Journal of Dairy Research

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Assessment of flow cytometric tools to characterize milk somatic cells in water buffalo

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Research Article

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Cite this article: Scatà MC, Grandoni F and De Matteis G (2025) Assessment of flow cytometric tools to characterize milk somatic cells in water buffalo. *Journal of Dairy Research* **92**, 69–72. https://doi.org/10.1017/S0022029925000329

Received: 4 September 2024
Revised: 21 November 2024
Accepted: 21 January 2025
First published online: 18 August 2025

Keywords:
flow cytometry; milk somatic cells; water buffalo

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Abstract

The aim of this Research Communication was to develop new flow cytometric tools for the fine identification and characterization of milk somatic cells in water buffalo (*Bubalus bubalis*). Four multicolour panels of antibodies were designed to identify different subsets of live leukocytes and epithelial cells in bulk milk samples. Panel 1, including the CD18/CD172a/CD14/CD16 markers and Live/Dead vitality dye, allowed us to identify total lymphocytes, polymorphonuclear neutrophils (PMN) and monocyte/macrophage subsets. Panel 2 (CD18/CD4/CD8/δ chain/CD335 and Live/Dead dye) allowed us to identify T helper ($CD4^+$), T cytotoxic ($CD8^+$), γδ lymphocytes and NK cells. Panel 3 (CD18/CD79a/CD21 and Live/Dead dye) allowed us to identify total and $CD21^+$ B lymphocytes. Finally, with Panel 4 (CD18/MHC-I/pan Cytokeratin and Live/Dead dye) the epithelial cells were distinguished from leukocytes. In conclusion, we propose a fine characterization of live milk somatic cell (live differential cell count (LDCC)) in buffalo species. In the future the determination of LDCC could used to identify new markers for detecting early inflammatory states of the mammary gland or for monitoring the technological properties of milks of different somatic cell composition.

Milk somatic cell count (SCC) is still used today as an indicator of udder health in dairy animals, and is a useful parameter for monitoring farm hygiene in bulk milk. However, SCC is not always a clear indicator of a potential infection because this parameter is temporally and individually variable during lactation. Milk somatic cells consist mainly of leukocytes and, in lower numbers, of epithelial cells representing part of immune defences of the mammary gland (Sordillo *et al.*, 1997). The viable leukocytes inside the udder offer different degrees of cellular protection against microbial invasion and may aid in the restructuring of the mammary gland that occurs during involution. In addition to the microbicidal functions of phagocytosis, leukocytes secrete a variety of immune-related components into milk including cytokines, chemokines, reactive oxygen species, antimicrobial proteins and peptides, in addition to assisting in the repair of damaged tissue caused by shedding and renewal processes (Ezzat Alnakip *et al.*, 2014). Milk leukocytes include different subsets such as polymorphonuclear neutrophils (PMN), macrophages and lymphocytes that perform specific functions during the immune response (Sordillo 2018). The relative proportion of lymphocytes, macrophages and PMN, called the differential cell count (DCC) can be performed using two different methods: microscopy and flow cytometry. The first is a simple and cost-effective method, the second is a flexible and powerful technology measuring a high cell number within a short time, maximizing the repeatability of test results, and increasing the accuracy. Several studies showed that DCC by flow cytometry is a useful and effective parameter for the early diagnosis of clinical and subclinical mastitis in different species such as sheep, bovine and dromedary camel (Albenzio and Caroprese, 2011; Pilla *et al.*, 2013; Alhafiz *et al.* 2022). Moreover, considerable research has focused on the use of DCC as a tool to understand the immune response to different pathogens of mastitis in quarter milk samples (Blagitz *et al.*, 2013, 2015). Previously, we showed that conventional flow cytometry can be used to effectively identify new immunological markers of udder health status in bovine composite milk samples (De Matteis *et al.*, 2020). Lately, Farschtschi *et al.* (2021) developed a flow cytometric immunophenotyping method in milk and blood to identify many biomarkers during systemic cattle diseases that are not directly affecting the mammary gland. Furthermore, they applied this cytometric assay to monitor the immune status over the lactation period in dairy cows (Farschtschi *et al.*, 2022). From our knowledge, few data are available in the literature concerning DCC in buffalo species. Previous studies showed that in buffalo milk a strong positive relationship exists between SCC and mastitis in buffalo and value of SCC > 200.000 cells/mL together with PMN cells higher than 50% should be used as a threshold value for identifying

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subclinical mastitis (Moroni *et al.*, 2006; Tripaldi *et al.*, 2010). Hussain *et al.* (2012) showed that the SCC and neutrophil counts were significantly higher, while the macrophage and lymphocytes were lower in the milk of mastitic Nili-Ravi buffaloes and cattle. In these studies, the DCC was performed only by microscopy. To fill this gap, the aim of the present study was to assess a flow cytometry immunophenotyping of milk somatic cells and the determination of their viability in buffalo milk (L-DCC). From our point of view these flow cytometric tools could lead to a significant increase in knowledge about animal health, welfare, and milk quality in buffalo species.

Materials and methods

Bulk tank milk samples were collected from lactating buffalo cows kept at the Research Center for Animal Production and Aquaculture of CREA. 50 mL of fresh milk were centrifuged at 800 x g for 20 min at 4 °C and the fat layer and supernatant were discarded. The cell pellets were washed twice with 50 mL of cold phosphate buffered saline solution (PBS) and centrifuged at 300 x g for 5 min. All samples were filtered through a 50 µm mesh to remove clumps debris and counted with LUNA-II™ Automated Cell Counter (Logos Biosystems by Aligned Genetics, Inc.). 1×10^6 cells were transferred into separate tubes and washed with 1 mL of cold PBS and centrifuged at 300 x g for 5 min. The cells were then incubated for 20 min at 4 °C in the dark, with saturating concentration of each monoclonal antibody of the four multicolour panels (online Supplementary Table S1), in a final volume of 100 µL of PBS. Optimal antibody concentrations were first determined by performing a series of single-colour titrations. All antibody panels were composed of primarily labeled antibodies to avoid a long working time and to reduce the cell loss that could occur with the many wash steps. In a preliminary trial in this study, we tested a pan-leukocyte marker, the anti-mouse CD45 antibody (clone 30 F-11 by ThermoFisher), to verify the cross reactivity in buffalo species.

Three antibodies (CD4, δ chain and MHC-I) were labeled in house using LYNX Rapid PE and RPE-Cy7 (Bio-Rad) as described in online Supplementary Table S1. Afterwards, 1 µL of the viability dye (Live/Dead ™ Fixable Near-IR Dead Cell Stain Kit, ThermoFisher) was added at 1:1000 final concentration and the samples were incubated for 10 min at RT. Finally, 2 mL of cold PBS was added to tubes and the samples were centrifuged at 300 x g for 5 min at 4 °C. For panels 3 and 4 it was necessary to permeabilize the cells because CD79a and cytokeratin are intracellular markers. This step was performed using the kit Cytofix/Cytoperm™ solution (BD Biosciences) following the manufacturer′s instructions. Briefly, the pellets were resuspended in 125 µL of fixation/permeabilization buffer (Cytofix/Cytoperm™ solution, BD Biosciences) and incubated at 4 °C for 20 min. After two cycles of washing with 800 µL of Perm/Wash buffer (BD Biosciences) at 400 x g for 10 min, the permeabilized cells were incubated with anti-CD79a (Panel 3) and anti-cytokeratin (Panel 4) at 4 °C for 30 min. After one wash with 800 µL of Perm/Wash buffer at 400 x g for 5 min, the cells were resuspended in the 150 µL of same buffer. All labeled samples were immediately acquired using a CytoFLEX flow cytometer; the data were analyzed using Kaluza software v. 2.1 (Beckman Coulter, Brea, CA, USA). A matrix of compensation was created for each panel of antibody using the VersaComp antibody Capture beads kit (Beckman Coulter) to correct the emission spectra overlap of the fluorochrome, removing the signal of any given fluorochrome from all detectors except the one devoted to measuring that dye.

Results and discussion

In this research communication we propose a fine characterization of live milk somatic cell (live differential cell count, LDCC) in water buffalo (*Bubalus bubalis*) and assess new flow cytometric tools for achieving this. For this purpose, we have developed four-multicolour panels (Panel 1-4) of antibodies to determine the composition of somatic cells in buffalo milk. In bovine species, high-resolution differential cell counts (HRDCCs) were assessed to provide in-depth immunophenotyping of blood and milk immune cells (Farschtschi *et al.*, 2021, 2022). The HRDCCs have proven to be a promising tool for more efficient milk diagnostics, ensuring milk quality and supporting cattle health and welfare.

In our study, unlike the HRDCCs, we have developed a rapid flow cytometric assay without the use of secondary antibody labeling. This reduces sample handling and avoids cell loss, thus improving the accuracy of the results. In each panel we added a marker of immune cells and a dye of viability to easily separate live leukocytes from debris and fat globules which may have remained in the cell pellet after the purification process. In our previous studies we tested the cross reactivity of five clones of anti-CD45 antibody markers (pan leukocyte marker) with water buffalo and none of them had shown positivity towards the buffalo (Grandoni *et al.*, 2017) Furthermore, in this study we observed that the clone 30 F-11 also did not recognize water buffalo CD45 antigen. Due to the lack of buffalo cross-reactivity of anti-CD45 antibody leukocyte markers we used anti-CD18 antibody as a marker of all leukocytes, as previously validated by Grandoni *et al.* (2023b). CD18 is the 95 kDa integrin β2. The β2 integrins (CD11/CD18) are the major adhesion molecule family of leukocytes and the β2 heterodimers are restricted to cells of the leukocyte lineage (Harris *et al.*, 2000). For panels 1-3, milk cells were first gated on FSC-Area and FSC-Height to exclude doublets and CD18 was plotted on SSC-A to separate all leukocytes. The CD18 positive cells were then plotted on Live/Dead *vs* SSC to distinguish live leukocytes ($CD18^+$/$Live/Dead^-$) from dead leukocytes ($CD18^+$/$Live/Dead^+$).

Panel 1 was designed as a five-colour panel with CD172a, CD14 and CD16 markers to CD18 and Live/Dead. The CD172a myeloid cell marker allowed us to differentiate myeloid from lymphoid cells, also distinguishing PMN, lymphocytes and monocytes/macrophages (Fig. 1A–E). The expression of CD172a, CD14 and CD16 is used to identify the three subsets of monocytes defined as classical (cM), intermediate (intM) and non-classical (ncM) monocytes in bovine and buffalo blood as identified by Hussen and Schuberth (2017) and Grandoni *et al.* (2023a). In buffalo milk, panel 1 allowed us to recognize two populations of monocytes/macrophages: a major population as $CD172a^+CD14^+CD16^+$ and a minor population as $CD172a^+CD14^{\pm}CD16^+$ (Fig. 1F). The co-expression of CD172a, CD14 and CD16 on monocytes agreed with data found by Elnaggar *et al.*, (2019) in buffalo blood samples. In a recent study Farschtschi and collaborators identify two subsets in bovine milk based only on the double expression of CD14 and CD16 markers: cM ($CD14^+CD16^-$) and ncM ($CD14^{\pm}$ $CD16^+$) but the lack of CD172a in their panel didn't allow us to compare these with our data (Farschtschi *et al.*, 2021). For a more reliable identification of subsets of monocytes/macrophages in buffalo milk other

https://doi.org/10.1017/S0022029925000329 Published online by Cambridge University Press

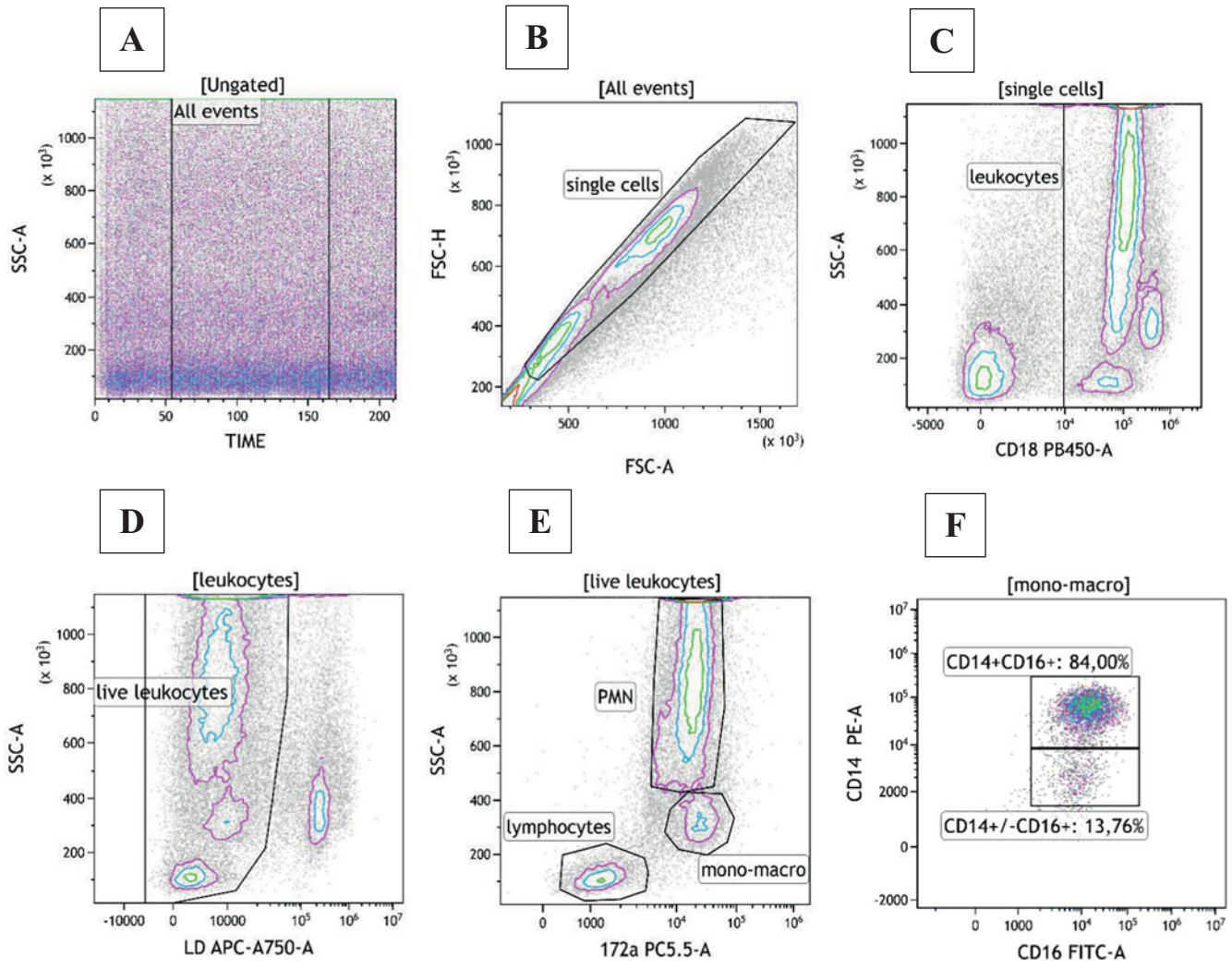

Figure 1. Gating strategy used in this study for the identification of leukocyte populations by flow cytometric analysis: **(A, B)** A dot plot FSC-A *vs.* FSC-H on All events was used to exclude doublets and a morphological gate was drawn to highlight single cells (singlets); **(C)** a dot plot CD18 PB450-A *vs.* SSC-A on singlets was used to identify leukocytes; **(D)** a dot plot LD APC-A750-A *vs.* SSC-A on leukocytes was used to identify live leukocytes; **(E)** a dot plot CD172a PC5.5-A *vs.* SSC-A on live leukocytes was used to differentiate myeloid from lymphoid cells, distinguishing in polymorphonuclear leukocytes (PMN), lymphocytes and monocytes/macrophages; **(F)** the dot plot CD16 FITC-A *vs.* CD14 PE-A on monocytes-macrophges was used to caracterize the subsets of monocytes in milk.

macrophage-specific markers could be added in the same mix as MHC-II and CD163.

Panel 2 was designed as a six-colours panel adding CD4, CD8, δ chain and CD335 markers to CD18 and Live/Dead. These markers allowed the identification of all subsets of T lymphocytes (T helper, T cytotoxic, T γδ) and NK cells. The proposed gating strategy is shown in supplementary Figure S1. Leukocytes were gated in a Live/Dead *vs* SSC dot plot (Fig. 1D) and a morphological gate was drawn to highlight lymphocytes (online Supplementary Figure S1, A). The lymphocytes were gated in δ chain *vs* CD335 plot, to identify γδ T and NK cells (online Supplementary Figure S1, B) as described by Grandoni *et al.* (2017) in buffalo blood. By gating the double negative population (other lymphocytes) on CD4 *vs* CD8 plot we were able to identify T helper ($CD4^+$) and T cytotoxic ($CD8^+$) lymphocytes (online Supplementary Figure S1, C).

In these bulk samples we have not identified NK cells. Similarly, Farschtschi *et al.* (2022) reported very low levels of NK cells (0.5 % of all viable lymphocytes) in composite milk samples (from all four quarters) in bovine animals. To understand if the lack of visualization of the NK was due to problems of the fluorescence channel we tried phycoerythrin (PE) dye, and we obtained the same result: no visualization of the NK cells. This could be due to the low concentration of these cells in the bulk milk sample. To characterize B lymphocytes, we used the four-colour panel 3 adding CD79a, and CD21 markers to CD18 and Live/Dead. The lymphocytes were gated (online Supplementary Figure S2, A) on CD21 *vs* CD79a plot to identify the different subsets of B lymphocytes (online Supplementary Figure S2, B).

Grandoni *et al.* (2023b) highlighted the presence of 5 different B lymphocyte subsets in whole blood due to the different expressions of the markers CD79a and CD21. Our data also confirms the presence of B lymphocytes in buffalo milk. Panel 4 was used to identify mammary epithelial cells (MEC). We added MHC-I marker to identify all somatic cells and CD18 marker to discriminate the leukocytes. Major histocompatibility complex (MHC) class I genes encode highly polymorphic molecules that are expressed on virtually every nucleated cell type and have been identified in all vertebrates (Ellis, 2004). Moreover, the anti-cytokeratin monoclonal

https://doi.org/10.1017/S0022029925000329 Published online by Cambridge University Press

antibody, previously used in the literature (Farschtschi *et al.*, 2021) was used to identify the epithelial cells. This antibody reacts with cytokeratin peptides 4, 5, 6, 8, 10, 13, 18. Cytokeratin is part of a subfamily of intermediate filament proteins that are represented in bovine epithelial cells (Baratta *et al.*, 2015). The gating strategy has been slightly modified compared to the previous ones as shown in online Supplementary Figure S3. Single cells were first gated on MHC-I *vs* SSC dot plot to separate all somatic cells (MHC-I$^+$) from debris and fat globules (online Supplementary Figure S3, A-C). All cells were gated on Live/Dead *vs* SSC dot plot (online Supplementary Figure S3, D) to separate the viable from dead cells and then the live cells were gated on CD18 *vs* SSC dot plot to identify live leukocytes (CD18$^+$) from other cells (CD18$^-$) (online Supplementary Figure S3, D-E). Finally, the CD18$^+$ cells were plotted on pan-Cytokeratin *vs* SSC to identify the citokeratin$^+$ milk epithelial cells (MEC: online Supplementary Figure S3, F). Our four flow cytometric panels allowed a thorough and simultaneous identification of viable milk cells in water buffaloes. In Italy, buffalo milk quality is of extreme importance for the dairy industry because the milk is mostly intended for the manufacture of mozzarella cheese. Recently, Costa *et al.*, (2020) showed that high milk SCC in buffalo milk is associated with altered composition and poor technological properties. A broader and more comprehensive identification of somatic cells in buffalo milk could improve knowledge on immunology of mammary gland and monitor both animal welfare and milk quality in Italian Mediterranean buffalo cows.

In conclusion, we have successfully used flow cytometry to achieve a live differential milk somatic cell count in water buffalo. Applying this immunophenotyping approach to composite or quarter milk samples could provide a valuable tool to monitor buffalo udder health during lactation or milk quality in relation to the composition of L-DCC different cell subsets. Moreover, these tools, after confirmation of the cross-reactivity of the antibodies for the species of use, could be easily applied to milk samples from other species.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029925000329.

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