

Complementary use of N-acetyl- β -D-glucosaminidase and somatic cell count for detecting experimental and naturally occurring mastitis in dairy goats

Jéssica Lobo Albuquerque Caldeira

Universidade Federal de Viçosa

Ugonna Henry Uzoka

Universidade Federal de Viçosa

Richard Costa Polveiro

Universidade Federal de Uberlândia

Luisa Maria de Souza Menezes

Universidade Federal de Viçosa

Mariana de Barros

Universidade Federal de Viçosa

Juliana Miwa Fujikura

Universidade Federal de Viçosa

Wagner Faria Barbosa

Universidade Federal de Viçosa

Grazieli Maboni

University of Georgia

Maria Aparecida Scatamburlo Moreira

masm@ufv.br

Universidade Federal de Viçosa

Research Article

Keywords: N-acetyl- β -D-glucosaminidase, Biomarker, Somatic cell count, Staphylococcus warneri, Intramammary infection, Dairy goats

Posted Date: August 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10500526/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Mastitis is an inflammatory condition of the mammary gland that can lead to several changes, including significant alterations in glandular enzymatic activity, depending on the causative pathogen.

Staphylococcus is frequently associated with intramammary mastitis in goats, with coagulase-negative *Staphylococcus* being the most prevalent group of pathogens responsible for subclinical mastitis in production animals. The diagnosis of mastitis in goats often relies on standardized tests designed for cattle, primarily the somatic cell count (SCC) test; however, the application of these techniques in goats is limited due to their biological differences. This study aimed to investigate the performance of the enzyme N-acetyl- β -D-glucosaminidase (NAGase) in 112 milk samples from animals experimentally infected with *S. warneri*, a coagulase-negative *Staphylococcus* species. Additionally, 49 milk samples from naturally infected animals at various stages of mastitis were analyzed. The NAGase activity was assessed using the fluorometric method. At the same time, SCC and Total Bacterial Count were determined using flow cytometry, with these tests serving as parameters for monitoring mastitis progression and infection. The results indicated that NAGase activity was significantly elevated in the milk of goats experimentally infected with *S. warneri*, with the enzyme reaching its peak two days after inoculation. The combined interpretation of NAGase and SCC improved differentiation between healthy and mastitic animals in naturally infected goats. In conclusion, this study demonstrates that NAGase activity can serve as an indicator of mastitis in goats, even in the early stages of infection, suggesting its potential utility as an adjunct biomarker for monitoring caprine mastitis when interpreted together with SCC.

Introduction

Mastitis, an inflammatory condition of the mammary gland, manifests in various forms depending on the causative pathogen, lesion severity, and parenchymal damage. Subclinical mastitis is prevalent in small ruminants, leading to alterations in milk composition and decreased production and thereby causing substantial economic losses [1, 2]. The genus *Staphylococcus*, particularly coagulase-negative *Staphylococcus* (CNS), is frequently implicated in intramammary infections (IMI) in small ruminants, notably contributing to subclinical mastitis in production animals [3, 4].

Staphylococcus warneri is a bacterium frequently present in the normal microbiota of the skin and mucous membranes of humans and animals [5]. However, it can also act as an opportunistic pathogen, causing infections in immunocompromised individuals [6, 7, 8, 9, 10]. Additionally, it has been associated with bovine abortions [11] and is frequently linked to mastitis in domestic ruminants [12, 13].

A distinctive characteristic of *S. warneri* is the presence of exoenzymes, such as the proteases SspA and SspB, which are commonly found in *S. aureus* but are generally absent in CNS strains [14, 15]. These extracellular proteases, known for their role in *S. aureus* virulence [16], may suggest similar pathogenic mechanisms in *S. warneri* [17], highlighting the clinical significance of this microorganism in infections.

Studies have emphasized the ability of *S. warneri* to form biofilms and exhibit resistance to multiple antibiotics, complicating the treatment of infections such as mastitis and increasing the risk of antimicrobial resistance dissemination [18, 19]. Moreover, *S. warneri*-induced mastitis in ruminants has been associated with alterations in milk composition, negatively impacting quality, including reduced protein content and increased levels of somatic cells and urea [20].

Inflammatory events within the mammary alveolus during infection reflect changes in milk composition. These alterations often involve significant shifts in mammary gland enzymatic activity, characterized by reductions in milk synthesis-related enzymes and elevations in native enzymes during inflammatory processes [21, 22]. Among milk enzymes, two distinct categories emerge: non-lysosomal enzymes like lactate dehydrogenase (LDH) and lysosomal enzymes including β -galactosidase, N-acetyl- β -glucosaminidase (NAGase), α -mannosidase, and β -glucuronidase, which demonstrate increased activity during inflammation [23]. LDH, NAGase, and phosphatases have served as indicators to evaluate mammary cell damage and milk quality in mastitis, displaying significant variations between healthy animals and those with subclinical and clinical mastitis, primarily documented in cattle [24, 25].

The diagnosis of mastitis in goats commonly relies on standardized methods developed for cattle, such as the California Mastitis Test (CMT), bacterial culture, and somatic cell count (SCC) [26]. Intramammary infections (IMI) typically lead to bacterial proliferation in the milk, resulting in elevated total bacterial count (TBC and SCC. However, while SCC is a valuable for mastitis control, its application in goats requires adjustments due to the unique biological characteristics of these animals. Notably, healthy goat mammary glands naturally exhibit higher SCC levels than cattle, attributed in part to the apocrine secretion of the goat mammary glands [27].

Consequently, SCC is not established as a reliable diagnostic criterion for goats, as several factors influence somatic cell counts in milk, including the lactation stage, estrus, breed, and milking method [28, 29, 30]. Other milk-derived biomarkers, including chemical elements, have also shown potential for monitoring experimental goat mastitis, supporting the complementary use of multiple biomarkers for udder health assessment [31]. In contrast, due to its increased activity in mammary glands during inflammatory processes, the NAGase enzyme emerges as a promising marker for mastitis diagnosis in cattle and goats. Although previous studies have demonstrated increased NAGase activity in goats with mastitis, most investigations were based primarily on naturally infected animals evaluated under field conditions [28, 32, 33]. In the present study, we combined a controlled experimental intramammary infection model using *Staphylococcus warneri* with longitudinal monitoring of NAGase activity, SCC, and TBC, together with evaluation of naturally infected field animals. Additionally, we explored the combined interpretation of NAGase and SCC to investigate their complementary value for differentiating mastitis severity categories in dairy goats.

Therefore, this study aimed to evaluate NAGase activity in goats experimentally infected with *Staphylococcus warneri* and in naturally infected field animals, as well as to investigate the potential complementary use of NAGase and SCC for the detection and classification of caprine mastitis.

Material and Methods

The experimental protocol, numbered 62/2018, was approved by the Ethics Committee on the Use of Animals (CEUA) from the Federal University of Viçosa (UFV), Brazil.

Experimental Infection

The sample size used in our study was determined based on practical and ethical limitations. Thus, we chose to allocate more animals to the infection group to obtain robust results for the proposed analyses while maintaining a negative control that ensured the necessary comparison without compromising animal welfare.

Seven healthy female goat of the Parda Alpina breed, closely related genealogically, were chosen from the Federal University of Viçosa goat breeding unit. All were primiparous, lactating, and had not received any antibiotics or other significant medications. Before the bacterial challenge, they underwent clinical assessment for signs of mastitis and milk abnormalities. Subsequently, they were individually housed in sanitized stalls with daily sanitation routines, including footbaths with quicklime for decontamination and designated hygiene areas.

During a two-week adaptation period, three intramammary-negative aerobic bacterial cultures were conducted on each goat. Following this, six of the goats had one side of their udders (right side) inoculated with a dose of 1.2×10^8 CFU/mL of the *S. warneri*, administered intramammarily in a 2 mL volume, following the method described by Moraes Peixoto et al. [34]. The isolate was previously obtained from a case of persistent subclinical caprine mastitis, identified through conventional microbiological characterization and molecular identification methods previously described by França (2021) [35]. A negative control goat received a 2 mL inoculation of sterile saline solution on the right side of the udder and underwent the same sampling procedures as the other animals, serving as a reference in the analyses. Throughout the experimental infection period, milk samples were subjected to microbiological monitoring using blood agar culture and Accumast® chromogenic agar to confirm bacterial growth and monitor intramammary infection dynamics.

Milk samples were collected in duplicate using sterile 50 mL centrifuge tubes during manual milking, before and after teat dipping, following udder manipulation and aseptic techniques with 70% alcohol. These samples were promptly transported to the laboratory for aliquoting and appropriate distribution. Samples were individually collected from the right and left udders, totaling 112 samples from infected animals, including those obtained on day 0 (before infection) and the subsequent seven days, which were analyzed for NAGase activity, somatic cell count, and total bacterial count.

Field animals

To investigate IMI in naturally infected goats and compare NAGase enzyme activity with the SCC parameter, we analyzed 49 milk samples from field animals, specifically Parda Alpina goats aged between 1 and 2 years, housed in the goat farm of the Universidade Federal de Viçosa, with a history of clinical or subclinical mastitis.

Based on the collected data, CMT and SCC, and the animals' clinical histories, the samples were categorized into four groups: (i) Healthy animals with no history of mastitis ($n = 10$), (ii) Animals with mild subclinical mastitis + ($n = 5$), (iii) Animals with moderate subclinical mastitis ++ ($n = 19$), and (iv) Animals with clinical mastitis +++ ($n = 15$). The classification of field animals reflected routine herd-monitoring conditions and was based on SCC, CMT, and clinical history, while microbiological culture data were available primarily for experimentally infected animals.

N-acetyl- β -D-glucosaminidase Activity

A modified plate-based fluorometric assay was employed to measure NAGase activity in milk samples [36]. Ten microliters of the test sample, positive control (an animal with clinical mastitis), negative control (a clinically healthy animal), and blank sample (distilled water) were pipetted in duplicate into a plate. These samples were then incubated with 40 μ l of the substrate 4-methylumbelliferyl-N-acetyl-D-glucosaminide (2.25 mM 4-MUAG, M-2133 Sigma-Aldrich®). The reaction was halted by adding 150 μ l of glycine buffer (0.2 M, pH 11).

The fluorescence of the product 4-methylumbelliferone (4-MU) generated by NAGase activity was measured using a fluorimeter equipped with a 355 nm excitation filter and a 460 nm emission filter. The fluorescence averages of the duplicate samples were then calculated and subtracted from the mean fluorescence of distilled water (blank sample).

NAGase activity was expressed as pmol of 4-MU/min/ μ l of milk at 20°C. Thus, enzyme activity was determined using the equation: NAGase activity of sample = (fluorescence of sample minus blank) $\times$ NAGase activity of control 1 / (fluorescence of control 1 minus blank). Control 1 in the equation was defined according to the standard curve, described below.

Standard curve

The analysis of NAGase activity relied on a standard curve established by measuring the fluorescence of 10 different known concentrations of 4-methylumbelliferone (4-MU) (ranging from 1 to 450 μ M 4-MU in glycine buffer). These preestablished curve values were used to assess NAGase activity in the milk of a goat with clinical mastitis (control 1), and the lower and upper limits of quantification for the analysis were determined from the linear section of the curve.

To determine the respective molarity of control 1, the fluorescence of control 1 minus the blank (water) was divided by the angular coefficient. This calculation was performed using ten replicates conducted at

three different times to ensure accurate determination of this value, which served as a parameter for analyzing NAGase activity in the test samples.

Somatic Cell Count and Total Bacterial Count

To monitor the progression of IMI and assess the increase in milk contamination resulting from experimental infection, milk samples were analyzed for SCC and TBC. These samples were stored in bottles containing Bronopol® preservatives and Azidiol tablets and then refrigerated between 2°C and 6°C until analysis.

SCC was determined using flow cytometry based on the Bentley Instruments, NexGen Somacount FCM model. TBC was assessed using electronic equipment from the same brand, specifically the Bactcount IBC model. Both analyses were conducted at Embrapa Gado de Leite in Juiz de Fora, Minas Gerais, Brazil.

For the milk samples obtained from field animals, SCC served as an indicator of mastitis. Animals were classified as mastitic or non-mastitic based on SCC values. Values equal to or greater than 2,000,000 cells/mL were considered mastitic, following the criterion established by Souza et al. [37], which is widely used in the literature for diagnosing mastitis in goats.

Statistical Analysis

To analyze NAGase activity, SCC, and TBC of experimental infection samples, we employed generalized additive models (GAM) with integrated smoothing estimation. Treatment (control or bacterial inoculation) was included in the models as a fixed effect, considering various error distributions. Model selection was based on the F-test ($P < 0.05$), adjusted R-squared, and visual analysis of residual plots.

SCC served as an indicator of mastitis for evaluating goats in field milk samples. Individuals were categorized as mastitic or non-mastitic based on SCC values, with a threshold of 2,000,000 cells/mL established as defined by Souza et al. [37]. Descriptive statistics, including mean, standard deviation, maximum, and minimum values, were calculated for NAGase and SCC activity in each group. Due to the magnitude of the observed values, logarithmic analyses (base 10) were incorporated. Additionally, a Pearson correlation and Student's t-test ($P < 0.05$) were employed to investigate the linear relationship between NAGase activity and SCC.

Furthermore, to elucidate how NAGase activity and SCC jointly identify different levels of goat mastitis (healthy, subclinical mastitis +, subclinical mastitis ++, and clinical mastitis), a decision tree model [38, 39] was developed for exploratory classification analysis. The classification was performed with 80% and 20% of the field data, respectively, which were previously logarithmized (base 10).

In addition, an exploratory evaluation of diagnostic performance was performed using SCC-based classification as the reference criterion for mastitis status in field animals. The discriminatory capacity

of NAGase activity alone and in combination with SCC was assessed based on the cutoff values identified during the classification analysis. Sensitivity and specificity estimates were interpreted as preliminary indicators of diagnostic performance due to the limited sample size and exploratory nature of the study. For exploratory classification purposes, mastitis status in field animals was defined based on SCC, CMT, and clinical evaluation criteria routinely adopted in dairy goat herd monitoring.

All analyses were conducted in R (R Core Team, 2022) and assisted by several R packages. Specifically, GAM, descriptive analyses, linear correlation, and decision tree fitting were performed using the “mgcv” [40], “tidyverse” [41], “stats” (a base R package), and “tree” [42] packages. Additionally, the graphs were plotted using the “ggplot2” package [43] and adjusted with CorelDraw X8 (<https://www.coreldraw.com/en/>).

Results

Experimental Infection

The results of the experimental infection are illustrated in Figure 1, along with Supplementary Tables 1 - 3. Microbiological monitoring confirmed bacterial growth in inoculated mammary glands throughout the infection period, whereas the control animal remained culture-negative during the experimental phase. As depicted in Figure 1A, a peak in NAGase activity was observed two days after inoculation, reaching 35235 pmoles of 4-MU/min/μl of milk, in contrast to the control group, which recorded 5017 pmoles of 4-MU/min/μl of milk, showing a significant difference ($P < 0.0001$). This disparity remained statistically relevant until the fifth day post-inoculation. The NAGase temporal analysis was adequately modeled by a generalized additive model using the quasipoisson error distribution (deviance explained = 82.2%). Significant smooth terms for plaque (treated as a random effect) ($P < 0.0001$) and for the time-treatment interaction ($P < 0.0001$) indicated an interaction between the factors.

Figure 1B demonstrates a peak in SCC two days after inoculation, reaching 9,746,450 cells/mL, in contrast to the control group, which recorded 571,624 cells/mL, showing a significant difference ($P < 0.0001$). This disparity remained statistically relevant until the seventh day post-inoculation ($P = 0.0465$). Modeling of SCC over time was optimized by using a quasi-family error distribution (deviance explained = 69.6%). Although the random effect of plaque was not significant ($P = 0.467$), the model was simplified, highlighting a significant temporal term per treatment ($P < 0.0001$).

As shown in Figure 1C, TBC reached its peak two days after inoculation, registering 3,491,131 cfu/mL, in contrast to the control, which presented 21,800 cfu/mL, revealing a statistically significant difference ($P < 0.0001$). This discrepancy remained until the fourth day post-inoculation, with statistical significance ($P = 0.0036$). Modeling TBC over time was effective when using a quasi-family error distribution (explained deviance = 80.6%). The random effect did not demonstrate significance ($P = 0.433$), similar to that observed for SCC.

Field Animals

Of the 49 individuals evaluated, 34 were identified as mastitic based on the established SCC threshold, as detailed in Table 1. It was observed that the level of SCC in mastitic goats was significantly high, presenting a magnitude 37.81 times higher compared to those in non-mastitic goats. In contrast, when analyzing NAGase activity, the difference between the groups was less pronounced, with an average of 10.21% higher in mastitic animals (Table 1). Examining the variation in values between maximum and minimum, both for SCC and NAGase, a greater amplitude was found in mastitic goats. However, the standard error was greater in the SCC for mastitic goats. The Pearson correlation (0.63) revealed a moderate and significant linear association ($df = 47$, $t = 5.5387$, $P < 0.0001$) between NAGase levels and SCC.

Exploratory assessment of the identified cutoff values demonstrated that the combined interpretation of NAGase activity and SCC improved differentiation between healthy and mastitic animals compared with the isolated use of SCC. Although these findings support the potential diagnostic value of NAGase as an adjunct biomarker, the performance estimates should be interpreted cautiously due to the limited number of animals evaluated.

Figure 2 demonstrates the decision tree algorithm that recognized a cutoff in NAGase activity ($4.04 = \log_{10}$ (10943.85 pmoles of 4-MU/min/ μ l of milk)) that separates healthy animals and animals with moderate subclinical mastitis ++ from animals with clinical mastitis and mild subclinical mastitis +. Two cutoff points were found in the SCC that separate healthy animals from subclinical ++ and subclinical + from clinical animals, which were, respectively, 6.32 ($\log_{10}$ (2071715 cells/mL)) and 6.39 ($\log_{10}$ (2448950 cells/mL)). With this set of cutoff values, the model differentiated the mastitis severity categories within the evaluated population and showed consistent classification patterns within the analyzed dataset. However, these findings should be interpreted as preliminary due to the limited sample size.

Discussion

Experimental Infection

NAGase, a lysosomal enzyme present in milk, serves as an indicator of inflammation in the mammary gland due to its origin in phagocytic cells, mainly neutrophils [4]. In this study, we observed a significant increase in NAGase activity after intramammary infection (IMI), as illustrated in Figure 1A.

The findings of our study align with those of previous research. Leitner et al. [44] analyzed 500 samples of goat milk from different herds and identified a striking correlation between the bacteriological status of the udder and NAGase activity. Furthermore, Barth et al. [28] found that intramammary infection significantly impacted NAGase activity in 58 goat milk samples when investigating the influence of infection on milk parameters. Additional studies have also demonstrated significantly higher NAGase activity in milk samples from infected udders in goats at different stages of lactation compared to the uninfected half of the udder [33, 45]. Thus, the results found in our study reinforce the potential of

NAGase as an effective tool for monitoring mammary health in goats, allowing the differentiation of infected from noninfected animals even in the early stages of infection.

Intramammary infections increase the influx of blood leukocytes, mainly neutrophils, which are attracted by chemotaxis to combat the pathogen involved in the infection. These leukocytes also contribute significantly to the increase in SCC in secreted milk, which is the most widely used indicator of mammary gland health in cattle, sheep, and goats. However, interpretation of SCC in goats can be difficult due to several physiological factors that influence the increase in these cell counts [4, 46, 47]. We observed that during the development of the experimental infection in our study, there was a significant increase in SCC after inoculation (Figure 1B), confirming the recruitment of immune cells to the site of infection and supporting the results obtained for NAGase. Although the precise threshold of SCC counts to detect mastitis in dairy goats is not clearly defined, studies have consistently indicated a significant elevation in cell counts when the mammary gland is infected [33, 43, 45,48], corroborating our findings as illustrated in Figure 1A.

Although the primary use of SCC for diagnosing mastitis in goats may have its limitations, it remains the predominant method in practice [47, 48], even if its interpretation is challenging. However, in controlled environments, with the minimization of biological variables, as evidenced in our research, SCC emerges as a reliable parameter to indicate infection.

Bacterial plate counting is the conventional method for determining the presence of bacteria in milk, providing information about viable cells and their multiplication. However, due to the time required to perform this technique, faster methods such as flow cytometry have gained popularity in milk analysis laboratories for determining total bacterial count (TBC) in goats and sheep [29, 48, 50]. In our study, we used TBC as a parameter to monitor the bacteriological status of animals. We observed a peak in bacterial cells on the second day after infection (Figure 1C), which coincided with the timing of the other analyses. This finding strongly suggests that the increase in NAGase and SCC was mainly due to bacterial proliferation (Figures 1A and 1B).

The relationship between intramammary infections caused by coagulase-negative *Staphylococcus* (CNS) and SCC is not well established in the literature. Most studies have been conducted in field herds, where physiological factors strongly influence variation in cell counts. Therefore, it is important to consider these factors when evaluating the relationship between the presence of CNS and SCC in goats [50].

However, in our study, which consisted of an experimental infection in a controlled environment, with all animals subject to the same management and conditions, it was possible to observe that an intramammary infection caused by a CNS pathogen strongly influenced changes in milk parameters, such as SCC and NAGase.

Field Animals

Our data from field animals demonstrate cutoffs with a Log₁₀ value of 6.39 (2,448,950 cells/mL), suggesting that higher SCC values can be used to distinguish mastitic animals from healthy ones. Several studies suggest that values higher than the defined SCC cutoff points are more appropriate to differentiate animals with and without mastitis [37, 46]. In line with this, our experimental infection data on SCC (Figure 1B) showed that animals exhibited increasing levels above the cutoff of 2000×10^3 cells/mL.

In a study conducted by Min et al. [52], which examined subclinical intramammary infections and their impact on milk composition in 35 goats, SCC values ranging from 2000 to 4000×10^3 cells/mL were reported in infected goats. However, they were unable to accurately correlate intramammary infection with increased SCC. This highlights the complexity of interpreting SCC in goats and the influence of several factors on SCC readings.

By analyzing the milk of 200 dairy goats from different herds, Kumar et al. [33] found significantly higher mean SCC and Log₁₀SCC values in infected udder halves (SCC = 3619.63 ± 35 , Log₁₀SCC = 6.31 ± 0.05) compared to uninfected udder halves (SCC = 1422.15 ± 17 , Log₁₀SCC = 5.67 ± 0.03), which is in line with the values observed in our study when distinguishing mastitic from non-mastitic animals. This suggests that higher SCC values may indeed be indicative of mammary infection in dairy goats.

A recent study by Nagahata et al. [53], who investigated 62 goats, observed that milk samples with SCC between 301 and 1000×10^3 and $>1010 \times 10^3$ cells/mL showed greater bacteriological positivity than those with SCC $< 300 \times 10^3$. This result suggests that high values of SCC may be associated with the presence of intramammary infection.

We cannot rule out the effects of seasonal fluctuations and lactation stage for the lower SCC values found in the literature [45]. As shown in Figure 2, subclinical + animals may be misclassified as healthy if only the SCC parameter is considered, demonstrating that this parameter alone is unreliable. It is important to consider multiple factors and perform a comprehensive assessment for improved detection of mastitis in goats.

Previous studies have demonstrated increased NAGase activity in goats with mastitis, although most were conducted under field conditions, where physiological and management-related factors may influence SCC and enzymatic activity [28,32,44,45]. In contrast, the controlled experimental infection model used here enabled temporal evaluation of inflammatory progression while minimizing biological and environmental variability. Our findings extend previous observations by demonstrating that the combined interpretation of NAGase activity and SCC improves discrimination among mastitis severity categories. These results are consistent with our recent study using the same *Staphylococcus warneri* experimental model, which also identified NAGase as a reliable indicator of mammary inflammation during disease progression and treatment [54].

Defining a threshold value for NAGase activity in the literature is challenging due to variation in measurement units in existing studies, although many employ fluorometric tests for this assessment. However, studies indicate a significant increase in NAGase activity in goats with mammary gland infections at different stages of lactation [28, 33, 44, 45, 53]. This finding highlights the potential of NAGase as a valuable indicator also in naturally infected goats. Therefore, there is an evident need to establish a standardized and effective method for its use, and our study contributes preliminary evidence supporting the complementary use of NAGase in mastitis assessment.

Finally, when analyzing Figure 2, it is evident that evaluating animals using just one parameter, whether NAGase or SCC, presents limitations in differentiating groups, especially due to the proximity of the SCC cutoff points. However, the combination of NAGase and SCC parameters provides a clearer distinction between the groups. Healthy animals are distinguished by lower values of both SCC and NAGase, while those with clinical mastitis exhibit elevated levels of these parameters. This data integration highlights the importance of combined analysis for improved differentiation between healthy and mastitic animals in dairy goats.

Although the present study was not designed as a definitive clinical validation trial, the exploratory classification analyses suggest that NAGase may provide complementary diagnostic information when interpreted together with SCC. Future studies involving larger populations, independent validation cohorts, and formal ROC-based analyses will be important to establish robust diagnostic cutoff values and fully characterize the sensitivity and specificity of this biomarker in caprine mastitis.

The correlations identified in our study primarily involve variables generally considered causally interconnected, such as milk cell concentration and the occurrence of mastitis, as well as the enzymatic activity performed by udder defense cells. We acknowledge several limitations of this study, including the relatively small sample size in the experimental trial, which may restrict extrapolation of the findings to broader production systems. In addition, comprehensive pathogen-level microbiological characterization was not available for all field samples, since classification under field conditions was primarily based on SCC, CMT, and clinical history. Furthermore, universally established reference values for NAGase activity in goats are still lacking. Nevertheless, the results presented here contribute to the initial understanding of the potential of new adjunct biomarkers, such as NAGase, in the detection and monitoring of caprine mastitis, a field that remains underexplored.

Conclusion

In this study, we evaluated complementary approaches for mastitis detection. Key findings highlight that the NAGase enzyme activity was significantly elevated in the milk of goats experimentally infected with *S. warneri*, with the enzyme reaching its peak two days after inoculation. In naturally infected animals, the combination of both NAGase and SCC techniques provided improved differentiation between healthy and mastitic animals. These findings suggest that NAGase may serve as a promising adjunct biomarker for the detection and monitoring of caprine mastitis, particularly when interpreted together with SCC.

Declarations

Author Contribution

Author contributions Conceptualization, methodology, formal data analysis, investigation, primary manuscript drafting, revision, editing, and data visualization: JLAC, UHU, RCP; Research investigation, methodology development, project administration, and manuscript writing, review, and editing: LMSM, MB, JMF; Investigation, manuscript review, editing and research supervision: WFB, GM; Resource management, methodology development, manuscript review, editing and research supervision: MASM.

Acknowledgement

The authors express grateful appreciation to all the members of the Laboratory and Bacterial Disease Laboratory (LDBAC) and the Veterinary Department of the Federal University of Viçosa for their kind assistance during this study.

Data Availability

All raw analytical data, processed datasets and metadata files supporting this publication are available from the corresponding author upon reasonable request.

References

1. Gosselin VB, Lovstad J, Dufour S, Adkins PRF, Middleton JR. Use of MALDI-TOF to characterize staphylococcal intramammary infections in dairy goats. *J Dairy Sci.* 2018;101:6262–70. <https://doi.org/10.3168/jds.2017-14224>.
2. Wang YM, Ma YQ, Bi SC, Ma XD, Guan R, Wang SH, et al. Therapeutic effect of ginsenoside Rg1 on mastitis experimentally induced by lipopolysaccharide in lactating goats. *J Dairy Sci.* 2019;102:2443–52. <https://doi.org/10.3168/jds.2018-15280>.
3. Gocmen H, Darbaz I, Ergene O, Esendal OM, Aslan S. The relationships between somatic cell count, total bacterial count and intramammary infection in milk samples of Damascus goats during postpartum days. *Small Rumin Res.* 2019;180:1–5. <https://doi.org/10.1016/j.smallrumres.2019.09.018>.
4. Novac CS, Andrei S. The impact of mastitis on the biochemical parameters, oxidative and nitrosative stress markers in goat's milk: A review. *Pathog.* 2020;9:1–23. <https://doi.org/10.3390/pathogens9110882>.
5. Nagase N, Sasaki A, Yamashita K, Shimizu A, Wakita Y, Kitai S, Kawano J. Isolation and Species Distribution of Staphylococci from Animal and Human Skin. *J Vet Med Sci.* 2002;64(3):245–50. <https://doi.org/10.1292/JVMS.64.245>.

6. Bryan CS, Parisi JT, Strike DG. Vertebral osteomyelitis due to *Staphylococcus warneri* attributed to a hickman catheter. *Diagn Microbiol Infect Dis*. 1987;8(1):57–9. [https://doi.org/10.1016/0732-8893\(87\)90048-4](https://doi.org/10.1016/0732-8893(87)90048-4).
7. Kamath U, Singer C, Isenberg HD. Clinical significance of *Staphylococcus warneri* bacteremia. *J Clin Microbiol*. 1992;30(2):261–4. <https://doi.org/10.1128/JCM.30.2.261-264.1992>.
8. Martínez-Lage JF, Martínez-Lage Azorín L, Almagro MJ. *Staphylococcus warneri* ventriculoperitoneal shunt infection: Failure of diagnosis by ventricular CSF sampling. *Child's Nerv Syst*. 2010;26(12):1795–8. <https://doi.org/10.1007/S00381-009-1050-0>.
9. Ivić I, Karanović J, Pavičić-Ivelja M. Sepsis with multiple abscesses caused by staphylococcus warneri: A case report. *Cent Eur J Med*. 2013;8(1):45–7. <https://doi.org/10.2478/S11536-012-0066-0>.
10. Campoccia D, Montanaro L, Visai L, Corazzari T, Poggio C, Pegreff F, Maso A, Pirini V, Ravaioli S, Cangini I, Speziale P, Arciola CR. Characterization of 26 *Staphylococcus warneri* isolates from orthopedic infections. <https://doi.org/10.1177/039139881003300903>. 2018;33(9):575–81.
11. Barigye R, Schaan L, Gibbs PS, Schamber E, Dyer NW. Diagnostic evidence of *Staphylococcus warneri* as a possible cause of bovine abortion. *J Vet Diagn Invest*. 2007;19(6):694–6. <https://doi.org/10.1177/104063870701900613>.
12. Hosseinzadeh S, Dastmalchi Saei H. Staphylococcal species associated with bovine mastitis in the North West of Iran: Emerging of coagulase-negative staphylococci. *Int J Veterinary Sci Med*. 2014;2(1):27–34. <https://doi.org/10.1016/J.IJVSM.2014.02.001>.
13. Zigo F, Farkasová Z, Vydrostková J, Regecová I, Ondrasovicová S, Vargová M, Sasáková N, Pecka-Kielb E, Bursová S, Kiss DS. Dairy Cows' Udder Pathogens and Occurrence of Virulence Factors in *Staphylococci*. *Animals*. 2022;12(4):470. <https://doi.org/10.3390/ANI12040470/S1>.
14. Nickerson NN, Prasad L, Jacob L, Delbaere LT, McGavin MJ. Activation of the SspA serine protease zymogen of *Staphylococcus aureus* proceeds through unique variations of a trypsinogen-like mechanism and is dependent on both autocatalytic and metalloprotease-specific processing. *J Biol Chem*. 2007;282(47):34129–38. <https://doi.org/10.1074/JBC.M705672200>.
15. Yokoi KJ, Kuzuwa S, Iwasaki SI, Yamakawa A, Taketo A, Kodaira KI. Aureolysin of *Staphylococcus warneri* M accelerates its proteolytic cascade, and participates in biofilm formation. *Biosci Biotechnol Biochem*. 2016;80(6):1238–42. <https://doi.org/10.1080/09168451.2016.1148576>.
16. Kolar SL, Ibarra A, Rivera J, Mootz FE, Davenport JM, Stevens JE, Horswill SM, A. R., Shaw LN. Extracellular proteases are key mediators of *Staphylococcus aureus* virulence via the global modulation of virulence-determinant stability. *MicrobiologyOpen*. 2013;2(1):18–34. <https://doi.org/10.1002/MB03.55>.
17. Liu C, Zhao X, Xie H, Zhang X, Li K, Ma C, Fu Q. Whole genome sequence and comparative genome analyses of multi-resistant *Staphylococcus warneri* GD01 isolated from a diseased pig in China. *PLoS ONE*. 2020;15(5):e0233363. <https://doi.org/10.1371/JOURNAL.PONE.0233363>.

18. Szczuka E, Krzysińska S, Kaznowski A. Clonality, virulence and the occurrence of genes encoding antibiotic resistance among *Staphylococcus warneri* isolates from bloodstream infections. *J Med Microbiol.* 2016;65(8):828–36. <https://doi.org/10.1099/JMM.0.000287>.
19. Hoque MN, Faisal GM, Das ZC, Sakif TI, Mahtab A, Hossain M, M. A., Islam T. Genomic features and pathophysiological impact of a multidrug-resistant *Staphylococcus warneri* variant in murine mastitis. *Microbes Infect.* 2023;105285. <https://doi.org/10.1016/J.MICINF.2023.105285>.
20. Vasil M, Pecka-Kiełb E, Elečko J, Zachwieja A, Zawadzki W, Zigo F, Illek J, Farkašová Z. Effects of udder infections with *Staphylococcus xylosus* and *Staphylococcus warneri* on the composition and physicochemical changes in cows milk. *Pol J Vet Sci.* 2016;19(4):841–8. <https://doi.org/10.1515/PJVS-2016-0105>.
21. Pyörälä S. Indicators of inflammation in the diagnosis of mastitis. *Vet Res.* 2003;34:565–78. <https://doi.org/10.1051/vetres:2003026>.
22. Fox PF, Kelly AL. Indigenous enzymes in milk: Overview and historical aspects—Part 1. *Int Dairy J.* 2006;16:500–16. <https://doi.org/10.1016/j.idairyj.2005.09.013>.
23. Albenzio M, Santillo A. Biochemical characteristics of ewe and goat milk: Effect on the quality of dairy products. *Small Rumin Res.* 2011;101:33–40. <https://doi.org/10.1016/j.smallrumres.2011.09.023>.
24. Chagunda MGG, Larsen T, Bjerring M, Ingvarsen KL. L-lactate dehydrogenase and N-acetyl- β -D-glucosaminidase activities in bovine milk as indicators of non-specific mastitis. *J Dairy Res.* 2006;73(4):431–40. <https://doi.org/10.1017/S0022029906001956>.
25. Nagargoje SD, Somagond YM, Panda BSK, Dang AK. Enzymes in mastitis milk. *Enzym Beyond Tradit Appl Dairy Sci Technol.* 2023;37–56. <https://doi.org/10.1016/b978-0-323-96010-6.00002-3>.
26. Contreras A, Sierra D, Sánchez A, Corrales JC, Marco JC, Paape MJ, et al. Mastitis in small ruminants. *Small Rumin Res.* 2007;68:145–53. <https://doi.org/10.1016/j.smallrumres.2006.09.011>.
27. Paape MJ, Poutrel B, Contreras A, Marco JC, Capuco AV. Milk somatic cells and lactation in small ruminants. *J Dairy Sci.* 2001;84(Suppl):E237–44. [https://doi.org/10.3168/jds.S0022-0302\(01\)70223-8](https://doi.org/10.3168/jds.S0022-0302(01)70223-8).
28. Barth K, Aulrich K, Müller U, Knappstein K. Somatic cell count, lactoferrin and NAGase activity in milk of infected and non-infected udder halves of dairy goats. *Small Rumin Res.* 2010;94:161–6. <https://doi.org/10.1016/j.smallrumres.2010.07.022>.
29. Koop G, Dik N, Nielen M, Lipman LJA. Short communication: Repeatability of differential goat bulk milk culture and associations with somatic cell count, total bacterial count, and standard plate count. *J Dairy Sci.* 2010;93:2569–73. <https://doi.org/10.3168/jds.2009-2705>.
30. Dimitrov D, Stoimenov G, Morrison O. Diagnosis of subclinical mastitis in dairy goats (Review). *J Med Dent Pract.* 2018;5:702–7. <https://doi.org/10.18044/medinform.201851.702>.
31. Uzoka UH, Fujikura JM, Polveiro RC, et al. Evaluation of chemical elements as potential biomarkers in the treatment of goat mastitis. *Vet Res Commun.* 2025;49:166. <https://doi.org/10.1007/s11259-025-10733-9>.

32. Maisi P. Analysis of physiological changes in caprine milk with CMT, NAGase and antitrypsin. *Small Rumin Res.* 1990;3:485–92. [https://doi.org/10.1016/0921-4488\(90\)90079-L](https://doi.org/10.1016/0921-4488(90)90079-L).
33. Kumar R, Gupta DK, Bansal BK, Singh RS. Alterations in udder health indicators in goats with intramammary infection. *Indian J Vet Med.* 2019;39:22–6.
34. Moraes Peixoto R, de Moraes Peixoto Araújo R, e Silva Peixoto LJ, Gonçalves Bomfim SA, Guedes da Silva TM, Sarmiento Silva TM, et al. Treatment of goat mastitis experimentally induced by *Staphylococcus aureus* using a formulation containing *Hymenaea martiana* extract. *Small Rumin Res.* 2015;130:229–35. <https://doi.org/10.1016/j.smallrumres.2015.07.009>.
35. França CA. (2021). Tolerance and persistence to enrofloxacin in *Staphylococcus spp.* of goat mastitis: characterization, determination of virulence factors and comparative genomics. Thesis (Doctorate) - University Federal Rural of Pernambuco, Doctoral Program in Biotechnology – Northeast Biotechnology Network (RENORBIO), Recife.
36. Hovinen M, Simojoki H, Pösö R, Suolaniemi J, Kalmus P, Suojala L, et al. N-acetyl - β -D-glucosaminidase activity in cow milk as an indicator of mastitis. *J Dairy Res.* 2016;83:219–27. <https://doi.org/10.1017/S0022029916000224>.
37. Souza FN, Blagitz MG, Penna CFAM, Della Libera AMMP, Heinemann MB, Cerqueira MMOP. Somatic cell count in small ruminants: Friend or foe? *Small Rumin Res.* 2012;107:65–75. <https://doi.org/10.1016/j.smallrumres.2012.04.005>.
38. Quinlan JR. Induction of decision trees. *Mach Learn.* 1986;1:81–106.
39. Shalev-Shwartz S, Ben-David S. Understanding machine learning: From theory to algorithms. Cambridge University Press; 2014.
40. Wood SN. (2017) Generalized additive models: An introduction with R, second edition. Generalized Additive Models: An Introduction with R, Second Edition 1–476. <https://doi.org/10.1201/9781315370279/generalized-additive-models-simon-wood>
41. Wickham H, Averick M, Bryan J, et al. Welcome to the {tidyverse}. *J Open Source Softw.* 2019;4:1686. 10.21105/joss.01686.
42. Ripley B. (2022) tree: Classification and Regression Trees.
43. Wickham H. ggplot2: Elegant graphics for data analysis. 2nd ed. New York: Springer; 2016.
44. Leitner G, Merin U, Silanikove N, Ezra E, Chaffer M, Gollop N, et al. Effect of subclinical intramammary infection on somatic cell counts, NAGase activity and gross composition of goats' milk. *J Dairy Res.* 2004;71:311–5. <https://doi.org/10.1017/S0022029904000305>.
45. Stuhr T, Aulrich K, Barth K, Knappstein K, Larsen T. Influence of udder infection status on milk enzyme activities and somatic cell count throughout early lactation in goats. *Small Rumin Res.* 2013;111:139–46. <https://doi.org/10.1016/j.smallrumres.2012.09.004>.
46. Jimenez-Granado R, Sanchez-Rodriguez M, Arce C, Rodriguez-Estevez V. Factors affecting somatic cell count in dairy goats: A review. *Span J Agric Res.* 2014;12:133–50. <https://doi.org/10.5424/sjar/2014121-3803>.

47. 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;1:460–76. <https://doi.org/10.2174/2210298101666210402123549>.
48. Koop G, De Vliegher S, De Visscher A, Supré K, Haesebrouck F, Nielen M, et al. Differences between coagulase-negative *Staphylococcus* species in persistence and in effect on somatic cell count and milk yield in dairy goats. *J Dairy Sci.* 2012;95:5075–84. <https://doi.org/10.3168/JDS.2012-5615>.
49. De Garnica ML, Linage B, Carriedo JA, De La Fuente LF, García-Jimeno MC, Santos JA, et al. Relationship among specific bacterial counts and total bacterial and somatic cell counts and factors influencing their variation in ovine bulk tank milk. *J Dairy Sci.* 2013;96:1021–9. <https://doi.org/10.3168/jds.2012-5915>.
50. Sierra D, Sánchez A, Contreras A, Luengo C, Corrales JC, de la fe C, et al. Short communication: Effect of storage and preservation on total bacterial counts determined by automated flow cytometry in bulk tank goat milk. *J Dairy Sci.* 2009;92:4841–5. <https://doi.org/10.3168/jds.2008-1988>.
51. Gosselin VB, Dufour S, Adkins PRF, Middleton JR. Persistence of coagulase negative staphylococcal intramammary infections in dairy goats. *J Dairy Res.* 2019;86:211–6. <https://doi.org/10.1017/S0022029919000311>.
52. Min BR, Tomita G, Hart SP. Effect of subclinical intramammary infection on somatic cell counts and chemical composition of goats' milk. *J Dairy Res.* 2007;74:204–10. <https://doi.org/10.1017/s0022029906002378>.
53. Nagahata H, Fuse M, Umehara K, Gondaira S, Higuchi H, Hisaeda K, et al. Evaluation of the health status of mammary glands and compositional changes in udder-half milk obtained from dairy goats for milk quality management. *Anim Sci J.* 2022;93:e13714. <https://doi.org/10.1111/asj.13714>.
54. Uzoka UH, Polveiro RC, Caldeira JLA, et al. Exploratory assessment of milk biomarkers during non-antibiotic treatment in experimental *Staphylococcus warneri* goat mastitis. *World J Microbiol Biotechnol.* 2026;42:344. <https://doi.org/10.1007/s11274-026-05052-3>.

Table

Table 1. Descriptive parameters of NAGase activity and somatic cell count (SCC) of goats considered mastitic or non-mastitic by the SCC threshold value (2,000,000 cells/ml, Souza et al. 2012).

Parameters	SCC $\geq$ 2000000				SCC $<$ 2000000			
	SCC		NAGASE		SCC		NAGASE	
	Normal	log10	Normal	log10	Normal	log10	Normal	log10
Mean	17202029	7.0291	15571.89	4.0113	4548667	5.3529	14128.86	4.0019
Stand. error	3360407	0.0729	2555.48	0.0704	145807	0.1385	3351.21	0.0947
Min	2192000	6.3408	1663.09	3.2209	40000	4.6021	2500.89	3.3981
Max	98452000	7.9932	57405.06	4.7590	1958000	6.2918	44709.31	4.6504
Amplitude	96260000	1.6523	55741.97	1.5380	1918000	1.6897	42208.43	1.2523
(n)*	34	34	34	34	15	15	15	15

*(n) represents the number of individuals considered as mastitic when the SCC threshold was applied.

Figures

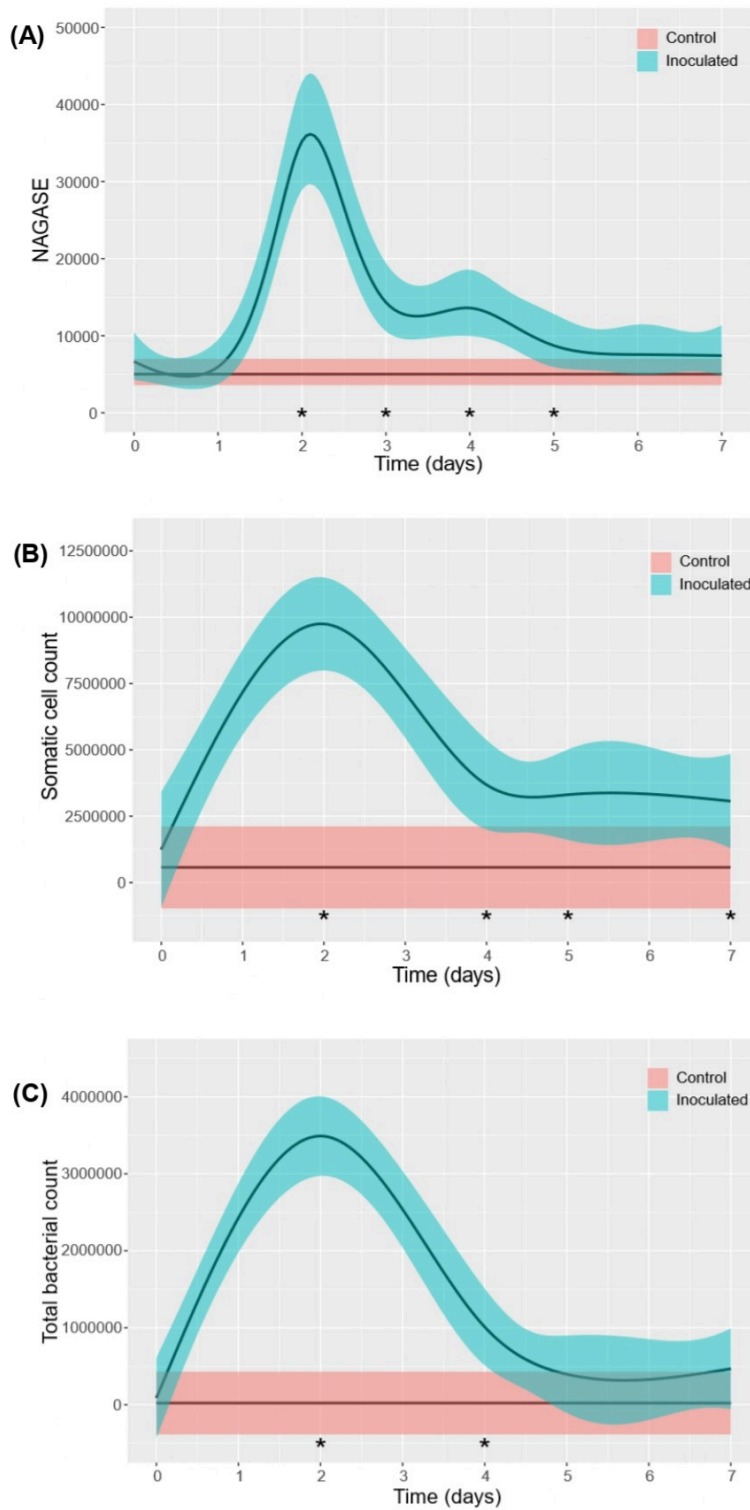


Figure 1

Temporal marginal means estimated using generalized additive models for goats experimentally infected with *Staphylococcus warneri*. **(A)** N-Acetyl- β -D-Glucosaminidase (NAGase) activity in milk over time. **(B)** Somatic cell count (SCC) over time. **(C)** Total bacterial count (TBC) over time. In all graphs, asterisks at the bottom indicate statistically significant differences between the control goat and

inoculated goats according to the Sidak method ($P < 0.05$). Shaded areas surrounding the trend lines represent 95% confidence intervals.

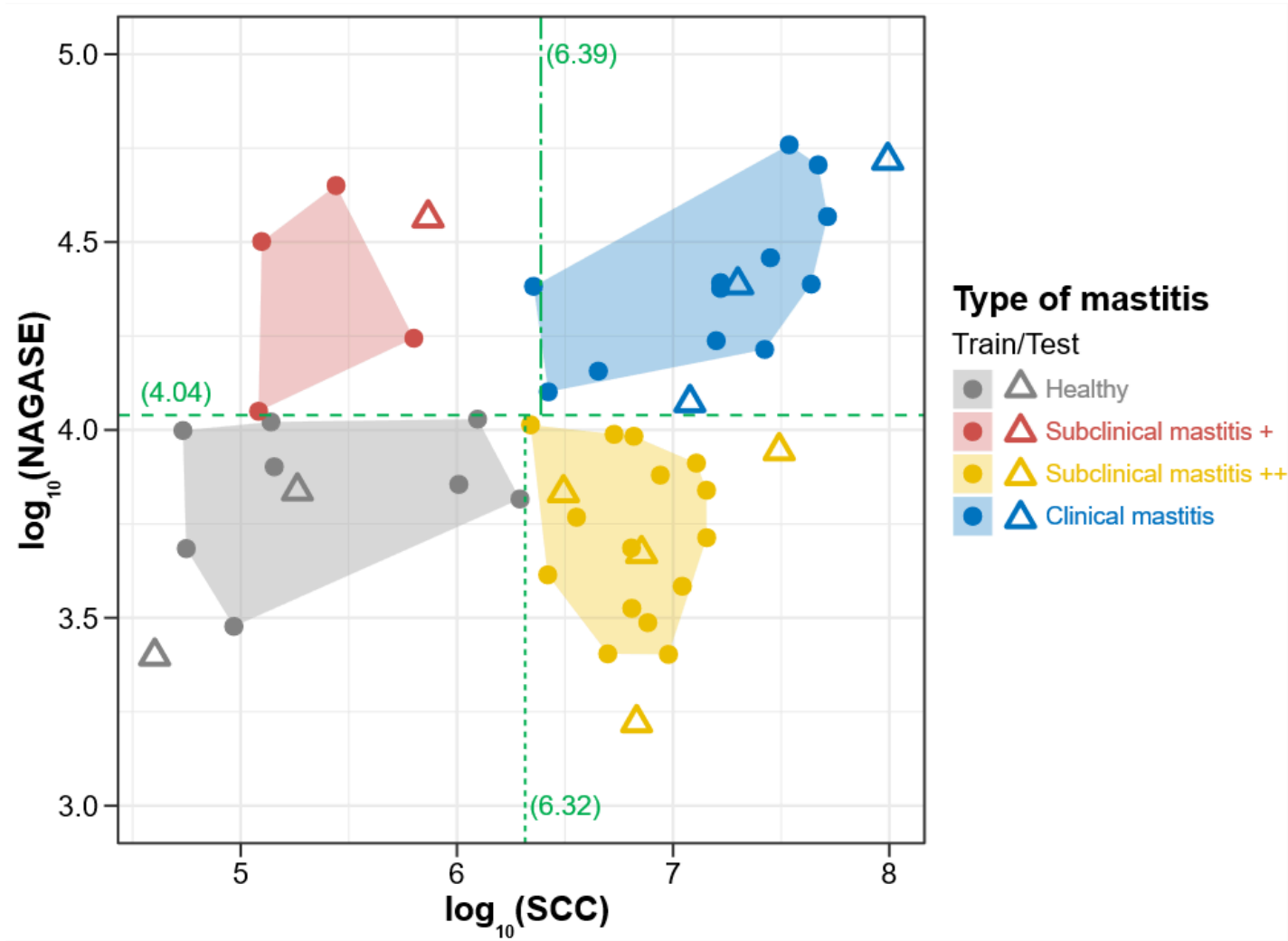


Figure 2

Population of field goats in relation to (logarithmic) values of NAGase activity and somatic cell count (SCC) observed in milk. The figure also shows the result of exploratory classification analysis using 80% and 20% of the data subsets, respectively. Thus, different green dashed lines indicate the cutoff point(s) in each variable found in the training of the decision tree model. Circled dots within each shaded polygon indicate the goats that participated in the training population. Triangular points indicate individuals who participated in the test population (validation).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTables.docx](#)