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RESEARCH ARTICLE

Cut-off value of somatic cell count and validation of differential somatic cell count by the fluoro-optical method in subclinical mastitis milk in three sheep breeds

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Somatic cell count (SCC) is a test used for mastitis control in ewes, but there is no consensus on the cut-off value. Other markers of subclinical mastitis (SCM) have been proposed as potentially more efficient, sensitive and specific, such as differential somatic cell count (DSCC). Several studies have been conducted on cow milk DSCC application, especially since the development of a method (Foss DSCC method) that allows the rapid and simultaneous determination of both SCC and DSCC in cow milk. The aim of this study was to investigate the Foss DSCC method in ewe milk. We first calculated a SCC cut-off for sheep half-udder milk samples from three dairy breeds to be used with bacteriological analysis to define SCM; second, we validated the Foss DSCC method following the validation conducted on bovine milk; and finally, the DSCC cut-off study was conducted for the sheep SCM milk. For this purpose, 4074 ewe half-udder milk samples from three breeds were analysed for bacteriological, SCC and DSCC investigation. The validation of the Foss DSCC method followed that previously conducted on cow milk, while the optimal cut-off values were chosen based on the Youden method after generating receiver operating characteristic curves and calculating the relative area under curve values. The specificity, repeatability and robustness of the Foss DSCC method for sheep milk were comparable to those of the method for bovine milk. The optimal cut-off resulted in 500 × 10^3 cells/mL and 71.5% for SCC and DSCC, respectively. For the first time, the Foss DSCC method was validated in sheep milk, and SCC and DSCC cut-off values were determined for three important dairy milk breeds of ewes in Italy. These results will allow developing further studies to improve mastitis screening and will help farmers, veterinarians and technicians to identify SCM in flocks.

Keywords Somatic cell count, Differential somatic cell count, Sub-clinical mastitis, Sheep milk.

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INTRODUCTION

Although sheep only provide 1% of the world's milk (Libera *et al*. 2021), they play an important role in the livestock economies of several countries, including China, the world's largest producer, Greece, Romania and Italy in Europe, as well as the Near East and North Africa countries (Balthazar *et al*. 2017). This species is considered profitable, especially in Mediterranean and Third Countries, due to the relatively low production costs and capital investment required for sheep farms, but it perfectly fits into the idea of organic farming in highly industrialised countries (Libera *et al*. 2021). Sheep milk consumption is not common, while it is an excellent matrix for cheese production due to its high level of protein, fat and calcium from casein units (Balthazar *et al*. 2017).

The quality and hygiene of livestock production are mandatory for its commercialisation. For milk, both quality and hygiene can be evaluated through the somatic cell count (SCC) (Fadillah *et al*. 2023). Somatic cells are composed mainly of inflammatory cells, such as lymphocytes, macrophages and polymorphonucleates (PMN), which play an essential role in the defence of the mammary gland (Kaskous *et al*. 2023). The decreased health status of the udder, such as intramammary infection, induces an increase in SCC, due to tissue damage and the release of a variety of chemoattractant compounds into the mammary system that attract inflammatory cells from the bloodstream (Alhussein and Dang 2018). This has a related negative effect on milk quality, such as coagulation properties, curd yield and cheese quality of cheese (Libera *et al*. 2021). However, a high SCC is not only a milk quality problem but also indicates udder health problems, such as the presence of udder infections due to zoonotic pathogens with risks for the consumer, influencing milk hygiene (Fadillah *et al*. 2023; Schadt 2023). Therefore, mastitis is not only one of the most relevant health and welfare problems in dairy ruminants but also the most expensive in sheep (Libera *et al*. 2021). However, in sub-clinical mastitis (SCM), no milk, udder or systemic abnormality can be observed, but there is an increase in SCC value (Libera *et al*. 2021). For this reason, SCC is an important marker of SCM, but there is a lack of consensus regarding a cut-off value for sheep milk able to discriminate between a healthy udder and those affected by a SCM (Libera *et al*. 2021). The physiological SCC level reported in the literature for healthy sheep milk is controversial, with a minimum of 10 to a maximum of 1000×10^3 cells/mL (Libera *et al*. 2021). This could be because several other factors can affect SCC values in this species, such as age, parity, breed, management system, number of born lambs, physiological lactation stage and season (Libera *et al*. 2021).

Due to the present limitations of SCC, other markers of SCM have been proposed as potentially more efficient, sensitive and specific (Libera *et al*. 2021). Among them, the differential somatic cell count (DSCC) has been under investigation especially for cow milk (Huang *et al*. 2023). Several studies have been conducted on cow milk DSCC application (Bobbo *et al*. 2020; Schwarz *et al*. 2020a, 2020b; Huang *et al*. 2023; Magro *et al*. 2023), especially since the development of a method (Foss DSCC method) that allows the rapid and simultaneous determination of both SCC and DSCC in cow milk (Damm *et al*. 2017). This tool is a fluor optic method showing low-cost, reliable, repeatable and high-throughput results (Damm *et al*. 2017). The Foss DSCC method can automatically calculate the percentage of PMNs and lymphocytes on the SCC, while macrophages can be calculated by subtracting the DSCC from 100% (Damm *et al*. 2017).

Although the use of the Foss DSCC method is routinely used for the SCC analysis of sheep milk, because the validation is done through interlaboratory proficiency tests, no validation has been conducted on the DSCC in sheep milk. The aim of this study was to investigate the Foss DSCC method in ewe milk to discriminate between healthy udders and those affected by a SCM. For this purpose, we first calculated an SCC cut-off for sheep half-udder milk samples from three dairy breeds to be used with bacteriological analysis to define SCM; second, we validated the Foss DSCC method following the validation conducted on bovine milk; and finally, the DSCC cut-off study was conducted for the sheep SCM milk.

MATERIALS AND METHODS

Animals

This study was approved by the Institutional Animal Care and Use Committee, University of Pisa (N: 11/2021 of 19.03.2021). Ten farms, located in Grosseto and Siena provinces of Tuscany, Italy, were included in this study. The inclusion criteria of the farms were as follows: (1) they owned a dairy flock of Lacaune, Sarda, or Comisana in semi-intensive dairy systems, (2) they had a history of bulk milk SCC above 10^6 cells/mL on average during the last year, which is the highest limit used in literature to distinguish between healthy and mastitis in sheep (Kaskous *et al*. 2023) (3) they were historically *Mycoplasma agalactiae*-free and (4) they were vaccinated for *Staphylococcus aureus*. Between 2021 and 2022, ewe half-udder milk samples were collected from 2037 animals. At the time of milk sampling, all the animals included in this study were milked twice a day, they showed no signs of clinical mastitis, and they were all healthy based on clinical examination performed by the local veterinarian.

Milk samples

Before sampling, teat ends were cleaned and disinfected with a commercial chlorhexidine-based disinfectant, and the first streams of foremilk were discarded. Two aliquots of around 40 mL of milk were collected from each half-udder in sterile plastic tubes and were suddenly refrigerated at 4°C in a dedicated box. The first aliquot of the milk sample was analysed for bacterial culture, while the second one was analysed for SCC and DSCC. Contextually to SCC and DSCC analyses, slides for microscopic differential cell count examination were performed. All analyses were performed by laboratories of Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, Grosseto, Italy, within 12 h after harvesting. The laboratories are accredited according to UNI CEI EN ISO/IEC 17025. Bulk sheep milk samples, routinely analysed by the laboratory, were used for the repeatability test of the validation of the Foss DSCC method on milk sheep.

Bacteriological examination

Milk samples were cultured following standard procedures (Adkins and Middleton 2018). Briefly, 10 microliters of milk sample was taken with a sterilised loop and plated onto

blood agar medium and incubated at 37°C under aerobic conditions. The plates were examined after 24 and 48 h for bacterial growth. The isolation of at least 10 colonies of the same type was considered to be significant as bacteriologically positive, while the isolation of three or more types of colonies was considered to be the result of environmental contamination, and the samples were eliminated from the study. Developed bacterial colonies were examined for taxonomical analyses: morphological characteristics of bacterial colonies, Gram staining, and oxidase and catalase results were considered. Gram-positive cocci were screened for catalase activity, growth on Baird Parker Agar Base + RPF Supplement and Modified Edwards Medium.

Somatic cell count determination

The determination of SCC in half-udder milk samples was performed through the fluoro-opto-electronic method with a Fossomatic 7 DC instrument (Foss Electric, Hillerød, Denmark) according to ISO UNI EN ISO 13366 (2006).

The determination of SCC in half-udder milk samples was performed through the fluoro-opto-electronic method using a Fossomatic 7 DC instrument (Foss Electric, Hillerød, Denmark) in accordance with UNI EN ISO 13366 (2006) at the laboratory of the Istituto Zooprofilattico Sperimentale Lazio e Toscana, accredited by Accredia, the Italian Accreditation Body (Laboratory No. 0201A).

The SCC analysis of bulk sheep milk with the Fossomatic 7 DC instrument was routinely used by the laboratory because it was previously validated through a biannual proficiency test organised by an independent organism (Associazione Italiana Allevatori Laboratorio Standard Latte, AIA, web site http://www.aia.it/lsl/index.htm) following UNI CEI EN ISO/IEC 17043 and ISO 13528 e/o UNI ISO 5725-2, and a proficiency test on sheep milk organised by the Centro di Referenza Nazionale per la qualità del latte e dei prodotti derivati degli ovini e dei caprini (C.Re.L.D.O.C., IZSLT, Rome) organised in 2021 (data not showed). The samples that presented a good separation (GOSE) less than 1 at the fluoro-opto-electronic method with a Fossomatic 7 DC instrument were eliminated from the study.

Validation of the Foss DSCC method for sheep milk

The determination of DSCC in half-udder milk samples was performed through the fluoro-opto-electronic method with a Fossomatic 7 DC instrument (Foss Electric, Hillerød, Denmark). In order to evaluate the specificity of the Foss DSCC method, the relation with another internal reference method for cell differentiation was investigated. Briefly, 250 samples of individual milk were casually chosen and smeared on microscopic slides at the same time as Foss DSCC analysis. The milk smears were performed following Paape *et al.* (1963) and Petersson *et al.* (2011) modified methods (Petersson *et al.* 2011; Paape *et al.* 1963). Samples were agitated by shaking 25 times in 7 s with a movement of approximately 30 cm, and then 0.01 mL of milk was transferred through a sterile loop to a microscope slide and distributed over one square centimetre. The smears were air-dried for 24 h on a level surface and degreased with ethylic alcohol 96%. The slides were stained using a haematological stain (Hemacolor®, Merck KGaA, Darmstadt, Germany), and 100 cells per slide were identified as polymorph nucleates, lymphocytes or macrophages at 100× magnification and counted as described by Pongrácz and Iváncsics (2007). Pearson's correlation coefficients (r) were calculated in order to investigate the relation between the two methods using the R software package.

To calculate the repeatability of the Foss DSCC method, 16 routinely available sheep-composite samples were analysed in 10 replicates, and the mean and the standard deviation were calculated. The robustness of the method was investigated considering the effect of the samples' variability on separation between cells and the milk matrix background in the obtained dot plots from the analysed samples for DSCC. The percentage of samples with GOSE less than 1 was calculated.

Statistics

The prevalence and confidence interval of 95% (CI 95%) of the isolated bacteria in SCM were calculated.

In order to generate receiver operating characteristic (ROC) curves and to calculate related values of area under the curve (AUC), sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), the optimal cut-off values and their confidence intervals (95% CI), the web tool for ROC curve analysis easyROC statistical software, version 1.3.1, was used. The cut-off value of SCC was chosen based on the Youden method, considering SCM when the bacteriological culture was positive, as reported in literature (Clements *et al.* 2003; Riggio *et al.* 2013; Persson *et al.* 2017). Interpretation of AUC was based on $0.9 \leq$ AUC excellent test, $0.8 \leq$ AUC < 0.9 considerable test, $0.7 \leq$ AUC < 0.8 fair test, $0.6 \leq$ AUC < 0.7 poor test and $0.5 \leq$ AUC < 0.6 fail test (Çorbacıoğlu and Aksel 2023).

According to the Shapiro–Wilk normality test, SCC and DSCC data followed a non-normal distribution. Therefore, the nonparametric Mann–Whitney test was applied, using the software R, to investigate the statistical difference for SCC median values between half-udders showing negative results of bacteriological culture (healthy) and those positive (affected by SCM) (R Core Team 2021). While for DSCC median values, the nonparametric Mann–Whitney test was applied, using the software R, to investigate the statistical difference between half-udders showing negative results (R Core Team 2021). In this case, bacteriological culture results (bacteriologically negative or positive), the calculated SCC value (under or equal or above to SCC cut-off value),

and the intersection of both results (bacteriologically negative and under or equal to SCC cut-off value or bacteriologically positive and above to SCC cut-off value) were used to discriminate between healthy and affected by SCM samples. The DSCC cut-off value was chosen based on the Youden method for the same discriminants used as gold standard tests (bacteriological culture results, the SCC calculated cut-off value, and the intersection of both results) using the web tool for ROC curve analysis easyROC.

RESULTS

From the 10 included dairy sheep farms, three farms bred Sarda ewes, four Lacaune sheep and three Comisana. Overall, 613 Sarda, 501 Lacaune and 923 Comisana sheep were involved.

A total of 4074 milk samples from half-udder were collected and analysed. From bacteriological examination, 2851 samples tested negative and 1053 tested positive for one or two bacteria (30.46%, CI 95% 29.05–31.87). The highest prevalence of positive samples was observed in Sarda sheep (42.33% CI 95% 39.57–45.10) followed by Lacaune (27.35%, CI 95% 24.59–30.11) and Comisana breed (24.27%, CI 95% 22.31–26.22) (Table 1). The most representative identified bacteria were Gram positive in all breeds, with a prevalence of 28.86% (Lacaune: 26.05%; Sarda: 23.24%; Comisana: 23.24%). Particularly, *Staphylococcus* spp. were the most isolated bacteria in all breeds, followed by *Streptococcus* spp. and Gram-negative bacteria, with differences depending on the breeds (Table 1). Among *Staphylococcus* spp., nonaureus staphylococci (NAS) were the most representative bacteria (22.04%).

Overall, 3552 samples (2633 negative, 919 positive) were considered for SCC statistical analysis, resulting in a GOSE of 1 and no contamination by environmental bacteria (negative or positive at bacteriological examination) and with an amount analysable by the instrument. The median SCC value of negative samples was 131.00 × 10^3 cells/mL, while for positive samples, it was 1068.00 × 10^3 cells/mL; there was a statistically significant difference for SCC median values between half-udders showing negative results of bacteriological culture (healthy) and those positive (affected by SCM) (Table 2). Even if the number of analysed samples was different, the mean of SCC in negative samples was similar in the three breeds. On the contrary, the mean of SCC in positive samples was lower in the Comisana breed (619.50 × 10^3 cells/mL), where a higher number of samples was analysed, than in the two other breeds. However, the significant difference of SCC results between bacteriological negative and positive samples was observed in all three breeds (Table 2).

The SCC cut-off value calculated by the ROC analysis for the whole population to discriminate between healthy and SCM half-udders was 500 × 10^3 cells/mL, with a fair

Table 1 Prevalence and confidence interval 95% (CI 95%) of identified bacteria in milk of half-udders collected from 10 dairy sheep farms in Tuscany (Italy) breeding three different breeds of ewes: Lacaune (n = 501), Sarda (n = 613) and Comisana (n = 923).

	L				S				C				Total			
	Total	Positive	Prevalence	CI 95%	Total	Positive	Prevalence	CI 95%	Total	Positive	Prevalence	CI 95%	Total	Positive	Prevalence	CI 95%
	n	*n*	%	%	*n*	*n*	%	%	*n*	*n*	%	%	*n*	*n*	%	%
Gram negative	1002	13	1.30	0.60–2.00	1226	89	7.26	5.18–8.71	1846	19	1.03	0.57–1.49	4074	121	2.97	2.45–3.49
Gram positive	1002	452	26.05	23.33–28.77	1226	430	35.07	32.40–37.74	1846	429	23.24	21.31–25.17	4074	1120	28.86	26.12–28.89
Staphylococcus spp.	1002	221	22.06	19.49–24.62	1226	376	30.67	28.09–33.25	1846	357	19.34	17.54–21.14	4074	954	23.42	22.12–24.72
CPS	1002	30	2.99	1.94–4.05	1226	0	0.00	-	1846	26	1.41	0.87–1.95	4074	56	1.37	1.02–1.73
NAS	1002	191	19.06	16.63–21.49	1226	376	30.67	28.09–33.25	1846	331	17.93	16.18–19.68	4074	898	22.04	20.77–23.32
Streptococcus spp.	1002	18	1.80	0.97–2.62	1226	22	1.79	1.05–2.54	1846	11	0.60	0.24–0.95	4074	51	1.25	0.91–1.59
Total	**1002**	**274**	**27.35**	**24.59–30.11**	**1226**	**519**	**42.33**	**39.57–45.10**	**1846**	**448**	**24.27**	**22.31–26.22**	**4074**	**1241**	**30.46**	**29.05–31.87**

C, Comisana; CPS, coagulase-positive staphylococcus; L, Lacaune; NAS, Nonaureus staphylococci; S, Sarda.

Table 2 Median and standard deviation (SD) of somatic cell count (SCC) in bacteriological negative and positive samples in three different breeds (Lacaune, Sarda and Comisana).

	Negative			Positive		
Breed	Number	Median (10^3 cells/mL)	SD (10^3 cells/mL)	Number	Median (10^3 cells/mL)	SD (10^3 cells/mL)
L	713	164.00^{A}	2258.54	200	1373.50^{B}	9084.60
S	677	129.00^{A}	4288.16	397	1124.00^{B}	7942.92
C	1243	115.00^{A}	2719.01	322	619.50^{B}	6404.56
Total	2633	131.00^{A}	3097.19	919	1068.00^{B}	7726.41

C, Comisana; L, Lacaune; S, Sarda; SD, standard deviation.
$^{A,B}P < 0.01$.

Table 3 Area under the curve (AUC), sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), optimal cut-off values, and their confidence intervals (95% CI), of somatic cell count (SCC) values of subclinical mastitis milk considering bacteriological positive and negative results in three different breeds (Lacaune, Sarda and Comisana).

Breed	Number of negative samples	Number of positive samples	Optimal cut-off point	AUC	Sensitivity (CI 95%)	Specificity (CI 95%)	Positive Predictive Value (CI 95%)	Negative Predictive Value (CI 95%)
L	713	200	501	0.848	0.850 (0.793–0.896)	0.822 (0.792–0.849)	0.572 (0.525–0.672)	0.951 (0.930–0.960)
S	677	397	468	0.778	0.688 (0.640–0.733)	0.79 (0.758–0.820)	0.658 (0.615–0.706)	0.812 (0.777–0.839)
C	1243	322	500	0.671	0.540 (0.484–0.596)	0.805 (0.782–0.827)	0.418 (0.384–0.474)	0.871 (0.844–0.887)
Total	2633	919	500	0.753	0.665 (0.633–0.695)	0.807 (0.792–0.822)	0.547 (0.522–0.581)	0.873 (0.857–0.884)

C, Comisana; L, Lacaune; S, Sarda.

AUC value of (0.753) and a Se and a Sp of 0.665 and 0.807, respectively (Table 3). Considering the breed, Lacaune showed a cut-off of 501 × 10^3 cells/mL, with a considerable AUC value (0.850), Sarda showed a cut-off of 468 × 10^3 cells/mL, with a fair AUC of 0.778 (fair), and Comisana had a cut-off of 500 × 10^3 cells/mL, with a poor AUC of 0.671. All the results for AUC, Se, Sp, PPV and PNV are reported in Table 3.

In order to check the relation between the Foss DSCC method and the microscopical method after haematological staining for cell differentiation, of the 250 smears performed, 198 samples that had an SCC value between 50 and 1500 × 10^3 cells/mL were examined through the two methods used (100 samples of Lacaune, 48 of Sarda and 50 of Comisana breed) following the Damm *et al*.'s (2017) validation (respectively, Figure 1a–d). The Pearson's coefficient *r* was 0.700 ($P < 0.00001$) considering all the samples analysed, 0.723 ($P < 0.00001$) for Lacaune samples, 0.815 ($P < 0.00001$) for Sarda samples, and 0.566 ($P < 0.00002$) for Comisana samples.

The repeatability was determined by analysis of 16 routinely available sheep-composite samples, run in 10 replicas. Samples with SCC ranging from 118 × 10^3 to 1273 × 10^3 cells/mL and DSCC from 53.89% to 83.71% represented the considered data set. All samples showed an acceptable repeatability (SD minum 0.55 maximum 2.17), and the average standard deviation was 1.15. Method-specific robustness was tested on 3953 samples of the three considered breeds, with SCC ranging from 50 to 81 141 × 10^3 cells/mL. As described by Damm *et al*. (2017), even though a lower signal-to-noise ratio may still give comparable DSCC, it might render the method less robust (Damm *et al*. 2017). For this reason, the separation between the cell signal and the background signal of the analysed samples was evaluated. Among 3953 samples analysed with Fossomatic 7 DC, 258 half-udder samples showed a GOSE = 0, with a percentage of 6.53% of the total sampling. Considering the breed, Lacaune samples showed a percentage of GOSE = 0 of 3.35% (33/936), Sarda of 6.08% (74/1218) and Comisana of 8.63% (151/1749).

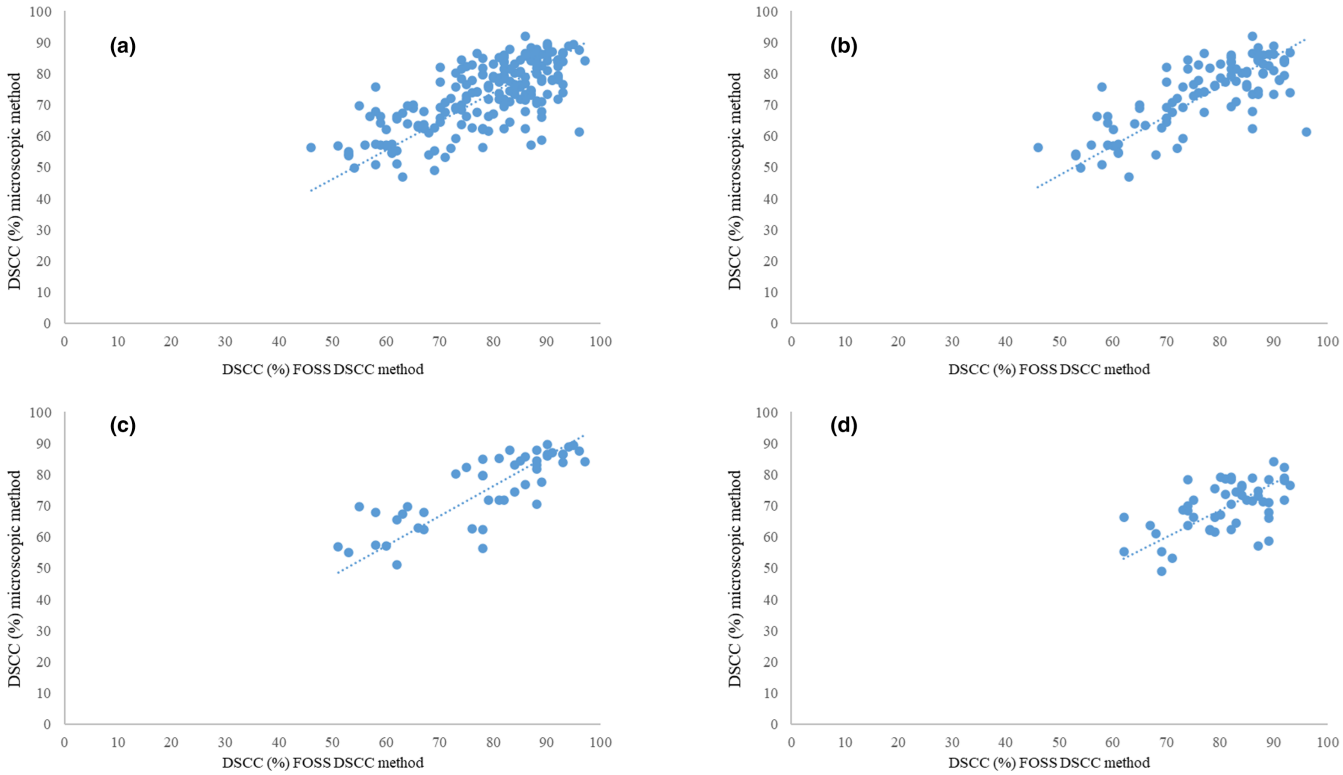

FIGURE 1 Results of the investigation of the relation between Foss differential somatic cell count (DSCC) method and light microscopy after haematological staining for cell differentiation. Data are illustrated in combination with linear trend lines. Each symbol represents the result of one half-udder milk sample, but overlapping is possible. (a) Results of total samples; (b) Results of Lacaune (100) samples; (c) Results of Sarda (48) samples; (d) Results of Comisana (50) samples.

Overall, 2465 half-udder milk samples with a range from 50 to 1500 $\times$ 10^3 cells/mL of milk (658 Lacaune, 714 Sarda and 1093 Comisana half-udder milk samples) were considered for DSCC further statistical analysis. Results were analysed considering bacteriological culture results or SCC previously obtained cut-off to discriminate between healthy and SCM half-udders (Table 4). When bacteriological culture was considered as the gold standard, the DSCC median value was 65–40% for negative samples and 72–75% for positive ones, with a highly significant difference when considering the whole sampling. However, similar results were obtained only in the Lacaune breed, where negative samples showed a DSSC value of 69.30% and positive samples 80.50%, with a highly significant difference. On the contrary, in Sarda and Comisana breeds, no statistical differences were observed in DSCC values between bacteriological negative and positive groups. When the cut-off of 500 $\times$ 10^3 cells/mL was used to identify the SCM samples, statistical differences were observed in all breeds, and the DSCC median was 78.00% in SCM samples. The significant differences were also observed when the intersection of the bacteriological culture and SCC estimated cut-off (500 $\times$ 10^3 cells/mL) results were used to identify SCM samples. However, in this case, in the Sarda breed, the statistical difference was lower ($P < 0.05$).

The optimal cut-off value and the AUC, Se, Sp, PPV and NPV of DSCC using bacteriological culture results or the calculated cut-off of SSC as the gold standard, or the intersection of both results are reported in Table 5. The cut-off value ranged from 68.1% to 75.1%. Considering all samples, the cut-off value was 71.2% using either bacteriological culture results or the SCC calculated cut-off, and the intersection of both results, but the AUC value was higher (0.77) only when the SCC calculated cut-off or the intersection results were used as the gold standard test to identify SCM. The lowest cut-off value was observed in the Sarda breed (68.1%), when the bacteriological culture was considered as the gold standard with a poor AUC value (0.51). However, when SCC was >500 $\times$ 10^3 cells/mL or the intersection of both results was used as a discriminant of SCM in this breed, the AUC values resulted fair (0.63) and the cut-off values were, respectively, 73.6% and 73.3%. Finally, the cut-off observed in the Comisana breed using bacteriological results as the gold standard or the intersection with estimated SCC cut-off value was the same as observed in the whole sampling (71.2%), but the AUC resulted, respectively, fail (0.59) and fair (0.85).

Table 4 Median and standard deviation (SD) of differential somatic cell count (DSCC) in bacteriological negative and positive samples, and in under or equal and above 500 × 10^3 cells/mL samples in three different breeds (Lacaune, Sarda and Comisana).

	Negative			Positive		
Breed	Number	Median (%)	SD (%)	Number	Median (%)	SD (%)
L	551	69.30^{A}	14.19	107	80.50^{B}	13.2
S	488	72.00	13.57	226	72.65	12.39
C	912	58.35	16.24	181	63.70	18.72
Total	1951	65.40^{A}	15.99	514	72.75^{B}	16.32
	<500 × 10^3 cells/mL			**>500 × 10^3 cells/mL**		
L	507	67.20^{A}	14.04	151	81.60^{B}	9.503
S	514	69.90^{A}	12.75	200	78.05^{B}	13.67
C	911	54.70^{A}	15.97	182	76.80^{B}	10.79
Total	1932	63.00^{A}	15.9	533	78.00^{B}	11.89
	Negative and <500 × 10^3 cells/mL			**Positive and >500 × 10^3 cells/mL**		
L	481	67.00^{A}	13.88	81	82.00^{B}	9.038
S	402	70.00^{a}	12.94	114	76.65^{b}	12.24
C	793	55.80^{A}	15.71	63	77.80^{B}	9.435
Total	1676	63.10^{A}	15.70	258	78.80^{B}	10.97

C, Comisana; L, Lacaune; S, Sarda.
$^{A,B}P < 0.01$; $^{a,b}P < 0.05$.

DISCUSSION

According to the literature (Alekish and Alshehabat 2014; Świderek *et al*. 2016; Zafalon *et al*. 2016), the most prevalent bacteria isolated in the present study were NAS (22.04%) (Table 1), already considered the main pathogen causing SMC in sheep (Onni *et al*. 2010; Ozenc *et al*. 2011; Vasileiou *et al*. 2019).

Recently, two reviews included studies that attempt to set a cut-off value of SCC to define the physiological level or the SCM level in ewe milk in the literature (Libera *et al*. 2021; Kaskous *et al*. 2023). Despite this topic being widely approached by literature, both reviews agreed that a clear threshold value was not defined; thus, a cut-off value is still under discussion (Libera *et al*. 2021; Kaskous *et al*. 2023). Moreover, among studies investigating SCC cut-off values in sheep milk, very few evaluated it based on a ROC curve analysis calculating the related values of AUC (Clements *et al*. 2003; Riggio *et al*. 2013; Alekish and Alshehabat 2014; Świderek *et al*. 2016; Zafalon *et al*. 2016); some of them were conducted on meat-producing sheep (Clements *et al*. 2003; Zafalon *et al*. 2016). As in our study, in the majority of the studies, the SCC cut-off value was investigated by assessing cases of SCM using bacteriological status as the gold standard or reference point (Clements *et al*. 2003; Świderek *et al*. 2016; Zafalon *et al*. 2016; Persson *et al*. 2017).

In the literature, in relation to the breeds, several studies suggested a different SCC physiological value with a wide range (from 39 to 1600 × 10^3 cells/mL) in ewe milk (Kaskous *et al*. 2023). We investigated three different sheep dairy breeds that are common in the region of investigation to define an SCC and DSCC cut-off to use for discriminating between healthy and SCM-affected milk. Using the Fossomatic 7 DC instrument after a previously validated SCC analysis on sheep milk, the SCC cut-off value of 500 × 10^3 cells/mL was estimated in the whole sampling, but very similar results were found in all three dairy breeds considered (501 × 10^3 cells/mL for Lacaune, 468 × 10^3 cells/mL for Sarda, and 500 × 10^3 cells/mL for Comisana). These results are in agreement with similar studies on Valle del Belice dairy sheep when the whole sampling or minor pathogen-positive samples were considered, and the SCC threshold was estimated to be 645 × 10^3 cells/mL with an AUC of 0.75 and 0.73 respectively (Riggio *et al*. 2013). Similar results to our study were also reported in Swedish meat-producing breeds with a cut-off of 513.5 and 414.5 × 10^3 cells/mL, respectively for weaning and after lambing milk (Persson *et al*. 2017). Even though few differences in the SCC cut-off values could be influenced by the breed investigated, as evidenced also in the Sarda breed in the present study (468 × 10^3 cells/mL), a cut-off of 500 × 10^3 cells/mL could be an acceptable value to differentiate between healthy and SCM milk in ewes. This result was also confirmed by the AUC test that resulted fair (0.753) for the whole sampling and considerable (0.848) for the Lacaune breed. The Sp was higher than Se, with the exception of the Lacaune breed, where very similar Se and Sp were observed (respectively 0.855 and 0.820). However, a cut-off value with a good Sp could be useful in a context

Table 5 Area under the curve (AUC), sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), optimal cut-off values, and their confidence intervals (95% CI), of Differential Somatic cell count (DSCC) values of subclinical mastitis milk in three different breeds (Lacaune, Sarda and Comisana).

	Breed	Number of negative	Number of positive	Optimal cut-off point %	AUC	Sensitivity (CI 95%)	Specificity (CI 95%)	Positive predictive value (CI 95%)	Negative predictive value (CI 95%)
Bacteriological culture	L	551	107	75.5	0.72	0.729 0.634–0.810	0.679 0.638–0.718	0.306 0.269–0.412	0.928 0.893–0.939
SCC > 500 × 10^3 cells/mL	L	507	151	75.5	0.83	0.844 0.778–0.896	0.75 0.710–0.786	0.504 0.454–0.619	0.941 0.912–0.951
Bacteriological culture and SCC > 500 × 10^3 cells/mL	L	481	81	75.5	0.83	0.852 0.756–0.921	0.753 0.712–0.791	0.367 0.320–0.540	0.968 0.942–0.974
Bacteriological culture	S	488	226	68.1	0.51	0.646 0.580–0.708	0.418 0.374–0.463	0.34 0.299–0.406	0.718 0.659–0.754
SCC > 500 × 10^3 cells/mL	S	514	200	73.6	0.63	0.629 0.560–0.694	0.605 0.563–0.646	0.381 0.341–0.451	0.809 0.761–0.834
Bacteriological culture and SCC > 500 × 10^3 cells/mL	S	402	114	73.3	0.60	0.605 0.509–0.696	0.587 0.537–0.636	0.294 0.253–0.382	0.84 0.780–0.866
Bacteriological culture	C	912	181	71.2	0.59	0.398 0.326–0.473	0.755 0.726–0.783	0.244 0.217–0.305	0.863 0.822–0.881
SCC > 500 × 10^3 cells/mL	C	911	182	69.1	0.83	0.743 0.675–0.804	0.791 0.763–0.816	0.423 0.384–0.509	0.937 0.915–0.946
Bacteriological culture and SCC > 500 × 10^3 cells/mL	C	793	63	71.2	0.85	0.794 0.673–0.885	0.817 0.788–0.843	0.256 0.223–0.409	0.98 0.964–0.984
Bacteriological culture	Total	1951	514	71.2	0.59	0.543 0.499–0.586	0.63 0.608–0.651	0.279 0.260–0.316	0.839 0.814–0.852
SCC > 500 × 10^3 cells/mL	Total	1932	533	71.2	0.77	0.748 0.710–0.784	0.687 0.666–0.707	0.401 0.379–0.449	0.907 0.889–0.914
Bacteriological culture and SCC > 500 × 10^3 cells/mL	Total	1676	258	71.2	0.77	0.756 0.699–0.807	0.692 0.669–0.714	0.274 0.254–0.337	0.948 0.932–0.953

C, Comisana; L, Lacaune; S, Sarda.

of treatment or culling of sheep with SCM with the awareness of the risk of false-negative results (Zafalon *et al*. 2016). Anyhow, a high statistically significant difference ($P < 0.001$) for SCC values between healthy half-udders and those affected by SCM was observed in the present study as previously reported by Persson *et al*. (2017).

In the present study, two aliquots of 40 mL of milk were collected, when possible, from half-udders of three breeds of dairy sheep, after the discarding of the first streams of foremilk. The first aliquot was used for bacteriological examination, while the second aliquot was used for SCC and DSCC examination. In bovine, a different SCC value was observed between cisternal and alveolar milk in the udder (Sarikaya and Bruckmaier 2006). This may have influenced the analysis, but unlike cattle, sheep have a greater cisternal capacity, and the contribution of cisternal milk to total udder milk in the Lacaune breed was 68%, with a range of 47–77%, whereas in the Sarda breeds, the values ranged from 76.8% to 84.89% for the left and right halves of the udder, respectively (Labussière 1988; Nudda *et al*. 2000; Rovai *et al*. 2008). Considering that, the milk analysed was cisternal milk, but in dairy sheep milk, no SCC value difference was observed between cisternal and alveolar milk when they are milked with an interval of up

to 12 h, as the ewes in the present study that were milked twice a day (Castillo *et al*. 2008). In 2017, Damm and colleagues introduced a new technique for differentiating somatic cells simultaneously to the measurement of SCC through the Foss DSCC method (Damm *et al*. 2017). This method was validated in dairy cow milk and compared with other methods (Damm *et al*. 2017). The method used as the gold standard to calculate the specificity of the DSCC test was an internal method developed using a fluorescence microscopy that showed a coefficient correlation of $r = 0.8456$ ($P < 0.001$) with the FOSS DSCC method when applied to 180 routinely available cow-composite samples (Damm *et al*. 2017). No correlation ($r = 0.3520$, $P = 0.0532$) was observed with the light microscopy method after centrifugation and haematological staining on 35 cow-composite samples, while a correlation of $r = 0.8051$ ($P < 0.001$) was observed with the internal method developed using a fluorescence microscopy (Damm *et al*. 2017). Even if the Foss DSCC method has been validated only for cow milk samples, the Fossomatic 7 DC instrument is also able to provide DSCC values for the milk of other dairy species. To the best of our knowledge, no study has evaluated the applicability of the Foss DSCC method on sheep milk yet. The first step of our project was the evaluation of the specificity of the Foss DSCC method on sheep milk, comparing this method with light microscopy examination, after haematological staining, as previously described in the literature (Pongrácz and Iváncsics 2007), on 198 half-udder ovine milk samples. The correlation between the two methods calculated in the present study in the whole sampling was lower ($r = 0.700$, $P < 0.00001$) than the correlation observed in cow milk samples using the fluorescence microscopy examination (Damm *et al*. 2017). However, when breeds were considered, the correlation ranged from 0.815 ($P < 0.00001$) for Sarda samples to 0.566 ($P < 0.00002$) for Comisana samples. The correlation observed in Sarda samples ($n = 48$) is in accordance with the correlation observed in 35 cow milk samples using the fluorescence microscopy method (Damm *et al*. 2017). In the cow samples, the correlation with the light microscopy method observed in the Damm *et al*. study (2017) was much lower than our correlation in all breed samples examined (Damm *et al*. 2017). In the light microscopy method chosen in our study, no centrifugation and washing of cells were applied; this could explain the difference observed. The centrifugation and washing of cells are well known to alter the distribution of cell types occurring in milk, as also reported by Damm *et al*. (2017). The lower correlation in Comisana breed ($r = 0.566$, $P < 0.00002$) and Lacaune ($r = 0.723$, $P < 0.00001$) milk observed with respect to Sarda ($r = 0.815$, $P < 0.00001$) breed in the present study could be due to the fat composition of milk of these breeds. The three breeds considered in the present study are well known to differ in fat milk content (Pulina *et al*. 2005). In the literature, the average fat content of Sarda, Lacaune, and Comisana is reported to be 6.69%, 7.14%, and 7.5–10.6% respectively (Pulina *et al*. 2005). In milk microscopy examination, the average fat content can make the reading difficult to perform (Moraes *et al*. 2018). Even if the correlation was significant in all different breed milk samples analysed, the lower correlations observed in Lacaune and Comisana breed milk seem to decrease with the increase of fat composition of these milk breeds. The repeatability observed for sheep milk in the present study was acceptable for all examined samples ($n = 16$) and showed an average SD (1.15) lower than that observed in bovine milk (2%) by Damm *et al*. (2017). In sheep milk analysed samples in the present study, a percentage of 6.53% of samples showed a GOSE = 0, with a range from 3.35% to 8.63% depending on the breed. The robustness of the Foss method is based on separation between background signal and cell signal, called GOSE (Damm *et al*. 2017). During the Foss method on 655 individual cow milk samples with variable factors such as season, region, country, dairy herd management, breed, and so on, no difficulty was observed (Damm *et al*. 2017). However, GOSE = 0 was also observed in studies on cow milk by Shwarz *et al*. (2020a, 2020b) and Magro *et al*. (2023), reporting a variability of samples with GOSE = 0 from 0.04% to 0.55%. In the present study, the higher percentage observed in sheep milk could be influenced by the high SCC value of some analysed samples that showed a median (±SD) of $1068.00 \pm 7726.41 \times 10^3$ cells/mL in positive samples. No reference sheep milk sample exists, so it was not possible to analyse a reference milk sample.

Since 2017, when Damm and colleagues introduced the Foss DSCC method for differentiating somatic cells in dairy cow milk (Damm *et al*. 2017), the combination of SCC and DSCC for the detection of SCM showed a higher sensitivity than SCC alone (Schwarz *et al*. 2020b). The association of these two indices with milk yield and quality has been demonstrated in some studies (Bobbo *et al*. 2020; Schwarz *et al*. 2020b), and their use has become a hot topic to identify SCM and to reduce mastitis-associated losses in cows (Huang *et al*. 2023). Even if it is difficult to establish a fully established threshold for DSCC across all SCC levels, for practical purposes, a cut-off of 65% was defined to categorise the cow udder health status in combination with the 200×10^3 cells/mL SCC threshold (Schwarz *et al*. 2020b).

The cut-off value for DSCC observed in the present study in sheep milk (71.2%) was higher than the threshold defined in cow milk (from 65.0% to 69.3%) (Schwarz *et al*. 2020a, 2020b). Previous studies observed a lower percentage of macrophages in ovine milk than in bovine milk (Albenzio *et al*. 2019). Being the DSCC value the percentage of PMN and lymphocytes on SCC, this could explain the higher DSCC cut-off value observed in ewe milk in the present study. Even if no study has been previously reported on the

validation of the FOSS DSCC method for sheep milk, recently a study defined the DSCC cut-off value in Valle del Berice sheep milk breed (Tolone *et al*. 2023). The DSCC cut-off value was defined by applying four SCC cut-offs previously reported in the literature (265, 500, 645, 1000 × 10^3 cells/mL) and no bacteriological examination of milk samples was conducted (Tolone *et al*. 2023). However, the DSCC cut-off ranged from 76.1 to 79.8 for the four SCC cut-offs chosen (Tolone *et al*. 2023). This is in accordance with the DSCC cut-off evaluated in the present study that resulted higher than the bovine DSCC cut-off (Schwarz *et al*. 2020b). The AUC values reported by Tolone and colleagues were considerable for every SCC cut-off used (Tolone *et al*. 2023). On the contrary, the AUC value reported in the present study for the DSCC cut-off ranged from fail to considerable depending on the breed and the test considered as the gold standard. However, studies conducted on cows suggested the use of the SCC and DSCC combination to improve Se and Sp of these tests to identify SCM (Bobbo *et al*. 2020; Schwarz *et al*. 2020a, 2020b). However, in the present study, to the best of our knowledge, the DSCC Foss method was validated in sheep milk for the first time, and SCC and DSCC cut-off values were determined for three important dairy milk breeds of ewes in Italy. Further studies should be conducted in sheep to study the combination of both values to distinguish not only healthy and sub-clinical mastitis affected udders, but, as in cows, also, if possible, the categorisation of udders in healthy, suspicious of mastitis, mastitis, chronic mastitis.

CONCLUSION

The validation of the Foss DSCC method and the definition of SCC and DSCC cut-off values in dairy sheep milk will allow developing further studies to improve mastitis screening and will provide a better understanding of this important pathology in sheep milk production. The use of the DSCC value as an indicator of SCM for sheep milk will provide a more precise definition of the udder health status than the SCC value standing alone. The rapid, simultaneous and cost-effective determination of DSCC and SCC will help farmers, veterinarians and technicians to identify SCM in flocks, which has a significant impact on milk production and quality.

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AUTHOR CONTRIBUTIONS

Marco Montagnani: Writing – original draft; investigation; formal analysis. **Letizia Ciofi:** Validation; writing – review and editing. **Elisa Gasparoni:** Investigation; formal analysis. **Francesca Vichi:** Investigation; formal analysis. **Andrea Santini:** Investigation; formal analysis. **Riccardo Pietrini:** Formal analysis; investigation. **Moira Pacini:** Formal analysis; investigation. **Tiziana Galli:** Validation; writing – review and editing. **Carlo Boselli:** Writing – review and editing. **Francesca Bonelli:** Supervision; writing – review and editing. **Gianluca Fichi:** Funding acquisition; conceptualization; supervision; writing – review and editing.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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