The presence of pathogens in milk of ewes in relation to the somatic cell count and subpopulations of leukocytes

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Abstract: Mastitis is a major health problem of the udder in dairy sheep breeds. For diagnosis of subclinical mastitis, somatic cell count (SCC) is commonly used. The presence of pathogens in the udder causes the increase of leukocytes and thus SCC in milk. Therefore, the aim of this study was to evaluate the presence of pathogens in the milk of ewes and the possible relationship with SCC. The changes of leukocytes subpopulation in milk samples with high SCC were evaluated as well. The experiment was carried out on a dairy farm with the Lacaune breed. This study was conducted on 45 ewes (98 milk samples) without signs of clinical mastitis. Based on somatic cell count, samples were divided to five SCC groups: SCC1 < 200 000 cells/ml (45 milk samples); 200 000 ≤ SCC2 < 400 000 cells/ml (10 milk samples); 400 000 ≤ SCC3 < 600 000 cells/ml (six milk samples); 600 000 ≤ SCC4 < 1 000 000 cells/ml (six milk samples); SCC5 ≥ 1 000 000 cells/ml (31 milk samples). No pathogens were observed in the majority of milk samples (60.20%). Coagulase-negative staphylococci (CNS) were the most commonly isolated pathogens from the milk of ewes (86.11%). *Staphylococcus epidermidis* had the highest incidence from CNS (35.48%). In the SCC5 group, up to 79.31% of bacteriological samples were positive. The percentage of leukocytes significantly increased ($P < 0.001$) in the samples with higher SCC ($\geq 200 \times 10^3$ cells/ml) in comparison to the group SCC1. Also, the percentage of polymorphonuclear cells (PMNs) was significantly higher with increasing SCC ($P < 0.001$). In conclusion, the presented results showed that the high SCC was caused by the presence of the pathogen in milk. Thus SCC < 200 000 cells/ml and leukocyte subpopulation, especially PMNs, could be considered as important tools in udder health program applied in dairy ewes.

Keywords: sheep; mastitis; flow cytometry

Mastitis is the major health problem of the udder in dairy animals. This disease is associated with economic losses due to a decrease in milk yield, degradation of milk quality and treatment costs (Eyduran et al. 2013; Aref et al. 2018). The mastitis in dairy ewes causes a problem for food safety (Silanikove et al. 2014). The incidence of clinical mastitis is relatively low; however, subclinical mastitis is a serious problem in dairy ewes' flocks (Moroni et al. 2007), where the prevalence of subclinical mastitis varies from less than 2% to 50% (Gelasakis et al. 2015; Tancin et al. 2017). In general, the somatic cells count (SCC) is used to diagnose subclinical mastitis in dairy ewes (Tvarozkova et al. 2019), though in much less range than in dairy cows. Also, the California mastitis test, measurement of electrical conductivity, milk colour values, milk composition and quality, may be used to detect mastitis (Aytekin et al. 2018). The SCC may vary depending on parity, stage of lactation, season or breed (Souza et al. 2012; Kuchtik et al. 2017; Milerski et al. 2020).

Due to the presence of mastitis pathogens, the increased migration of leukocytes from blood to the mammary gland causes an increase in SCC (Albenzio and Caroprese 2011). Damm et al. (2017) reported that the change in number and present types of leukocytes could provide information about the intramammary infection. The primary role in the immunity of udder and the effective response against pathogens play the immune cells, particularly PMNs (neutrophils) (Souza et al. 2012). Mainly PMNs are dominant types of leukocytes that increased during infection of the mammary gland (Lindmark-Mansson et al. 2006). Earlier studies revealed that numbers of leukocytes, mainly neutrophils, were crucial for the elimination of intramammary infection (De Matteis et al. 2020).

The major types of pathogens causing subclinical mastitis in ewes are coagulase-negative staphylococci (CNS), although other pathogens, such as *Staphyloccocus* (*S.*) *aureus*, *Streptococcus* spp. And Gram-negative bacteria can also cause mastitis (Persson et al. 2017). The species of pathogens could change the leukocytes differential in milk (Bagnicka et al. 2011). The presence of major pathogens *Streptococcus* (*Str.*) *agalactiae, Str. intermedius, S. aureus*) in milk, samples caused a high percentage of eosinophils and neutrophils in the mentioned study (Bagnicka et al. 2011). The change in SCC and subpopulations of leuko-

cytes could help to study the pathology of intramammary infection.

The aim of this study was to study the possible relationship between the level of SCC and the presence of mastitis pathogens in the milk of ewes at half udder level. Additionally, the different subpopulations of leukocytes in milk in relation to SCC were studied as well.

MATERIAL AND METHODS

Animals and sampling

The experiment was conducted on a farm with ewes of the Lacaune breed. The animals were fed in a stable with hay and grass or alfalfa silage and with concentrate intake in the parlour during milking. Ewes were machine milked two times a day. The samples were taken during morning milking. Overall, 98 milk samples from 45 ewes without signs of clinical mastitis were evaluated in the study. The milk samples were collected at half udder level. The milk samples were collected by discarding the first squirts of milk and disinfection of the teat end with 70% alcohol. Samples were taken to sterile tubes for bacterial examination, evaluation of SCC and for the analysis of leukocyte subpopulations (Zoche-Golob et al. 2015).

Bacteriological examination of milk samples

For bacterial cultivation, 100 μl of the sample was applied to blood agar (MkB Test a.s., Rosina, SR). These samples were made from 100 μl of milk from udder half level and 900 μl of phosphate-buffered saline (PBS) (MkB Test a.s., Rosina, SR). All plates were aerobically incubated at 37 °C. After 24 hours, we evaluated plates for grown pathogens. The grown colonies were identified on the basis of Gram staining, cells morphology, type of hemolysis, the activities of catalase (3% H_2O_2; Merck, Darmstadt, Germany), esculin hydrolysis and cytochrome oxidase C (Bactident Oxidase; Merck, Darmstadt, Germany). Presumptive *S. aureus* was detected by using the clumping factor test (Staph Plus Kit; DiaMondiaL, Vienna, Austria). On modified Rambach agar, esculin positive streptococci were cultivated to identify *Str. uberis* or *Enterococcus* sp. according to Watts et al. (1993).

To characterise esculin negative streptococci, Lancefield serotyping (Strept Kit; DiaMondiaL, Vienna, Austria) was used. MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) was used to identify the species of gram-negative rods and all grown colonies also. Briefly, a loopful of the colony material was dissolved in 300 µl distilled water (300 µl), and subsequently, ethanol (900 µl) was added for the extraction of protein. After centrifugation of cell suspension (at 17 000 × *g* for 2 min) the supernatant was discarded. The remaining ethanol was discarded after repeated centrifugation. The pellet was resuspended in formic acid-water (5–50 µl) depending on the pellet size, and acetonitrile was added finally in an equal volume. The supernatant (1 µl) was transferred to the MALDI-TOF MS target plate after centrifugation at 17 000 × *g* for 2 min and allowed to dry at room temperature before applying 1 µl of matrix solution (saturated solution α-cyano-4-hydrosy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) (Jurinke et al. 2004).

Samples with contagious pathogens (*S. aureus*, *Str. agalactiae*) with one or more colony-forming unit (CFU) were reported as positive. Other pathogens were reported as positive if at least five CFU were found. Samples were reported as contaminated if more than two pathogens were isolated from one milk sample and the growth of contagious pathogens were not identified. Identified pathogens were divided to groups: CNS (*S. auricularis*, *S. caprae*, *S. epidermidis*, *S. equorum*, *S. chromogenes*, *S. microti*, *S. petrasii*, *S. piscifermentans*, *S. warneri*, *S. xylosus*) and Gram⁻ (*Mannheimia haemolytica*, *Providencia rettgeri* and *Providencia stuartii*) to indicate their presence in different somatic cells groups described below.

Determination and categories of the SCC

Milk samples underwent heat treatment at 40 °C for 15 minutes. Subsequently, SCC was determined using the Somacount 150 (Bentley Instruments, Chaska, MN, USA).

The milk samples at half udder level were divided based on somatic cell count to five SCC groups: SCC1 < 200 000 cells/ml (45 milk samples); 200 000 ≤ SCC2 < 400 000 cells/ml (10 milk samples); 400 000 ≤ SCC3 < 600 000 cells/ml (six milk samples); 600 000 ≤ SCC4 < 1 000 000 cells/ml (six milk samples); SCC5 ≥ 1 000 000 cells/ml (31 milk

samples) using MS Excel (Microsoft, Redmond, WA, USA).

Evaluation of leukocyte subpopulations

The percentage of leukocytes and their subpopulations was detected by the flow-cytometric method. For flow cytometry, samples were processed as described by Vasicek et al. (2019) with a slight modification. The cells for analysis were obtained by centrifugation of milk sample (50 ml) at 1 500 × *g* and 4 °C for 30 minutes. The cream was carefully removed, and the supernatant was discarded. Each cell pellet was resuspended in 5 ml of ice-cold PBS (Biowest, Riverside, MO, USA) and centrifuged at 460 × *g* at 4 °C for 15 minutes. The cell concentration was detected using an automated cell counter EVE™ (NanoEntek, Waltham, MA, USA). Cells at the concentration of 10^6 were stained with the mouse monoclonal antibodies specific again ovine antigens (all from Washington State University, Pullman, WA, USA) at the dilution rate of 1 : 100 for 15 min on ice. In order to distinguish between leukocyte subpopulations and epithelial cells (Figure 1) following antibodies were used: anti-CD18 (HUH82A, Ig2a or BAQ30A, IgG1) for all leukocytes, anti-CD21 (BAQ15A, IgM) for B lymphocytes, anti-CD2 (MUC2A, IgG2a) for T lymphocytes, anti-CD4 (GC50A, IgM) for T helper lymphocytes, anti-CD8 (CACT80C, IgG1) for T cytotoxic lymphocytes, anti-CD14 (CAM66A, IgM) for monocytes/macrophages and anti-CD11b (S-MM12A, Ig1) for live and nonviable polymorphonuclear cells (PMNs, granulocytes). Proper secondary rat anti-mouse antibodies (anti-IgG2a, anti-IgM and anti-IgG1) conjugated with a fluorochrome (FITC, PE and APC, respectively) were used at the dilution rate of 1 : 50 in additional staining of cells for 15 min on ice. In order to eliminate the unspecific antibody binding, heat-inactivated sheep serum, which was prepared at our laboratory from ovine peripheral blood, was applied to samples. The dead cells were detected and excluded from the analysis using 7-AAD staining (Thermo Fisher Scientific, Waltham, MA, USA) by adding 5 µl of the dye to each sample according to the producer's manual. At least 10 000 cells were analysed at the flow rate of 35 µl/min in each sample using flow cytometer FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) equipped
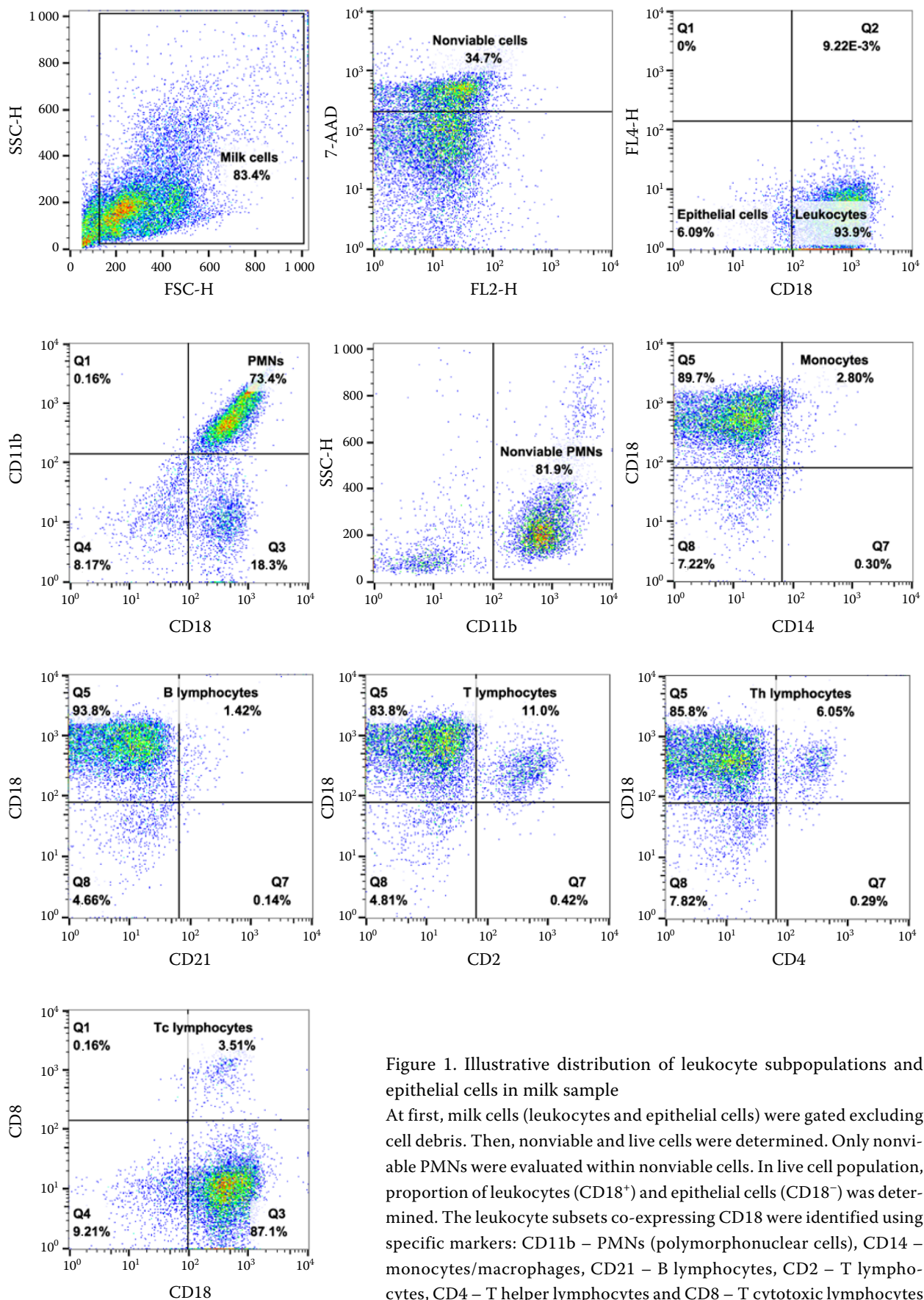
Figure 1. Illustrative distribution of leukocyte subpopulations and epithelial cells in milk sample

At first, milk cells (leukocytes and epithelial cells) were gated excluding cell debris. Then, nonviable and live cells were determined. Only nonviable PMNs were evaluated within nonviable cells. In live cell population, proportion of leukocytes (CD18⁺) and epithelial cells (CD18⁻) was determined. The leukocyte subsets co-expressing CD18 were identified using specific markers: CD11b – PMNs (polymorphonuclear cells), CD14 – monocytes/macrophages, CD21 – B lymphocytes, CD2 – T lymphocytes, CD4 – T helper lymphocytes and CD8 – T cytotoxic lymphocytes

with an argon-ion laser (488 nm) and a red diode laser (635 nm). The four different bandpass filters were used to acquire the fluorescent signals as follows: 530 nm for FITC (FL1), 585 nm for PE (FL2), > 670 nm for 7-AAD (FL3) and 661 nm for APC (FL4). The set-up of the instrument and the quality control was performed using four-color calibration beads (BD Calibrite beads; BD Biosciences, Franklin Lakes, NJ, USA). The unstained samples and/or samples stained only with secondary antibodies were used in order to properly gated the positive cell populations. The leukocytes subpopulations were counted within the CD18⁺ cells. The CD18⁻ cells were considered as epithelial cells.

Statistical analysis

The obtained results were evaluated using the SigmaPlot software v11.0 (Systat Software Inc., Dusseldorf, Germany) with one-way ANOVA (Holm-Sidak method) and expressed as the means ± SEM. SCC1 group was considered as control (healthy) udder, and it was compared to the others SCC groups for significant differences (at level $P < 0.05$) in observed parameters.

RESULTS AND DISCUSSION

The overall prevalence of the bacteriological positive milk samples was 36.73% from separate udder halves of ewes. Vasileiou et al. (2018) detected 26% occurrence of subclinical mastitis and other study found 36.1% prevalence of subclinical mastitis (Tvarozkova et al. 2020). The negative bacteriological samples formed 60.20% of the total tested samples. Only 3.06% of examined samples were classified as contaminated samples. The major pathogens identified from samples were CNS. CNS were also the most frequent pathogens identified in other studies (Holko et al. 2019). *S. epidermidis* was recorded to be the highest occurrence among the CNS in the present study (35.48%). According to Queiroga et al. (2018), *S. epidermidis* was the most frequent CNS causing subclinical mastitis. In the study of Zigo et al. (2017) and Vasileiou et al. (2018) *S. chromogenes* was the most frequent CNS pathogens. An important finding was that no contagious pathogens were found in the present study. Though in another recent our work, *S. au-*

reus was detected in 5.3% of positive bacteriological samples (Tvarozkova et al. 2020). In the study of Ergun et al. (2009), the occurrence of *S. aureus* was observed in 3.1% of milk samples without clinical signs. In the present study, other identified pathogens were Gram-negative species, specifically *Mannheimia* (*M.*) *haemolytica* (5.56%), *Providencia rettgeri* (5.56%) and *Providencia stuartii* (2.78%). *M. haemolytica* caused 11% of cases of intramammary infection in a study by Mavrogianni et al. (2007). Vasileiou et al. (2018) observed the occurrence of *M. haemolytica* in 2.5% of samples with subclinical mastitis.

In Table 1 the presence of pathogens in different SCC groups are shown. Only 4.44% of bacteriological positive samples were identified in SCC1 (< 200 × 10³ cells/ml), and 79.31% of positive bacteriological samples were identified in SCC5 (≥ 1 000 × 10³ cells/ml), which confirmed the relationship between SCC and the presence of pathogens (Tvarozkova et al. 2019). Swiderek et al. (2016) determined the lowest average SCC in milk samples without pathogen. Similarly, Persson et al. (2017) detected that high SCC was associated with intramammary infection.

As mentioned above, the presence of pathogens was frequently isolated in milk samples of the SCC5 group. Leitner et al. (2000) reported an increase in the SCC and percentage of leukocytes during intramammary infection. We also demonstrated that the total percentage of leukocytes was significantly lower ($P < 0.001$) in the SCC1 group (< 200 × 10³ cells/ml) compared to other SCC groups (Table 1). The percentage of PMNs was significantly lower ($P < 0.001$) in SCC1 group than in other SCC groups (Table 1). The increase of PMNs in infected glands was reported by Leitner et al. (2012). Souza et al. (2020) and Albenzio and Caroprese (2011) found a lower percentage of PMNs in milk from the healthy udder. The mean percentage of T lymphocytes was significantly higher ($P < 0.01$) in the SCC1 group in comparison with the SCC5 group. No differences were recorded in the mean percentages of B lymphocytes, T helper lymphocytes, T cytotoxic lymphocytes and the ratio of T helper lymphocytes/T cytotoxic lymphocytes (CD4⁺/CD8⁺) among SCC groups (Table 1). Similarly, in the study of Leitner et al. (2012), no significant differences were observed in mean values of T helper lymphocytes and T cytotoxic lymphocytes in infected samples and samples without

Table 1. Proportion of immune and epithelial cells in sheep in different somatic cell count groups

SCC class	SCC1 (n = 45)	SCC2 (n = 10)	SCC3 (n = 6)	SCC4 (n = 6)	SCC5 (n = 31)
Bacteriological positive samples	6.66% (CNS – 4.44%, contaminated – 2.22%)	60% (CNS)	28.58% (CNS – 14.29%, Gram⁻ – 14.29%)	42.86% (CNS)	86.21% (CNS – 65.52%, Gram⁻ – 13.79%, contaminated – 6.90%)
Leukocytes – CD18⁺ (%)	32.02 ± 3.74[a]	71.87 ± 8.44[b]	88.42 ± 5.03[b]	90.75 ± 3.19[b]	85.49 ± 3.08[b]
PMNs – CD11b (%)	13.92 ± 2.75[a]	48.07 ± 10.27[b]	71.11 ± 10.23[b]	73.56 ± 9.31[b]	72.27 ± 5.00[b]
B lymphocytes – CD21⁺ (%)	1.65 ± 0.39	1.47 ± 0.32	3.42 ± 2.13	1.14 ± 0.23	1.59 ± 0.44
T lymphocytes – CD2⁺ (%)	11.72 ± 2.01[c]	10.09 ± 4.24	6.11 ± 1.86	7.52 ± 3.19	3.29 ± 0.93[d]
Th lymphocytes – CD4⁺ (%)	4.47 ± 0.67	4.39 ± 1.25	8.40 ± 5.83	6.13 ± 2.43	3.75 ± 0.77
Tc lymphocytes – CD8⁺ (%)	5.10 ± 0.67	5.06 ± 1.66	5.54 ± 3.38	5.03 ± 2.61	3.58 ± 0.72
CD4⁺/CD8⁺ (%)	1.04 ± 0.11	1.10 ± 0.27	1.23 ± 0.37	1.60 ± 0.34	1.53 ± 0.23
Monocytes/Macrophages – CD14⁺ (%)	2.51 ± 0.36	1.70 ± 0.41	5.11 ± 2.67	3.35 ± 0.64	2.50 ± 0.37
Epithelial cells – CD18⁻ (%)	67.68 ± 3.29[a]	28.02 ± 8.42[b]	11.08 ± 4.69[b]	9.02 ± 3.24[b]	14.41 ± 3.08[b]
Nonviable cells – 7-AAD⁺ (%)	27.82 ± 1.66	30.50 ± 6.35	41.85 ± 4.64	38.06 ± 11.11	20.05 ± 2.10
Nonviable cells PMNs – CD11b⁺ (%)	15.44 ± 1.86[a]	58.05 ± 8.69[b]	79.08 ± 6.54[b]	74.52 ± 11.08[b]	72.02 ± 5.50[b]

PMNs = polymorphonuclear cells (granulocytes); SCC = somatic cell count, cells/ml; Tc lym = T cytotoxic lymphocytes; Th lym = T helper lymphocytes
[a,b]Are statistically different at $P < 0.001$; [c,d]are statistically different at $P < 0.01$ within the same row

pathogens. No differences were reported between the mean percentage of B lymphocytes and T lymphocytes in the infected udder than in milk from the healthy udder (Souza et al. 2012). On the other hand, Albenzio et al. (2012) detected the increase in lymphocytes as SCC increased. In the percentage of macrophages and monocytes, we did not observe significant differences. Also, Albenzio and Caroprese (2011) did not detect significant differences in the percentage of macrophages between low SCC and high SCC. Similarly, Leitner et al. (2012) did not observe a significant increase of macrophages and monocytes in positive bacteriological samples compared with negative bacteriological samples. The decrease of epithelial cells was detected in SCC groups with high SCC ($\geq 200 \times 10^3$ cells/ml) compared with SCC1 ($P < 0.001$). Aref et al. (2018) reported a higher count of epithelial cells in milk from the uninfected udder. In the percentage of nonviable cells, we did not observe significant differences among SCC groups. Similarly, in another study, no significant differences were determined in the number of nonviable cells (Vasicek et al. 2019). The percentage of nonviable PMNs was significantly lower in SCC1 compared with other SCC groups ($P < 0.001$) (Table 1). Also, Albenzio et al. (2012) recorded an increase in the percentage of nonviable PMNs in milk with increasing SCC. On the other hand, we found that there were no differences between viable and nonviable PMNs (Table 1).

CONCLUSION

This study presented the relation between the presence of pathogens and somatic cell count and the content of subpopulations of leukocytes in the milk of ewes. In 79.31% of milk samples from the udder, half level with SCC $\geq 1\ 000\ 000$ cells/ml were found pathogens. CNS were the most common pathogens in milk samples. The milk samples with SCC $\geq 200\ 000$ cells/ml had a significantly higher percentage of leukocytes ($P < 0.001$) compared with milk samples with SCC $< 200\ 000$ cells/ml. The main subpopulation of leukocytes, which was significantly higher in milk samples with SCC $\geq 200\ 000$ cells/ml in comparison with milk samples with SCC $< 200\ 000$ cells/ml, were PMNs ($P < 0.001$). The presence of the pathogen in milk caused the increase in SCC, percentage of leuko-

cytes, mainly PMNs. The evaluation of subpopulations of leukocytes has great potential and could be used as a promising tool to identify ewes affected by an inflammatory infection.

Conflict of interest

The authors declare no conflict of interest.

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