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Lactate dehydrogenase: Detecting high bacterial and somatic cells counts in goats from whole milk samples

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ABSTRACT

The efficacy of using only somatic cell count (SCC) in goats to study the milk quality as a single test has been widely questioned, with total bacterial count being considered as ancillary test in several studies. Therefore, the main objective of this study was to identify new biomarkers of interest that correlate with high SCC and TBC values in milk samples. The concentration of five selected proteins according to their association with infected and injured udders (milk amyloid A, lactoferrin, pentraxin 3, N-acetyl-beta-D-glucosaminidase and lactate dehydrogenase) were determined in bulk raw milk together with SCC and TBC values. Only LDH showed a significant positive correlation with respect to SCC or TBC, no correlation between the other proteins with respect to both parameters were detected. Following, LDH activity and SCC and TBC values were analyzed in a wide diversity of goat milk samples performing a receiver operating characteristic (ROC) curve analysis. LDH showed a significant positive correlation with both SCC and TBC values. LDH activity differed significantly between milk samples with a high and/or low SCC and TBC with a sensitivity that can reach values of 91.3 % and a specificity of 83.2 %. Our results, highlight LDH activity measurement as a biomarker of interest in raw milk for the detection of low-quality milk samples which could potentially be used for the diagnosis of subclinical mastitis in goats.

1. Introduction

Milk quality and safety is related to somatic cell count (SCC) and total bacterial count (TBC). High SCC or TBC represent a significant problem in dairy industry, leading to important economic losses with hygienic and legal consequences. In addition, it has an impact on food safety and public health (Koop et al., 2012; Matuozzo et al., 2020; Nabih et al., 2018; Viguier et al., 2009). One of the factors leading to an increase in those parameters in milk is the subclinical mastitis (SCM) (Stuhr and Aulrich, 2010; Viguier et al., 2009; Koop et al., 2012).

Measurement of TBC in bulk milk is a reliable technique and is commonly used to evaluate the bacteriological quality of milk. The bulk milk bacterial cells can enter from the environment during milking or from the growing of bacteria because of insufficient cleaning and sanitation of the system, but in some cases, they can also enter from the mammary gland of the goat. Therefore, sometimes TBC of bulk milk can be used to monitor the SCM situation in a farm (Koop et al., 2012).

SCM has been associated with high SCC values in milk becoming as the most frequently used technique as a first approach for the diagnosis of SCM in goats (Katsoulos et al., 2010; Koop et al., 2012; Persson and Olofsson, 2011). However, the SCC values in goat milk has special connotations, since they do not accurately correlate with clinical status or SCM and its interpretation may result challenging (McDougall et al., 2014; Stuhr and Aulrich, 2010; Viguier et al., 2009).

One of the main drawbacks of using SCC in goats is its great fluctuation along the lactation in a healthy udder as well as during parity and estrus or other physiological factors (Barth et al., 2010; Haenlein, 2002; Hiss et al., 2008). Besides, goat milk contains many cytoplasmic particles from its apocrine secretion which could overestimate the counts when using automatic counters (Haenlein, 2002; Leitner et al., 2004; Rovai et al., 2014).

In addition, expensive equipment are needed nowadays for

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laboratory analyses of SCC and TBC in milk samples (Dohoo et al., 2011; Rovai et al., 2014). All these features highlight the need to develop an accurate, easy-to-use and inexpensive system for the detection of low quality goat's milk not only based on SCC.

Detection of specific proteins, which concentration increases in milk from injured or infected udder, have been pointed as an approach of interest for this goal (Katsafadou et al., 2019; Matuozzo et al., 2020; Persson et al., 2014). Among them, acute phase proteins, such as milk amyloid A (**MAA**) or haptoglobin (**Hp**) (Ceciliani et al., 2012; Gonzalez et al., 2008), and antimicrobial proteins, such as lactoferrin (**LF**) or pentraxin 3 (**PTX3**) (Cremonesi et al., 2012; Olumee-Shabon et al., 2013), have aroused the interest of different research studies. In addition, the change in the activity of selected enzymes (N-acetyl-D-glucosaminidase, **NAG-ase**; lactate dehydrogenase, **LDH**; or adenosine triphosphate, **ATP**) secreted by epithelial cells of the mammary gland or cells from the surrounding tissue has also been examined as a potential surrogate marker of SCM in goats (Cremonesi et al., 2012; Olumee--Shabon et al., 2013; Zhang et al., 2014).

In order to optimize faster control measures, to reduce and to limit high SCC and TBC values in the goat's milk, this study aimed, firstly, to compare the concentration of PTX3, LF, MAA, LDH and NAGase with TBC and SCC in bulk milk from different goat flocks. Secondly, the best candidate was chosen based on the best correlations previously obtained with the above-mentioned parameters. LDH turned out to be the best candidate and was validated in a wider study with individual and bulk samples as biomarker of high TBC and SCC values in milk samples from goats.

2. Materials and methods

2.1. Experimental design and milk samples

The goat flocks included in this study belonged to the breeds Malagueña and Murciano-Granadina, and were under official milk control by Cabrandalucia (Andalusian Federation of Purebred Caprine Herd Associations). Three experiments were set up to address the two aims of this study. In the first one, bulk milk samples were collected from 49 dairy goat flocks (one bulk milk sample/flock) to determine the concentration of the five selected proteins (PTX3, LF, MAA, LDH and NAGase) to be compared with SCC and TBC results.

According to the results from the first experiment, LDH was chosen as the best candidate and two additional experiments were conducted to validate its use as biomarker of high SCC and TBC levels in goat milk samples. As a first approach, two dairy goat flocks from the previous experiment with the highest SCC and TBC values were identified with the purpose of selecting animals with high SCC and TBC records in order to assess the power of LDH. Individual milk samples from a total of 42 healthy animals (21 animals/selected dairy herd) were collected every two-weeks at morning milking during about the first three months (12 weeks) of lactation (milk of the first week was discarded because of the colostrum). Dynamics of the biomarker over the three first months of lactation as well as the correlation of the LDH with respect to SCC and TBC were calculated.

The last experiment was carried out to determinate the cut-off points for LDH activity for the detection of high SCC and TBC in goat milk samples regardless of its origin (individual animal or bulk tank)A total of 581 milk samples (bulk milk or individual milk samples) received for routine analyses, were included in this study.

After milking, bulk milk samples (50 mL) were collected aseptically from each tank (4 °C) using a sterile metal collector. Milk samples obtained from individuals (approximately 50 mL from the 2 udders) were collected in sterile tubes after cleaning the teats with 70 % ethanol and discarding foremilk. Samples were maintained and transported at 4 °C and processed immediately after arriving to the laboratory.

All samples were subjected to SCC and TBC examination and were classified as positive (high) or negative (low) to SCC or TBC values based on different classification criteria.

2.2. Somatic cell count (SCC) and total bacteria count (TBC)

SCC and TBC were determined in milk samples (20 mL) by flow cytometry using an automatic Fossomatic™ 7 and a BactoScan™ FC (Foss, Hillerød, Denmark) calibrated with cow's and goat's milk (Koop et al., 2012).

2.3. Classification criteria

Samples were classified into positive or negative based on SCC or TBC values. Regarding SCC, three criteria were established (SCC-1 to SCC-3: > 1 million somatic cells/mL, > 1.5 million somatic cells/mL and > 2 million somatic cells/mL, respectively) according to the literature consulted (Koop et al., 2012; Leitner et al., 2008; Stuhr and Aulrich, 2010). Milk samples with values included within any of these categories were considered as positive whereas samples with lower values were classified as negative ones.

Similarly, three criteria were established for TBC (TBC-1 to TBC-3: > 50,000 bacterial cells/mL, > 200,000 bacterial cells/mL and > 500,000 bacterial cells/mL, respectively), with the last criterion corresponding with the maximum bacterial cells/mL allowed in milk in Europe (Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004, Annex III, Section IX, Chapter I-III, point 3b). Milk samples with TBC values included within any of these categories were considered as positive, whereas samples with lower values were classified as negative ones.

2.4. Protein concentrations and enzyme activity assays

Analyses of milk concentration of PTX3, LF and MAA proteins was carried out by indirect ELISAs and the activity of LDH and NAGase were determined using a kinetic method and a spectrophotometric method, respectively. The measurements were performed using commercially available kits (Table 1) and following manufacturer's instructions. All samples were analyzed in duplicate. Measurements were carried out by expert person and were blind.

2.5. Statistical analysis

Protein concentrations, enzyme activities, SCC and TBC values were evaluated for approximate normality of distribution by the D' Agostino & Pearson omnibus normality test (GraphPad Prism v5.0; GraphPad software, San Diego, CA, USA). For variables not normally distributed, Kruskal-Wallis and Mann-Whitney test were used. Differences in variables following a normal distribution were performed by Student's *t*-test. Correlation coefficients were assessed by the Spearman test

Table 1
Commercial kits used in milk goat samples to measure the selected biomarkers for detection of injured or infected udder.

Parameter[1]	Commercial kit	Distributor	Milk Dilution	Unit
PTX3	Goat Pentraxin 3 ELISA	BlueGene	1/2000	µg/mL
LF	Goat Lactoferrin ELISA	BlueGene	1/50,000	µg/mL
MAA	Milk Amyloid A Assay	Tridelta	1/500	µg/mL
LDH	Lactate Dehydrogenase Activity Colorimetric Assay	BioVision	1/25	mU/mL
NAGase	*B-N*-Acetylglucosaminidase Assay	Sigma	1/10	mU/mL

[1] PTX3: pentraxin 3; LF: lactoferrin; MAA: milk amyloid A; LDH: lactate dehygrogenase; NAGase: *N*-acetyl-beta-D-glucosaminidase.

(GraphPad Prims v5.0, San Diego, CA, USA) and were considered relevant if $P \leq 0.05$. Receiver operating characteristic (**ROC**) curve analysis was performed using MedCalc v12.7.8 (https://www.medcalc.org/). Sensitivity, specificity and area under the curve (**AUC**) were determined from the resulting ROC analysis with 95 % confidence interval. Statistical significance was defined at $P \leq 0.05$. The positive likelihood ratio (**LR+**) and the negative LR (**LR-**) were also calculated.

3. Results

3.1. Protein concentration in bulk milk samples

The concentration of PTX3, LF, MAA, LDH and NAGase was analyzed in bulk milk from 49 goat farms. Table 2 shows the mean values for each biomarker ± standard deviation as well as the correlation among the different parameters analyzed.

No correlation was detected between the proteins with respect to SCC or TBC, whereas only LF showed a significant positive correlation with respect to SCC ($r = 0.38$; $P < 0.05$). On the other hand, we observed a significant positive correlation between LDH and both SCC ($r = 0.52$; $P < 0.05$) and TBC ($r = 0.29$; $P < 0.05$) (Table 2).

3.2. Dynamics of the LDH over the time

According to the results obtained from the first experiment, we selected two flocks (A and B) and collected individual milk samples from 21 healthy animals (without clinical mastitis, no apparent signs of local inflammation or systemic involvement) from each one every two-weeks during the first three months of lactation and subjected them to LDH activity and SCC and TBC analyses (Table 3).

Fig. 1 shows individual and mean values ± standard deviation for LDH activity obtained from both flocks. We did not observe significant differences in the LDH activity throughout the study in the time frame of the study, although we detected high individual variability (Fig. 1).

We observed a significant positive correlation between SCC and TBC with respect to LDH activity in both herds (Table 4).

3.3. LDH activity cut-off point for the detection of high SCC and TBC levels

LDH activity was analyzed in a total of 581 milk samples by means of ROC curve analysis, which allowed determining optimal cut-offs for predicting low or high SCC and TBC values. Samples were classified as positive or negative according to the classification criteria above mentioned.

All the calculated ROC curves were significant for all the tested criteria. Table 5 show all the information related to ROC curves, including the AUC and the sensitivity and specificity.

Focusing on the AUC, several criteria reached an AUC > 0.85, which corresponded to excellent discrimination power. We also found acceptable discrimination power for the remaining criteria (AUC > 0.70).

Taking the TBC values into consideration as an independent criterion, the maximum sensitivity (91.3 %) and specificity (62.5 %) were reached at a cut-off of 33.5 mU/mL of LDH to discriminate between milk samples with 50,000 bacterial cells/mL or more (TBC-1). Likewise, the maximum LR+ (2.4) and minimum LR- (0.1) were reached by this criterion.

When we considered the combination of both parameters, we obtained the best accuracy values in terms of sensitivity (91.2 %) for the criterion TBC-1 + SCC-1, which agrees with the minimum LR- (0.14), those results were reached at a cut-off of 37.2 mU/mL of LDH. In terms of specificity (83.2 %), the best results were reached for the criterion TBC-2+SCC-3, which agrees with one of the maximum LR+ (4.0).

4. Discussion

Due to the problematic and discussed efficacy of using only SCC for the determination of the quality of goat milk, the main goal of this study was to identify a new biomarker based on SCC and TBC for this species. The concentration or activity of five proteins which concentration increases in milk from injured or infected udder was analyzed and correlated with respect to SCC and TBC in bulk milk samples from different goat flocks. Despite previous studies have demonstrated the effect of the bacteriological status of udders and SCC on an increase in the concentration or activity of the proteins and enzymes included in the present study (Gonzalez et al., 2008; Hiss et al., 2008; Barth et al., 2010; Cremonesi et al., 2012; Olumee-Shabon et al., 2013; Stuhr et al., 2013; Zhang et al., 2014), we did only observe correlation between LF and SCC and between LDH activity and SCC and TBC.

Several factors, such as the stage of lactation or the parity among others, have been pointed out to vary the concentration of the selected molecules under study. Since we performed our first approach using bulk milk samples, several of the above-mentioned factors could be suffering a dilution effect in our results. In addition, the different methodological approaches carried out in each particular study may be also associated with the discrepancies observed.

The significant positive correlation observed between LDH activity with respect to SCC and TBC highlights LDH as a candidate of interest in the diagnosis of milk quality in dairy goats. Indeed, this enzyme is already used for the diagnosis of SCM in dairy cows (Friggens et al., 2007; Hiss et al., 2008). Nevertheless, although several studies have been performed to demonstrate the usefulness of LDH in goats, the results are controversial and no conclusive findings have been previously obtained (Ying et al., 2002; Katsoulos et al., 2010; Stuhr and Aulrich, 2010).

LDH activity in individual milk samples from 42 healthy animals (from two different flocks) during the first three months of lactation was analyzed. Although we found a high individual variability, we did not observe significant differences in the LDH activity along the sampling timeframe. Other studies suggest that the stage of lactation is significantly associated with the LDH activity (Persson et al., 2014; Stuhr et al., 2013). Interestingly, we observed a significant positive correlation between LDH activity and SCC and TBC in both studied flocks. Although

Table 2
Correlation between the different parameters measured in bulk milk samples, lactate dehygrogenase (LDH) and *N*-acetyl-beta-D-glucosaminidase (NAGase) activities (mU/mL), milk amyloid A (MAA), lactoferrin (LF) and pentraxin 3 (PTX3) concentrations (μg/mL) and somatic cell count (SCC; cells/mL; x103) and total bacteria count (TBC; bacterial cells/mL; $x10^3$) (n = 49). Values are expressed as mean ± standard deviation. *: $P \leq 0,05$.

Biomarker	Bulk milk	Correlations between different parameters measured in bulk milk samples.							
SCC	1,287.94 ± 576.21		LDH	TBC	SCC	PTX3	LF	NAGase	MAA
TBC	317.75 ± 764.05	LDH	–	0.29(*)	0.52(*)	0.07	0.05	−0.08	0.53(*)
LDH	136.40 ± 88.87	TBC		–	0.40(*)	0.24	0.18	0.09	−0.14
PTX3	0.83 ± 0.23	SCC			–	0.25	0.38(*)	−0.14	−0.06
LF	6.38 ± 1.82	PTX3				–	0.67(*)	−0.18	−0.04
NAGase	0.20 ± 0.13	LF					–	−0.07	−0.12
MAA	35.90 ± 34.76	NAGase						–	0.53(*)
		MAA							–

Table 3
Correlation between lactate dehydrogenase (LDH) activity with respect to total bacterial count (TBC) and somatic cell count (SCC) measured in individual milk samples from goats belonging to flocks A and B. * $P \leq 0.05$.

		Flock A												
	TBC[1]							SCC						
	W1	W3	W5	W7	W9	W11	Total	W1	W3	W5	W7	W9	W11	Total
LDH	0.43*	−0.07	0.30	0.51*	0.42*	0.48*	0.59*	0.63*	−0.17	0.65*	0.49*	0.45*	0.64*	0.63*
TBC	–	–	–	–	–	–	–	0.58*	0.27	0.53*	0.43*	0.76*	0.54*	0.88*
		Flock B												
	TBC							SCC						
	W1	W3	W5	W7	W9	W11	Total	W1	W3	W5	W7	W9	W11	Total
LDH	0.58*	0.54*	0.41	0.17	0.75*	0.54*	0.82 *	0.69*	0.59*	0.22	0.11	0.77*	0.58*	0.78*
TBC	–	–	–	–	–	–	–	0.42	0.33	0.40	0.68*	0.92*	0.90*	0.93*

[1] W: week.

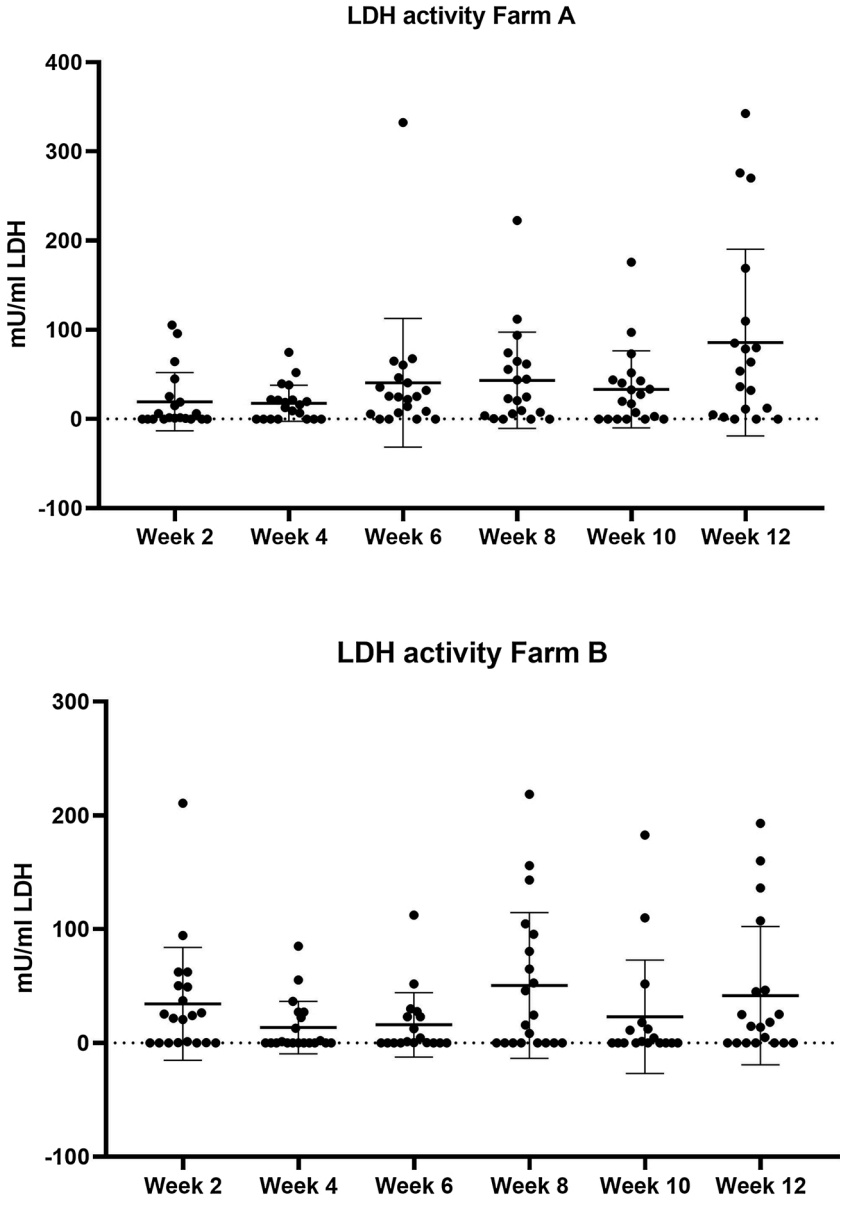

Fig. 1. Lactate dehydrogenase (LDH) activity over the three first months of lactation period in both analyzed flocks. Individual and mean values ± standard deviation are shown. No significant differences were observed.

this positive correlation between LDH and SCC has been previously described in cows and sheep (Chagunda et al., 2006; Friggens et al., 2007; Sani et al., 2018), it has not been previously observed in goats.

Finally, to investigate the potential of LDH activity as a biomarker of high-quality milk in goats, LDH activity was analyzed in a total of 581 milk samples including bulk and individual samples collected at different lactation periods, irrespective of age, breed, stage and number of lactations. The idea of this part of the study was to demonstrate

Table 4
Lactate dehydrogenase (LDH) concentration cut-off data for using it as a biomarker for the detection of high TBC and SCC levels in goat's milk with sensitivities and specificities based on established criteria. Sensitivity, specificity and area under the curve (AUC) were determined from the resulting receiver operating characteristic (ROC) analysis with 95 % confidence interval.

Criteria[1]	Total samples	Positive samples	Negative samples	Cut-off LDH Concentration	Sensitivity	Specificity	AUC	LR+[2]	LR-[3]
TBC-1	564	335 (59.40 %)	229 (40.60 %)	33.45	91.34	62.45	0.83	2.43	0.14
TBC-2	564	102 (18.09 %)	462 (81.91 %)	69.45	71.57	53.25	0.67	1.53	0.53
TBC-3	564	58 (10.28 %)	506 (89.72 %)	69.45	77.59	51.78	0.68	1.61	0.43
TBC-1+SCC-1	562	318 (56.58 %)	244 (43.42 %)	37.2	91.19	63.11	0.83	2.47	0.14
TBC-1+SCC-2	562	216 (38.43 %)	346 (61.57 %)	49.14	90.74	54.62	0.81	2.00	0.17
TBC-1+SCC-3	562	114 (20.28 %)	448 (79.72 %)	106.82	77.19	74.55	0.83	3.03	0.31
TBC-2+SCC-1	562	97 (17.26 %)	465 (82.74 %)	85.12	67.01	61.08	0.69	1.72	0.54
TBC-2+SCC-2	562	67 (11.92 %)	495 (88.08 %)	138.65	55.22	82.63	0.74	3.18	0.54
TBC-2+SCC-3	562	43 (7.65 %)	519 (92.35 %)	142.06	67.44	83.24	0.79	4.02	0.39
TBC-3+SCC-1	562	54 (9.61 %)	508 (90.39 %)	69.45	79.63	51.57	0.70	1.64	0.39
TBC-3+SCC-2	562	41 (7.30 %)	521 (92.70 %)	142.06	58.54	82.34	0.78	3.31	0.50
TBC-3+SCC-3	562	23 (4.09 %)	539 (95.91 %)	142.06	78.26	81.82	0.85	4.30	0.27

[1] TBC: Total bacterial count.
[2] LR+: positive likelihood ratio.
[3] LR-: negative likelihood ratio.

whether LDH can be used to detect high SCC or TBC values regardless of the origin of the sample. Although a dilution effect could be expected for SCC or TBC values in bulk milk samples, a proportional similar effect could be expected in those samples regarding LDH activity.

Analyzing LDH activity with respect to the different established SCC and TBC criteria, all the calculated ROC curves yielded a significant result. Furthermore, AUC ranged from 0.7 to > 0.87 which corresponded from acceptable to excellent discrimination power, respectively. This suggests that LDH activity could discriminate significantly between milk samples with high or low levels of SCC and TBC values. Other studies also suggested that LDH activity may be a marker of interest for the diagnosis of SCM in goats, and therefore, know the quality of the milk (Katsoulos et al., 2010; Stuhr et al., 2013). Stuhr et al. (2013) evidenced the role of LDH activity as a marker of interest to discriminate between infected and non-infected goat milk samples. Furthermore, Katsoulos et al. (2010) determined the use of LDH activity in milk serum to differentiate between bacteriology negative and positive goat milk samples.

The best sensitivity result (91.3 %) was obtained when using the TBC criterion as reference. When both parameters, SCC and TB, were combined the optimum cut-off for LDH activity was set at 37.2 mU/mL, showing a sensitivity of 91.2 % (TBC-1 + SCC-1 criterion) and a specificity of 83.2 % (TBC-2+SCC-3 criterion). These results showed the capacity of discrimination of LDH activity between samples with $\geq 5 \times 10^4$ bacteria/mL and $\geq 2 \times 10^6$ somatic cells/mL. Differences observed in the LDH activity by the authors above mentioned and ours could be associated with different analytical techniques and with variations due to biological samples, which emphasizes the importance of performing harmonization studies among different laboratories.

Our study highlights the possibility of measuring LDH activity in raw goat milk regardless the origin of the sample with good sensitivity and specificity when compared with SCC and TBC values. Our results open the possibility of using this parameter to determine the presence of high SCC and TBC in milk directly in the field developing a rapid and direct diagnostic tool. Different cut-off points could be applied according to the flock needs or preferences.

Data availability

Data will be made available on request.

Author contributions

Conceptualization, L.G.-G. and J.G-L.; methodology, A.G.-R., L.G.-G., F.C-T and B. B.-D.; validation, L.G.-G. and J.G-L.; formal analysis, A.G.-R., L.G.-G., F.C-T and B. B.-D.; investigation, A.G-R, L.G.-G., I.L., C.-T. and R. J.-A. ; resources, L.G.-G., I.L., C.-T., R.J.A. and J.G.-L.; data curation, L.G.-G and A.G.-R.; writing—original draft preparation, L.G.-G, A.G.-R.; writing—review and editing, I.L., L.G.-G, A.G.-R, C.T., J.G.-L. and R.J.A.; visualization, I.L., L.G.-G., A.G.-R. and J.G.-L.; supervision, L.G.-G. and J.G.-L.; project administration, C.T., I.L. and R.J.A; funding acquisition, I.L., C.T. and J.G.-L. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

None declared. The authors declare no conflicts of interest. None of the authors of this manuscript has a financial or personal relationship with other people or organizations that could inappropriately influence the content of this work.

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