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

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Supplementary file

Material & Methods

Reagents and milk samples

ATTO620NHS ester, CD4FITC antibody conjugate, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) were purchased from Sigma-Aldrich (Germany).

Raw cow milk was obtained from quarters of 40 cows from local farms. The tests were performed during the summer. The regulation in force while the analyses were performed was Commission regulation EC No 1662/2006. Approximately 10 ml of each milk sample were collected aseptically. Milk was transported in sterile tubes under refrigerated conditions. All of the analyses were performed on the day of the collecting of the milk samples.

Microbial analyses

First of all, after collecting the row milk samples, microbiological examinations were performed. The analyses were made by dairy laboratory "St. George" (Burgas, Bulgaria), conforming to standard ISO/IEC 17025:2017. For total bacteria count (TBC) in milk was used horizontal method, skim milk plate count agar with colony count at 30 °C by the pour plate technique (ISO 4833-1:2013). Coliforms were counted by a horizontal method for enumeration and colony-counting technique based on their ability to ferment lactose with

production of acid and gas (ISO 4832:2006). For beta-glucuronidase-positive *Escherichia coli* (*E. coli*) was used a colony-count technique at 44 °C and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (ISO 16649-2:2014). Coagulase-positive staphylococci (CPS) were determined by technique using Baird-Parker medium (ISO 6888-1:1999). The samples were tested for *Salmonella* by four successive stages including pre-enrichment, enrichment, plating out on selective solid media, and confirmation (ISO 6579-1:2017).

Somatic cell counting – total count, neutrophils and $CD4^+$ cells

Neutrophil cells and $CD4^+$ cells were analyzed by immunofluorescence staining. Milk sample was gently mixed and 50 µl of it was loaded in a microcentrifuge tube. After that, 10 µl of the anti-neutrophil antibody – ATTO620 conjugate (1 mg/ml) and 2 µl of the CD4FITC antibody conjugate (1 mg/ml) were added, and 40-min incubation was performed at 4°C. Then, 8 µl of the well-mixed sample were loaded in a chip for automatic cell counting by Lactoscan SCC. After the counting, the rest of the sample was diluted properly with Saline-EDTA (0.9 % NaCl and 7.5 mM EDTA) to obtain about 100 000 cells/ml and 150 µl of it was loaded into a well of polystyrene flat bottom microplate. The analysis was performed by a flow cytometer Guava easyCyteTM 8HT.

Lactoscan SCC and Guava easyCyteTM – parameters

All measurements were performed on the automatic cell counter Lactoscan SCC and the flow cytometer Guava easyCyteTM 8HT. Optimization of the parameters for both apparatus was performed prior the analyses. The automatic cell counter Lactoscan SCC was equipped with

two lasers, blue laser at 470 nm and red at 627 nm. The parameters of the instrument are shown in Table S1.

Table S1. Lactoscan SCC parameters for neutrophil and $CD4^+$ cells counting in cow milk. The values are for adjustment of light source 627 nm (for anti-neutrophil antibody – ATTO620 conjugate) and light source 470 nm (for CD4FITC antibody conjugate).

	Laser 627 nm	Laser 470 nm
Exposition	2.110	2.110
Gain	33	3
Power	100%	100%
Focus	2880	2720

The samples were also analyzed by the flow cytometer (Guava easyCyteTM 8HT), program guava® ExpressPlus. The program allowed adjustment up to three fluorescence parameters (GRN – green, YLW – yellow, RED – red) in combination with forward scatter (FSC) and side scatter (SSC). The samples were run at medium speed and mixing. The counted events for each analysis was 3 000. The instrument used software for the analysis of the data. Target cells – neutrophils and $CD4^+$ were gated on dot plots representing cell size based on FSC and granularity based on SSC.

Statistical analysis

Each sample was analyzed triplicate. Analysis and visualization of the data were done with Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Differences were considered statistically significant at $P<0.05$.

Results & Discussion

Some of the most major mastitis agents (*Staphylococcus aureus*, *Escherichia coli* and *Coliform* organisms) were selected for the microbiological analyzes (Ameen *et al.* 2019; Zhang *et al.* 2020). *Staphylococcus aureus* mainly produces subclinical and chronic mastitis, but it also may cause per-acute mastitis and lead to gangrene of the quarters. Bacterial toxins are thought to cause the appearance of mastitis and gangrene. *Escherichia coli* cause inflammation of the mammary gland in dairy cows with local and sometimes severe systemic clinical symptoms. It has been found that the severity of *E. coli* mastitis is mainly determined by the host defence status, rather than by *E. coli* pathogenicity (Burvenich *et al.* 2003). *Coliform* bacteria are found in the environment of a dairy cow (Paape & Guidry 1969; Bright *et al.* 1987; Hohmann *et al.* 2020). *Coliform* mastitis is typically acute, but there are also cases of chronic and subclinical infections.

The detection methods in this study were according to the relevant ISO standard methods (Fig. S1). Each milk sample was tested for *Salmonella* but there was no sample with positive result.

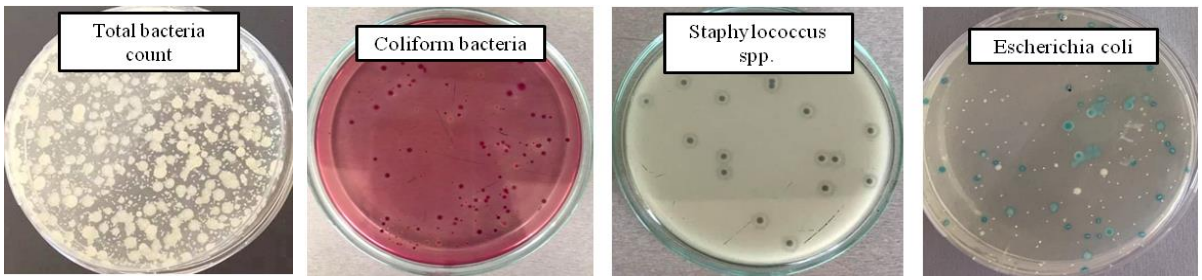

Fig. S1. Microbial analyzes of milk samples according to relevant ISO method (Total bacteria count – ISO 4833-1:2013, Coliforms – ISO 4832:2006; Coagulase-positive staphylococci – ISO 6888-1:1999, beta-glucuronidase-positive *Escherichia coli* – ISO 16649-2:2014).

Table. S2. Bacteria and somatic cell count in three groups of milk samples.

Milk	SCC, x 10^5 cells/mL	TBC, cfu/mL	*Staphilococcus spp.*	*Escherichia coli*	*Coliforms*
Healthy (n=16)	1.3 – 4.2 GM: 3.0	29 000 ± 15%	Negative (< 10 cfu/mL)	Negative (< 10 cfu/mL)	Negative (<< 1000 cfu/mL)
Dirty (n=12)	1.7 – 4.3 GM: 3.2	918 180 ± 5%	Positive (100 ± 35% cfu/mL)	Positive (1 200 ± 30% cfu/mL)	Positive (220 000 ± 30% cfu/mL)
Mastitic (n=75)	2.81 – 16.5 GM: 6.0	120 000 ± 15%	Positive	Positive	Positive

*GM – geometric mean; SCC – somatic cell count (determined by Lactoscan SCC); TBC – total bacteria count.

In mastitc milk samples the neutrophil count was significantly higher than that in healthy group of milks. Similar results were obtained in other studies (Riollet *et al.* 2001; Alhussien *et al.* 2015).

Reference

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ISO 4832:2006 Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coliforms. Colony-count technique

ISO 4833-1:2013 Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony count at 30 degrees C by the pour plate technique

ISO 6579-1:2017 Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of Salmonella - Part 1: Detection of Salmonella spp.

ISO 6888-1:1999 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) - Part 1: Technique using Baird-Parker agar medium

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