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Neutrophil and CD4$^+$ milk cell count related to natural incidence of mastitis in Jersey cattle

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Research Article

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Abstract

This Research Communication describes the relation between somatic cells and microbial content in milk from Jersey cattle. Milk samples were classified in groups: healthy, dirty and mastitic (from *Staphylococcus* spp., *Escherichia coli, Coliforms).* The somatic cells in each of those groups were analysed by two methods – flow cytometric and automatic fluorescent cell counting. Those methods were compared. Total somatic cell count (SCC), neutrophil count, and lymphocytes with cluster of differentiation 4 (CD4$^+$cells) were determined. There was a positive relationship between microbes and somatic cells. It was noticed that the neutrophil count was generally increased together with SCC, whilst the CD4$^+$ cell count was higher in healthy milk samples (about 8%) compared to mastitic ones (about 3%). Lower number of CD4$^+$ cells (from 1 to 4%) was determined in samples positive for *Staphylococcus spp.* but with lower SCC (from 2.7 to 4.0 × 10^5 cells/ml). Also, the number of CD4$^+$ cells in *Staphylococcus* spp.-positive samples increased (to 4.8%) together with higher SCC, something that was not observed in the other mastitic samples. Knowledge of those relations could be useful for veterinary medical tests in the initial phase of inflammation.

Product quality can be compromised both by the presence of mastitis and also from antibiotic residuals. These veterinary cases make the milk unfit for human consumption (Alhussien and Dang, 2020). Cobirka *et al.* (2020) presented a review about the epidemiology and classification of mastitis, discussing three microorganisms as the major pathogens that cause mastitis: *Escherichia coli, Staphylococcus aureus* and streptococci. Intra-mammary infections lead to a high somatic cell count (SCC) in milk. Some relationships have been observed: if SCC increases, the percentage of neutrophils increases too (Alhussien and Dang, 2020). CD4$^+$ lymphocytes are necessary for the antigen-specific recruitment of neutrophils to mycobacterial infection, possible acting *via* chemotactic cytokines (Yang, 2018). There are only a few studies about the potential relationship between SCC (total SCC, neutrophils and CD4$^+$ cells) and mastitis (clinical or subclinical) caused by *E. coli* and *S. aureus* (Rainard *et al.*, 2018).

We have recently demonstrated the utility of an automated flourescent cell counter for the simultaneous determination of SCC and neutrophil percentage in bovine milk (Chengolova *et al.*, 2021). Now we extend that work to determine the relationship of pathogenic microorganisms (*Staphylococcus aureus*, *Escherichia coli* and *Coliforms*) in milk samples to the SCC, neutrophil and CD4$^+$ cell percentage.

Material and methods

Quarter udder cow milk samples were classified as healthy when SCC was ≤ 400 000 cells/ml, and the total bacteria count (TBC) was ≤100 000 cfu/ml. Microbial analyses were made with selective nutrient media as described in the relevant ISO standard according to the current regulation in the Republic of Bulgaria. (see the online Supplementary File).

The analyses of total and differential cell count were performed by an automatic cell counter Lactoscan SCC (Milkotronic Ltd., Bulgaria) and a flow cytometer Guava easyCyteTM 8HT (Luminex, USA) (online Supplementary Table S1). The SCC was determined using nucleic acid fluorescent dye Sofia Green from the test kit of Lactoscan SCC and according to manufacturer's instructions. The neutrophils were visualized by specific antibody (obtained in our laboratory, as described in our previous paper Becheva *et al.*, 2017) which was conjugated to fluorescent dye ATTO620 conjugate. The anti-neutrophil antibody – ATTO620 conjugate was obtained using the conjugation procedure according to manufacturer's instructions (ATTO-TEC, Germany). CD4$^+$ cells were coloured by commercial conjugate – CD4FITC antibody. The cell staining techniques are detailed in the Supplementary File.

Results and discussion

Determination of bacteria and somatic cell count in milk

In this research 103 quarter cow milk samples were analysed. SCC, neutrophil and CD4+ cells were determined. Microbiological analyses were also made – total bacteria count (TBC), *Staphylococcus aureus*, *Escherichia coli* and *Coliforms*.

Table 1. Influence of *Staphylococcus aureus, Escherichia coli* and *Coliforms* on SCC, neutrophil and $CD4^+$ cell count

Milk group	(*n*)	SCC, × 10^5 cells/ml		Neutrophils, %		$CD4^+$ cells, %	
		Flow cytometer	Lactoscan SCC	Flow cytometer	Lactoscan SCC	Flow cytometer	Lactoscan SCC
Healthy	16	1.4–4.0	1.3–4.2	15.7 ± 7%	17.4 ± 6%	7.9 ± 5%	8.2 ± 7%
Mastitic (from *Staphylococcus* spp.) (*n* = 21)	4	2.7–4.0	2.8–3.9	40 ± 5%	42 ± 8%	1.0 ± 1%	0.8 ± 1%
	7	4.1–8.3	4.0–7.8	65 ± 8%	65 ± 12%	2.2 ± 3%	1.8 ± 4%
	10	8.3–13.0	8.0–12.1	71 ± 4%	73 ± 10%	4.8 ± 3%	3.9 ± 2%
Mastitic (from *Escherichia coli*) (*n* = 11)	2	3.0–3.4	3.2–3.8	38 ± 2%	39 ± 5%	3.0 ± 2%	2.6 ± 4%
	6	4.0–8.2	4.1–7.9	52 ± 6%	55 ± 13%	3.1 ± 6%	2.7 ± 6%
	3	8.3–12.0	8.3–10.0	74 ± 3%	72 ± 6%	2.8 ± 3%	3.2 ± 2%
Mastitic (from *Coliforms*) (*n* = 43)	3	3.2–3.9	3.4–4.0	34 ± 2%	35 ± 2%	2.6 ± 2%	2.5 ± 6%
	28	4.3–7.6	4.2–7.9	63 ± 9%	60 ± 12%	2.8 ± 8%	2.7 ± 4%
	12	8.6–18.0	8.1–16.5	71 ± 6%	70 ± 9%	2.4 ± 8%	2.6 ± 2%

SCC, somatic cell count; *n*, number of milk samples.

First of all, the cow milk samples were categorized in three groups: Healthy, Dirty and Mastitic (online Supplementary Table S2). The Healthy group showed low total SCC (from 130 000 to 420 000 cells/ml) negative tests for differential bacteria count (*Staphylococcus aureus*, *Escherichia coli* and *Coliforms*) and low TBC (29 000 colony forming units per ml – cfu/ml). The threshold of 400 000 cells/ml for the Healthy group was chosen because healthy cows should have an SCC below 200 000 cells/ml, and cows with counts over 400 000 cells/mL should be considered as having an intramammary infection (Cobirka *et al*., 2020). The Dirty group, on the other hand, had almost the same range of SCC but showed extremely high TBC (918 180 cfu/ml ± 25%) and positive tests for differential bacteria count. Obviously, the proportion of *Coliforms* in dirty milk samples was significant. That was probably due to poor cow hygiene. That group of milk samples was excluded from the subsequent somatic cell analyses.

The Mastitic group had very large differences in SCC (from 280 000 to 1 650 000 cells/ml) and bacteria were detected. Also, it was observed that the count of *Staphylococcus* spp. was the highest compared to the *E. coli* and *Coliforms* in the Mastitic group.

It should be noticed that there are significant differences between the infections caused by *S. aureus* and *E. coli*. Intra-mammary infection by *E. coli* is acute in nature and generally clears within a few days (Goldberg *et al*., 1995). In contrast, infection by *S. aureus* is often less severe but results in a chronic infection (Newbould, 1970). The *Coliform* organisms (*Escherichia coli, Klebsiella* spp. *and Enterobacter aerogenes*) also cause mastitis. The endotoxins contained in the bacterial cell wall have a pathogenic effect (Hohmann *et al*., 2020).

Influence of different pathogenic microorganisms on SCC, neutrophil and $CD4^+$ cell count

At the first stage of the study, 12 of the samples were classified as 'Dirty' so they were excluded from analysis, which as a consequence was made with 91 samples. The Healthy group comprised 16 samples and the Mastitic group 75 samples (detailed in online Supplementary Table S2). It was of interest to study the influence of the different mastitis causing pathogens on the proportion of different cell types – SCC, neutrophil and $CD4^+$ cell count (Table 1).

Total and differential cell counts in the milk samples were determined by flow cytometer and automatic cell counter Lactoscan SCC. Table 1 shows the results obtained by both methods and they are relatively similar with negligible differences, suggesting that the Lactoscan device could be used for counting of SCC, neutrophils and $CD4^+$ cells in milk. It was, however, apparent that the results obtained by the flow cytometer were higher than those obtained by Lactoscan, especially for the samples with high total number of microorganisms. Probably that was due to the difference in principles of the both methods and flow cytometric technique counted not only somatic cells but microorganisms or clotted huge proteins. However, the results remain similar.

It was noticed that if SCC increases, the percentage of neutrophils also increases. In this research the neutrophil count varied significantly from 17% in uninfected to 80% in mastitic milk samples. It is known that neutrophils are a key factor in the cows' defence against intra-mammary infection (Putz *et al*., 2020). The highest neutrophil count in our samples was associated with *Staphylococcus aureus* mastitic samples. Blagitz *et al*. (2015) reported that *Streptococcus dysgalactiae* infected udders also had higher SCC and neutrophils but the number of the $CD4^-$ had been higher, therefore, the number of the $CD4^+$ decreased. Our observations on $CD4^+$ cell in the samples showed increase in their count in samples in Mastitic group. Other authors reported similar observation. Rasheed *et al*. (2020) presented a review about the milk samples with mastitis, where the number of $CD4^+$ cells was higher than $CD4^+$ cells in healthy milk samples. Besides that, the authors reported that the number of $CD4^+$ cells was higher than $CD8^+$ T-cells in the milk samples with staphylococcal mastitis, whereas in cows with streptococcal mastitis, parallel increase in both $CD4^+$ and $CD8^+$ T-cells were observed. In our case in the samples with staphylococcal mastitis the count of $CD4^+$ cells increased compared to healthy milk samples, but in other mastitic samples the $CD4^+$ cell count was lower than healthy milk samples. Taylor *et al*. (1997) described that the lower value of $CD4^+$ cells might be caused by the suppressive $CD8^+$ lymphocytes, which inhibit proliferation of CD4 lymphocytes. In our study, $CD4^+$ cell count was higher in healthy milk

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samples (about 8%) compared to mastitic ones (about 3%). Lower number of $CD4^+$ cells (from 1 to 4%) was determined in samples positive for *Staphylococcus* spp. but those samples had lower SCC (from 2.7 to 4.0×10^5 cells/ml). Also, the number of $CD4^+$ cells in *Staphylococcus* spp.-positive samples increased (to 4.8%) together with elevated SCC. That was not observed in the other mastitic samples.

In conclusion, the findings of the present study suggest that the Lactoscan fluorescence cytometer is a good alternative to the flow cytometer for counting SCC, neutrophils and $CD4^+$ cells. It was shown that the number of neutrophils and CD4 lymphocytes in the milk are a more reliable sign for differentiation between early and late phases of infection than SCC or bacteriological examination. The proportion of different cell types has been shown to be influenced by the type of pathogens.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000510

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