JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Inflammatory biomarkers in milk for monitoring udder health status in Murciano-Granadina dairy goats
Running Title (within 10 words)	Biomarkers in milk to detect subclinical mastitis in dairy goats
Author	María Carmen Beltrán[1], Martín Rodríguez[1], Ernesto A. Gómez[2], Nemesio Fernández[1], Sebastià Balasch[3] and Cristòfol Peris[1]
Affiliation	[1]Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Camino de Vera, s/n, 46022 València, Spain [2]Centro de Investigación y Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias, 12400 Segorbe, Castellón, Spain [3]Departamento de Estadística e Investigación Operativa Aplicadas y Calidad, Universitat Politècnica de València, Camino de Vera, s/n, 46022 València, Spain
ORCID (for more information, please visit https://orcid.org)	María Carmen Beltrán (https://orcid.org/0000-0002-5335-2179) Martín Rodríguez (https://orcid.org/0000-0001-6079-9238) Ernesto A. Gómez (https://orcid.org/0000-0002-8774-420X) Nemesio Fernández (https://orcid.org/0000-0001-7150-2021) Sebastià Balasch (https://orcid.org/0000-0001-6632-2166) Cristòfol Peris (https://orcid.org/0000-0003-3529-1269)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment and supplies). Include name and number of grant if available.	This study was funded by project RTA2017-00049-C02-02 (Agencia Estatal de Investigación).
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Gómez EA, Fernández N, Beltrán MC, Peris C. Data curation: Rodríguez M, Beltrán MC, Peris C. Formal analysis: Balasch S, Beltrán MC, Peris C. Methodology: Balasch S, Beltrán MC, Peris C. Software: Balasch S, Beltrán MC, Fernández N. Validation: Rodríguez M, Gómez EA, Beltrán MC, Peris C. Investigation: Rodríguez M, Gómez EA, Fernández N, Beltrán MC, Peris C. Writing - original draft: Beltrán MC, Peris C. Writing - review & editing: Rodríguez M, Gómez EA, Fernández N, Balasch S, Beltrán MC, Peris C.
Ethics approval and consent to participate	Not applicable.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading and reprints)	Fill in information in each box below
First name, middle initial, last name	María Carmen Beltrán
Email address – this is where your proofs will be sent	mbeltran@dca.upv.es
Secondary Email address	
Address	Camino de Vera, s/n, 46022 València, Spain
Cell phone number	
Office phone number	+34 96 3877727
Fax number	+34 96 3877439

Inflammatory biomarkers in milk for monitoring udder health status in Murciano-Granadina dairy goats

María Carmen Beltrán[1], Martín Rodríguez[1], Ernesto A. Gómez[2], Nemesio Fernández[1], Sebastià Balasch[3] and Cristòfol Peris[1]

[1]*Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Camino de Vera, s/n, 46022 Valencia, Spain*

[2]*Centro de Investigación y Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias, 12400 Segorbe, Castellón, Spain*

[3]*Departamento de Estadística e Investigación Operativa Aplicadas y Calidad, Universitat Politècnica de València, Camino de Vera, s/n, 46022 Valencia, Spain*

María Carmen Beltrán (e-mail: mbeltran@dca.upv.es; ORCID: https://orcid.org/0000-0002-5335-2179)

Martín Rodríguez (e-mail: mrodriguez@dca.upv.es; ORCID: https://orcid.org/0000-0001-6079-9238)

Ernesto A. Gómez (e-mail: gomez_em@gva.es;ORCID: https://orcid.org/0000-0002-8774-420X)

Nemesio Fernández (e-mail: nfernand@dca.upv.es; ORCID: https://orcid.org/0000-0001-7150-2021)

Sebastià Balasch (e-mail: sbalasch@eio.upv.es; ORCID: https://orcid.org/0000-0001-6632-2166)

Cristòfol Peris (e-mail: cperis@dca.upv.es; ORCID: https://orcid.org/0000-0003-3529-1269)

Corresponding author:

María Carmen Beltrán, Universitat Politècnica de València; Camino de Vera, s/n, 46022 València, Spain;

Phone: +34 963877727, Fax: +34-96-3879439; E-mail: mbeltran@dca.upv.es

Abstract

The aim of this study was to investigate the potential use of some inflammatory biomarkers (somatic cell count, milk amyloid A and lactoferrin) as a tool to monitor the udder health status in dairy goats. Individual milk samples from 582 Murciano-Granadina goats belonging to six commercial herds were used in this study: half-udder milk samples for subclinical mastitis diagnosis and composite milk samples to determine gross composition, pH, somatic cell count (SCC), milk amyloid A and lactoferrin concentrations. Factors affecting the udder health traits analysed in milk (farm, parity, days in milk and subclinical mastitis) were statistically analysed using a General Lineal Model procedure. A logistic regression analysis was also applied to develop predictive models to detect subclinical mastitis in dairy goats. Results indicate that milk from goats with subclinical intramammary infections (n= 216) showed higher log SCC (6.31±0.05 vs 5.95±0.04 cells/ml; $p< 0.001$) and milk amyloid A (40.93±3.56 vs 31.62±2.83 μg/ml; $p< 0.05$) concentrations than that from uninfected animals. Higher log SCC ($p< 0.05$) and lactoferrin ($p< 0.05$) concentrations were also detected in milk from goats with subclinical mastitis caused by major pathogens such as *Staphylococcus aureus* and gram-negative bacteria. However, the non-infectious factors considered significantly affected the concentrations of the three inflammatory biomarkers in milk, compromising their suitability to be used as indicators of mastitis. Thus, the logistic regression model based on the complementary use of the SCC, milk amyloid A and lactoferrin for subclinical mastitis diagnosis presented a predictive performance too low to be used for practical purposes (area under ROC curve= 0.673±0.046; performance= 64.27 %). However, the use of the lactoferrin and lactose concentrations in milk could be a very sensitive tool to detect intramammary infections caused by major pathogens with higher inflammatory responses (area under ROC curve= 0.941±0.028; performance= 87.16 %). Therefore, further research is needed to investigate other indicators present in milk in order to establish a more accurate and effective methodology to detect subclinical mastitis in dairy goats.

Keywords: goat milk, subclinical mastitis, lactoferrin, milk amyloid A, somatic cell count

Introduction

In dairy goats, subclinical mastitis has negative implications on milk production and quality, leading to serious economic losses due to its high incidence and difficult detection [1, 2, 3]. Bacteriological examination of aseptically collected milk is considered the gold standard for the diagnosis of intramammary infections (IMI). However, in practice, it is not widely applied because it is time-consuming and expensive. Thus, the udder health status is traditionally monitored by visual inspection of the udder and its secretions, the SCC being used as a complementary diagnostic tool.

The use of SCC for the detection of subclinical mastitis could be accurate and very useful for cows [4, 5] and even ewes [6, 7]. However, its application in dairy goats is more complex due to the high influence of non-infectious factors such as lactation stage, parity or oestrus, among others on this parameter [2, 8, 9], leading to false positive outcomes in the mastitis diagnosis. The increases in the SCC caused by these factors are much more pronounced in dairy goats than in cows and sheep, reaching values consistent with intramammary infections even in the absence of the disease, which greatly complicates the use of SCC as the sole diagnostic tool for subclinical mastitis diagnosis [2, 10]. Therefore, a simple, practical and reliable method for the detection of subclinical mastitis in dairy goats is necessary.

During mammary gland inflammation, numerous antibacterial and immune defence proteins, including acute phase proteins (APP), are released into milk, which could potentially serve as mastitis biomarkers. Their application as alternative or complementary diagnostic tools has been the subject of several studies in recent decades, with milk amyloid A (MAA) and lactoferrin (LF) being among the non-enzymatic proteins most investigated in relation to mastitis [11, 12]. However, this research has mainly focused on dairy cows, and the availability of related studies on small ruminants, especially goats, is very limited.

Milk amyloid A (MAA) is an APP produced extrahepatically by the epithelial cells of the mammary gland [13, 14] and is considered a highly specific biomarker for subclinical mastitis identification because its concentration in milk significantly increases following intramammary infection [15, 16, 17, 18] in response to the udder inflammation. On the other hand, lactoferrin (LF) is an iron-binding glycoprotein synthesized by specific granules in polymorphonuclear leukocytes and glandular epithelial cells, considered a major part of the non-specific disease resistance complex in the mammary gland and other epithelial tissues [18, 19, 20]. Although LF is not an APP, its concentration in milk increases notably during the inflammatory response and is considered by several authors as a potential biomarker of mastitis [21, 22, 23].

In recent years, the relationship between the milk concentration of inflammatory biomarkers such as MAA or LF and subclinical IMI has been studied in dairy goats [19, 20, 24, 25]. Although such results confirm that milk from animals with subclinical mastitis shows higher MAA and LF concentrations than milk from uninfected goats [19, 24], they also highlight that other non-infectious factors such as the number and lactation status seem to affect the concentration of these potential biomarkers [20, 25], compromising their suitability as a tool for monitoring the udder health status in dairy goats. Thus, the scarcity of works and the inconclusive nature of the results obtained suggest that further research is needed. Therefore, the aim of this study was to evaluate infectious and non-infectious factors affecting MAA and LF concentration in goat's milk in order to assess the potential use of these proteins to improve subclinical mastitis diagnosis traditionally based on the SCC.

Material and Methods

Goats and milk samples

A total of 582 Murciano-Granadina goats from six commercial herds belonging to the Murciano-Granadina Goat Breeders Association of the Valencian Community (AMURVAL) were used in this study. In all these farms, the AMURVAL technical staff regularly carried out, over the last 5 years: 1) the official milk recording every 45 days, as a tool for breeding, nutrition and management decisions; 2) specific subclinical mastitis controls, taking half-udder milk samples from animals with high SCC and/or unexplained production losses.

In order to have variability in udder health status, the goats used for this study were selected as follows. Each farm was sampled three times a year, taking milk samples from 30-35 goats each time (90-100 different goats from each farm). These animals were pre-selected based on the information available from the previous official milk recording. For each sampling, the goats were first classified into three groups according to the SCC (low: $< 500 \times 10^3$ cells/ml, medium: 501-1,500 $\times 10^3$ cells/ml and high: > 1,500 cells/ml). Then, 10 to 12 animals were randomly selected from each of these groups (30-35 goats in total), keeping parity and lactation status balanced between groups. The 30-35 selected goats were then specifically sampled in the next official milk recording. Although each farm was sampled three times, the goats were different each time (each animal was only sampled once).

All milk samples from the goats selected for our study were collected by the AMURVAL technical staff during the official milk recording. Two types of samples were taken from each animal: 1) First, before milking, a milk sample was collected aseptically from each mammary gland to assess the presence of intramammary infection according to the National Mastitis Council standards [26]. Before sampling, the goats underwent a clinical examination of the udder to detect abnormalities that could suggest clinical mastitis. Then, the teat tips were

cleaned with a cotton pad soaked in 70% ethanol and, after removing three or four squirts, a 5 ml milk sample was collected in a sterile tube from each mammary gland (two samples per goat). Milk samples from udder halves were stored at 4°C and bacteriological examination for the diagnosis of subclinical mastitis was performed within 24 hours after sampling.

2) After machine milking, individual milk yield (MY) was recorded and a composite milk sample (500 ml) was taken for each goat directly from the volumetric milk meter. This sample was analysed to determine the chemical composition and concentration of the different inflammatory biomarkers considered.

Goat's milk analysis

Bacteriological examination of the udder halves milk samples was performed according to the test procedures described by the National Mastitis Council [26]. Briefly, 10 µl of milk were plated onto 5 % sheep blood agar (Columbia agar plates, Scharlab, Barcelona, Spain) and then incubated aerobically at 37ºC for up to seven days. Isolated colonies were identified by gross morphology, haemolysis and gram stain test. Coagulase test (Scharlab) and latex agglutination test (Microgen STAPH latex test, Microgen Bioproducts, Camberley, UK) were used for *Staphylococcus aureus* identification. Positive cultures were considered when more than five colonies were isolated, with the presence of more than two species leading to invalid culture. Cultures with absence of growth after seven days of incubation were considered negative. Finally, animals were classified according to the bacteriological analysis results. Thus, goats not showing clinical signs but having a positive bacteriological examination (unilateral or bilateral) were classified as subclinical or infected goats, and goats having no clinical signs and negative culture were classed as non-infected or healthy goats.

Composite goat milk samples were analysed at the Interprofessional Dairy Laboratory of the Valencian Community (LILCOVAL) for gross composition (Milkoscan FT 6000, Foss, HillerØd, Denmark), SCC (Fossomatic 5000, Foss) and pH (pH-meter Basic 20, Crison, Barcelona, Spain). The MAA concentration was analysed using a commercial sandwich ELISA method (milk amyloid A Mast ID ELISA, Tridelta Development Ltd, Maynooth, County Kildare, Ireland) according to the manufacturer's instructions. LF concentration was determined at the Institute for Animal Science, Physiology and Hygiene Unit of the University of Bonn (Germany), using a competitive ELISA method developed in the same laboratory [20].

Statistical analyses

Data were analysed using Statgraphics Centurion XIX software (Statpoint Technologies, Warrenton, VA, USA). A General Linear Model was employed to investigate the random effect of the farm (1 to 6) and the

fixed effects of parity (1: 1-2, 2: 3-4; 3: ≥ 5), DIM (1: < 90 days, 2: 90-190 days, 3: > 190 days) and the udder health status (infected or non-infected) on the quality parameters analysed in milk. The logarithm of the SCC (log SCC) was used to normalize the SCC distribution. The corresponding mean comparisons were performed using the LSD test.

In order to develop predictive models of dichotomous outcomes to detect subclinical IMI from the udder health traits analysed in milk (pH, lactose, log SCC, milk Amyloid A and LF), a logistic regression analysis (stepwise option) was applied using two different approaches. First, the animals were classified solely based on the results of the bacteriological examination of the half-udder milk samples. For a more accurate detection, a second analysis was performed in which subclinical mastitis status was confirmed by a positive culture and an increased SCC (≥ 1,500 x 10^3 cells/ml), while goats having negative cultures and low SCC (≤ 500 x 10^3 cell/ml) were considered uninfected. Receiver Operating Characteristic (ROC) curve analysis using Minitab statistical software (Minitab, LLC, State College, PA, USA) was applied to evaluate the predictive performance of the logistic regression models obtained to correctly classify infected versus non-infected goats.

Results

The targeted sampling provided goat's milk samples with high variability for all the parameters under study. Table 1 summarizes the descriptive statistics and Pearson product-moment correlations between milk quality traits (MY, fat, protein and total solids) and udder health traits (pH, lactose, log SCC, MAA and LF) in Murciano-Granadina dairy goats.

Overall, the correlations found between log SCC, MAA and LF were relatively low, with r-values ranging from 0.12 to 0.47, although they were statistically significant ($p< 0.01$). Significant negative correlations were also detected between the three inflammatory biomarkers and MY ($p<0.001$), as well as between log SCC and LF with lactose content ($p< 0.001$), respectively.

Effect of subclinical mastitis on the inflammatory biomarker concentrations in Murciano-Granadina goat's milk

A total of 1,154 half-udder milk samples were analysed for subclinical mastitis diagnosis, since ten of the 582 selected goats had only one functional mammary gland. Bacteriological analysis revealed that 286 mammary glands (24.78 %) were infected. Regarding the mastitis aetiology, positive cultures were mostly pure (98.95 %), in which a single species of microorganism was isolated, the most prevalent aetiological agents being coagulase-negative staphylococci (CNS, 54.19 %) and *Corynebacterium* spp. (31.12 %). Other microorganisms

were also detected, although to a lesser extent, such as *S. aureus* (6.30 %), gram-negative bacteria (4.19 %) and *Streptococcus* spp. (3.15 %).

According to the bacteriological analysis results, a total of 216 goats (37.11 %) were diagnosed with subclinical IMI, most of them (65.74 %) unilateral. The percentage of goats with subclinical mastitis on the different farms ranged from 22.92 to 61.39 %. Differences between farms in terms of microbial agents involved in the diagnosed subclinical IMI were also detected (Figure 1).

Table 2 summarizes the milk quality and udder health status traits analysed in the composite milk samples. Although no significant differences were detected for MY ($p > 0.05$), subclinical mastitis significantly affected milk quality characteristics. Thus, milk from goats diagnosed with subclinical IMI had higher values for log SCC ($p < 0.001$), MAA ($p < 0.05$) and pH ($p < 0.05$) than those obtained in milk from uninfected goats (Table 2), as well as a lower lactose concentration ($p < 0.01$). Lower LF concentrations were also detected in uninfected goats' milk, although differences did not become statistically significant ($p > 0.05$).

The effect of the microbial agent involved in the subclinical IMI on milk characteristics was also investigated. As shown in Table 3, higher log SCC and increased LF concentrations were detected in milk from goats infected with *S. aureus* and gram-negative bacteria ($p < 0.05$) than in milk from animals infected with CNS. However, the aetiology of the IMI did not significantly affect MAA concentration ($p > 0.05$).

Non-infectious factors affecting the inflammatory biomarkers (SCC, MAA, and LF) concentrations in Murciano-Granadina goat's milk.

As shown in Table 4, the milk concentration of the biomarkers considered was significantly affected by parity of the animals. In general, it was observed that log SCC increased progressively with the lactation number ($p < 0.001$). Significant differences were also found in milk from younger goats (first and second parity), which showed higher lactose content ($p < 0.05$) and lower values for LF ($p < 0.05$) concentration.

On the other hand, the DIM factor significantly affected all the variables analysed in milk (Table 5). Regarding the three inflammatory biomarkers, an increase in the log SCC values was detected after 90 days of lactation ($p < 0.05$), while MAA and LF concentrations increased significantly ($p < 0.05$) in milk from late lactation goats (> 190 days). As expected, lower MY ($p < 0.001$) and reduced lactose content ($p < 0.001$) were detected as lactation progressed.

Suitability of the inflammatory biomarkers to predict subclinical mastitis in Murciano-Granadina dairy goats

Logistic regression analysis was applied to develop predictive models able to detect subclinical mastitis in dairy goats from udder health traits analysed in milk (Table 6). In the first approach, the results indicated that log SCC and MAA were the best predictive variables. However, although the complementary use of the MAA and LF concentrations improved the predictive ability of the log SCC, the performance of this regression model to correctly classify the animals (infected versus uninfected) was relatively low (64.27 %) to be used for practical purposes (Figure 2a). In the second approach, when log SCC was included as a classificatory variable for subclinical IMI diagnosis, the best predictive model included LF and lactose concentrations (Table 6) showing a higher area under the ROC curve (Figure 2b), and therefore a greater ability to detect infected goats with higher inflammatory responses (predictive performance= 87.16 %).

Discussion

The subclinical form of mastitis compromises udder health status, affecting milk production and/or milk quality, leading to significant economic losses for farmers and a potential risk to consumer health [27]. The lack of clinical symptoms and the low specificity of log SCC as a diagnostic tool in dairy goats leads to subclinical mastitis, in general, being poorly detected. Thus, most IMIs persist throughout lactation, even during successive lactations, with infected animals acting as reservoirs that spread infection [28], making the development of rapid and sensitive tools for subclinical mastitis diagnosis in dairy goats necessary.

With the aim of improving mastitis detection in dairy goats, the alternative or complementary potential use of the MAA and LF was investigated in this study. Results herein supported previous studies conducted on dairy goats indicating that subclinical IMI increased the concentrations of log SCC, MAA and LF in milk [18, 20, 24].

However, as indicated by other authors in dairy cows [17, 23, 29, 30], different inflammatory reactions of the mammary gland were found depending on the microorganism involved in the infectious process. Thus, higher log SCC and LF concentrations were detected in milk from goats infected with major pathogens such as *S. aureus* than in milk from goats infected by CNS, microorganisms usually leading to mild clinical or subclinical mastitis with minor stimulation of the immune system [23].

As expected, CNS were the most frequently isolated microorganisms in the udder halves milk samples analysed. It is known that CNS appear to be the most prevalent pathogens and account for 58-93% of the bacteria causing IMI in dairy goats across various countries and geographical zones [2, 31]. Although CNS are usually considered minor pathogens as they do not usually generate significant alterations in the udder or in the milk, it should be noted that they tend to become chronic in herds, progressively damaging the

glandular tissue and impairing milk quality [2]. Results herein agree with those observed by other authors in dairy goats and could explain the fact that goats with subclinical IMI caused by CNS showed MAA and LF concentration values similar to those found in milk from non-infected goats.

Another aspect to consider regarding the aetiology of IMI is that, although APPs such as serum amyloid A (SAA) increase in serum concentrations during inflammatory response, the duration of this elevation could vary between species and diseases [14, 32, 33]. Gram-positive bacteria are well known for causing chronic mastitis instead of acute mastitis and require more hours after infection to increase cytokine and APP concentrations [23]. However, the higher concentration persists for a longer period when compared to gram-negative bacteria. Thus, the unknown period elapsed between onset of the infection and the milk sampling could explain the similar MAA concentrations obtained in this study in milk from SM goats, regardless of the microorganism involved in the infectious process.

In addition to the aetiological agent causing mastitis, it should be noted that, as occurring for the SCC, the inflammatory biomarkers MAA and LF were also affected by non-infectious factors such as DIM, PARITY and FARM, which undoubtedly compromises their use as predictors of subclinical IMI in dairy goats. Thus, some authors [25] observed that MAA increases physiologically as lactation progresses, as does SCC, even in absence of infection, and concluded that this APP was not suitable for the diagnosis of subclinical mastitis in dairy goats. Increased LF concentrations were detected by the same authors in late lactation *S. aureus* infected goat milk, but also in late lactation uninfected goat milk, although to a slightly lower extent. On the other hand, higher values of LF at the colostral period (first week postpartum) and a significant increase in the concentration of this inflammatory biomarker in milk from the ninth month of lactation in dairy goats were detected in a previous study [20]. It should be noted that LF is a glycoprotein of the immune defence system with several biological functions including inhibition of microorganisms, transport of iron, immune modulation and enhancing cell differentiation and regulation of several other functions [12, 19, 21]. Thus, increased LF concentrations during the colostral period and towards the end of lactation is more likely to be due to physiological alterations than to innate responses to pathogen invasion [20]. As indicated by other authors in dairy cows [29], the LF concentration was also affected by the age of the animals, with increased values in goats with three or more lactations.

Therefore, given the low specificity of such inflammatory biomarkers, the performance of the predictive logistic regression model based on the log SCC, MAA and LF was unsuitable for practical purposes, especially in late lactation goats. On the other hand, the higher area under the ROC curve in the predictive model based on the

use of lactose and LF (Figure 2b) suggests a higher probability of correctly diagnosing infected goats with a great inflammatory response, as occurs with major pathogens such as *S. aureus* or gram-negative bacteria. However, when regular milk sampling was performed to monitor the udder health status, such as the milk recording usually kept monthly, the time elapsed from the acute phase of prevalent subclinical IMI is unknown, and in some cases the MAA and LF concentrations might have been normalized in milk despite the survival of the pathogen inside the mammary gland. Thus, the potential use of these biomarkers could be reduced to the detection of subclinical mastitis leading to strong inflammatory responses, less frequent in dairy goat herds, and to the coincidence in time of the acute phase of the IMI with sampling.

Conclusions

There are many factors affecting the milk concentration of the SCC, MAA and LF in goat's milk. Although milk from goats with subclinical IMI had higher SCC, MAA and LF concentrations than milk from uninfected animals, with aetiological agents significantly affecting inflammatory responses, these biomarkers were also affected by factors other than mastitis, which compromises their suitability for use as a tool to monitor the udder health status in dairy goats. For practical purposes, the usefulness of these biomarkers is quite limited and, therefore, the alternative methods for detection of SM in dairy goats remain open for investigation.

Acknowledgements

This study was funded by project RTA2017-00049-C02-02 (Agencia Estatal de Investigación). The authors acknowledge the Murciano-Granadina Goat Breeders Association of the Valencian Community (AMURVAL) for their support in collecting the milk samples required for this study.

Author's contributions

Conceptualization: Gómez EA, Fernández N, Beltrán MC, Peris C.

Data curation: Rodríguez M, Beltrán MC, Peris C.

Formal analysis: Balasch S, Beltrán MC, Peris C.

Methodology: Balasch S, Beltrán MC, Peris C.

Software: Balasch S, Beltrán MC, Fernández N.

Validation: Rodríguez M, Gómez EA, Beltrán MC, Peris C.

Investigation: Rodríguez M, Gómez EA, Fernández N, Beltrán MC, Peris C.

Writing - original draft: Beltrán MC, Peris C.

Writing - review & editing: Rodríguez M, Gómez EA, Fernández N, Balasch S, Beltrán MC, Peris C.

292 **References**

293 1. Leitner G, Merin U, Silanikove N. Changes in milk composition as affected by subclinical mastitis in
294 goats. *J. Dairy Sci.* 2004, 87, 1719–1726. https://doi.org/10.3168/jds.S0022-0302(04)73325-1
295
296 2. Contreras A, Sierra D, Sánchez A, Corrales JC, Marco JC, Paape MJ, Gonzalo C. Mastitis in small
297 ruminants. *Small Rumin. Res*. 2007, 68, 145–153. https://doi.org/10.1016/j.smallrumres.2006.09.011
298
299 3. Gelasakis AI, Angelidis AS, Giannakou R, Filioussis G, Kalamaki MS, Arsenos G. Bacterial subclinical
300 mastitis and its effect on milk yield in low-input dairy goat herds. *J. Dairy Sci*. 2016, 9(5), 3698-3708.
301 https://doi.org/10.3168/jds.2015-10694
302
303 4. Schukken YH, Wilson DJ, Welcome F, Garrison-Tinofsky L, Gonzales RN. Monitoring udder health
304 and milk quality using somatic cell counts. *Vet. Res*. 2003, 34, 579–596.
305 https://doi.org/10.1051/vetres:2003028
306
307 5. Dalen G, Rachah A, Nørstebø H, Schukken YH, Reksen O. The detection of intramammary infections
308 using online somatic cell counts. *J. Dairy Sci*. 2018, 102, 5419-5429. https://doi.org/10.3168/jds.2018-
309 15295
310
311 6. Gonzalo C, Carriedo JA, Blanco MA, Beneitez E, Juárez MT, De La Fuente LF, San Primitivo F. Factors
312 of Variation Influencing Bulk Tank Somatic Cell Count in Dairy Sheep. *J. Dairy Sci.* 2005, 88, 969-974.
313 https://doi.org/10.3168/jds.S0022-0302(05)72764-8
314
315 7. Paape MJ, Wiggans GR., Bannerman DD, Thomas DL, Sanders AH, Contreras A, Moroni P, Miller RH.
316 Monitoring goat and sheep milk somatic cell counts. *Small Rumin. Res*. 2007, 68, 114–125.
317 https://doi.org/10.1016/j.smallrumres.2006.09.014
318
319 8. Bergonier D, De Cremoux R, Ruppc R, Lagriffould G, Berthelot X. Mastitis of dairy small ruminants
320 Vet. Res. 2003, 34, 689–716. https://doi.org/10.1051/vetres:2003030

9. Mehdid A, Díaz JR, Martí A, Vidal G, Peris C. Effect of estrus synchronization on daily somatic cell count variation in goats according to lactation number and udder health status. *J. Dairy Sci*. 2013, 96, 4368-437. https://doi.org/10.3168/jds.2012-6393

10. Kaskous S, Farschtschi S, Pfaffl MW. Physiological aspects of milk somatic cell count in small ruminants- A review. *Dairy*. 2023, 4, 26-42. https://doi.org/10.3390/dairy4010002

11. Viguier C, Arora S, Gilmartin N, Welbeck K, O'Kennedy R. Mastitis detection: current trends and future perspectives. *Trends Biotechnol*. 2009, 27, 486–493. https://doi.org/10.1016/j.tibtech.2009.05.004

12. Giagu A, Penati M, Traini S, Dore S, Addis MF. Milk proteins as mastitis markers in dairy ruminants–a systematic review. *Vet. Res. Commun.* 2022, 46, 329-351. https://doi.org/10.1007/s11259-022-09901-y

13. O'Mahony AM, Healy M, Harte D, Walshe KG, Torgerson PR, Doherty ML. Milk amyloid A: Correlation with cellular indices of mammary inflammation in cows with normal and raised serum amyloid A. *Res. Vet. Sci.* 2006, 80, 155-161. https://doi.org/10.1016/j.rvsc.2005.05.005

14. Ceciliani F, Ceron JJ, Eckersall PD, Sauerwein H. Acute phase proteins in ruminants. *J. Proteomics*. 2012, 75, 4207-4231. https://doi.org/10.1016/j.jprot.2012.04.004

15. Safi S, Khoshvaghti A, Jafarzadeh SR, Bolourchi M, Nowrouzian I. Acute phase proteins in the diagnosis of bovine subclinical mastitis. *Vet. Clin. Pathol.* 2009, 38, 471–476. https://doi.org/10.1111/j.1939-165x.2009.00156.x

16. Miglio A, Moscati L, Fruganti G, Pela M, Scoccia E, Valiani A, Maresca C. Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes. *J. Dairy Res.* 2013, 80, 496–502. https://doi.org/10.1017/s0022029913000484

17. Bochniarz M, Szczubiał M, Brodzki P, Krakowski L, Dąbrowsk R. Serum amyloid A as a marker of coẁ s mastitis caused by Streptococcus sp. *Com. Immunol. Microbiol. Infect. Dis.* 2020, 72, 101498. https://doi.org/10.1016/j.cimid.2020.101498

18. Matuozzo M, Spagnuolo MS, Hussein HA, Gomaa AM, Scaloni A, D'Ambrosio C. Novel biomarkers of mastitis in goat milk revealed by MALDI-TOF-MS-based peptide profiling. *Biology*. 2020, 9, 193, https://doi.org/10.3390/biology9080193

19. Chen PW, Chen WC, Mao FC. Increase of lactoferrin concentration in mastitic goat milk. *J. Vet. Med. Sci.* 2004, 66, 4, 345-350. https://doi.org/10.1292/jvms.66.345

20. Hiss S, Meyer T, Sauerwein H. Lactoferrin concentrations in goat milk throughout lactation. *Small Rumin. Res.* 2008, 80, 87-90. https://doi.org/10.1016/j.smallrumres.2008.07.027

21. Cheng JB, Wang JQ, Bu DP, Liu L, Zhang CG, Wei HY, Zhou LY, Wang JZ. Factors affecting the lactoferrin concentration in bovine milk. *J. Dairy Sci*. 2008, 91, 970-976. https://doi.org/10.3168/jds.2007-0689

22. Shimazaki KI, Kawai K. Advances in lactoferrin research concerning bovine mastitis. *Biochem. Cell Biol.* 2017, 95, 69–75. https://doi.org/10.1139/bcb-2016-0044

23. Dalanezi FM, Schmidt EMS, Joaquim SF, Guimarães FF, Guerra ST, Lopes BC, Cerri, RLA, Chadwick C, Langoni H. Concentrations of Acute-Phase Proteins in milk from cows with clinical mastitis caused by different pathogens. *Pathogens*. 2020, 9, 706. https://doi.org/10.3390/pathogens9090706

24. Sequeira, A.; Coelho, A. C.; Abreu, R.; Alegría, N.; Mendoça, Á.; Quintas, H. 2014. Diagnóstico de mastites subclínicas caprinas pelo método do doseamento de Amilóide A do leite. In OVICAPRI-Simpósio de Ovinos e Caprinos. Vila Real. Available in http://hdl.handle.net/10198/17427

25. Pisanu S, Cacciotto C, Pagnozzi D, Uzzau S, Pollera C, Penati M, Bronzo V, Addis MF. Impact of *Staphylococcus aureus* infection on the late lactation goat milk proteome: New perspectives for monitoring and understanding mastitis in dairy goats. *J. Proteomics.* 2020, 221, 103763. https://doi.org/10.1016/j.jprot.2020.103763

26. Firdaus I. Laboratory Handbook on Bovine Mastitis. National Mastitis Council (NMC) 2017. Available at https://www.academia.edu/50651208/Laboratory_Handbook_on_Bovine_Mastitis

27. Barrón-Bravo OG, Gutiérrez-Chávez AJ, Ángel-Sahagún CA, Montaldo HH, Shepard L, Valencia-Posadas M. Losses in milk yield, fat and protein contents according to different levels of somatic cell count in dairy goats. *Small Rumin. Res.* 2013, 113, 421–431. https://doi.org/10.1016/j.smallrumres.2013.04.003

28. Margatho G, Quintas H, Rodríguez-Estévez V, Simóes J. Infectious and non-infectious factors affecting somatic cell count and new diagnostic approaches of intramammary infections in dairy goats: a review. *Curr. Chin. Sci.* 2021, 1, 4, 460-476. https://doi.org/10.2174/2210298101666210402123549

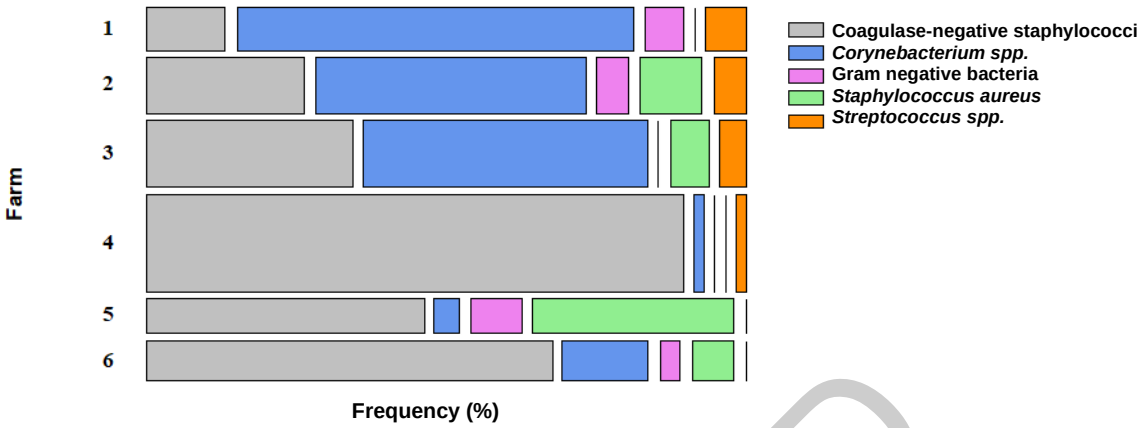
29. Hagiwara SI, Kazuhiro K, Anri A, Nagahata H. Lactoferrin concentrations in milk from normal and subclinical mastitic cows. *JVMS*. 2003, 65, 3, 319-323. https://doi.org/10.1292/jvms.65.319

30. Pyörälä S, Hovinen M, Simojoki H, Fitzpatrick J, Eckersall PD, Orro T. Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens. *Vet. Rec.* 2011, 168, 535–542. https://doi.org/10.1136/vr.d1120

31. Silanikove N, Merin U, Leitner G. On effects of subclinical mastitis and stage of lactation on milk quality in goats. *Small Rumin. Res.* 2014, 122 (1-3), 76-82. https://doi.org/10.1016/j.smallrumres.2014.07.018

406 32. Simojoki H, Salomäki T, Taponen S, Livanainen A, Pyörälä S. Innate immune response in
407 experimentally induced bovine intramammary infection with *Staphylococcus simulans* and *S. epidermidis.*
408 *Vet. Res.* 2011, 42, 49. https://doi.org/10.1186/1297-9716-42-49
409
410 33. Sadiq MB, Mansor R, Syed-Hussain SS, Saharee AA, Zakaria Z, Syahirah AA, Bousnane ZA, Jaezah
411 Adlina ZA, Salleh A, Wan Mohd Sukri WI, Mustaffa-Kamal F, Ramanoon SZ. Clinical observation, acute
412 phase protein levels, and histopathological changes of mammary gland in experimentally infected goats
413 with *Staphylococcus aureus Comp. Clin. Path*. 2019, 28, 1069-1075. https://doi.org/10.1007/s00580-019-
414 02926-x
415

416 **Tables and Figures**



417

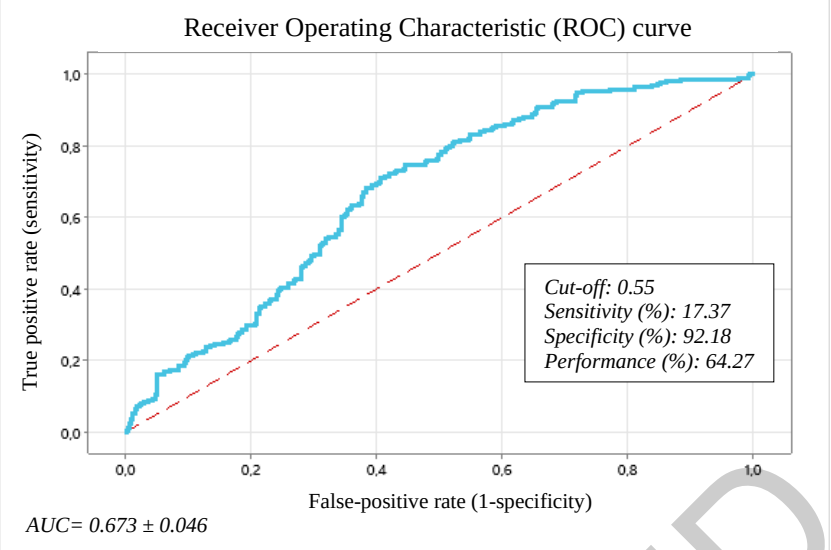
418 **Figure 1**. Mosaic plot for farm according to the aetiological agents causing subclinical mastitis in the

419 different commercial dairy goat herds.

420

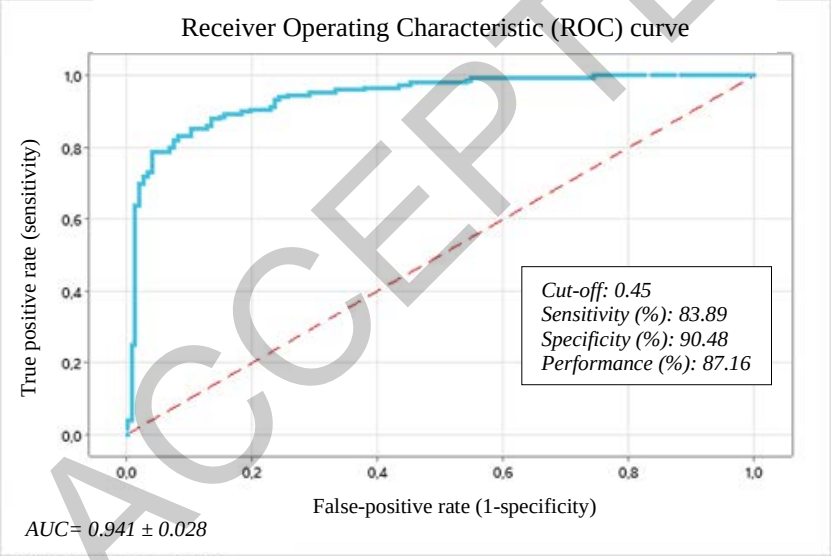
421

422 a)



423

424 b)



425

426 **Figure 2**. Receiver Operating Characteristic (ROC) curves for subclinical mastitis detection of a) the predictive
427 model using log SCC, MAA and LF concentrations in milk (Approach 1), and b) the predictive model based
428 on lactose and LF concentrations (Approach 2); AUC: area under the ROC curve ($IC_{95\%}$); Performance:
429 predictive ability of the logistic model to correctly classify the animals.

430

Table 1. Descriptive statistics and Pearson product-moment correlations between milk quality and udder health status traits in Murciano-Granadina dairy goats (n= 582)

	Milk quality traits				Udder health status traits				
	MY (litres/milking)	Fat (%)	Protein (%)	Total solids (%)	Lactose (%)	pH	Log SCC (cells/ml)	MAA (µg/ml)	LF (µg/ml)
Mean	1.52	5.44	3.89	14.73	4.59	6.67	6.05	34.78	27.03
SD	0.69	1.22	0.63	1.73	0.33	0.11	0.63	44.45	35.15
Correlations									
MY		-0.32 (***)	-0.43 (***)	-0.36 (***)	0.17 (***)	-0.16 (***)	-0.33 (***)	-0.24 (***)	-0.33 (***)
Fat			0.60 (***)	0.95 (***)	-0.16 (***)	-0.05 (ns)	0.22 (***)	0.27 (***)	0.19 (***)
Protein				0.78 (***)	-0.23 (***)	0.12 (**)	0.27 (***)	0.45 (***)	0.47 (***)
Total solids					-0.01 (ns)	-0.01 (ns)	0.18 (***)	0.36 (***)	0.26 (***)
Lactose						-0.19 (***)	-0.46 (***)	-0.06 (ns)	-0.26 (***)
pH							0.10 (*)	0.19 (***)	0.12 (**)
Log SCC								0.13 (**)	0.47 (***)
MAA									0.39 (***)

MY: Milk Yield; Log SCC: Logarithm of the Somatic Cell Count; MAA: Milk Amyloid A; LF: Lactoferrin; significant differences: p< 0.05 (*), p< 0.01 (**), p< 0.001 (***), p> 0.05 (ns)

Table 2. Quality parameters (mean ± SE) of the Murciano-Granadina goat's milk samples according to the udder health status

	Uninfected goats (n= 366)	Infected goats* (n= 216)	*p-value*
MY (litres/milking)	1.53 ± 0.04	1.43 ± 0.05	0.131
Fat (%)	5.54 ±0.08	5.49 ± 0.10	0.720
Protein (%)	3.90 ± 0.04	3.93 ± 0.05	0.662
Lactose (%)	4.62 ± 0.02	4.52 ± 0.03	**0.004**
Total solids (%)	14.88 ± 0.11	14.76 ± 0.14	0.503
pH	6.66 ± 0.01	6.68 ± 0.01	**0.030**
Log SCC (cells/ml)	5.95 ± 0.04	6.31 ± 0.05	**< 0.001**
MAA (µg/ml)	31.62 ± 2.83	40.93 ± 3.56	**0.044**
LF (µg/ml)	27.54 ± 2.30	33.49 ± 2.90	0.112

MY= Milk Yield; Log SCC= Logarithm of the Somatic Cell Count; MAA= Milk Amyloid A; LF: Lactoferrin; *Infected goats= 142 unilateral and 74 bilateral

Table 3. Quality parameters (mean ± SE) of the Murciano-Granadina goat's milk samples according to the aetiology of the subclinical mastitis

Parameter	Aetiology					
	CNS (n= 120)	*S. aureus* (n= 17)	*Corynebacterium* (n= 65)	*Streptococcus* (n= 7)	Gram-negative (n= 7)	p-value
MY (litres/milking)	1.48 ± 0.07	1.18 ± 0.15	1.45 ± 0.10	1.21 ± 0.22	0.93 ± 0.23	0.079
Fat (%)	5.45 ± 0.15	5.79 ± 0.31	5.38 ± 0.19	5.23 ± 0.47	6.77 ± 0.46	0.062
Protein (%)	3.88 ± 0.07^{a}	4.07 ± 0.16^{a}	3.94 ± 0.09^{a}	3.85 ± 0.23^{a}	4.73 ± 0.23^{b}	**0.012**
Lactose (%)	4.57 ± 0.04^{b}	4.22 ± 0.10^{a}	4.55± 0.06^{b}	4.30 ± 0.14^{ab}	4.33 ± 0.14^{ab}	**0.003**
Total solids (%)	14.73 ± 0.21^{a}	14.89 ± 0.44^{a}	14.67 ± 0.27^{a}	14.20 ± 0.66^{a}	16.70 ± 0.64^{b}	**0.047**
pH	6.68 ± 0.01	6.67 ± 0.03	6.68 ± 0.02	6.80 ± 0.04	6.69± 0.04	0.081
Log SCC (cells/ml)	6.23 ± 0.06^{a}	6.87 ± 0.13^{b}	6.15 ± 0.08^{a}	6.52 ± 0.20^{ab}	6.82 ± 0.20^{b}	**< 0.001**
MAA (µg/ml)	37.97 ± 6.23	29.63 ± 13.04	52.74 ± 7.89	39.43 ± 20.86	51.69 ± 18.88	0.481
LF (µg/ml)	25.32 ± 4.39^{a}	52.84 ± 9.30^{b}	40.78 ± 5.61^{b}	31.90 ± 14.89^{ab}	62.71 ± 13.48^{b}	**0.010**

MY: Milk Yield; Log SCC= Logarithm of the Somatic Cell Count; MAA= Milk Amyloid A; LF: Lactoferrin; CNS= Coagulase-negative staphylococci; $^{a, b}$: Different letters in the same row indicate statistically significant differences ($p < 0.05$)

Table 4. Quality parameters (mean ± SE) of the Murciano-Granadina goat's milk samples according to Parity

Parameter	Parity			p-value
	1–2 (n= 303)	3–4 (n= 204)	≥ 5 (n= 75)	
MY (litres/milking)	1.53 ± 0.04[b]	1.57 ± 0.04[b]	1.33 ± 0.07[a]	**0.014**
Fat (%)	5.32 ± 0.07[a]	5.53 ± 0.09[ab]	5.69 ± 0.14[b]	**0.024**
Protein (%)	3.90 ± 0.03	3.93 ± 0.04	3.91 ± 0.07	0.866
Lactose (%)	4.65 ± 0.02[b]	4.57 ± 0.02[a]	4.49 ± 0.04[a]	**< 0.001**
Total solids (%)	14.67 ± 0.10	14.87 ± 0.12	14.91 ± 0.20	0.303
pH	6.67 ± 0.01	6.67 ± 0.01	6.67 ± 0.01	0.915
Log SCC (cells/ml)	5.94 ± 0.04[a]	6.13 ± 0.04[b]	6.32 ± 0.07[c]	**< 0.001**
MAA (µg/ml)	42.43 ± 2.61	34.88 ± 3.12	31.51 ± 5.10	0.053
LF (µg/ml)	24.45 ± 2.11[a]	32.95 ± 2.53[b]	34.15 ± 4.14[b]	**0.010**

MY: Milk Yield; Log SCC: Logarithm of the Somatic Cell Count; MAA: Milk Amyloid A; LF: Lactoferrin; [a, b, c]: Different letters in the same row indicate statistically significant differences ($p < 0.05$)

Table 5. Quality parameters (mean ± SE) of the Murciano-Granadina goat's milk samples according to DIM (days in milk)

Parameter	DIM (days)			p-value
	< 90 (n= 128)	91–190 (n= 305)	≥ 191 (n= 149)	
MY (litres/milking)	1.72 ± 0.06[c]	1.43 ± 0.04[b]	1.28 ± 0.05[a]	**< 0.001**
Fat (%)	5.04 ± 0.12[a]	5.41 ± 0.07[b]	6.09 ± 0.11[c]	**< 0.001**
Protein (%)	3.76 ± 0.06[a]	3.79 ± 0.04[a]	4.18 ± 0.05[b]	**< 0.001**
Lactose (%)	4.69 ± 0.03[b]	4.52 ± 0.02[a]	4.51 ± 0.03[a]	**< 0.001**
Total solids (%)	14.29 ±0.16[a]	14.52 ± 0.10[a]	15.63 ± 0.15[b]	**<0.001**
pH	6.69 ± 0.01[b]	6.67 ± 0.01[ab]	6.65 ± 0.01[a]	**0.016**
Log SCC (cells/ml)	5.94 ± 0.06[a]	6.22 ± 0.04[b]	6.23 ± 0.05[b]	**< 0.001**
MAA (µg/ml)	32.84 ± 4.35[a]	26.86 ± 2.69[a]	49.12 ± 3.86[b]	**< 0.001**
LF (µg/ml)	27.40 ± 3.53[ab]	27.57 ± 2.17[a]	36.58 ± 3.15[b]	**0.032**

MY: Milk Yield; Log SCC: Logarithm of the Somatic Cell Count; MAA: Milk Amyloid A; LF: Lactoferrin; [a, b, c]: Different letters in the same row indicate statistically significant differences ($p < 0.05$)

Table 6. Logistic regression models of dichotomous outcomes to predict subclinical mastitis in Murciano-Granadina dairy goats

Subclinical Mastitis Status	Term	Coefficient	Odd-ratio	p-value
Approach 1 (Culture test)	Intercept	-6.75		**0.000**
	Log SCC	1.010	2.745	**0.000**
	MAA	0.007	1.007	**0.002**
	LF	-0.006	0.994	0.081
Approach 2 (Culture test and Log SCC)	Intercept	13.04		**0.000**
	Lactose	-3.322	0.036	**0.000**
	LF	0.103	1.108	**0.000**

Approach 1= Infected goats: positive culture test (n= 216); Uninfected goats: negative culture test (n= 366); Approach 2= Infected goats: positive culture test and increased somatic cell count (SCC $\geq$1,500 x 10^3 cells/ml; n= 149); Uninfected goats: negative culture test and low somatic cell count (SCC $\leq$500 x 10^3 cells/ml; n= 154); Log SCC: Logarithm of the somatic cell count; MAA: Milk Amyloid A; LF: Lactoferrin