow of inflammation-related blood proteins into the mammary gland, leading to increased non-casein protein content, and thus TP in milk is higher in the course of mastitis (8, 20). In our study, a less CAS than TP in milk was recorded for CM cows (73.8%) but not for SCM cows (>80%). In contrast, Coulon *et al.* (8) noticed a lower CAS/TP ratio during subclinical mastitis. The results of this study support the hypothesis that despite a decrease in CAS levels during mastitis, TP increases because of elevated levels of other proteins associated with a strong immune response in the udder. A study by Batavani *et al.* (3) showed that inflammation of the udder causes a significant increase in the concentration of immunoglobulins and albumin in milk. Immunoglobulins prevent adhesion of pathogens to epithelial membranes, inhibit proliferation and agglutination of bacteria and neutralise toxins, while their main function is to opsonise microorganisms for phagocytosis. An increase in milk immunoglobulin and albumin content during mastitis has been reported in cows, sheep and goats (8, 21, 34).

The level of LAC in milk is rather stable and varies much less than fat or TP, so any unexpected decrease in the content of this sugar can be an indicator of a negative metabolic balance or an infection of the udder (2, 9). Changes in the percentage of LAC in mastitis are the result of damage to the secretory cells responsible for its production. Also, the pathogens which cause mastitis contribute to lower LAC levels by using it as a substrate for their growth. Antanaitis *et al.* (2) proved a close relationship between the LAC level in milk and the microorganisms responsible for subclinical mastitis. The greatest fall in LAC was observed in milk from cows suffering from mastitis caused by infectious pathogens such as *S. agalactiae* and *Staphylococcus aureus* (2, 8). Other authors have noted a lower LAC when the udder was infected by environmental and opportunistic microorganisms including non-aureus staphylococci, coliform bacteria and even fungi (4). In our study, there was also a reduction in LAC levels in the milk of cows suffering from streptococcal mastitis. Lactose content in the milk from CM cows was 16.5% lower than this content in the milk from healthy cows. There was no significant difference between CM cows' milk and SCM cows' milk LAC. The strong correlation between milk sugar content and udder infection means that LAC can be considered a potential biomarker of mastitis and an indicator of health in cows (2, 4). Costa *et al.* (9) set a threshold milk LAC level of 4.553%, below which cows had a higher incidence rate of the disorder than cows with LAC ≥ 5.045%.

The least stable component of milk in terms of quantity is fat (26, 32). This is confirmed by the results of our study. Its level in the milk of diseased cows was lower by 42.5% in SCM sufferers and by as much as nearly 60% in CM cattle compared to the level in the milk of healthy animals. A decrease in the fat content of milk was also noted by other authors (6, 11, 33). In the course of mastitis, the degradation of fat in milk, in the same manner as the degradation of proteins, is caused by numerous enzymes which appear in the focus of inflammation. Harjanti *et al.* (16) found that lipolysis of milk globule membranes by leucocyte lipases or by plasmin through the hydrolysis of lipoproteins reduces the synthetic and secretory capacity of the mammary gland and causes a decrease in fat concentration during mastitis. In addition, lipases attack triglycerides and release free FA, which produce unpleasant odours in milk and dairy products (30). This is confirmed by the study of Ogola *et al.* (27) in which free FA levels increased substantially in milk of infected quarters. The negative correlation between milk composition and mammary inflammation is shown by numerous studies (6, 11, 16). In our study, there was a decrease in the level of fat but also in those of SFA and UFA. In milk from cows with mastitis, SFA content was lower by 42.5% in samples from the SCM group and by 58.7% in those from the CM group compared to this content in samples of milk from the healthy group. Equally significant was the decrease in UFA level by 54.3% in SCM cows' milk and by 66.7% in CM cows' milk from the level in healthy cows' milk.

Unfortunately, there was also a major loss of PUFA, which are highly desirable for their health-giving properties. Polyunsaturated fatty acid content in the milk of diseased cows was lower by 50% in SCM sufferers and by 66.7% in cows with CM. Similarly, a study by Chang *et al.* (6) on subclinical mastitis found reduced total FA in milk (saturated, monounsaturated and polyunsaturated fatty acids). However, the results of other authors' studies show a wide variation in the level of FA in the milk of unhealthy cows. Mavangira *et al.* (24) indicated that mastitis may even increase the content of PUFA. In turn, other studies indicate that the lipid composition of milk did not change at all in cases of mastitis caused by coagulase-negative staphylococci (31) or changed only 48–72 h after colimastitis induction (25).

In the present study, changes in the content of TP, CAS, LAC and FAT were found in the milk of cows suffering from mastitis caused by *Streptococcus* spp. compared to the milk of healthy cows. It should be emphasised that the falls in the levels of the main milk components were significant not only in the clinical but also in the subclinical form of mastitis. This is very unfavourable, because the reduction in TP, LAC and FAT content results in poor quality dairy products and impairs line processes, and the lack of visible symptoms in the course of subclinical mastitis means that the disease often goes unnoticed and remains untreated. It is also worth stressing that the parameters determined in the study can serve as indicators of the health of the udder in cows (e.g. LAC) and the suitability of milk for certain processing operations (*e.g.* CAS/TP ratio).

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