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Factors affecting N-acetyl-β-D-glucosaminidase as an indicator for mastitis detection in dairy sheep

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ABSTRACT

The study of new indirect methods for mastitis detection is of great relevance both at the economic level of the farm and dairies, and in terms of consumer health, and animal welfare. These methods help us to monitor the disease and speed up the decision-making process on treatment of the affected animal and the destination of the milk. The main aim of this work was to study the effect of intramammary infection and other non-infectious factors on the activity of the enzyme N-acetyl-β-D-glucosaminidase (**NAGase**) in milk, in order to evaluate its use as an indicator for the early diagnosis of mastitis in sheep that could be less expensive, easier to measure and a better marker of inflammation or complementary to existing methods such as somatic cell count (**SCC**). Seven biweekly samplings were carried out, in which NAGase activity, SCC and milk were analyzed. Glands were classified according to their sanitary status based on the results of the SCC and bacteriological analysis. Non-infectious factors such as lactation stage, parity number and milking session had a statistically significant effect on NAGase values, finding the highest NAGase values at the onset and end of the study, in infectious mastitic glands of multiparous females and at morning milking. However, among the NAGase variation factors studied, the health status of the gland was the factor that caused the highest variation in enzyme levels, with infectious mastitic glands showing higher values than healthy glands. The predictive ability of NAGase was also studied by means of several logistic regression models, with the one that included NAGase together with lactation stage and parity obtaining the best results if sensitivity is to be prioritized, or the model that included NAGase, lactation stage, parity, milking and production if specificity is to be prioritized. From the results obtained, it can be concluded that the use of NAGase as an intramammary infection detection method in sheep can be useful when non-infectious factors that cause changes in the concentration of the enzyme are also considered.

Implications

Early detection of mastitis is of great importance to achieve more profitable farms with better sanitary control. The objective of this work was to study whether the enzyme N-acetyl-β-D-glucosaminidase can be a variable of choice for the detection of mastitis in dairy sheep. The results showed that inflammation and intramammary infection caused an increase in N-acetyl-β-D-glucosaminidase, so it could be useful in the early detection of the disease. Other factors such as lactation stage, parity, milking and production affected N-acetyl-β-D-glucosaminidase values to a lesser extent, so it was also necessary to include them in the algorithm.

Introduction

Mastitis is one of the biggest problems for sheep milk production. It causes serious economic problems as a consequence of the drop in production and cheese yield (Martí de Olives et al., 2013), the premature culling of affected animals, treatment costs, losses of milk not considered fit for consumption, as well as the decrease in the growth of lambs due to the lower production and quality of milk from mastitic ewes (Moroni et al., 2007). They also pose a health risk to the consumer due to the presence of microorganisms and the risk of the presence of antibiotic residues in milk (Bergonier and Berthelot, 2003) and cause an animal welfare prob-

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lem (Gougoulis et al., 2010). In addition, the decrease in milk production negatively affects the environmental impact, since with more productive animals, we can obtain the same amount of milk with fewer animals and thus pollute the environment less (Capper et al., 2009).

N-acetyl-β-D-glucosaminidase (**NAGase**) is a lysosomal intracellular enzyme found in milk-producing epithelial cells and leukocytes. Due to the damage to cell membranes and the change in vascular permeability that occurs as a consequence of the inflammatory reaction in the gland during mastitis, NAGase is released into the milk, which is why this type of enzyme increases markedly in the milk during the disease (Kitchen et al., 1978).

A large number of studies in cattle have shown that there is an increase in NAGase activity in milk from infectious mastitic glands compared to healthy glands. Åkerstedt et al. (2011) obtained NAGase levels for healthy glands of between 0.8 and 6.1 U/L, while the mean value for glands with infectious clinical mastitis was 25.0 U/L. Kalmus et al. (2013) and Hovinen et al. (2016) concluded that enzyme activity was related to the severity of clinical signs: the more severe, the higher the NAGase levels. Nyman et al. (2014) in cattle found that intramammary infection status was statistically significantly associated with NAGase values, although it explained only 2% of the variation. It was also observed that parity, milk production, lactation stage, milk urea concentration and time of sampling affected the variable. Therefore, they conclude that it is necessary to study whether the diagnostic properties of NAGase would be improved if these non-infectious factors were taken into account when detecting animals with the disease. Although most published articles have observed a positive correlation between increased activity of this enzyme and intramammary infection (**IMI**), there are some publications in which no such relationship was found (Piccinini et al., 2007).

Despite the importance of this disease in sheep, there are few studies on this enzyme compared to in cattle. Moroni and Cuccuru (2001) observed differences between NAGase activity in healthy and infected glands. Leitner et al. (2004a) obtained statistically significant differences in NAGase values between healthy and infected glands, with values even varying depending on the pathogen that caused IMI. However, the effect of other variables and physiological factors on activity in sheep remains to be studied.

The aim of this work was to study the effect of IMI and other non-infectious factors, such as lactation stage, parity number and milking session, on NAGase enzyme activity in sheep milk, as well as its relationship with somatic cell count (**SCC**) and milk production. In addition, taking all these factors into account, the predictive ability of NAGase was studied in order to find the best algorithm to carry out the detection of IMI in a faster and easier way than other disease detection methods currently used, such as bacteriological culture or testing by PCR.

Material and methods

Animal management

This observational study was carried out at the Miguel Hernández University, in the facilities of the Small Ruminants Teaching and Experimental Farm of the Polytechnic School of Orihuela. The study included 82 Manchega ewes, 24 primiparous and 58 multiparous. The production system was intensive, with housing throughout the year in a 100 m^2 pen (1.19 m^2/animal), with access to an open-air area throughout the day. The average temperature at the beginning of the study was 15 °C, progressively increasing to an average of 23 °C at the end of the study. The reproductive rhythm practiced on the farm was one lambing per year, introducing the males at the beginning of September at a rate of one male for every 10 females and remaining with them for 28 days. Lambing took place in February, and the lambs were weaned and fed artificially. Mothers began to be mechanically milked twice a day (0800 and 1600 h). The milking routine was simple: attachment of the cluster without teat predipping, machine milking and machine stripping, and a postdipping of teats with a bath of iodine solution. The milking parameters used were: Pulsation ratio: 50%, pulsation rate: 180 puls/min, and vacuum level: 36 KPa. The animals were fed throughout the trial daily with 2.5 kg of complete ration (Unifeed) divided into two doses a day and had free access to straw and water. The diet provided to the animals was mainly based on corn, soybean, barley and wheat. The care of the animals used in this study was carried out in accordance with European regulations (Directive 2010/63/EU of the European Parliament and of the Council of animals used for scientific purposes).

Study design

Sampling methodology

During the 3.5 months of the study, a sampling was carried out every 15 days, the first check being two weeks after lambing, for a total of seven samplings. In the morning session, prior to milking, a 5 mL aseptic sample (teat cleaning with ethanol and collection in a sterile tube) was collected for microbiological analysis. Subsequently, each mammary gland was milked in a volumetric meter to record production and take a 100 mL sample of the total milk, which was used for SCC and NAGase enzyme activity analysis (at both milkings). The individual ewe was considered the experimental unit.

Analyses in the laboratory

To perform bacteriological analysis, 20 μL of milk were seeded on sheep blood-agar plates and incubated at 37 °C. Bacterial colony growth was counted at 24, 48 and 72 h, taking into account growth rate, culture appearance, density and characteristic features of the colonies. Following the recommendations of the methodology proposed by the National Mastitis Council (Harmon et al., 1990), positive cultures were considered those in which the growth of five or more identical colonies per plate was observed. In cases in which the growth of three or more different colonies was observed, the culture was classified as contaminated. Gram staining was used to identify the bacterial genus, and the catalase test was used for colonies compatible with Gram-positive microorganisms. To identify the bacterial species in staphylococci, the Apistah kit (BioMerieux, France) was used. These analyses were always carried out by the same person.

The SCC (× 1 000 cells/mL) was analyzed from azidiol-preserved samples by the fluoro-opto-electronic method (Model Fossomatic 5000, Foss Electric, Hillerød, Denmark) at LICOVAL (Laboratorio Interprofesional Lechero de la Comunidad Valenciana). The laboratory was accredited and complied with the ISO standard 8196-3. The inter-assay agreement was 5%, with analyses whose replications had a CV less than 4% (intra-assay) being accepted as valid. Analysis of NAGase activity (μM/min per mL) was performed using a fluorescence spectrophotometer (Fluostar Optima, BMG Labtech, Germany) according to the method described by Kitchen et al. (1978). Analyses were always performed by the same person. The inter-assay agreement was 15%, with analyses whose replications had a CV less than 15% (intra-assay) being accepted as valid.

Definition of the health status of the gland

Based on the experience of the research group and the literature reviewed (Las Heras et al., 2002; Burriel, 1997), glands were classified according to their health status based on the following criteria:

glands were considered to have infectious mastitis if the SCC was higher than 400 000 cells/mL and the bacteriological culture was positive in two or more consecutive samplings. Glands in which the SCC remained above the same value, but the bacteriological analysis was negative, were considered to have non-specific mastitis. Glands in which, despite positive bacteriological culture, the SCC remained below 400 000 cells/mL, were classified as latent infection. And finally, if the SCC was lower than 400 000 cells/mL and the bacteriological culture was positive only in an isolated sampling, the glands were considered healthy. In this study, clinical symptoms were not considered to define the presence or absence of the disease, only the presence of microorganisms and the SCC were taken into account.

Description of critical methods

To ensure the reproducibility of the published research, we present in the Supplementary Materials the codes of the statistical models referred to in the Inferential analysis between NAGase and explanatory variables and in the Predictive models for IMI detection. First, the R code of the mixed linear regression model is presented to complete the understanding of the inferential analysis. Second, the predictive model code is presented in detail along with additional measures that provide information for the classification of mastitis. The full description of the statistical models is explained in the Statistical Methodology section.

Validation of methods used

Validation of the predictive regression models was addressed by internal validation of the Area Under Curve. The internal validation process of the predictive ability was carried out using K-fold cross-validation, where the sample was divided into K = 10 folds. The assessment of the calibration performance of the predictive models was carried out. Regarding inter and intra-ewe variability, given the repeated measurements along samplings in each gland, nested random intercepts were considered in the inferential analysis. Therefore, SD measures and residual deviation were reported.

Statistical methodology

The distribution of the variables was evaluated by box plots and histograms. SCC (x 1 000 cells/mL) and NAGase (μM/min per mL) values were transformed using the natural logarithm to correct for skewness of their distribution and ensure normality of the data. These transformed variables were used in the regression models, while in the case of descriptive or non-parametric tests, the original scale variable was used to facilitate interpretation. Descriptive analyses were performed using the mean (SD) and median (1st, 3rd quartile), while absolute and relative frequencies were used for qualitative variables.

The correlation between quantitative variables was assessed using the bootstrapping correlation coefficient by means of R library rmcorr (Bakdash and Marusich, 2023), given the non-independence of the observations. Confidence intervals were calculated using bootstrapping since it does not require distributional assumptions. The correlation between NAGase and SCC was calculated globally, separated by the two milking times (morning, evening) and taking into account the SCC levels (< 200 000, between 200 000 and 400 000 and > 400 000 cells/mL) to assess the degree of correlation at each of the times. The relationship between NAGase and production was also studied.

Multiple univariate comparisons were performed using mixed regression models. Subsequently, two-by-two multiple comparisons were performed using Tukey's test and the Bonferroni correction. For the inferential analysis of the factors considered (lactation stage, parity, milking and health status), a mixed linear regression analysis was performed considering that NAGase is a continuous quantitative variable and the correlation between repeated measurements of the same animal (covariance structure). The effects considered in this study were: lactation stage (Sampling: 1, 2, 3, 4, 5, 6 and 7), parity number (primiparous or multiparous), milking session (Milking: morning or evening), gland health status (healthy, infectious mastitis, non-specific mastitis or latent infection) and their first-order interactions. The random effect of gland (right or left) nested to the ewe was indicated in the model to explain the clustering of mammary glands within animals. SD of intra-ewe and inter-ewe was reported. An unstructured covariance structure was used to account for repeated animal measurements. The final model provided the best fit for the data in each variable studied compared to different models that considered other covariances and hierarchical structures (evaluated using the Akaike information criterion).

In order to evaluate the predictive ability of NAGase and the other factors considered (lactation stage, parity, milking and udder production), logistic regression models were carried out to predict IMI. To compare the predictive ability of NAGase with SCC, logistic regression models with SCC have been performed as well. The predictive ability of the models was evaluated using the Area Under Curve (**AUC**), Nagelkerke's R^2 and the threshold value for which the sensitivity, specificity, precision, positive predictive value and negative predictive value were calculated. To assess the calibration performance of predictive models, plot calibration curves and statistics regarding slope were obtained. Analyses were carried out using the nlme (Pinheiro et al., 2021), rms (Harrell, 2022), Calibration Curves (De Cock et al., 2023) and R statistical software (R Core Team, 2021) libraries. A *P*-value < 0.05 was considered statistically significant.

Results

Mastitis prevalence

The prevalence of intramammary infection obtained in this study was between 13 and 20%, being between 7 and 9% glands with infectious mastitis and between 6 and 11% glands with latent infection (Table 1). This prevalence remained stable throughout the entire study. Considering gland health status, both the percentage of infectious mastitic glands and the percentage of latently infected glands remained stable in all samplings, between 7–8% and 7–10%, respectively (Table 1). However, in sampling 1, the lowest percentage of healthy glands (55%) and the largest proportion of non-specific mastitis (30%) were observed, while from the second sampling onwards, the percentage of healthy glands rose to around 80% and that of non-specific mastitis decreased to 0 and 3% (Table 1). It was found that practically all the animals with non-specific mastitis in the first sampling showed an increase in SCC bilaterally, values that decreased to normal in the second sampling. Taking into account the parity number, the percentage of infected glands in primiparous ewes was between 0 and 6%, while in multiparous ewes, this percentage was higher (19–23%).

Pathogens isolated during the study

Table 2 shows the NAGase and SCC values according to the pathogen that caused the infection. The most frequently isolated pathogens were *staphylococci* (96.9%), with *S. xylosus* and *S. lentus* being the most frequently found (33.8 and 21.5%, respectively). The highest values were obtained in glands infected by *S. aureus* (223.7 μM/min per mL and 13 881 000 cells/mL), followed by those infected by *S. xylosus* with values of 123.9 μM/min per mL and 466 500 cells/mL. The lowest values were found in glands infected with *S. lentus*, being lower than those of healthy glands (16.6 μM/

Table 1
Prevalence in ewes of gland health status throughout the study and overall NAGase values (μM/min per mL).

Sampling (n° of glands)	Healthy	Infectious mastitis	Non-specific mastitis	Latent infection
1 (n = 314)	173 (55%)	28 (9%)	95 (30%)	18 (6%)
2 (n = 322)	260 (81%)	28 (9%)	9 (3%)	24 (7%)
3 (n = 322)	260 (81%)	28 (9%)	9 (3%)	24 (7%)
4 (n = 320)	260 (83%)	24 (8%)	0 (0%)	30 (9%)
5 (n = 316)	250 (81%)	26 (8%)	0 (0%)	34 (11%)
6 (n = 316)	250 (81%)	22 (7%)	12 (4%)	26 (8%)
7 (n = 290)	236 (83%)	24 (9%)	0 (0%)	24 (8%)
NAGase	40.1	152.7 (113)	109.8 (103.4)	49
Mean (SD)	(32.5)	116.1	86	(36.5)
Median	32.4	(74.5, 202.6)	(38.4, 140.3)	42.8
(1st, 3rd Q.)	(19, 51.5)			(4.3, 62.3)

Abbreviations: NAGase = N-acetyl-β-D-glucosaminidase; Q = quartile.

Table 2
N-acetyl-β-D-glucosaminidase and somatic cell count (Median (1st, 3rd Q.) of milk from morning milking of ewes depending on the type of pathogen causing the infection.

Pathogen	NAGase (μM/min per mL)	n	SCC (× 1 000 cells/mL)	n
Healthy glands	34.2 (20.3, 57.8)	847	69 (43, 121.3)	900
Enterobacteriaceae	71.5 (63.6, 80)	4	260 (194, 277.8)	4
Staphylococcus spp.	55.1 (32, 106.4)	28	86 (52, 241)	33
S. aureus	223.7 (167.7, 284.6)	3	13 881 (10 947, 23 192)	6
S. xylosus	123.9 (66.3, 197.2)	41	466.5 (181.5, 2 197.5)	44
S. caprae	59.4 (14.6, 104.5)	11	80 (50, 1 099)	9
S. haemolyticus	45.4 (33.8, 52.2)	3	115 (84.5, 135.5)	3
S. sciuri	45.2 (45.2, 45.2)	1	58 (52.5, 66)	3
S. lentus	16.6 (13.3, 34.6)	24	61.5 (39.5, 92.8)	28

Abbreviations: NAGase = N-acetyl-β-D-glucosaminidase; Q = quartile; SCC = somatic cell count; n = number of glands.

min per mL, 61 500 cells/mL and 34.2 μM/min per mL, 69 000 cells/mL, respectively).

In the Supplementary Material, we can see three figures showing the values of NAGase, SCC and bacteriological culture of the sheep with the most frequent occurrence of *S. aureus* (Fig. S1), *S. xilosus* (Fig. S2) and *S. lentus* (Fig. S3). We can observe the difference in response that the pathogen produced in each of the animals. The SCC values were highest in the glands with *S. aureus* (Fig. S1), followed by those infected with *S. xilosus* (Fig. S2) and finally, the values of the glands infected with *S. lentus* (Fig. S3), in which no differences with the values of the healthy glands were observed. Regarding NAGase, no major differences were seen between the first two animals, but with the animal with *S. lentus*, the values were much lower. With respect to the bacteriology results, we observed that there were no changes in the classification throughout the lactation, all the samples that were positive in the first control remained positive in the last control, as did the negative samples. In general, high SCC levels corresponded with positive bacteriology, but in both Fig. S1 and Fig. S2, we can see that in some controls, there was an increase of SCC above 400 000 cells/mL with negative bacteriology.

N-acetyl-β-D-glucosaminidase variation factors

In Table 3, we can see the results of the inferential analysis between NAGase and the non-infectious factors. The mixed regression model code is presented in Supplementary Material S1. The results showed that the effect of lactation stage was statistically significant, with the highest NAGase values observed in the first sampling, decreasing considerably in sampling 3 (d = −0.36, $P < 0.001$), and then gradually increasing until reaching a maximum in sampling 7 (d = 0.18, $P = 0.011$) (Fig. 1). Regarding milking, NAGase values were statistically significantly lower in the evening than in the morning (d = −0.09, $P = 0.004$) (Fig. 2). As for parity, a statistically significant interaction with gland health status was observed, such that, in primiparous females, a statistically significant positive difference was detected (d = 0.63, $P = 0.025$) of infectious mastitic (81.39 μM/min per mL) versus healthy (37.32 μM/min per mL) glands, with no statistically significant difference of the latter from latently infected (55.14 μM/min per mL) and non-specific mastitis (52.04 μM/min per mL) glands ($P = 0.575$ and $P = 0.169$, respectively) (Fig. 2). In contrast, in multiparous ewes, infectious mastitic (128.94 μM/min per mL) and non-specific mastitis (100.04 μM/min per mL) glands had statistically higher log (NAGase) values (d = 0.67, $P = 0.026$ and d = 0.60, $P < 0.001$, respectively) than healthy ones (29.77 μM/min per mL). The results when comparing healthy glands and those with latent infection (41.89 μM/min per mL) were inconclusive ($P = 0.983$). The between-female variance parameter was 0.24 (95% CI [0.15, 0.38]), and the within-animal variance parameter was 0.003 [5 e-5, 0.12]. Given that the residual variance of the model was 0.54 [0.37, 0.79], this results in an intraclass correlation coefficient of $\rho_{ewe} = 0.309$. Therefore, the proportion of the total variance explained by the grouping structure of female ewes is low, meaning that results are not very dependent on the ewes to which the observations belong.

The Q-Q plot (Fig. S4) shows that residuals are heavier-tailed compared to the normal distribution, as specified in the Table 3-footer. The most notable deviation points to low NAGase values. This is explained by the observational nature of the study given that there are much more healthy glands than infectious or non-specific mastitis or latent infections (see Table 1, with percentages of healthy glands around 80% throughout the study). This is representative of what usually happens on farms, and healthy glands have lower values of NAGase. That explains the asymmetrical distribution of NAGase and the deviation observed on the left part of the Q-Q plot. The highest extreme values may be due to the animal being in a state of immunosuppression, which makes it easier for the amount of bacteria inside the animal to be higher than in other animals, causing more cellular damage and higher than usual

Table 3
Inferential analysis between N-acetyl-β-D-glucosaminidase and explanatory variables in sheep milk[1].

Outcome: log(NAGase)	Estimate (d)	SE	95% Confidence Interval		*P*-value
(Intercept)	3.75	0.1	[3.55,	3.94]	< 0.001
Sampling – 2	−0.05	0.07	[−0.18,	0.09]	0.476
Sampling – 3	−0.36	0.07	[−0.49,	−0.23]	< 0.001
Sampling – 4	−0.19	0.07	[−0.32,	−0.05]	0.006
Sampling – 5	−0.06	0.07	[−0.20,	0.07]	0.346
Sampling – 6	0.01	0.07	[−0.12,	0.14]	0.896
Sampling – 7	0.18	0.07	[0.04,	0.33]	0.011
Milking – Evening	−0.09	0.03	[−0.15,	−0.03]	0.004
Parity – Multiparous	−0.34	0.05	[−0.43,	−0.25]	< 0.001
Gland Health Status - Infectious mastitis	0.63	0.28	[0.08,	1.19]	0.025
Gland Health Status - Non-specific mastitis	0.21	0.15	[−0.09,	0.50]	0.169
Gland Health Status - Latent infection	0.20	0.35	[−0.49,	0.89]	0.575
Multiparous:Infectious mastitis	0.67	0.30	[0.08,	1.25]	0.026
Multiparous: Non-specific mastitis	0.60	0.18	[0.25,	0.94]	< 0.001
Multiparous: Latent infection	0.01	0.36	[−0.69,	0.71]	0.983
Var Ewe:Gland(Intercept)	0.003		[5 e-5,	0.12]	
Var ewe(Intercept)	0.24		[0.15,	0.38]	
Residual	0.54		[0.37,	0.79]	

Abbreviations: NAGase = N-acetyl-β-D-glucosaminidase; Var = Variance.
[1] Model diagnosis: normality and homoscedasticity of residuals can be found in Supplementary Fig. S4. Q-Q plot shows that residuals have heavier tails than the normal distribution, which could lead to biased estimates. This could be problematic for the prediction of data points, but is not the aim of the study.

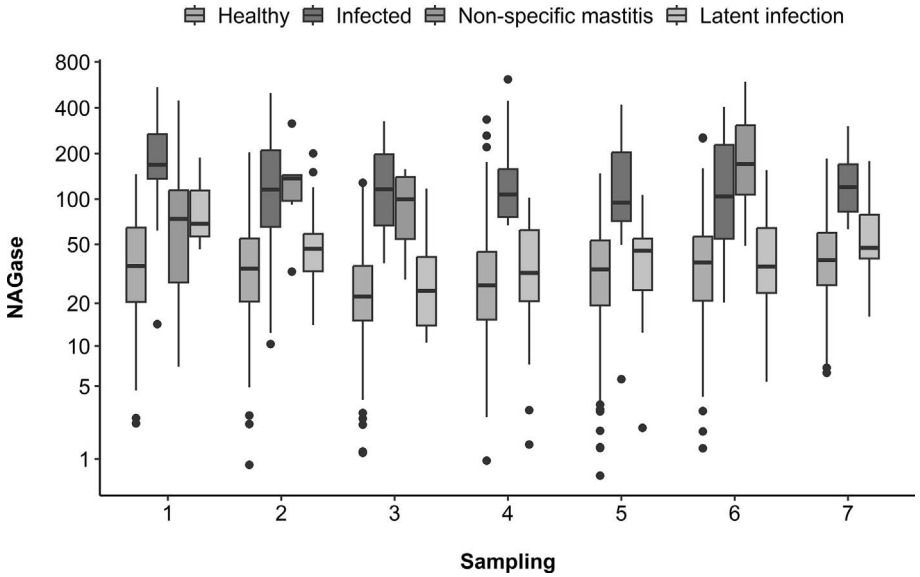

Fig. 1. N-acetyl-β-D-glucosaminidase values (μM/min per mL) throughout the study according to the health status of the sheep glands. Abbreviations: NAGase = N-acetyl-β-D-glucosaminidase.

NAGase values. The 60% of the samples with low NAGase values considered outliers came from glands with a higher than average milk production, and more than 70% of the samples had SCC values lower than or equal to 100 000 cells/mL, this may indicate that there is a dilution effect that has caused the decrease in the values of both NAGase and SCC. The rest of the values can be considered as outliers typical of a sample with variability.

Univariate comparisons were made of the health status of the glands in each of the conditions (Table 4). In the seven samplings of the study, statistically significant differences ($P < 0.001$) were found between the NAGase values of infectious mastitic and healthy glands, obtaining the highest estimated difference in the first one. Multiparous females showed greater differences between health states than primiparous females. Differences between pairs of health statuses were higher at the evening milking than in the morning milking.

Correlation between N-acetyl-β-D-glucosaminidase, somatic cell count and production

Overall, NAGase and SCC were positively correlated, although to a weak degree (r = 0.37) (Table 5). The correlation coefficients for each milking session were higher in the afternoon milking, except in the range between 200 000 and 400 000 cells/mL. Depending on the SCC interval, it was observed that the higher the SCC, the higher the correlation coefficient (Supplementary Fig. S5). In the evening, milking of the global sample was obtained a moderate correlation between the two variables (r = 0.48). In Supplementary Fig. S6 is represented SCC and NAGase values along samplings for primiparous and multiparous ewes. This figure shows that when bacteriology is positive, primiparous sheep show higher values of SCC and NAGase. This also happens in multiparous sheep, although in

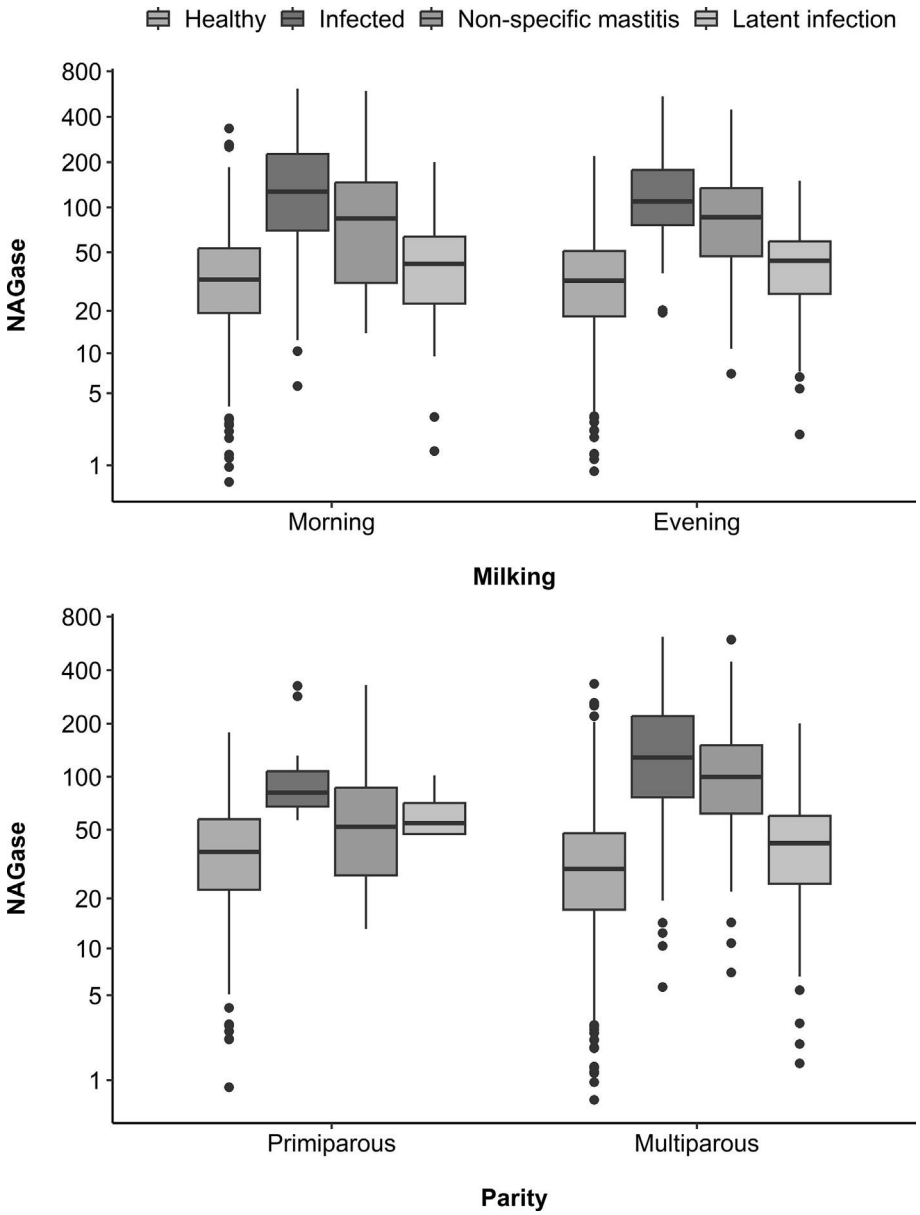

Fig. 2. N-acetyl-β-D-glucosaminidase values (μM/min per mL) according to parity (lower panel) and milking (upper panel) depending on the health status of the ewe's glands. Abbreviations: NAGase = N-acetyl-β-D-glucosaminidase.

a milder form. Regarding milk production, there was a statistically significant negative association with NAGase (−0.39).

Predictive models

Predictive model code and additional measures for predictive ability assessment are presented in detail in Supplementary Material S1. All predictive models run resulted in an AUC between 0.74 and 0.80 (Table 6). The models with the highest AUC are models 1, 2, 3, 5 and 7. In model 1, NAGasa and all study variables (lactation stage, parity, milking and production) were included, in model 2, production was excluded, in 3, milking was excluded and in 5, milking and production were excluded at the same time. Models 2, 3 and 5 obtained predictive ability results similar to model 1, which included all factors, indicating that these factors do not improve the predictive ability of the model. Adding the SCC to model 1 (model 7) also did not improve the predictive ability since the same AUC value was obtained (0.80). Replacing NAGase with SCC in model 1 (model 6) slightly worsened the predictive ability of the previous models, as a lower AUC value (0.78) was obtained. The predictive model that excludes the variable parity number from model 1 (model 4) reports the lowest AUC, indicating that this variable does provide information for the classification of IMI.

Regarding the evaluation of the R^2 index, model 7 is the one that presented the highest value of explained variance (31%), followed by model 6 (27%) and models 1, 2, 3, and 5 (26%). As with the AUC, model 4 is the one that presented the lowest value with 19%. It was corroborated that removing the milking and/or udder production variables from the model hardly reduced the explained variance, since it remained at 26%. However, taking out the variable parity number reduced the explained variance of the R^2 by 7 units (from 26 to 19%) and the addition of SCC increased it by 5 units (from 26 to 31%).

For each of the models, three cut-off values were calculated to classify the samples as infected or healthy. The first threshold is the one that generated the highest sum of sensitivity and speci-

Table 4
Difference and 95% confidence interval in log(NAGase) according to gland health status, throughout the study, parity number and milking session in ewes.

Sampling	Infectious mastitis - Healthy	Non-specific mastitis - healthy	Latent infection - healthy	Infectious mastitis - Non-specific mastitis	Infectious mastitis - Latent infection	Non-specific mastitis - Latent infection
1	1.88*** [1.35, 2.42]	0.60*** [0.22, 0.98]	1.15* [0.10, 2.19]	1.28*** [0.68, 1.89]	0.74 [−0.35, 1.82]	−0.54 [−1.63, 0.55]
2	1.19*** [0.77, 1.61]	0.83* [0.10, 1.57]	0.44* [0.02, 0.86]	0.36 [−0.48, 1.19]	0.75** [0.19, 1.31]	0.39 [−0.45, 1.23]
3	1.62*** [1.21, 2.02]	1.08*** [0.40, 1.76]	0.16 [−0.27, 0.59]	0.53 [−0.21, 1.28]	1.45*** [0.88, 2.02]	0.92* [0.12, 1.71]
4	1.38*** [0.98, 1.78]		−0.12 [−0.46, 0.22]		1.50*** [−1.99, −1.01]	
5	1.09*** [0.57, 1.60]		0.04 [−0.33, 0.40]		1.05*** [0.44, 1.66]	
6	0.90*** [0.30, 1.50]	1.41*** [0.69, 2.12]	−0.20 [−0.66, 0.27]	−0.50 [−1.28, 0.27]	1.10*** [0.38, 1.81]	1.60*** [0.80, 2.40]
7	0.86*** [0.53, 1.19]		0.05 [−0.20, 0.31]		0.81*** [0.41, 1.21]	
Primiparous	0.64* [0.11, 1.17]	0.30* [0.02, 0.58]	0.11 [−0.69, 0.92]	0.34 [−0.26, 0.93]	0.53 [−0.43, 1.49]	0.19 [−0.64, 1.03]
Multiparous	1.25*** [1.02, 1.48]	0.86*** [0.62, 1.10]	0.18* [0.01, 0.36]	0.39** [0.07, 0.70]	1.07*** [0.79, 1.35]	0.68*** [0.39, 0.97]
Morning	1.04*** [0.76, 1.32]	0.59*** [0.29, 0.88]	0.04 [−0.19, 0.27]	0.45* [0.06, 0.83]	0.99*** [0.64, 1.34]	0.54*** [0.17, 0.91]
Evening	1.31*** [1.03, 1.59]	0.78*** [0.53, 1.03]	0.14 [−0.09, 0.37]	0.53*** [0.17, 0.86]	1.17*** [0.82, 1.52]	0.64*** [0.31, 0.97]

P-values from multiple comparisons were corrected using the Bonferroni method.
Superscript "*" means $P < 0.05$.
Superscript "**" indicates that $P < 0.01$.
Superscript "***" indicates that $P < 0.001$.
All other markers did not show statistically significant differences.
Abbreviations: NAGase = N-acetyl-β-D-glucosaminidase.

Table 5
Correlation between N-acetyl-β-D-glucosaminidase and somatic cell count based on to somatic cell values in sheep milk[1].

Bootstrapping Correlation Coefficient r, [95% Confidence Interval]	Milking Morning	Milking Evening	Global
Global SCC	0.29 [0.06, 0.52]	0.48 [0.28, 0.63]	0.37 [0.16, 0.53]
SCC < 200	0.03 [−0.04, 0.10]	0.07 [−0.03, 0.16]	0.01 [−0.04, 0.06]
200 < SCC < 400	−0.43 [−0.91, 0.11]	−0.27 [−0.84, 0.39]	−0.06 [−0.36, 0.23]
SCC > 400	0.03 [−0.46, 0.50]	0.33 [0.14, 0.57]	0.20 [−0.09, 0.49]

Abbreviations: NAGase = N-acetyl-β-D-glucosaminidase; SCC = somatic cell count.
[1] The scatterplot of NAGase and SCC values can be found in Supplementary Fig. S5.

ficity. In the following two, two combinations of sensitivity and specificity were reported, enhancing one of the two in each case. Model 7, which included all variables, presented the highest combined sensitivity (73%) and specificity (71%) for a cut-off value of 0.15, followed with very similar values by models 1, 2, 3 and 5. Model 1, which included NAGase with all variation factors, reported a sensitivity of 64% and a specificity of 79% for a cut-off value of 0.21. Equal values were obtained for model 2, excluding production (cut-off value 0.20, sensitivity 64% and sensitivity 79%). In the models in which milking was excluded (model 3) and production and milking at the same time (model 5), the values were very similar, but with a small increase in sensitivity at the expense of specificity (cut-off values 0.16 and 0.16, sensitivity of 72 and 73% and specificity of 71 and 70%). Model 6, which included the SCC with all variation factors, had a slightly lower combined sensitivity and specificity value than the previous models with the highest specificity value (96%) and the lowest sensitivity value (45%) for a cut-off value of 0.30. Finally, model 4, which included NAGase with all values except parity, had the lowest overall sensitivity (67%) and specificity (72%) values with a cut-off value of 0.16 (Table 6). Calibration curves were similar in all models (Fig. S7). Given that an internal validation was performed, we focused on the calibration slope which resulted in a value of 1.00 [0.86, 1.14]. This is the target value and given that the calibration curve is over the diagonal the predicted risks correspond well to observed proportions. Nevertheless, there seems to be a systematic pattern, which may be due to the low prevalence of IMI, showing slightly underestimated risks for extreme predicted probabilities. Further studies are needed to improve calibration using external validation procedures.

Discussion

Animal health status

The prevalence of IMI at gland level found in this study (13–20%) was similar to that observed in other previous works such as that of McDougall et al. (2002), who observed a prevalence of 19% at the beginning of lactation or Al-Majali and Jawabreh (2003) who obtained 10%. Considering the influence of lactation stage on prevalence, IMI percentages remained stable in all samplings, which agrees with Leitner et al. (2001), who observed that in 90% of cases, the health status of the glands remained stable throughout lactation. However, McDougall et al. (2002) found that a very high percentage of infected ewes healed spontaneously (93.8%). With regard to the healthy glands and those with non-specific mastitis, in the first sampling, the percentages were 55.1 and 30.3%, while in the second sampling, almost all the non-specific mastitis became healthy with percentages of 81 and 3%, respectively, these values were maintained throughout the study. Almost all the animals that progressed from non-specific mastitis to being healthy, presented in the first sampling an increase in

Table 6
Predictive models for the detection of intramammary infections in ewes.

Regression models	AUC [95% CI]	AUC int. valid	R^2	Threshold Value	Se	Sp	Accuracy	PPV	NPV
1) Mod: log(NAGase) + Lactation stage + parity + milking + Pudder_l	0.80	0.80	26%	0.21	64%	79%	77%	92%	35%
	[0.77,			0.11	80%	59%	62%	94%	26%
	0.83]			0.27	50%	88%	83%	91%	43%
2) Mod: log(NAGase) + Lactation stage + parity + milking	0.80	0.80	26%	0.20	64%	79%	77%	92%	35%
	[0.77,			0.11	80%	59%	62%	94%	26%
	0.83]			0.21	62%	80%	77%	92%	35%
3) Mod: log(NAGase) + Lactation stage + parity + Pudder_l	0.80	0.80	26%	0.16	72%	71%	71%	93%	30%
	[0.77,			0.12	80%	60%	63%	94%	26%
	0.83]			0.21	62%	80%	77%	92%	35%
4) Mod: log(NAGase) + Lactation stage + milking + Pudder_l	0.74	0.74	19%	0.16	67%	72%	71%	93%	30%
	[0.71,			0.10	80%	50%	54%	93%	22%
	0.78]			0.19	56%	80%	76%	91%	33%
5) Mod: log(NAGase) + Lactation stage + parity	0.80	0.80	26%	0.16	73%	70%	70%	94%	30%
	[0.77,			0.12	80%	60%	63%	94%	26%
	0.83]			0.21	62%	80%	78%	92%	36%
6) Mod: log(SCC) + Lactation stage + parity + milking + Pudder_l	0.78	0.77	27%	0.30	45%	96%	88%	96%	44%
	[0.75,			0.11	80%	54%	58%	94%	23%
	0.81]			0.19	57%	80%	76%	91%	34%
7) Mod: log(SCC) + log(NAGase) + Lactation stage + parity + milking	0.80	0.79	31%	0.15	73%	71%	71%	93%	31%
+ Pudder_l	[0.77,			0.11	80%	59%	62%	94%	26%
	0.83]			0.19	60%	81%	77%	92%	35%

Abbreviations: AUC = Area Under Curve; CI = Confidence interval; AUC int. valid = Area Under Curve internally validated using K-fold cross-validation; Se = Sensitivity; Sp = Specificity; PPV = Positive Predictive Value; NPV = Negative Predictive Value; Mod = Model; NAGase = N-acetyl-β-D-glucosaminidase; SCC = somatic cell count; Pudder_l = Production in liters.

SCC bilaterally in both glands, so we suspected that the inflammation of the first control could be due to some physiological reaction of the animal. This could be due to the fact that the first control was performed very close to lambing, and there could still be some physiological inflammatory reaction in the gland due to lambing and the adaptation of mechanical milking, together with a lower milk production that increased the concentration of SCC. The prevalence of mastitis was higher in multiparous (19–23%) than in primiparous females (0–6%), coinciding with the findings of other studies such as that of Leitner et al. (2003) or of Al-Majali and Jawabreh (2003).

Almost all of the isolated pathogens were staphylococci (96.9%), coinciding with McDougall et al. (2002) who found that 100% of subclinical mastitis were caused by staphylococci. S. aureus was the bacteria that caused the highest levels of NAGase (223.7 μM/min per mL) and SCC (13 881 000 cells/mL), which is consistent with Contreras et al. (2007), who described that this bacteria could cause severe clinical signs. Enterobacteriaceae also elicited high levels of NAGase and SCC (71.5 μM/min per mL and 260 000 cells/mL), although these were lower than those obtained by S. xylosus-infected glands with values of 123.9 μM/min per mL and 466 500 cells/mL. The rest of the coagulase-negative staphylococci elicited lower NAGase and SCC values than those produced by the major pathogens, which is consistent with that described by Leitner et al. (2001).

N-acetyl-β-D-glucosaminidase variation factors

In accordance with other studies, we found that mammary gland health status had a statistically significant effect on NAGase activity. The highest values of the enzyme were found in infectious mastitic glands (116.12 μM/min per mL), followed by those of glands with non-specific mastitis (85.95 μM/min per mL), by those of latently infected glands (42.75 μM/min per mL) and finally those of healthy glands (32.35 μM/min per mL). This is because infectious mastitis, as defined, was accompanied by inflammation and IMI that could increase cell destruction producing high levels of NAGase. The non-specific ones presented lower values, since they only presented inflammation (high SCC), and in those that presented latent infection, their values were lower than the previous ones because they presented lower SCC levels and a possibly not very severe IMI that did not cause high damage to the lactocytes. Since NAGase is present in both lactocytes and somatic cells, it can be considered both an indicator of inflammation (mastitis, presence of SCC) and of the presence of microorganisms (IMI with or without inflammation). These results are in agreement with those observed for NAGase in cattle by Kitchen et al. (1984), who found statistically significant differences according to whether the gland was considered healthy (negative bacteriology and SCC < 500 000 cells/mL, 18.3 μmol/L per min), with latent infection (positive bacteriology and SCC < 500 000 cell/mL, 20.6 μmol/L per min), with non-specific mastitis (negative bacteriology and SCC > 500 000 cell/mL, 55.8 μmol/L per min) or specific mastitis (positive bacteriology and SCC > 500 000 cell/mL, 62.1 μmol/L per min). In sheep, Leitner et al. (2001) observed statistically significant differences in their values, obtaining in healthy glands values of 0.855 μmol/L per min, in infectious mastitic glands infected with coagulase-negative *staphylococci* 3.37 μmol/L per min and in those infected with streptococci 4.89 μmol/L per min. Later in 2003, Leitner et al. again found the same effect due to IMI, but with values of 1.145 μmol/L per min in healthy glands and 3.655 μmol/L per min in infectious mastitic glands.

When studying the trend of NAGase values throughout lactation, we saw that the highest values were observed at the beginning and end of the study, and the lowest values were found in the intermediate samplings. This may be due to the increase in polymorphonuclear leukocytes that occurs after lambing and before the dry period (Fruganti et al., 1985), which could occur as a consequence of physiological and hormonal changes experienced by the ewe at these times that could make the mammary gland more susceptible to infection. It may also be due, as with SCC, to a dilution effect. The SCC evolution curve is inversely related to the milk production curve, Fuertes et al. (1998) observed that the SCC reached its minimum value in the 5th week postpartum, coinciding with the maximum milk production. This trend coincides with that reported in cows by Hovinen et al. (2016),

who observed that NAGase activity was highest during the first 30 days of lactation (1.04 pmol of 4-MU/min per μL milk at 20 °C), decreasing gradually until 100 days postpartum (0.56 pmol of 4-MU/min per μL milk at 20 °C).C), whereafter it tends to increase at the end of lactation in glands with mastitis. In sheep, Moroni and Cuccuru (2001) found that, for milk from both healthy and infected glands, enzyme activity was higher at the end of lactation than at the beginning (20.28 vs 15.62 nmol/mL per min for healthy and 45.30 vs 24.75 nmol/mL per min for infected). These results are different from those of Leitner et al. (2001), who were inconclusive regarding NAGase activity due to lactation stage for any of the 3 health states studied (healthy, coagulase- negative staphylococci-infected and streptococcus-infected glands). Although in all samplings, we observed statistically significant differences between healthy glands and those with infectious mastitis or non-specific mastitis, in the first one, we found the largest differences, making it the time that best discriminates diseased animals from healthy ones.

With respect to the effect of milking, although the highest values of NAGase activity were recorded at the morning milking, the largest differences occurred in the evening milking. In any case, the differences were so small that they are not biologically relevant; therefore, we could indistinctly use either of the two milking sessions to measure the enzyme. This coincides with that observed in sheep by Leitner et al. (2003), whose results were inconclusive regarding NAGase activity according to milking time and by Åkerstedt et al. (2011) in cattle, who observed small differences between milkings.

The last non-infectious factor studied was parity, which showed a statistically significant interaction with health status. Statistically significantly, both primiparous and multiparous females had the highest NAGase values for infectious mastitic glands, although the NAGase values for primiparous females were lower than those for multiparous females. This result is similar to that observed in cattle by Chagunda et al. (2006), who observed that in infected glands, the lowest values were found in primiparous cows. In primiparous females, only the differences between healthy glands and those with infectious mastitis or non-specific mastitis were statistically significant (no statistically significant differences with glands with latent infection); however, in multiparous females, the differences were statistically significant between healthy glands and the rest of the health statuses, and greater than in primiparous females. This could be explained by the fact that multiparous ewes have had a greater exposure to mammary pathogens throughout their lives, so there is a greater reaction of their immune system. These NAGase differences due to parity should be considered in mastitis prediction models in order to optimize their detection capability.

Correlation between N-acetyl-β-D-glucosaminidase, somatic cell count and production

The correlation between the two variables was higher the higher the SCC values were. A moderate positive correlation (r = 0.48) was obtained in the evening milking of the global sample, which was significantly higher than in the morning milking of the same sample (r = 0.29). This may be due to the fact that the shorter interval between milkings results in a lower production but with a higher concentration of components. The SCC evolution curve is inversely related to the milk production curve, Fuertes et al. curve, Fuertes et al. (1998). This result is similar to those found in other studies in cattle, such as that of Chagunda et al. (2006), in which they found correlations of r = 0.41 between the two parameters in healthy glands being higher in glands with mastitis (r = 0.59). Other works have obtained higher correlations in glands with subclinical mastitis (r = 0.73), but without finding the correlation between them in healthy glands (Hovinen et al., 2016), which corroborates that an important part of NAGase activity comes from damaged udder epithelial cells and leukocytes (Kitchen et al., 1978). In sheep, Leitner et al. (2001) obtained a correlation coefficient of 0.85 between NAGase and SCC.

Analyzing the relationship between production and NAGase, we observed that there was a negative correlation between NAGase values and milk production. Leitner et al. (2003) observed that milk production of ewes with both healthy glands was higher than with one infectious mastitic gland, compared to infectious mastitis in both glands (2.05, 1.78 and 1.44 kg/day, respectively). These data were corroborated by Leitner et al. (2004b) in another study in which they obtained similar data (2.88, 2.80, and 1.81 kg/day, respectively). Martí de Olives et al. (2013) detected that the decrease in milk production was affected by the severity of IMI, being more pronounced as SCC levels increased.

Predictive models

All models obtained the same predictive ability (AUC 0.80) except model 6 with a similar value (AUC 0.78) and model 4 in which parity number was excluded, where the AUC decreased to 0.74, indicating that this variable provides relevant information when classifying IMI. Models 1, 2, 3, 5 and 7 obtained the best values for sensitivity (64–73%) and specificity (70–79%), although with lower values than those found in other studies in cattle such as Hovinen et al. (2016), who for a cut-off value of 0.76 pmol 4-MU/min per μL milk obtained a sensitivity of 85% and a specificity of 84% to differentiate healthy quarters from those with subclinical mastitis. In sheep, there are no studies on predictive models of NAGase, but Clements et al. (2003) found that the higher the SCC threshold used, the higher the specificity for mastitis detection with the California Mastitis Test increased, while the sensitivity decreased. Berthelot et al. (2006) analyzed SCC in sheep throughout lactation and obtained a specificity of 75%, considering that an udder was healthy if in all samplings, the SCC was lower than 500 000 cells/mL and a sensitivity of 82% if at least two samplings had SCC greater than 1–1.2 million cells/mL.

We must keep in mind that all diagnostic methods have their limitations and there is no 'Gold Standard' test for the detection of inflammation or infection (Adkins and Middleton, 2018). In the case of bacteriology, some of its limitations would be the possibility of contamination of the sample and the subjectivity of the operator who examines it or possible false negatives due to non-continuous excretion. For other methods such as SCC or NAGase, some of these limitations could be that the values of the variables are affected by the pathogen that causes the infection and by external factors such as lactation stage, parity number, milking session, etc. This may cause the limitation of not reaching 100% sensitivity and specificity. As can be seen in Supplementary Figure S3, it is possible that sometimes glands with positive bacteriology (as with SCC), such as those infected with S.lentus, do not increase the variable and IMI cases are not detected with this method, but in practice, this would not be a serious problem at an economic and livestock level as these types of IMI are not causing a stronger affection of the gland by the pathogen. Although it has its limitations, we consider that the use of NAGase can be valid for the detection of IMI that causes a greater involvement of the gland.

According to the results obtained, any of the models 1, 2, 3, 5 and 7 could be used, as they are very similar for both AUC, internally validated AUC, and sensitivity and specificity values. Models 1 and 2 have the highest specificity, while model 5, which includes NAGase, lactation stage and parity, includes fewer variables than the others, and has the highest sensitivity and the same AUC as other more complex models. The addition of the SCC to the more complex NAGase model (model 1) made the R^2 slightly higher

because the model was more complex as it included more variables; however, the inclusion of the SCC did not improve the predictive ability of model 1. The criterion of choice will depend on the characteristics of the farms, taking into account the cost of installing electronic production meters and the cost of increasing false positives and the possible costs of treatment or confirmatory analysis. This will depend on the usual average herd prevalence, as higher prevalence may make higher sensitivity desirable. By prioritizing specificity over sensitivity, a lower false positive rate is obtained with a consequent reduction in veterinary costs for unnecessary treatment. When using model 5, the results are similar to those of more complex models, leading to a reduction in analysis costs. This model, being the simplest of all because it includes fewer variables, could be the most suitable algorithm for use at the farm level together with biosensors such as those proposed for cattle by Kumar et al. (2020) or Nirala and Shtenberg (2021) that would help us in the early detection of the disease.

Conclusions

The findings obtained in this study suggest that the NAGase enzyme can be used as an indirect detection method for mastitis in sheep, since, although it is also affected by other non-infectious factors, it is IMI that causes the greatest changes in enzyme levels. We consider that all NAGase models, except model 4, can be good options for IMI detection as they have a predictive ability equal or superior to the SCC model. The choice will depend on the needs of farms depending on whether they need to prioritize specificity or sensitivity.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101111.

Ethics approval

The procedures were carried out in accordance with Spanish and European animal protection laws. This project was approved by the Research Ethics Committee of the Miguel Hernández University with the ethical approval code DTA-JDS-001-09, according to the Spanish Royal Decree RD 53/2013 and the EU Directive 2010/63/EU for the protection of animals used for experimental research and other scientific purposes.

Data and model availability statement

None of the data were deposited in an official repository. The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author(s).

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

CRediT authorship contribution statement

Y. Miralles: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **V. Fornés:** Formal analysis, Writing – review & editing, Validation. **A. Roca:** Resources, Investigation. **R. Muelas:** Resources, Investigation. **J.R. Díaz:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **G. Romero:** Writing – review & editing, Supervision, Resources, Conceptualization.

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