sciendo

DOI: 10.2478/fv-2024-0026

FOLIA VETERINARIA, 68, 3: 44—51, 2024

THE USE OF FLOW CYTOMETRY IN THE ANALYSIS OF SOWS' COLOSTRUM AND MILK

Jaroslav Novotný[1], Dagmar Mudroňová[2], Zuzana Krepelková[1], Katarína Bárdová[1], Alexia D'Alfonso[3]

[1]Clinic for Swine, [2]Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
Slovakia
[3]Le Palmier, Triq il-Ħmistax ta' Settembru 1598, Mqabba MQB1422
Malta

jaroslav.novotny@uvlf.sk

ABSTRACT

The flow cytometry method is used in many fields, not only scientific, but also clinical. In science, flow cytometry is used in immunology, molecular biology, microbiology or plant biology. In medicine, has its use, in the diagnosis of tumours, in reproductive and prenatal diagnosis, in transplants or in haematology. In our experimental work, we used this method to analyse colostrum and milk of sows. The aim of this study was to determine the number of somatic cells, the number of leukocytes and the number of T-lymphocytes. Colostrum samples were collected within 24 hours of delivery and then milk samples were collected at weekly intervals during 3 weeks. Statistical analysis revealed significant differences in the number of leukocytes (CD45+) in colostrum and milk. The most significant difference was noted between colostrum and milk in weeks 2 and 3 of the experiment ($P < 0.0001$), with the highest values found in colostrum. On the other hand, we found that the levels of helper T-cells (CD4+) and cytotoxic T-cells (CD8+) gradually increased over time ($P > 0.05$), with the highest values observed in the third week after farrowing. We can conclude that flow cytometry can be successfully used for the examination of somatic cells in sows' milk.

Key words: colostrum; flow cytometry; milk; sows

INTRODUCTION

The quality and volume of colostrum and milk produced by sows has a major impact on the survival and growth of piglets. New-born piglets go from a sterile or extremely low density intrauterine microbiome environment to an antigen-rich external environment, so that they need an adequate immunologic response to survive [25]. At birth, piglets have very limited body reserves [11]. Due to the epitheliochorial structure of the placenta, they do not receive antibodies prenatally [24]. As such, they are born agammaglobulinemic, with limited cell-mediated immunity and no effector and memory T-lymphocytes. Therefore, piglets are extremely dependent on the acquisition of maternal immunity via colostrum [2].

Sow colostrum contains leukocyte cells, such as granulocytes (neutrophils, 40 %), T-lymphocytes (30 %), B-lymphocytes (13–16 %), and macrophages (7–11 %) [6, 7]. Neutrophils are thought to play a major role in the protection of the sow, but do not contribute to the development of the immune system of piglets. Since the primary report about the presence of both CD3+/CD4+ (helper/inducer) and CD3+/CD8+ (cytotoxic/suppressor) T-cells in colostrum, T-lymphocytes reflect the physiologic and immunologic conditions of the sow and could be involved in the early immunologic response of piglets [9, 12, 13, 27].

Milk is known to be a high-value nutritional biological fluid composed of water, proteins, fat, sugars, minerals, etc. Other important components existing naturally in raw milk are somatic cells (SC), and the predominant cell type, besides shed epithelial cells, in most species are leucocytes, including macrophages, polymorphonuclear neutrophils cells (PMNs), and lymphocytes [3].

Porcine flow cytometry has long been handicapped by the lack of available antibodies, and in particular fluorochrome-conjugated antibodies, but the availability of these tools has greatly improved [8], allowing more accurate detection and sorting of different cells [5, 18]. Compared with conventional microscopy observation, flow cytometry allows counting SCs and identifying SC types with fewer samples and less time, thus allowing characterizing SCs and determining the roles of SCs in the milk. Recent advances of SC studies by flow cytometry in medicine diagnostic field give opportunities to study SCs in dairy field at the subpopulation level [1, 21]. The combination of immunofluorescence and flow cytometry allows the differentiation of cell types using specific antibodies [10, 22].

The aim of this study was to evaluate the number of somatic cells, the number of leukocytes and T-lymphocytes in the colostrum and milk of sows by flow cytometry.

MATERIALS AND METHODS

Animals

In the experiment, we evaluated six lactations in five sows (P09, P13, P19, P20, P21) of crossbreeds Duroc, Landrace, Pietrain, of different ages, farrowing in different seasons at the Clinic for Swine of the University of Veterinary Medicine and Pharmacy (UVMP) in Košice.

Experimental design

Colostrum samples were collected within twenty-four hours after delivery. Subsequently, milk samples were collected every week at the same time for the next three weeks after farrowing. Before sampling, the piglets were separated from the sows; the mammary glands were thoroughly cleaned and degreased with alcohol to avoid contamination of the samples. In order to obtain a sufficient volume of colostrum/milk, 30 I.U. of oxytocin (Biovet JSC) was administered intramuscularly. Samples were always taken from the first three anterior pairs of mammary glands to the sterile beakers. After collection, the samples were sent directly to the laboratory for further processing.

Diagnostic testing – flow cytometry

The initial stage of the analysis was the preparation of the milk sample for processing through the flow cytometer. This was done by removing all the fat component in the milk and receiving the cellular fraction. Two millilitres of milk were centrifuged at a high speed of 2500 revolutions per minute (rpm) for fifteen minutes (Centrifuge MPW-225e, MPW, Poland). Afterwards, the separated fat content was removed from the tube of the milk sample and the sediment was suspended in the phosphate-buffered saline (PBS; MP Biomedicals, France). This procedure was performed for multiple times until the milk sample contains no fat. The succeeding centrifugations were done at the speed 1500 rpm for 10 minutes. Two ml of PBS were added to the tube of the milk sediment that is left after the fat component was removed.

Counting of somatic cells

The absolute counting of the cells through the flow cytometer is possible by the use of 123count eBeads (ThermoFisher Scientific, Life Technologies, USA). They are 7 micrometres (μm) microparticles containing encapsulated dyes which are compatible with blue and violet dyes excitation sources. They emit fluorescence between the range of 500 nm and 750 nm. The eBeads represent a suspension of 1000 beads per microlitre (μl).

The samples of isolated milk cells were diluted in the ratio of 1:100 and 1:1000. The eBeads were mixed on a vortex for 15 to 30 seconds for uniform mixing of the beads prior the addition to the milk cell suspension. This is an important step to ensure an accurate counting result. We mixed 100 μl of diluted milk cell suspension with 100

µl of 123count eBeads. The number of milk somatic cells and beads were analysed using a BD FACS Canto flow cytometer (Fig. 6; Becton Dickinson Biosciences, USA) equipped with BD FACS Diva™ software. The eBeads were excited by a blue 488 nm laser and subsequently the position of milk cells and beads were determined on a dot plot FSC-A versus SSC-A (Fig. 1a) and the counts on a dot plot for PE (phycoerythrin; 575 ± 26 nm) and FITC (fluorescein isothiocyanate; 530 ± 30 nm) fluorescence (Figure 1b). The number of milk somatic cells were calculated by the formula:

$$\text{Absolute number (cells.ml}^{-1}\text{)} = \frac{\text{(cell number x beads volume)}}{\text{(beads number x milk volume)}} \times \text{beads concentration (100 x 10}^{4}\text{.ml}^{-1}\text{)}$$

Leukocyte population analysis

The proportions of leukocyte populations (%) in the milk samples were analysed by flow cytometry after direct labelling with monoclonal antibodies (MoAb) conjugated with fluorochromes. For the identification of all leukocytes, was used an anti-porcine anti-CD45 MoAb conjugated with FITC (AbD Serotec, UK). The subpopulations of T-lymphocytes – T-helper cells (CD4+CD8-) and T-cytotoxic cells (CD4-CD8+) were identified by the help of double-staining with anti-porcine anti-CD4 MoAb-FITC (AbD Serotec, UK) and anti-CD8 MoAb-PE (AbD Serotec, UK). The numbers of milk cells were adjusted to 1.10^6 in 50 µl. Samples of milk cell suspensions (50 µl) were mixed with 10 µl of anti-CD45 MoAb in one tube, and in the second tube with 4 µl of anti-CD4 MoAb and 2 µl of anti-CD8 MoAb. The tubes were incubated for 20 minutes in dark conditions at the room temperature. Then, the samples were washed with 2 ml of PBS with 5 min centrifugation at 1500 rpm. The washing was repeated. The sediments were suspended in 200 µl of PBS and analysed on the above described flow cytometer. The position of milk cells was gated as is described for counting of milk cells (Fig. 1a). Then only single cells were selected for further analysis (Fig. 2a). Proportion of all leukocytes (CD45+) was determined based on the high green fluorescence emission (Fig. 2b). Proportions of CD4+ and CD8+ lymphocytes were analysed on dot plot for FITC-A and PE-A (Fig. 2c).

Statistical processing of results

Statistical processing of the results was performed by one-way analysis of variance in the statistical program GraphPad Prism. Values of $P < 0.05$ were considered significant.

RESULTS

Statistical analysis of the somatic cells counts in colostrum and milk in the monitored weeks of the experiment showed insignificant differences. The lowest values (7.45 $\log_{10}$ cells.ml^{-1}) were recorded in the colostrum of sow P20, on the contrary, the highest values were recorded in sow P13 (10.3 $\log_{10}$ cells.ml^{-1}) in milk from the 3rd week of the experiment (Fig. 3).

The highest value of leukocytes (8.79 $\log_{10}$ cells.ml^{-1}) was recorded in the colostrum of sow P13, the lowest value (3.40 $\log_{10}$ cells.ml^{-1}) was recorded in the same sow in the third week of the experiment (Fig. 4). No significant differences were noted when the animals were statistically compared. When comparing leukocyte values (CD 45+) over time, a significant decrease was found between colostrum collection after farrowing and weeks 2 and 3 of lactation ($P < 0.001$; Fig. 5). A significant difference was also found between the 1st and 2nd week of lactation ($P < 0.05$; Figure 5) and the 1st and 3rd week of lactation ($P < 0.01$; Fig. 5).

By comparing cytotoxic T-lymphocytes (CD8+) and T-helper lymphocytes (CD4+) in colostrum and milk of sows, higher values of cytotoxic T-lymphocytes were found. An insignificant increase in values was detected during the experiment, with the lowest values recorded in colostrum (Fig. 6).

DISCUSSION

Somatic cells (SC) are an important component naturally present in milk. The amount of SCs, usually called somatic cell count (SCC), in milk is used as an important indicator of cow milk quality and health status, but not commonly measured in lactating gilts or sows [14]. SCs are involved in protecting the mammary gland from infection as part of the innate immune system [23]. SC are made up of four main cell types, namely macrophages, polymorphonuclear neutrophils (PMNs), lymphocytes and epithelial cells [4, 12, 16, 19]. These types of leukocytes play an important role in the immune system, where their goal is to

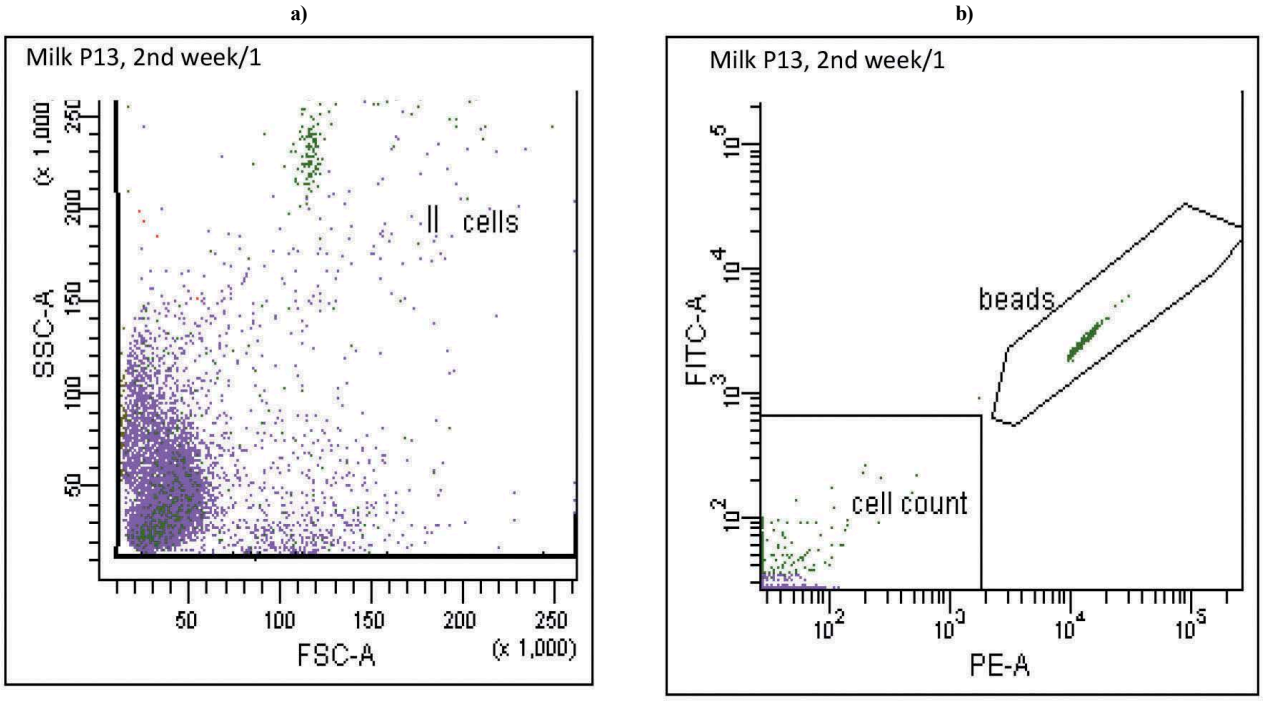

Fig. 1. Determination of: a) the position of milk cells and beads on a dot plot for size (FSC-A) and granularity (SSC-A); b) the counts of milk cells (cell count) and beads emitting green (FITC-A) and orange (PE-A) fluorescence

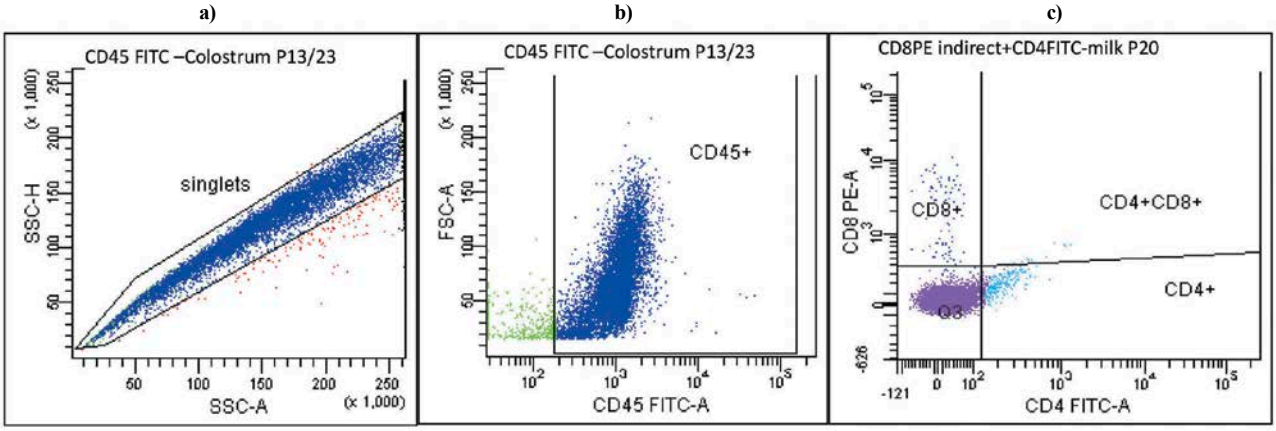

Fig. 2. Identification of milk leukocyte populations: a) gating of single cells; b) determination of the all leukocytes (CD45+) on dot plot for green fluorescence (FITC-A) versus FSC-A; c) determination of T-helper (CD4+) and T-cytotoxic (CD8+) lymphocytes on dot plot for FITC-A versus PE-A

destroy foreign substances and restore affected tissues. It clearly follows from this fact that their increased number in milk means that the mammary gland is affected by inflammation. Other types of leukocytes (eosinophils, basophils) and erythrocytes (red blood cells) can also be found among other blood cells. Erythrocytes can occur in colostrum, in milk they appear in cases of severe inflammation or injury, most often the teats ducts. Epithelial-derived somatic cells are exfoliated epithelial cells from different parts of the mammary gland, which arise by detachment during regenerative processes. However, as the SCC parameter is temporally and individually variable during lactation, it is not always a clear indicator of potential infection. Immune cells, especially neutrophils, have been shown to play an important role in mammary immunity, in effective defence against invading pathogens and in the fight against infectious diseases [17, 20]. Our experimental observation showed that values of SCC in colostrum was in the range of 2.80 x 10^7 to 5.90 x 10^9 cells.ml^{-1} (from 7.45 $\log_{10}$ cells.ml^{-1} to 9.77 $\log_{10}$ cells.ml^{-1}, Fig. 3), which is disproportionately higher than 5.8 x 10^4 to 2.9 x 10^6 cells.ml^{-1} observed in sow′s colostrum by M a u r e r et al. [14]. This difference could be caused by the different time of collection after farrowing, different location of collection (the best quality

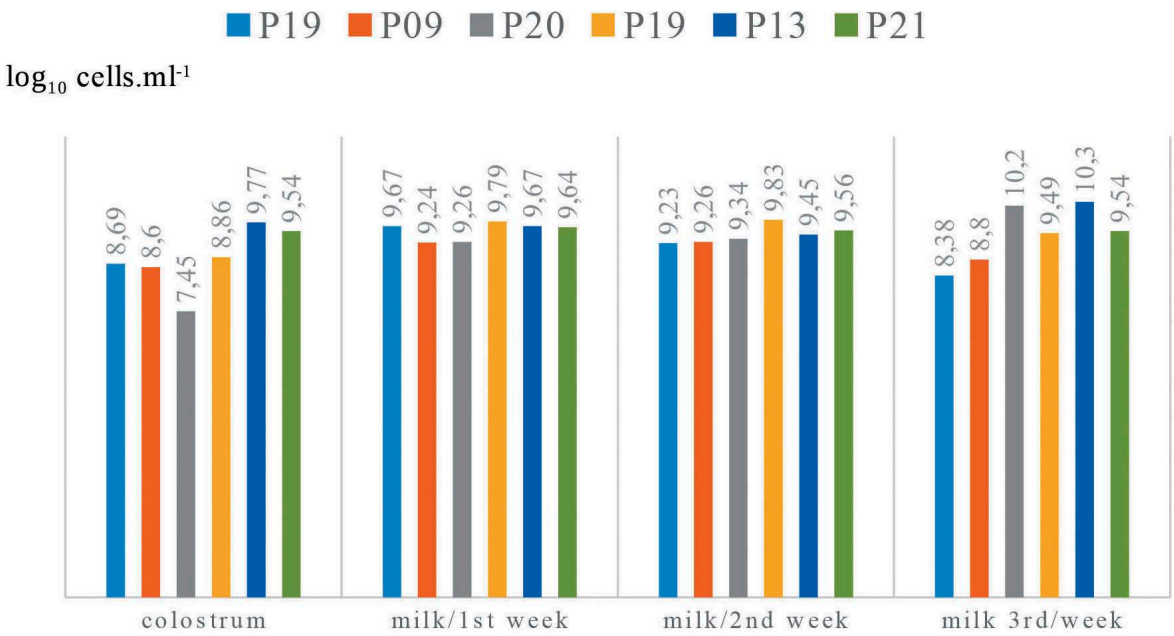

Fig. 3. Numbers of cellular elements in the colostrum and milk in sows (log_{10}cells.ml^{-1})

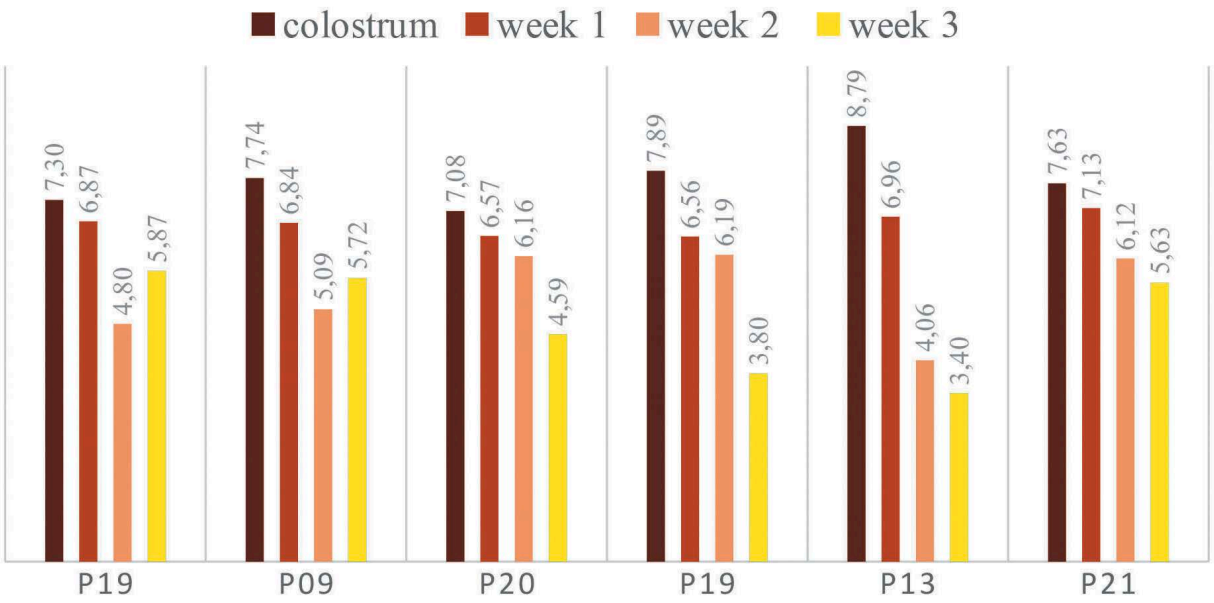

Fig. 4. Leucocytes in sow milk (CD45+), comparison of experimental animals

teats are located cranially), different morphology of teats, and surely different age and breed of sows.

The colostrum of the sow contains a significant number of leukocytes. The predominant cell types in porcine colostrum are neutrophils and lymphocytes [3, 26]. Lymphocytes from maternal colostrum are able to cross the intestinal epithelial barrier of neonatal piglets and to migrate via the blood to different tissues (e.g. lymph nodes, spleen, liver, lungs). Furthermore, research has shown that these cells are functional and show antigen-specific activity in these organs [15]. The proportion of lymphocytes in the colostrum differs from that in the peripheral blood of sows. The proportion of cytotoxic (CD8+) T-cells is significantly higher in porcine colostrum than in peripheral blood [9]. In contrast, the proportion of helper (CD4+) T-cells in the colostrum of sow is lower than in peripheral blood.

P o m o r s k a et al. [19] monitored leukocyte subsets in porcine milk secretions and determined the percentage of monocytes/macrophages (CD45+CD14+), T-cells (CD3+) and their subpopulations (CD4+, CD8+) and double pos-

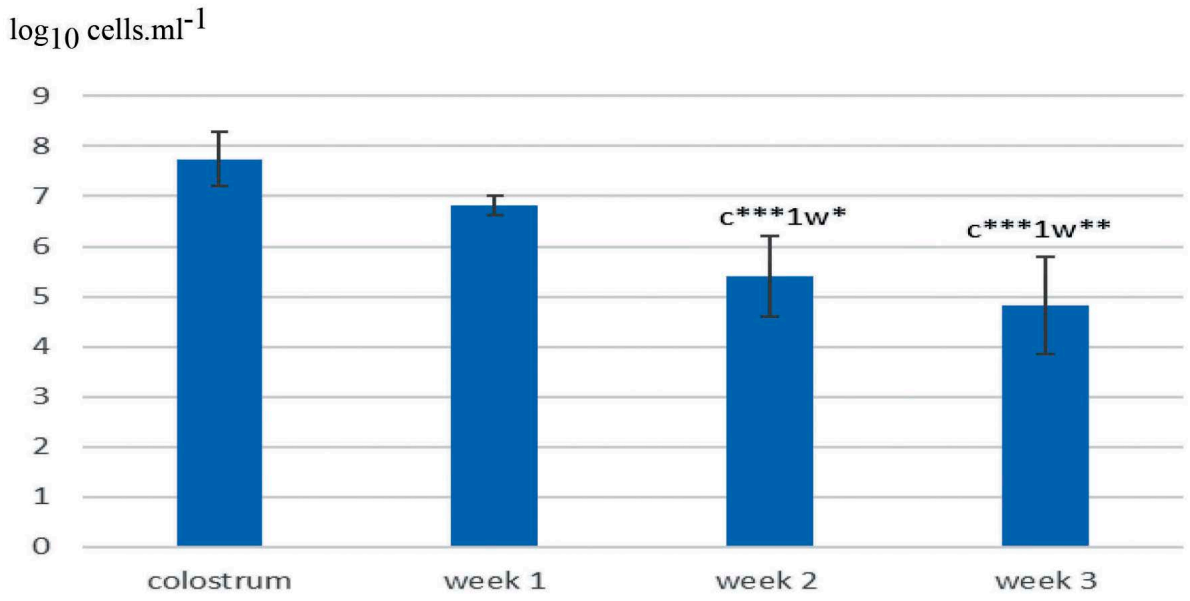

Fig. 5. Average numbers of leucocytes (CD45+) in the sow´s colostrum and milk taken 1, 2 and 3 weeks after the parturition c – significantly different from colostrum; 1w – significantly different from week 1; * P < 0.05; ** P < 0.01; *** P < 0.001

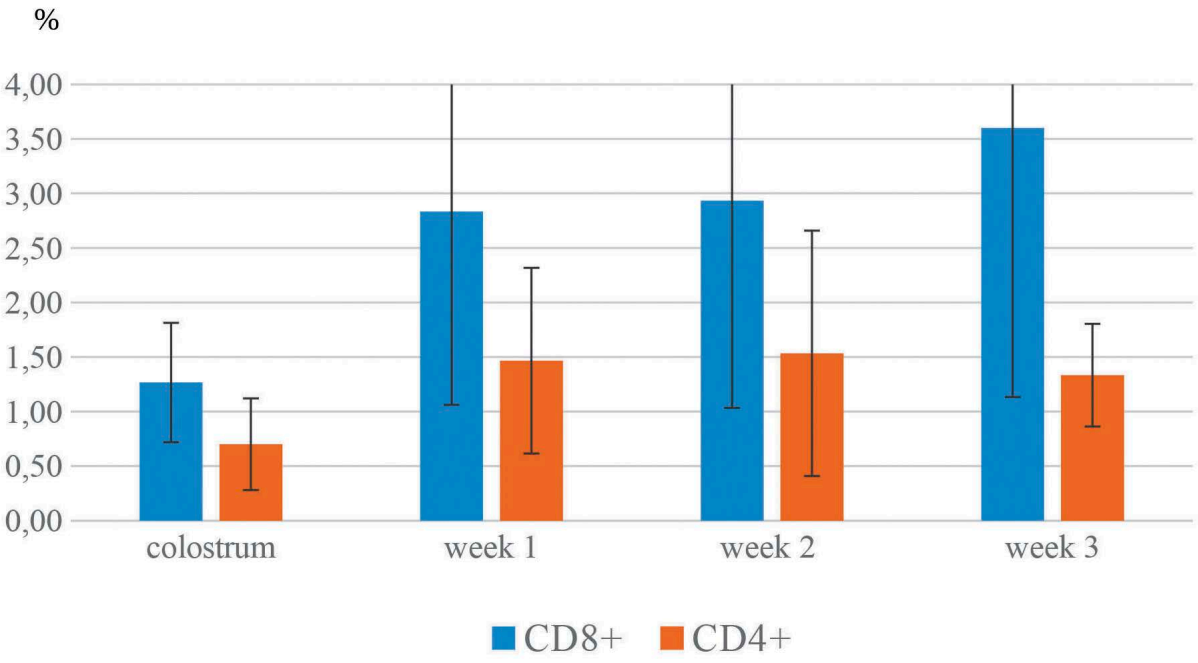

Fig. 6. Percentage of T-cytotoxic (CD8+) and T-helper (CD4+) lymphocytes in milk

itive (DP) CD4+CD8+) in colostrum and milk using flow cytometry. The percentage of both CD8+ and CD4+ cells was relatively stable during lactation, except for a significant decrease on the last day of lactation. On the other hand, our findings showed that the levels of CD4+ and CD8+ increased gradually over time, with the highest amount in the third week after birth. This means that both helper T-cells (CD4+) and cytotoxic T-cells (CD8+) were present at increased levels each week. Cytotoxic T-cells were present in greater numbers than helper T-cells, which was confirmed by the study of P o m o r s k a et al. [19], as CD8+ cells also predominated over CD4+ cells throughout lactation. This may be due to the nature of the cells whose function is to fight disease. These findings confirm that these T-lymphocytes play an important role in the immune protection of piglets.

CONCLUSIONS

The use of flow cytometry in the analysis of sow colostrum and milk can be useful for the experimental determination of SCC, leukocytes and T-lymphocytes despite the high content of milk fat. These results will also help to understand the importance of colostrum and milk in relation to piglet immunity. From the point of view of further experiments, however, it is necessary to analyse samples from a larger number of animals, preferably purebred pigs with a precise specification of the sampling site.

REFERENCES

1. **Albenzio, M., Caroprese, M., 2011:** Differential leucocyte count for ewe milk with low and high somatic cell count. *J. Dairy Res*., 78, 43–48. DOI: 10.1017/S0022029910000798.
2. **Bandrick, M., Ariza-Nieto, C., Baidoo, S. K., Molitor, T. W., 2014:** Colostral antibody-mediated and cell-mediated immunity contributes to innate and antigen-specific immunity in piglets. *Dev. Comp. Immunol*., 43, 1, 114–120. DOI: 10.1016/j.dci.2013.11.005.
3. **Boutinaud, M., Jammes, H., 2002:** Potential uses of milk epithelial cells: A review. *Reprod. Nutr. Dev.*, 42, 133–147. DOI: 10.1051/rnd:2002013.
4. **Craig, J. R., Dunshea, F. R., Cottrell, J. J., Wijesiriwardana, U. A., Pluske, J. R., 2019:** Primiparous and multiparous sows have largely similar colostrum and milk composition profiles throughout lactation. *Animals*, 9, 2, 35. DOI: 10.3390/ani9020035.
5. **Dosogne, H., Vangroenweghe, F., Mehrzad, J., Massart-Leen, A. M., Burvenich, C., 2003:** Differential leukocyte count method for bovine low somatic cell count milk. *J. Dairy Sci.*, 86, 828–834. DOI: 10.3168/jds.S0022-0302(03)73665-0.
6. **Evans, P. A., Newby, T. J., Stokes, C. R., Bourne, F. J., 1982**: A study of cells in the mammary secretions of sows. *Vet. Immunol. Immunopathol*., 3, 515–527. DOI: 10.1016/0165-2427(82)90017-4.
7. **Forner, R., Newby, T. J., Stokes, C. R., Bourne, F. J., al., 2021:** Distribution difference of colostrum-derived B and T cells subsets in gilts and sows. *PLOS ONE*, 16, 889. DOI: 10.1371/journal.pone.0249366.
8. **Gerner, W., Talker, S.., Koinig, H., Sedlak, C., Mair, K. H., Saalmüller, A., et al., 2015:** Phenotypic and functional differentiation of porcine alphabeta T cells: Current knowledge and available tools. *Mol. Immunol.*, 66, 3–13. DOI: 10.1016/j.molimm.2014.10.025.
9. **Hlavova, K., Stepanova, H., Faldyna, M., 2014:** The phenotype and activation status of T and NK cells in porcine colostrum suggest these are central/effector memory cells. *Vet. J*., 202, 3, 477–482. DOI: 10.1016/j.tvjl.2014.09.008.
10. **Kelly, A. L., Tiernan, D., O'Sullivan, C., Joyce, P., 2000:** Correlation between bovine milk somatic cell count and polymorphonuclear leukocyte level for samples of bulk milk and milk from individual cows. *J. Dairy. Sci.*, 83, 300–304. DOI: 10.3168/jds.S0022-0302(00)74878-8.
11. **Le Dividich, J., Rooke, J. A., Herpin, P., 2005:** Nutritional and immunological importance of colostrum for the new-born pig. *J. Agr. Sci.,* 143, 469–485. DOI: 10.1017/S0021859605005642.
12. **Le Jan, C., 1994:** A study by flow cytometry of lymphocytes in sow colostrum. *Res. Vet. Sci*., 57, 3, 300–304. DOI: 10.1016/0034-5288(94)90121-x.
13. **Magnusson, U., Rodriguez-Martinez, H., Einarsson, S., 1991:** A Simple, rapid method for differential cell counts in porcine mammary secretions. *Vet. Rec*., 129, 22, 485–490. DOI: 10.1136/vr.129.22.485.
14. **Maurer, R. T., Gourley, K. M., Rathbun,T. J., Woodworth, J. C., DeRouchey, J. M., Tokach, M. D., et al., 2020:** Evaluating sow colostrum somatic cell count as an indicator for colostrum composition or litter characteristics. *J. Anim. Sci*., 30, 98 (Suppl. 3), 237–238. DOI: 10.1093/jas/skaa054.415. PMCID: PMC7701755.
15. **Nechvatalova, K., Kudlackova, H., Leva, L., Babickova, K., Faldyna, M., 2011:** Transfer of humoral and cell-mediated immunity via colostrum in pigs. *Vet. Immunol. Immunopathol*., 142, 1–2, 95–100. DOI: 10.1016/j.vetimm.2011.03.022.
16. **Olechnowicz, J., Jaskowski, J. M., 2012:** Somatic cells count in cow′s bulk tank milk. *J. Vet. Med. Sci.*, 12, 74, 681–686. DOI: 10.1292/jvms.11-0506.
17. **Paape, M. J., Bannerman, D. D., Zhao, X., Lee, J. W., 2003:** The bovine neutrophil: Structure and function in blood and milk. *Vet. Res*., 34, 597–627. DOI: 10.1051/vetres:2003024.
18. **Piepers, S., DeVliegher, S., Demeyere, K., Lambrecht, B. N., DeKruif, A., Meyer, E., et al., 2009:** Technical note: Flow cytometric identification of bovine milk neutrophils and simultaneous quantification of their viability. *J. Dairy Sci.*, 92, 626–631. DOI: 10.3168/jds.2008-1393.

19. **Pomorska-Mol, M., Markowska-Daniel, I., Bednarek, D., 2010:** Flow cytometric analysis of leukocytes in porcine mammary secretions. *Bull. Vet. Inst.*, Pulawy, 54, 2, 188–192. Available at https://api.semanticscholar.org/CorpusID:9631670.
20. **Rainard, P., Riollet, C., 2006:** Innate immunity of the bovine mammary gland. *Vet. Res*., 37, 369–400. DOI: 10.1051/vetres:2006007.
21. **Reuter, K., Ehmcke, J., Stukenborg, J. B., Simoni, M., Damm, O. S., Redmann, K., et al., 2014:** Reassembly of somatic cells and testicular organogenesis in vitro. *Tissue Cell*, 46, 86–96. DOI: 10.1016/j.tice.2013.12.001.
22. **Riollet, C., Rainard, P., Poutrel, B., et al., 2001:** Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. *J. Dairy Sci.*, 84, 1077–1084. DOI: 10.3168/jds.S0022-0302(01)74568-7.
23. **Rupp, R., Boichard, D., Bertrand, C., Bazin, S., 2000:** Overview of milk somatic cell counts in the French dairy cattle breeds. *Prod. Anim*., 13257–13267. DOI: 10.20870/productions-animales.2000.13.4.3785.
24. **Salmon, H., Berri, M., Gerdts, V., Meurens, F., 2009:** Humoral and cellular factors of maternal immunity in swine. *Dev. Comp. Immunol.*, 33, 3, 384–393. DOI: 10.1016/j.dci.2008.07.007.
25. **Stinson, L. F., Boyce, M. C., Payne, M. S., Keelan, J. A., 2019:** The not-so-sterile womb: Evidence that the human foetus is exposed to bacteria prior to birth. *Front. Microbiol*., 10, 1124. DOI: 10.3389/fmicb.2019.01124.
26. **Wuryastuti, H., Stowe, H. D., Bull, R. W., Miller, E. R., 1993:** Effects of vitamin E and selenium on immune responses of peripheral blood, colostrum, and milk leukocytes of sows. *J. Anim. Sci.*, 71, 2464–2472. DOI: 10.2527/1993.7192464x.
27. **Zhang, S., Chen, F., Zhang, Y., Lv, Y., Heng, J., Min, T., et al., 2018:** Recent progress of porcine milk components and mammary gland function. *J. Anim. Sci*., 9, 77. DOI: 10.1186/s40104-018-0291-8.

Received June 12, 2024
Accepted July 17, 2024