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Research article

Deeper exploration of inflammatory cell populations in milk to monitor udder health in dairy cows

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ABSTRACT: The present study explored the predictive values of milk leukocyte differentials (MLD) as a basis for improving the diagnosis of intramammary infections (IMIs) and subclinical mastitis. Quarter milk samples were collected for bacteriological analysis, quarter somatic cell count (qSCC), and MLD. The MLD were assessed using the cytospin technique, direct microscopic smears, and flow cytometry. The predictive values of each single leukocyte population and useful potential indices that could better reflect immune complexity were also calculated. Changes in the percentage of any leukocyte alone failed to substantially improve the predictive value of qSCC in diagnosing IMIs. Although certain parameters increased the area under the receiver operating characteristic curve (ROC curve) as a result of increased specificity values, a slight reduction in sensitivity was observed. The so-called CD8 complex was a unique parameter which improved both the sensitivity (78.79 %) and the specificity (80.77 %) in IMI diagnosis, resulting in the highest area under the ROC curve (0.87). To diagnose subclinical mastitis, the percentage of macrophages and the sum of the percentage PMNLs and T CD8$^+$ cells divided by the percentage of macrophages showed the highest predictive values (sensitivity = 79.63, specificity = 73.68, and area under the ROC curve = 0.83) in the differentiation of the inflammatory condition status of cows. In conclusion, this study provides further insights into using T CD8$^+$ lymphocytes in diagnosing bovine IMIs, combined with PMNLs and macrophages. The antidromic trend of macrophages *vs.* PMNLs and T CD8$^+$ lymphocytes due to the increasing qSCCs was crucial to differentiating quarters under both inflammatory and non-inflammatory conditions.

Keywords: differential cell count, somatic cell count, diagnosis, mastitis, dairy cattle

Introduction

The definition of bovine mastitis has not always been consistent. Markedly, the terminology intramammary infection (IMI) and subclinical mastitis are often used interchangeably. Because of this concern, IMIs require the presence of an infectious pathogen, which usually entails an increase in milk somatic cell count (SCC). Conversely, subclinical mastitis indicates an inflammatory process and does not entail an infection, although it is often caused by a bacterial infection (Andersen et al., 2010). Under this scenario, the bacteriological examination is crucial to define IMIs. At the same time, the SCC is extensively used as the gold standard for measuring inflammation and is, therefore, included as a component of the definition of mastitis (Pyörälä, 2003; Andersen et al., 2010).

Somatic cell count measures all somatic cell types in milk but it does not distinguish the different cell populations present. Thus, it has been proposed that differential cell counting could provide a more reliable udder health status of the mammary gland (Pyörälä, 2003; Koess and Hamann, 2008; Takano et al., 2018). Light microscopy and flow cytometry can obtain differential cell counts (DCCs). Microscopic DCC is a simple and cost-effective method although several researchers prefer flow cytometry analysis on account of its greater accuracy (Koess and Hamann, 2008; Pilla et al., 2013; Takano et al., 2018).

The distribution and counts of leukocytes are critical to mammary gland defenses. Therefore, as regards the complexity of the immune system and participation of all cell types in immune responses, the quantification of a single cell type may not provide the most reliable data for identifying what is different for diagnosis decisions (Leitner et al., 2015). Given this background, the present study aimed to explore the predictive values of several parameters using milk leukocyte differentials to improve the identification of IMIs and subclinical mastitis that could better stimulate immunity during infection or/and inflammation.

Materials and Methods

This study complied with the Ethical Principles in Animal Research. It was approved by the Bioethics Commission of the Faculdade de Medicina Veterinária e Zootecnica – Universidade de São Paulo (Process n. 1685/2009).

Animals and sampling

The present study collected 112 quarter milk samples from 28 clinically healthy Holstein dairy cows [mean daily milk yield = 24.19 ± 0.71 kg; mean parity = 2.65 ± 0.16; mean days in milk (DIM) = 196.6 ± 12.13] from a commercial dairy herd. Immediately postpartum (up to 21 DIM) animals were not used for this study.

Prior to the milk sampling, the strip cup test was performed to detect any abnormal secretions. Next, pre-dipping was carried out, and one towel was used for each teat. After discarding the first three streams the ends of the teats were scrubbed with 70 % ethanol using a piece of cotton. Quarter milk samples were aseptically collected for microbiological analysis as the NMC (1999) described. Following this, milk samples were collected by hand milking in sterile polypropylene vials (cat. n. CLS4558-300EA, Corning) for the quarter SCC (qSCC; 40 mL) and the differential cell counts by direct microscopic smears (10 mL), and in sterile polypropylene bottles (cat. n. 3120-0500, Nalgene™ PPCO Centrifuge Bottles, ThermoFisher Scientific) for the differential cell counts by cytospin and cytometric flow analyses (200 mL). Milk samples were kept at 4 °C until arrival at the laboratory. Next, milk samples were randomized and codified, and the additional milk analyses were conducted without knowing the quarter's status.

Bacteriological culture

The bacteriological culture was carried out by culturing 0.01 mL of each milk sample on 5 % sheep blood agar plates (Becton Dickinson GmbH). The plates were incubated for 24-72 h at 37 °C, followed by observation of colony morphology, Gram staining, and biochemical testing (Oliver et al., 2004). The sample was considered culture-positive when $\geq$ 1 colony grew ($<$ 100 cfu mL^{-1}).

Determination of milk qSCC

For qSCC measurement, milk samples were collected in tubes containing microtablets of bronopol (2-bromo-2-nitropane-1,3-diol) and were performed using an automated somatic cell counter (Somacount 300, Bentley Instruments®), as previously described (IDF, 1995).

Direct microscopic smears

Milk leukocyte differentials were determined by direct microscopic smears using fresh milk (Blagitz et al., 2013; Takano et al., 2018). The milk smears in duplicate were stained with the Rosenfeld dye (Rosenfeld, 1947), a combination of May-Grunwald and Giemsa dyes, and the polymorphonuclear and mononuclear leukocytes were differentiated at a magnification of × 100.

Separation of milk cells for flow cytometry and cytospin centrifuge

For flow cytometric and cytospin analysis, 200 mL of milk from each mammary quarter was diluted with 200 mL of PBS. Milk cells were separated as previously described by Blagitz et al. (2015) and Souza et al. (2022). In brief, milk samples were centrifuged at 1,000 × g for 15 min, and the cream layer and supernatant were discarded. The cell pellet was washed once using 30 mL of PBS and centrifuged at 400 × g for 10 min. Next, the cells were resuspended in 1 mL of RPMI-1640 nutritional medium (R7638, Sigma Aldrich) supplemented with 10 % fetal bovine serum (Cultilab) and counted using a Neubauer chamber. Next, the milk cells were placed in 1 mL of RPMI-1640 nutrition medium (R7638, Sigma Aldrich) supplemented with 10 % fetal bovine serum (Cultilab). Cell viability was assessed using trypan blue exclusion and counted utilizing a hematocytometer. The milk cells were then adjusted to 2 × 10^6 viable cells mL^{-1} using the nutrition medium and 10 % fetal bovine serum to dilute the milk cell suspensions so as to achieve the target concentration.

Cytospin technique

The cytospin technique for differential leukocyte counts was used as previously described by Della Libera et al. (2004) and Takano et al. (2018). In short, 200 µL of milk cell suspension in triplicate was centrifuged at 28 × g for 6 min using a cytocentrifuge (Cytospin 3 SHANDON®). Next, the smears were stained with the Rosenfeld dye (Rosenfeld, 1947), a combination of May-Grunwald and Giemsa dyes, and 400 leukocytes per sample were differentiated into lymphocytes, macrophages and polymorphonuclear leukocytes at a magnification of × 100.

Identification of milk leukocytes by flow cytometry

Identification of milk leukocyte populations was based on their cytoplasmatic granularity and mean fluorescence intensity following 2-step fluorescent immunolabeling with primary anti-bovine monoclonal antibodies (mAbs) and the secondary antibodies (Ab) coupled to the long-wavelength fluorescent probes (Table 1). In brief, 100 µL of milk cells (2 × 10^5 viable cells) were washed with PBS and incubated with the primary mAbs for 30 min on ice to detect CD21 (tube A), and combinations of CD3, CD4 and CD8 (tube B), and CH138 and CD14 (tube C) in polypropylene tubes suitable for flow cytometry as previously described (Della Libera et al., 2015; Souza et al., 2020). After washing with PBS, the cells were incubated for 30 min at room temperature with the secondary Abs. Following this, the cells were washed with PBS and analyzed by flow cytometry (FACSCalibur, BD Bioscience). Twenty thousand milk cells were

Table 1 – Monoclonal antibodies used for labeling and differentiation of bovine milk leukocytes in flow cytometric analysis.

Description	Primary antibody							Secondary antibody						
	Name	Type	Amount	Specificity	Host	Company	Isotype	Name	Type	Amount	Specificity	Host	Company	Isotype
Polymorphonuclear Leukocytes	CH138A	CH138A	1 µL	Bovine	Mouse	VMRD[1]	IgM	M31504	IgM – PE	1 µL	Mouse	Goat	Invitrogen[2]	IgM
CD14	MM61A	CD14	1 µL	Bovine	Mouse	VMRD[1]	IgG1	A10541	IgG1 – APC	1 µL	Mouse	Goat	Invitrogen[2]	IgG1
T Lymphocyte	MM1A	CD3	1 µL	Bovine	Mouse	VMRD[1]	IgG1	M32018	IgG1 – PE-Cy5	1 µL	Mouse	Goat	Invitrogen[2]	IgG1
CD4 T Lymphocyte	ILA11	CD4	1 µL	Bovine	Mouse	VMRD[1]	IgG2a	M32204	IgG2a – PE	1 µL	Mouse	Goat	Invitrogen[2]	IgG2a
CD8 T Lymphocyte	BAQ111A	CD8	1 µL	Bovine	Mouse	VMRD[1]	IgM	M31501	IgM – FITC	1 µL	Mouse	Goat	Invitrogen[2]	IgM
B Lymphocyte	BAQ15A	CD21	1 µL	Bovine	Mouse	VMRD[1]	IgM	M31501	IgM – FITC	1 µL	Mouse	Goat	Invitrogen[2]	IgM

PE = R-Phycoerythrin; APC = Allophycocyanin; FITC = fluorescein isothiocyanate; PE-Cy5 = Phycoerythrin cyanine 5; [1]VMRD Pullman Inc. Corp®; [2]Invitrogen.

analyzed in each sample, excluding most cell debris. A gating strategy to differentiate polymorphonuclear leukocytes (PMNLs) and macrophages was used because CD14 can also be expressed to a lesser extent on bovine milk PMNLs (Souza et al., 2020). An unstained control, secondary antibody control, and single-stained milk samples were also prepared as compensation controls. FlowJo software (TreeStar Inc.) was used to examine the data.

Statistical analysis

All the statistical analyses to determine the predictive values [i.e., sensitivity, specificity, and area under the curve of the receiver operating characteristics (ROS)] of all parameters were calculated using the quarter milk microbiological culture outcomes (Dingwell et al., 2003; Ferronatto et al., 2018) as a gold standard. The predictive values of the percentage of each leukocyte population, single-cell ratios and double interactions ratio were also calculated using widely accepted milk qSCC thresholds of 100,000 cells mL^{-1} (Bansal et al., 2005; Pilla et al., 2013) and 200,000 cells mL^{-1} (Pyörälä, 2003; Schukken et al., 2003) as a gold standard. To determine the PMNLs, macrophage and lymphocyte counts, the percentage of each leukocyte population obtained by the flow cytometry method was multiplied by the qSCC. The predictive values of qSCC (total milk cells) and the milk leukocyte differentials were assessed by cytospin and direct microscopic smears. The percentage and counts of several cell types were determined by flow cytometry, certain single-cell ratios, such as $CD4^+/CD8^+$ T lymphocyte ratio, widely used in specific infectious diseases (Marco et al., 2018); and other single-cell ratios proposed for the diagnosis of mastitis: 1) T cells/B cells ratio (Schwarz et al., 2013); 2) PMNLs/Lymphocytes ratio (Pilla et al., 2012); 3) Phagocytes/Lymphocytes ratio (Pilla et al., 2012); and 4) PMNLs/Lymphocytes ratio (Gonçalves et al., 2017). Furthermore, potential useful indices were also calculated: double ratio interaction $\frac{\%\ \text{Lymphocytes} \div \%\ \text{Macrophages}}{\%\ \text{PMNLs} \div \%\ \text{Lymphocytes}}$; double CD8 interaction ratio $\frac{(\%\ \text{Lymphocytes} - \%\ \text{CD8}) \div \%\ \text{Macrophages}}{(\%\ \text{PMNLs} + \%\ \text{CD8}) \div \%\ \text{Lymphocytes}}$; an internal complex relationship that could better reflect immunity complexity $\frac{(\%\ \text{Macrophages} \times \%\ \text{PMNLs}) \times (\text{Macrophages counts} \times \text{PMNL counts})}{[(\%\ \text{Mononuclear cells} \div \%\ \text{PMNLs}) \div (\%\ \text{PMNL} + \%\ \text{Macrophages})] \div \%\ \text{Lymphocytes}}$ (Leitner et al., 2015), and the so-called CD* complex, a novel proposed index calculated in the present study: $\frac{(\%\ \text{PMNLs} \times \%\ \text{T CD8}) \times (\text{PMBLs counts} \times \text{T CD8 counts})}{\%\ \text{Macrophages} \times \text{Macrophages counts}}$. This final index was positioned in accordance with the antidromic trend of macrophages *vs.* PMNLs and T $CD8^+$ lymphocytes at increasing qSCCs.

The ROC area under the curve was calculated by determining the point at the minimum distance from the left-upper corner of the unit square and the point where Youden's index is at its maximum (Habibzadeh et al., 2016). In the generalized linear regression models, lactation stage and parity were considered independent while a diagnostic variable was considered dependent. The prediction accuracy of the models was evaluated using the area of curvature ROC and the model's optimal sensitivity and specificity. The "pROC" (Robin et al., 2011) and tidy (Wickham et al., 2019) packages for the R programming language were used to develop the two ROC curvature alternatives.

The correlations between qSCC and the percentage of each leukocyte population determined by flow cytometry were determined using Spearman correlation for nonparametric data. They were carried out using the GraphPad Prism 9.0 software® (GraphPad Software, Inc.). The statistical significance was set at $p \leq 0.05$.

Results

Overall, out of the 112 investigated milk samples, 30.36 % (n = 34) of the total milk samples that were classified as culture-positive, and major and minor mastitis pathogens accounted for 38.24 % (n = 13; *Streptococcus dysgalactiae* = 11; *Staphylococcus aureus* = 2) and 61.76 % (n = 21; *Corynebacterium bovis* = 18; *Staphylococcus chromogenes* = 3) of total microbiological culture positive milk samples, respectively. The mean qSCC was 540,081 ± 110,601 cells mL^{-1}, while the median qSCC was 90,000 cells mL^{-1} (ranged from 1,000 to 7,094,000 cells mL^{-1}).

The predictive values of all investigated parameters were summarized in Figures 1 and 2 and Table 2. In the current study, the T cell/B cell ratio and T $CD4^+/CD8^+$ ratio could not be recommended as a tool for diagnosing IMIs (Table 2, and Figures 1 and 2). It was observed that the

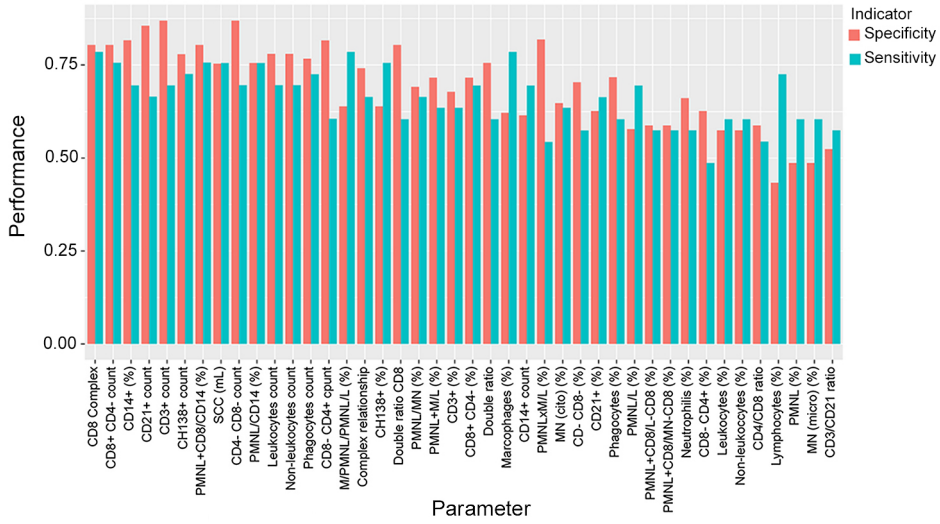

Figure 1 – Sensitivity and specificity of the milk leukocyte differentials considering the cutoff point that maximizes sensitivity and specificity to differentiate bacteriologically positive (with intramammary infections) and negative (healthy) udder quarters. SCC = quarter milk somatic cell count; PMNL = polymorphonuclear leukocytes; M = macrophages; L = Lymphocytes; CH138^{+} (%) = percentage of PMNL determined by flow cytometry; Neutrophils (%) = percentage of neutrophils determined by cytospin; PMNL (%) = percentage of PMNL determined by direct microscopic smears; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by cytospin; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; CD14^{+} (%) = percentage of macrophages determined by flow cytometry; CD14^{+} (counts) = count of macrophages determined by flow cytometry; Lymphocytes (%) = percentage of lymphocytes determined cytospin; Macrophages (%) = percentage of macrophages determined cytospin; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % CD8^{+})/% Macrophages)/[(% PMNLs + % CD8^{+})/% Lymphocytes]; CD8 complex = ([% PMNLs × % T CD8^{+}) × (PMNLs counts × T CD8^{+} counts)/(% Macrophages × Macrophages counts); and complex relationship = ([% Macrophages × % PMNLs] × [Macrophage counts × PMNL counts])/([% Mononuclear cells/% PMNLs]/[% PMNL^{+} % Macrophages]/% Lymphocytes.

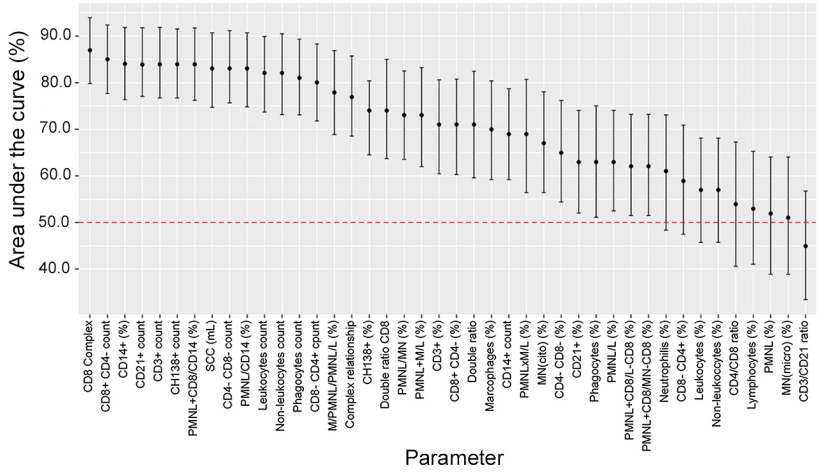

Figure 2 – Distribution of the area under the curve values and their respective 95 % confidence intervals of the milk leukocyte differentials to differentiate bacteriologically positive (with intramammary infections) and negative (healthy) udder quarters. SCC = quarter milk somatic cell count; PMNL = polymorphonuclear leukocytes; M = macrophages; L = Lymphocytes; CH138^{+} (%) = percentage of PMNL determined by flow cytometry; Neutrophils (%) = percentage of neutrophils determined by cytospin; PMNL (%) = percentage of PMNL determined by direct microscopic smears; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by cytospin; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; CD14^{+} (%) = percentage of macrophages determined by flow cytometry; CD14^{+} (counts) = count of macrophages determined by flow cytometry; Lymphocytes (%) = percentage of lymphocytes determined by cytospin; Macrophages (%) = percentage of macrophages determined by cytospin; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % CD8^{+})/% Macrophages)/[(% PMNLs + % CD8^{+})/% Lymphocytes]; CD8 complex = ([% PMNLs × % T CD8^{+}) × (PMNLs counts × T CD8^{+} counts)/(% Macrophages × Macrophages counts); and complex relationship = ([% Macrophages × % PMNLs] × [Macrophage counts × PMNL counts])/([% Mononuclear cells/% PMNLs]/[% PMNL^{+} % Macrophages]/% Lymphocytes.

Table 2 – Predictive values and cutoff points that maximize the specificity and sensitivity of the different parameters to diagnosed intramammary infections used in the present study.

Variable	Optimal cutoff point	Area under the curve	Specificity	Sensitivity
			%	
$CD14^+$ (%)	7.83	84	82.05	69.70
$CD14^+$ count	11576	69	61.54	69.70
$CD21^+$ (%)	13.2	63	62.82	66.67
$CD21^+$ count	44712	84	85.90	66.67
CD3/CD21 ratio	0.93	45	52.56	57.58
$CD3^+$ (%)	11.95	71	67.95	63.64
$CD3^+$ count	32937	84	87.18	69.70
$CD4^-$ $CD8^-$ (%)	4.24	65	70.51	57.58
$CD4^-$ $CD8^-$ count	10448	83	87.18	69.70
CD4/CD8 ratio	0.36	54	58.97	54.55
$CD8^-$ $CD4^+$ (%)	1.94	59	62.82	48.48
$CD8^-$ $CD4^+$ count	5634	80	82.05	60.61
CD8 Complex (%)	126980	87	80.77	78.79
$CD8^+$ $CD4^-$ (%)	5.02	71	71.79	69.70
$CD8^+$ $CD4^-$ count	8186	85	80.77	75.76
$CH138^+$ (%)	13.25	74	64.10	75.76
$CH138^+$ count	27155	84	78.21	72.73
Complex Relationship	34223670	77	74.36	66.67
qSCC (mL^{-1})	145000	83	75.64	75.76
Double ratio	7.55	71	75.64	60.61
Double ratio CD8	3.76	74	80.77	60.61
Leukocytes (%)	58.42	57	57.69	60.61
Leukocyte count	97921	82	78.21	69.70
Lymphocytes (%)*	3.15	53	43.24	72.73
(M/PMNL)/(PMNL/L)(%)	1.62	78	64.10	78.79
Macrophages (%)*	38.73	70	62.16	78.79
Mononuclear Leukocytes (%)#	65.31	59	61.49	56.06
Neutrophils#	53	61	66.22	57.58
Non-leukocytes (%)	41.57	57	57.69	60.61
Non-leukocyte count	70112	82	78.21	69.70
Phagocytes count	53935	81	76.92	72.73
Phagocytes (%)	32.96	63	71.79	60.61
PMNL/CD14 (%)	1.82	83	75.64	75.76
PMNL/L (%)	0.48	63	57.69	69.70
PMNL/MN (%)	0.44	73	69.23	66.67
$PMNL^+CD8^+/CD14^+$ (%)	2.91	84	80.77	75.76
PMNL $+CD8^+/L - CD8^+$ (%)	0.92	62	58.97	57.58
PMNL + $CD8^+$/MN – $CD8^+$ (%)	0.92	62	58.97	57.58
PMNL + M/L (%)	1.17	73	71.79	63.64
PMNL × M/L (%)	2.80	69	82.05	54.55
Polymorphonuclear Leukocytes (%)*	16.39	52	48.65	60.61

qSCC = quarter milk somatic cell count; PMNL = polymorphonuclear leukocytes; $CH138^+$ = PMNL determined by flow cytometry; M = macrophages; L = Lymphocytes; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by cytospin technique; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % $CD8^+$)/% Macrophages)/[(% PMNLs + % $CD8^+$)/% Lymphocytes]; CD8 complex = ([% PMNLs × % T $CD8^+$) × (PMNLs counts × T $CD8^+$ counts)/(% Macrophages × Macrophages counts); and complex relationship = ([% Macrophages × % PMNLs] × [Macrophage counts × PMNL counts])/([% Mononuclear cells/% PMNLs]/[% PMNL+ % Macrophages]/% Lymphocytes. *Measured cytospin; #determined by direct microscopic smears.

percentage of any leukocyte alone did not substantially improve the predictive values of the diagnosis of IMIs. In this regard, although certain parameters increased the area under the ROC curve due to an increase in the specificity values (i.e., T $CD8^+$ counts, T cells counts, B cells counts, PMNLs counts, percentage of macrophages, and the sum of the percentage PMNLs and T $CD8^+$ cells divided by the percentage of macrophages), they were associated with a slight reduction in sensitivity (Figures 1 and 2). In this regard, the so-called CD8 complex better reflects the immunity complexity and was the only parameter that improved both sensitivity and specificity, resulting in the highest area under the ROC curve and sensitivity values used in IMI diagnosis (Figures 1 and 2).

Additionally, the percentage of macrophages and the sum of the percentage PMNLs and T $CD8^+$ cells divided by the percentage of macrophages showed the highest predictive values in the differentiation of the inflammatory condition status of dairy cows using both somatic cell counts thresholds (100,000 and 200,000 cells mL^{-1}; Figures 3, 4, 5 and 6, and Tables 3 and 4). The predictive values of all investigated parameters pertaining to the milk qSCC thresholds were summarized in Figures 3, 4, 5 and 6, and Tables 3 and 4.

Correlations between the qSCC and the different leukocyte populations determined by flow cytometry are shown in Figure 7. The qSCC correlation with the percentage of T lymphocytes, $CD8^+$ $CD4^-$ T lymphocytes, $CD4^+$ $CD8^-$ T lymphocytes, $CD4^-$ $CD8^-$ T lymphocytes, lymphocytes B, macrophages and neutrophils was $r = 0.36$ ($p = 0.0001$), $r = 0.29$ ($p = 0.002$), $r = 0.38$ ($p < 0.0001$), $r = 0.22$ ($p = 0.02$), $r = 0.18$ ($p = 0.06$), $r = -0.65$ ($p < 0.0001$) and $r = 0.51$ ($p < 0.0001$), respectively.

Discussion

In the present study, while the predictive values of many parameters used to diagnose IMIs (pertaining to the widely used bacteriological outcomes as a gold standard) were explored, only a few parameters showed slight improvements in the predictive values when compared to the widely used qSCC. Thus, the differential somatic cell count did not robustly increase the predictive values of qSCC, which could be explained, at least in part, by the redundancy of host immune defenses (Nish and Medzhitov, 2011; Leitner et al., 2015), reinforced by the correlations between qSCC and distinct cell populations (Figure 3). In agreement with the findings of the present study, Schwarz et al. (2019), Lozada-Soto et al. (2020), and Zecconi et al. (2021) had reported quite similar ROC curve values when compared to the total leukocyte counts and differential leukocyte counts at the end of the lactation period and in fresh cows. Analogously, Schwarz et al. (2020), using many milk samples found fairly comparable predictive values of SCC alone when compared to DCC alone (using a combined proportion of PMNLs and overall lymphocytes) and in combination

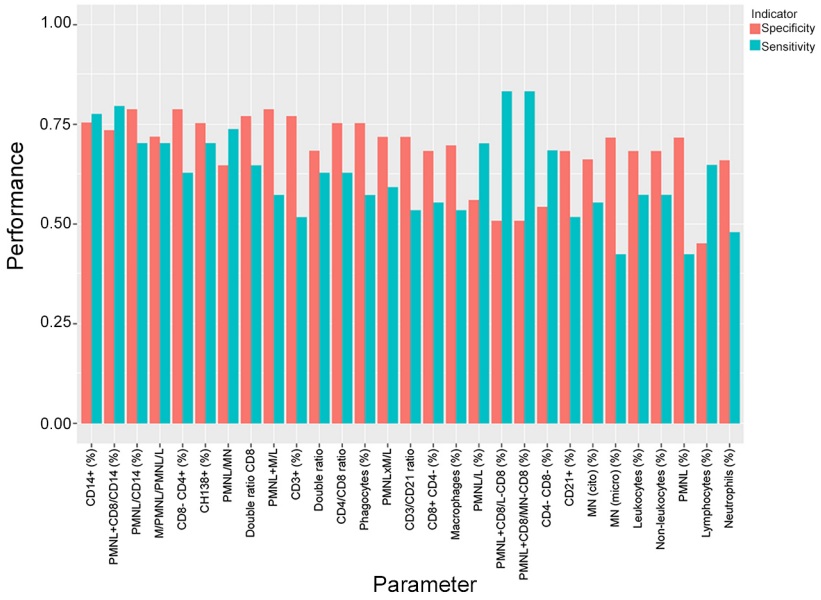

Figure 3 – Sensitivity and specificity of the milk leukocyte differentials considering the cutoff points that maximizes their sensitivity and specificity to differentiate udder quarters under healthy and inflammatory conditions (SCC threshold = 100,000 cells mL^{-1}). SCC = somatic cell count. PMNL = polymorphonuclear leukocytes; M = macrophages; L = Lymphocytes; $CH138^+$ (%) = percentage of PMNL determined by flow cytometry; Neutrophils (%) = percentage of neutrophils determined by a cytospin; PMNL (%) = percentage of PMNL determined by direct microscopic smears; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by a cytospin; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; $CD14^+$ (%) = percentage of macrophages determined by flow cytometry; Lymphocytes (%) = percentage of lymphocytes determined by cytospin; Macrophages (%) = percentage of macrophages determined by cytospin; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % $CD8^+$)/% Macrophages)/[(% PMNLs + % $CD8^+$)/% Lymphocytes].

with SCC. Furthermore, although several studies have indicated statistical significance between diseased and healthy quarters using differential cell counts, it did not signify discrimination, as achieved significance failed to show nonoverlapping data distribution (Leitner et al., 2015).

The association between increased cell count and PMNL influx to the mammary gland during the infectious process is notorious (Paape et al., 2003; Souza et al., 2012; Gonçalves et al., 2017). Furthermore, a recently published study indicated an even more significant increase in the T lymphocyte $CD8^+$ subpopulation (Souza et al., 2020), which could aid differentiation between healthy and diseased udder quarters. Overall, identifying T $CD8^+$ lymphocytes in milk samples could have great implications for IMI diagnosis and prognosis (Sordillo et al., 1997; Park et al., 1993; Alnakip et al., 2014; Souza et al., 2020). While the study size was limited, these data corroborated the findings of the current study, wherein the identification of T $CD8^+$ lymphocytes in milk samples improved the predictive values of the variables evaluated in the diagnosis of IMIs. For example, the combination of the percentage and the number of T $CD8^+$ lymphocytes and PMNLs, as these populations increased during IMIs, divided by the percentage and counts of macrophages, which represent the major population in healthy quarters while the percentage decreases during infection, resulted in a calculated novel index, the so-called CD8 complex. Consequently, the overall strategy resulted in the highest predictive values found in the present study. However, further longitudinal studies are needed, as the immune response is not static (Leitner et al., 2015).

Conversely, the CD3/CD21 failed to distinguish culture-negatives from positives, resulting in the poorest predictive values for IMI diagnosis. In this regard, Schwarz et al. (2013), analyzing the proportions of $CD2^+$ T and $CD21^+$ B lymphocytes suggested the use of CD2/CD21 index as a new marker to determine udder health, which, at least in part, was not supported by this study, using CD3 mAb instead of CD2 mAb to identify T lymphocytes.

Furthermore, the percentage of T lymphocytes and PMNLs increased while the percentage of macrophages drastically dampened in udder quarters with subclinical mastitis. Taken altogether, these findings resulted in the highest predictive values of the percentage of milk macrophages measured by flow cytometry ($CH138A^-$/$CD14^+$) and the sum of the percentage of PMNLs and T $CD8^+$ cells divided by the percentage of macrophages used for diagnosing subclinical mastitis (considering the

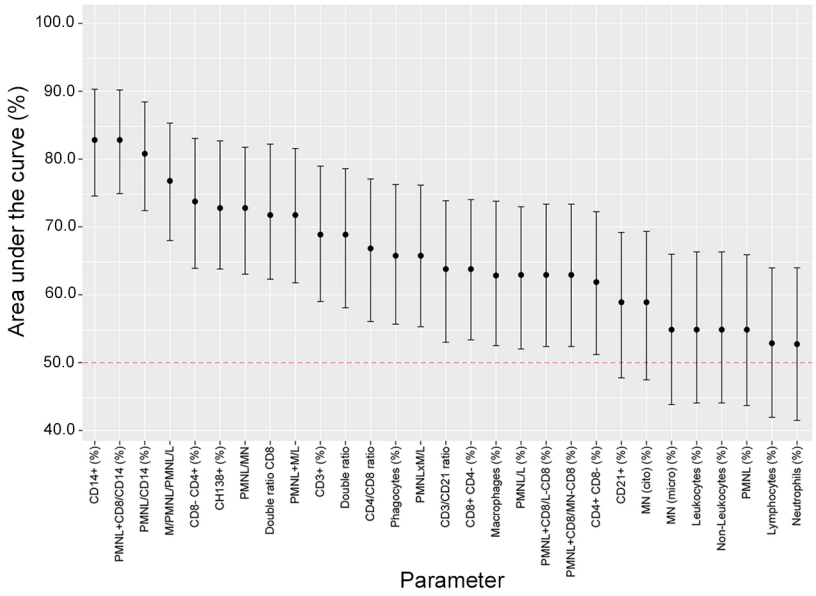

Figure 4 – Distribution of the area under the curve values and their respective 95 % confidence intervals of the milk leukocyte differentials to differentiate udder quarters under healthy and inflammatory conditions (SCC threshold = 100,000 cells mL^{-1}). SCC = somatic cell count; PMNL = polymorphonuclear leukocytes; M = macrophages; L = Lymphocytes; CH138^{+} (%) = percentage of PMNL determined by flow cytometry; Neutrophils (%) = percentage of neutrophils determined by cytospin; PMNL (%) = percentage of PMNL determined by direct microscopic smears; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by cytospin technique; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; CD14^{+} (%) = percentage of macrophages determined by flow cytometry; Lymphocytes (%) = percentage of lymphocytes determined cytospin; Macrophages (%) = percentage of macrophages determined by cytospin; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % CD8^{+})/% Macrophages)/[(% PMNLs + % CD8^{+})/% Lymphocytes].

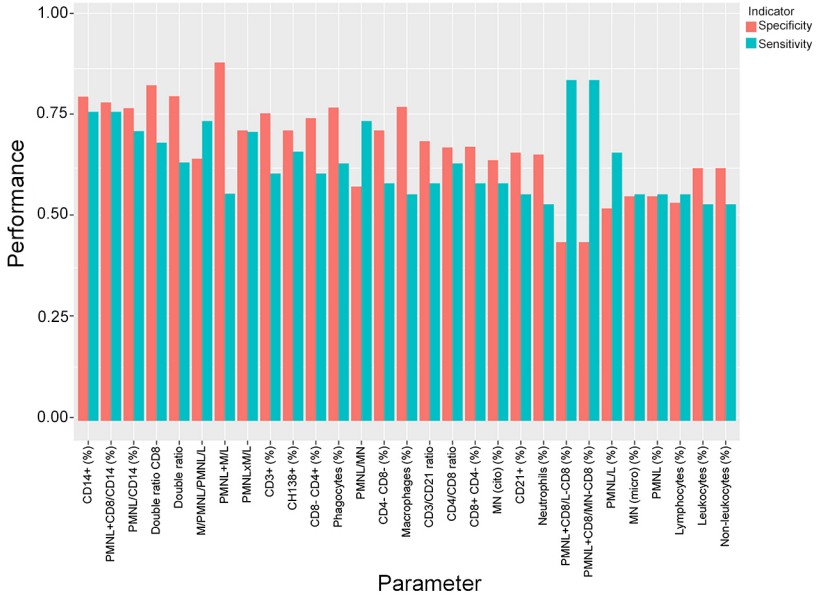

Figure 5 – Sensitivity and specificity of the milk leukocyte differentials considering the cutoff point that maximizes sensitivity and specificity to differentiate udder quarters under healthy and inflammatory conditions (SCC threshold = 200,000 cells mL^{-1}). SCC = somatic cell count; PMNL = polymorphonuclear leukocytes; M = macrophages; L = Lymphocytes; CH138^{+} (%) = percentage of PMNL determined by flow cytometry; Neutrophils (%) = percentage of neutrophils determined by cytospin; PMNL (%) = percentage of PMNL determined by direct microscopic smears; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by cytospin; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; CD14^{+} (%) = percentage of macrophages determined by flow cytometry; Lymphocytes (%) = percentage of lymphocytes determined by cytospin technique; Macrophages (%) = percentage of macrophages determined by cytospin technique; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % CD8^{+})/% Macrophages)/[(% PMNLs + % CD8^{+})/% Lymphocytes].

Table 3 – Predictive values and cutoff points that maximize the specificity and sensitivity of the different parameters to diagnosed subclinical mastitis used in the present study.

Variable	Optimal cutoff point**	Area under the curve**	Specificity**	Sensitivity**
			%	
$CD14^+$ (%)	13.84	83	75.44	77.78
$CD21^+$ (%)	14.45	59	68.42	51.85
CD3/CD21 ratio	1.06	64	71.93	53.70
$CD3^+$ (%)	13.10	69	77.19	51.85
$CD4^-$ $CD8^-$ (%)	2.02	62	54.39	68.52
CD4/CD8 ratio	0.51	67	75.44	62.96
$CD8^-$ $CD4^+$ (%)	1.83	74	78.95	62.96
$CD8^+$ $CD4^-$ (%)	4.72	64	68.42	55.56
$CH138^+$ (%)	13.45	73	75.44	70.37
Double ratio	4.70	69	68.42	62.96
Double ratio CD8	2.39	72	77.19	64.81
Lymphocytes (%)*	3.82	53	45.28	64.81
(M/PMNL)/(PMNL/L)(%)	1.63	77	71.93	70.37
Macrophages (%)*	32.63	63	69.81	53.70
Mononuclear Leukocytes (%)#	87.87	55	71.7	42.59
Neutrophils#	53.00	53	66.04	48.15
Non-leukocytes (%)	43.46	55	68.42	57.41
Phagocytes (%)	33.42	66	75.44	57.41
PMNL/CD14 (%)	1.15	81	78.95	70.37
PMNL/L (%)	0.37	63	56.14	70.37
PMNL/MN (%)	0.24	73	64.91	74.07
$PMNL^+CD8^+/CD14^+$ (%)	1.00	83	73.68	79.63
PMNL $+CD8^+$/L – $CD8^+$ (%)	0.59	63	50.88	83.33
PMNL + $CD8^+$/MN – $CD8^+$ (%)	0.59	63	50.88	83.33
PMNL + M/L (%)	1.18	72	78.95	57.41
PMNL × M/L (%)	4.31	66	71.93	59.26
Polymorphonuclear Leukocytes (%)*	12.13	55	71.70	42.59

**Using the threshold of 100,000 cells mL^{-1} to diagnosis subclinical mastitis; qSCC = quarter milk somatic cell count; PMNL = polymorphonuclear leukocytes; $CH138^+$ = PMNL determined by flow cytometry; M = macrophages; L = Lymphocytes; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by a cytospin; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % $CD8^+$)/% Macrophages)/[(% PMNLs + % $CD8^+$)/% Lymphocytes]. *Measured by cytospin; #determined by direct microscopic smears.

Table 4 – Predictive values and cutoff points that maximize the specificity and sensitivity of the different parameters to diagnosed subclinical mastitis used in the present study.

Variable	Optimal cutoff point**	Area under the curve**	Specificity **	Sensitivity**
			%	
$CD14^+$ (%)	9.70	85	80.56	76.92
$CD21^+$ (%)	14.45	60	66.67	56.41
CD3/CD21 ratio	1.06	65	69.44	58.97
$CD3^+$ (%)	13.10	71	76.39	61.54
$CD4^-$ $CD8^-$ (%)	4.06	67	72.22	58.97
CD4/CD8 ratio	0.51	64	68.06	64.10
$CD8^-$ $CD4^+$ (%)	2.13	70	75.00	61.54
$CD8^+$ $CD4^-$ (%)	4.90	64	68.06	58.97
$CH138^+$ (%)	16.10	71	72.22	66.67
Double ratio	7.56	75	80.56	64.10
Double ratio CD8	3.58	78	83.33	69.23
Lymphocytes (%)*	4.71	53	54.41	56.41
(M/PMNL)/(PMNL/L)(%)	1.63	75	65.28	74.36
Macrophages (%)*	26.80	66	77.94	56.41
Mononuclear Leukocytes (%)#	80.91	54	55.88	56.41
Neutrophils#	53.00	59	66.18	53.85
Non-leukocytes (%)	43.86	52	62.50	53.85
Phagocytes (%)	32.56	69	77.78	64.10
PMNL/CD14 (%)	1.82	81	77.78	71.79
PMNL/L (%)	0.41	58	52.78	66.67
PMNL/MN (%)	0.26	69	58.33	74.36
$PMNL^+CD8^+/CD14^+$ (%)	2.12	83	79.17	76.92
PMNL + $CD8^+$/L – $CD8^+$ (%)	0.59	59	44.44	84.62
PMNL + $CD8^+$/MN – $CD8^+$ (%)	0.59	59	44.44	84.62
PMNL + M/L (%)	1.01	75	88.89	56.41
PMNL × M/L (%)	4.32	74	72.22	71.79
Polymorphonuclear Leukocytes (%)*	19.09	54	55.88	56.41

**Using the threshold of 100,000 cells mL^{-1} to diagnosis subclinical mastitis; qSCC = quarter milk somatic cell count; PMNL = polymorphonuclear leukocytes; $CH138^+$ = PMNL determined by flow cytometry; M = macrophages; L = Lymphocytes; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by cytospin; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % $CD8^+$)/% Macrophages)/[(% PMNLs + % $CD8^+$)/% Lymphocytes]. *Measured by cytospin; #determined by direct microscopic smears.

widely used qSCC thresholds as a gold standard). In contrast, the percentage of milk macrophages determined by cytospin had poor predictive value. In this regard, determining leukocyte populations by flow cytometry resulted in higher predictive values for the percentage of both macrophages and PMNLs applied to diagnosing IMIs and subclinical mastitis. Overall, these findings reinforce the idea of the poor repeatability of traditional methods, such as the cytospin technique and direct microscopic smears, due to the subjective evaluation of the relatively low number of milk cells (Koess and Hamman, 2008; Takano et al., 2018). Indeed, in a previous study (Takano et al., 2018), the measurement of distinct milk populations by flow cytometry resulted in the highest strength of the linear correlation (r-value) between milk qSCCs and the percentage of milk PMNLs (r = 0.48) and macrophages (r = –0.65) than those obtained by the cytospin technique (PMNLs, r = 0.43; macrophages, r = –0.11) and microscopic smears (PMNLs, r = 0.39).

Although there is no consensus in the literature on the predominant leukocyte population in healthy udders, as a number of studies have suggested that lymphocytes are the main leukocyte population (Dosogne et al., 2003; Schwarz et al., 2011a; Schwarz et al., 2011b; Pilla et al., 2012), the present study using a precise high-throughput flow cytometry method with specific monoclonal antibodies to differentiate leukocytes populations

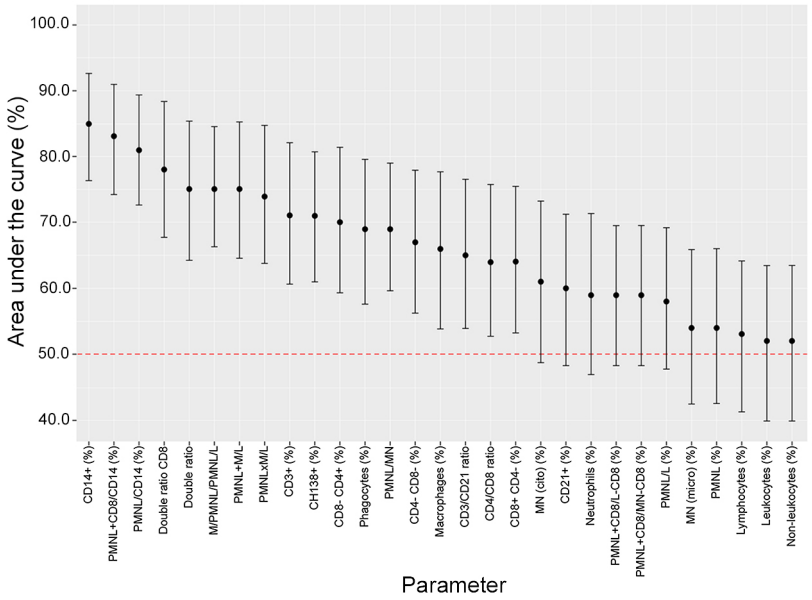

Figure 6 – Distribution of the area under the curve values and their respective 95 % confidence intervals of the milk leukocyte differentials to differentiate udder quarters under healthy and inflammatory conditions (SCC threshold = 200,000 cells mL^{-1}). SCC = somatic cell count; PMNL = polymorphonuclear leukocytes; M = macrophages; L = Lymphocytes; CH138^{+} (%) = percentage of PMNL determined by flow cytometry; Neutrophils (%) = percentage of neutrophils determined by cytospin; PMNL (%) = percentage of PMNL determined by direct microscopic smears; MN = Mononuclear Leukocytes; MN (cito) = Mononuclear Leukocytes determined by cytospin; MN (micro) = Mononuclear Leukocytes by direct microscopy smears; CD14^{+} (%) = percentage of macrophages determined by flow cytometry; Lymphocytes (%) = percentage of lymphocytes determined by cytospin; Macrophages (%) = percentage of macrophages determined by cytospin; double ratio = ([% Lymphocytes/% Macrophages)/[% PMNLs/% Lymphocytes]; double ratio CD8 = ([(% Lymphocytes – % CD8^{+})/% Macrophages)/[(% PMNLs + % CD8^{+})/% Lymphocytes].

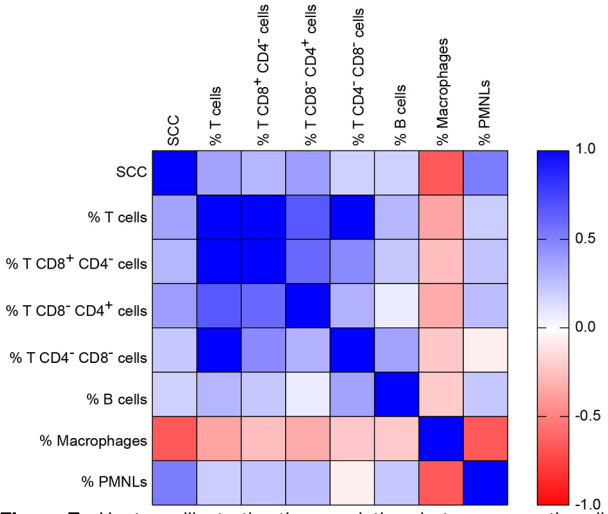

Figure 7 – Heatmap illustrating the correlations between somatic cell counts and the differential leukocytes subpopulations determined by flow cytometry in bovine milk (r value). SCC = quarter somatic cell counts; PMNLs = polymorphonuclear leukocytes.

corroborated several studies which have reported that macrophages are the main population in milk from healthy udder quarters (Sarikaya et al., 2005; Damm et al., 2017; Gonçalves et al., 2017; Takano et al., 2018).

In this regard, although many studies have investigated the fluctuations of each leukocyte percentage by microscopic differential somatic cell counts (Koess and Hamann, 2008; Schwarz et al., 2011a; Gonçalves et al., 2017) or flow cytometry (Pillai et al., 2001; Dosogne et al., 2003; Koess and Hamann, 2008; Schwarz et al., 2011b; Pilla et al., 2013; Schwarz et al., 2019), the role of the distinct T cell subpopulations (e.g., T CD4^{+} and T CD8^{+} lymphocytes) in the diagnosis of IMIs has long been neglected. In addition, even when using flow cytometry, the mAb utilized can affect the DCC outcomes (Souza et al., 2020). In this study, PMNL, monocyte/macrophage, and lymphocyte subsets were accurately identified, whereas others evaluating milk DCC by flow cytometry did not use CD14 and CH138A mAb in combination (Pillai et al., 2001; Rivas et al., 2001; Dosogne et al., 2003; Koess and Hamann, 2008; Schwarz et al., 2011a; Pilla et al., 2013). Not doing so can lead to erroneous identification of some PMNL, which can also express CD14 on their surface (Paape et al., 1996; Sládek et al., 2002; Ibeagha-Awemu et al., 2008). Even worse, a number of studies did not use a specific mAb to accurately differentiate and identify many milk cell types (Pillai et al., 2001; Dosogne et al., 2003). Other studies used CD11b mAb to identify PMNL or to differentiate PMNL (CD11b^{+}/CD14^{-}) and macrophages

($CD11b^-/CD14^+$; Koess and Hamann, 2008; Schwarz et al., 2011a; Pilla et al., 2013) though lymphocytes and macrophages can also express CD11b (Riollet et al., 2001; Duan et al., 2016). Furthermore, comparing the DCC of milk obtained among studies can be complicated because the type of material of the sample bottle and the method of preparation could impact the leukocyte populations (Schröder and Hamann, 2005), beyond the effect of different milk fractions on milk cell populations (Sarikaya et al., 2005).

In conclusion, this study further provided the first insights into the T $CD8^+$ lymphocytes in diagnosing bovine IMIs. Combined with PMNLs and macrophages, it improved the predictive value of differential cell counts in the diagnosis of IMIs. Furthermore, due to an increase in the percentage of PMNLs and T cells, the markedly dampened percentage of macrophages was crucial to the differentiation of udder quarters under both inflammatory and non-inflammatory conditions.

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Authors' Contributions

Conceptualization: Blagitz MG, Souza FN, Della Libera AMMP. **Data acquisition**: Blagitz MG, Souza FN, Ramos Sanchez EM, Batista CF. **Data analysis**: Souza CMS, Blagitz MG, Lima DM, Souza FN, Heinemann MB. **Investigation**: Blagitz MG, Souza FN, Batista CF. **Design of methodology**: Blagitz MG, Souza FN, Ramos Sanchez EM, Heinemann MB, Fernandes ACF, Della Libera AMMP. **Data curation**: Blagitz MG, Souza FN. **Formal analysis**: Blagitz MG, Souza FN. **Funding acquisition**: Della Libera AMMP. **Supervision**: Della Libera AMMP. **Writing-original draft**: Souza CMS. **Writing-review and editing**: Blagitz MG, Souza FN, Alves AJ, Heinemann MB, Ramos Sanchez EM, Torres HF, Fernandes ACF, Della Libera AMMP.

References

Alnakip EM, Quintela-Baluja M, Böhme K, Fernández-No I, Caamaño-Antelo S, Calo-Mata P, et al. 2014. The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. Journal of Veterinary Medicine 2014: 659801. https://doi.org/10.1155/2014/659801

Andersen S, Dohoo IR, Olde Riekerink R, Stryhn H, Mastitis Research Workers' Conference. 2010. Diagnosing intramammary infections: evaluating expert opinions on the definition of intramammary infection using conjoint analysis. Journal of Dairy Science 93: 2966-2975. https://doi.org/10.3168/jds.2009-2726

Bansal BK, Hamann J, Grabowskit NT, Singh KB. 2005. Variation in the composition of selected milk fraction samples from healthy and mastitic quarters, and its significance for mastitis diagnosis. Journal of Dairy Research 72: 144-152. https://doi.org/10.1017/S0022029905000798

Blagitz MG, Souza FN, Santos BP, Batista CF, Parra AC, Azevedo LFF, et al. 2013. Function of milk polymorphonuclear neutrophil leukocytes in bovine mammary glands infected with *Corynebacterium bovis*. Journal of Dairy Science 96: 3750-3757. https://doi.org/10.3168/jds.2012-6370

Blagitz MG, Souza FN, Batista CF, Diniz SA, Azevedo LFF, Silva MX, et al. 2015. Flow cytometric analysis: Interdependence of healthy and infected udder quarters. Journal of Dairy Science 98: 2401-2408. https://doi.org/10.3168/jds.2014-8727

Damm M, Holm C, Blaabjerg M, Bro MN, Schwarz D. 2017. Differential somatic cell count-A novel method for routine mastitis screening in the frame of Dairy Herd Improvement testing programs. Journal of Dairy Science 100: 4926-4940. https://doi.org/10.3168/jds.2016-12409

Della Libera AMMP, Araújo WP, Kitamura SS, Rosenfeld AMF, Birgel EH. 2004. Milk cytology of healthy buffaloes (*Bubalus bubalis*) bred in São Paulo state, Brazil. Ciência Rural 34: 1087-1092 (in Portuguese, with abstract in English). https://doi.org/10.1590/S0103-84782004000400019

Della Libera AMMP, Souza FN, Batista CF, Santos BP, Azevedo LFF, Sanchez EMR, et al. 2015. Effect of bovine leukemia virus infection on milk neutrophil function and the milk lymphocyte profile. Veterinary Research 46: 1-8. https://doi.org/10.1186/s13567-014-0125-4

Dingwell RT, Leslie KE, Schukken YH, Sargeant JM, Timms LL. 2003. Evaluation of the California mastitis test to detect an intramammary infection with a major pathogen in early lactation dairy cows. Canadian Veterinary Journal 44: 413-415.

Dosogne H, Vangroenweghe F, Mehrzad J, Massart-Leën AM, Burvenich C. 2003. Differential leukocyte count method for bovine low somatic cell count milk. Journal of Dairy Science 86: 828-834. https://doi.org/10.3168/jds.S0022-0302(03)73665-0

Duan M, Steinfort DP, Smallwood D, Hew M, Chen W, Ernst M, et al. 2016. CD11b immunophenotyping identifies inflammatory profiles in the mouse and human lungs. Mucosal Immunology 9: 550-563. https://doi.org/10.1038/mi.2015.84

Ferronatto JA, Ferronatto TC, Schneider M, Pessoa LF, Blagitz MG, Heinemann MB, et al. 2018. Diagnosing mastitis in early lactation: use of Somaticell®, California mastitis test and somatic cell count. Italian Journal of Animal Science 17: 723-729. https://doi.org/10.1080/1828051X.2018.1426394

Gonçalves JL, Lyman RL, Hockett M, Rodriguez R, Santos MV, Anderson KL. 2017. Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis. Journal of Dairy Research 84: 309-317. https://doi.org/10.1017/S0022029917000267

Habibzadeh F, Habibzadeh P, Yadollahie M. 2016. On determining the most appropriate test cut-off value: the case of tests with continuous results. Biochemia Medica 26: 297-307. https://doi.org/10.11613/BM.2016.034

Ibeagha-Awemu EM, Lee J-W, Ibeagha AE, Bannerman DD, Paape MJ, Zhao X. 2008. Bacterial lipopolysaccharide induces increased expression of toll-loke receptors (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. Veterinary Research 39: 11. https://doi.org/10.1051/vetres:2007047

International Dairy Federation [IDF]. 1995. Milk: Enumeration of Somatic Cell. IDF, Brussels, Belgium. (Standard 148A).

Koess C, Hamann J. 2008. Detection of mastitis in the bovine mammary gland by flow cytometry at early stages. Journal of Dairy Research 75: 225-232. https://doi.org/10.1017/S0022029908003245

Leitner G, Blum SE, Rivas AL. 2015. Visualizing the indefinable: three-dimensional complexity of 'infectious diseases'. PLoS ONE 10: e0123674. https://doi.org/10.1371/journal.pone.0123674

Lozada-Soto E, Maltecca C, Anderson K, Tiezzi F. 2020. Analysis of milk leukocyte differential measures for use in management practices for decreased mastitis incidence. Journal of Dairy Science 103: 572-582. https://doi.org/10.3168/jds.2019-16355

Marco SA, Brown C, Pancoast TC. 2018. Diagnostic utility of CD4/CD8 ratio in bronchoalveolar lavage. Clinical Pulmonary Medicine 25: 67-73. https://doi.org/10.1097/CPM.0000000000000247

National Mastitis Council [NMC]. 1999. Laboratory Handbook on Bovine Mastitis. National Mastitis Council, Madison, WI, USA.

Nish S, Medzhitov R. 2011. Host defense pathways: role of redundancy and compensation in infectious disease phenotypes. Immunity 34: 629-636. https://doi.org/10.1016/j.immuni.2011.05.009

Oliver SP, Gonzalez RN, Hogan JS, Jayarao BM, Owens WE. 2004. Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality. National Mastitis Council, Madison, WI, USA.

Paape MJ, Liluis EM, Wiitanen PA, Kontio MP, Miller RH. 1996. Intramammary defense against infections induced by *Escherichia coli* in cows. American Journal of Veterinary Research 57: 477-482.

Paape MJ, Bannerman DD, Zhao X, Lee JW. 2003. The bovine neutrophil: structure and function in blood and milk. Veterinary Research 34: 597-627. https://doi.org/10.1051/vetres:2003024

Park YH, Fox LK, Hamilton MJ, Davis WC. 1993. Suppression of proliferative response of BoCD4$^+$ T lymphocytes by activated BoCD8$^+$ T lymphocytes in the mammary gland of cows with *Staphylococcus aureus* mastitis. Veterinary Immunology and Immunopathology 36: 137-151. https://doi.org/10.1016/0165-2427(93)90103-b

Pilla R, Schwarz D, König S, Piccinini R. 2012. Microscopic differential cell counting to identify inflammatory reactions in dairy cow quarter milk samples. Journal of Dairy Science 95: 4410-4420. https://doi.org/10.3168/jds.2012-5331

Pilla R, Malvisi M, Snel GGM, Schwars D, Konig S, Czerny CP, et al. 2013. Differential cell count as an alternative method to diagnose dairy cow mastitis. Journal of Dairy Science 96: 1653-1660. https://doi.org/10.3168/jds.2012-6298

Pillai SR, Kunze E, Sordillo LM, Jayarao BM. 2001. Application of differential inflammatory cell count as a tool to monitor udder health. Journal of Dairy Science 84: 1413-20. https://doi.org/10.3168/jds.S0022-0302(01)70173-7

Pyörälä S. 2003. Indicators of inflammation in the diagnosis of mastitis. Veterinary Research 34: 565-578. https://doi.org/10.1051/vetres:2003026

Riollet C, Rainard P, Poutrel B. 2001. Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. Journal of Dairy Science 84: 1077-1084. https://doi.org/10.3168/jds.S0022-0302(01)74568-7

Rivas AL, Quimby FW, Blue J, Coksaygan O. 2001. Longitudinal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry, and cytology. Journal of Veterinary Diagnostic Investigation 13: 399-407. https://doi.org/10.1177/104063870101300506

Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. 2011. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 12: 77. https://doi.org/10.1186/1471-2105-12-77

Rosenfeld G. 1947. Panchronic dye for hematology and cytology. New combination of May-Grunwall and Giemsa components in one quick-use dye = Corante pancrômico para hematologia e citologia. Nova combinação dos componentes de May-Grunwall e do Giemsa num só corante de emprego rápido. Memórias do Instituto Oswaldo Cruz 20: 329-335 (in Portuguese).

Sarikaya H, Werner-Misof C, Atzkern M, Bruckmaier RM. 2005. Distribution of leukocyte populations, and milk composition, in milk fractions of healthy quarters in dairy cows. Journal of Dairy Research 72: 486-492. https://doi.org/10.1017/S0022029905001317

Schröder AC, Hamann J. 2005. The influence of technical factors on differential cell count in milk. Journal of Dairy Research 72: 153-158. https://doi.org/10.1017/s0022029905000804

Schukken YH, Wilson DJ, Welcome F, Garrison-Tikofsky L, Gonzalez RN. 2003. Monitoring udder health and milk quality using somatic cell counts. Veterinary Research 34: 579-596. https://doi.org/10.1051/vetres:2003028

Schwarz D, Diesterbeck US, König S, Brügemann K, Schlez K, Zschöck M, et al. 2011a. Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. Journal of Dairy Research 78: 448-455. https://doi.org/10.1017/S0022029911000574

Schwarz D, Diesterbeck US, König S, Brügemann K, Schlez K, Zschöck M, et al. 2011b. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. Journal of Dairy Science 94: 5033-5044. https://doi.org/10.3168/jds.2011-4348

Schwarz D, Rivas AL, Konig S, Diesterbeck US, Schlez K, Zschock M, et al. 2013. CD2/CD21 index: a new marker to evaluate udder health in dairy cows. Journal of Dairy Science 96: 5106-5119. https://doi.org/10.3168/jds.2013-6804

Schwarz D, Lipkens Z, Piepers S, De Vliegher S. 2019. Investigation of differential somatic cell count as a potential new supplementary indicator to somatic cell count for identification of intramammary infection in dairy cows at the end of the lactation period. Preventive Veterinary Medicine 172: 104803. https://doi.org/10.1016/j.prevetmed.2019.104803

Schwarz D, Santschi DE, Durocher J, Lefebvre DM. 2020. Evaluation of the new differential somatic cell count parameter as a rapid and inexpensive supplementary tool for udder health management through regular milk recording. Preventive Veterinary Medicine 181: 105079. https://doi.org/10.1016/j.prevetmed.2020.105079

Sládek Z, Rysánek D, Faldyna M. 2002. Activation of phagocytes during initiation and resolution of mammary gland injury induced by lipopolysaccharide in heifers. Veterinary Research 33: 191-204. https://doi.org/10.1051/vetres:2002007

Sordillo LM, Shafer-Weaver K, De Rosa D. 1997. Immunobiology of the mammary gland. Journal of Dairy Science 80: 1851-1865. https://doi.org/10.3168/jds.S0022-0302(97)76121-6

Souza FND, Sanchez EMR, Heinemann MB, Gidlund MA, Reis LC, Blagitz MG, et al. 2012. The innate immunity in bovine mastitis: the role of pattern-recognition receptors. American Journal of Immunology 8: 166-178. https://doi.org/10.3844/ajisp.2012.166.178

Souza FN, Blagitz MG, Batista CF, Takano PV, Gargano RG, Diniz SA, et al. 2020. Immune response in nonspecific mastitis: what can it tell us? Journal of Dairy Science 103: 5376-5386. https://doi.org/10.3168/jds.2019-17022

Souza RM, Souza FN, Batista CF, Piepers S, De Visscher A, Santos KR, et al. 2022. Distinct behavior of bovine-associated staphylococci species in their ability to resist phagocytosis and trigger respiratory burst activity by blood and milk polymorphonuclear leukocytes in dairy cows. Journal of Dairy Science 105: 1625-1637. https://doi.org/10.3168/jds.2021-20953

Takano PV, Blagitz MG, Mira CS, Batista CF, Della Libera AMMP, Souza FN. 2018. Comparative study of distinct techniques to determine differential leukocyte counts in milk. Pesquisa Veterinária Brasileira 38: 773-778 (in Portuguese, with abstract in English). https://doi.org/10.1590/1678-5150-PVB-5252

Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, et al. 2019. Welcome to the tidyverse. Journal of Open Source Software 4: 1686. https://doi.org/10.21105/joss.01686

Zecconi A, Meroni G, Sora V, Mattina R, Cipolla M, Zanini L. 2021. Total and Differential cell counts as a tool to identify intramammary infections in cows after calving. *Animals* 11: 727. https://doi.org/10.3390/ani11030727