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Comprehensive evaluation of milk biomarkers as indicators of intramammary infection in dairy goats across lactation

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We evaluated the milk biomarkers cathelicidin (Cath), haptoglobin (Hp), milk serum amyloid A (M-SAA), N-acetyl-β-D-glucosaminidase (NAGase), lysozyme (LZ), and the half-udder milk somatic cell count (H-SCC), for their potential association with intramammary infections (IMI) in dairy goats across lactation stages. Half-udder milk from 105 goats was collected during early (39 ± 13 days in milk, DIM), mid (136 ± 12 DIM), and late (269 ± 12 DIM) lactation stages and analyzed for bacteriological culture (BC), H-SCC, and potential biomarker reactivity. Predictive performance was evaluated with BC as the outcome, and Dairy Herd Improvement records provided contextual production data. Cath positivity, assessed by western blot, showed the strongest association with positive BC in early lactation and performed well also in mid-lactation. A dynamic H-SCC threshold was reliably correlated with positive BC in early and mid-lactation. Hp positivity, assessed by Western blot, was associated with BC in mid-lactation, while NAGase, assessed by enzymatic assay, showed no consistent association. M-SAA, assessed by ELISA, suffered technical limitations due to a lack of dilution linearity, and LZ, assessed by enzymatic assay, showed no association with BC. Our data suggest stage-dependent associations and support further validation of Cath, Hp, and SCC as potential components of combined diagnostic panels, particularly in early and mid-lactation. However, a semi-quantitative Western blot assessment for Cath and Hp, as well as technical limitations for M-SAA, warrant cautious interpretation and further analytical validation.

Keywords Mastitis, Biomarkers, Udder health, Small ruminants, Cathelicidin, Haptoglobin, NAGase, Milk serum amyloid A, Lysozyme

Mastitis caused by intramammary infection (IMI) is a prevalent issue in dairy goat farming[1], representing the primary cause of reductions in both the quantity and quality of milk and dairy products and resulting in relevant economic losses. Subclinical mastitis, in particular, is highly prevalent and difficult to detect, as infected animals often lack noticeable signs[2]. Clinical evaluations of the udder and mammary secretions combined with the milk somatic cell count (SCC), or the California Mastitis Test (CMT), are typically used to monitor the disease, accompanied when feasible by bacteriological culture (BC) to identify causative microorganisms[3]. SCC, however, lacks specificity in small ruminants, particularly goats, complicating its interpretation[4]. SCC is physiologically high in goat milk, ranging from 270,000 to 2,000,000 cells/mL in healthy udders[5], and increasing throughout lactation[6,7] with most literature suggesting thresholds of 500,000 to 1,000,000 cells/mL of milk[4]. Unlike dairy cows, where thresholds are typically 200,000–400,000 cells/mL, this variability underscores the need for refined diagnostics in goats.

IMIs are mainly due to bacteria[6]. Non-*aureus* staphylococci and mammaliicocci (NASM), previously referred as coagulase-negative staphylococci, are the most frequently isolated genera and typically cause chronic, subclinical mastitis, with *Staphylococcus caprae* as the most frequently isolated species[8]. *S. aureus* and *Mannheimia haemolytica* are the main causative agents of acute mastitis in small ruminants, although *S. aureus*

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can also persist in subclinical forms, requiring sensitive diagnostics[9]. Other major pathogens include Gram-negative bacteria such as *Serratia* spp., while *Corynebacterium* spp. and other minor pathogens can be isolated from the milk of goats with subclinical mastitis[10]. Given these pathogen profiles, BC remains the preferred tool for identifying the etiological agent and defining management strategies for IMI prevention and control[3]. However, limitations including low sensitivity, timing issues, and cost, make it unsuitable for routine goat mastitis screening[11].

To address these challenges, researchers have explored milk proteins as alternative biomarkers relying on their association with infectious mastitis to enhance diagnostic sensitivity and specificity[3]. The objective is to identify new molecules that outperform currently available tests, improving diagnostic reliability and scientific understanding of goat udder health. Several non-enzymatic antimicrobial and immune defense proteins, including cathelicidins (Cath), haptoglobin (Hp), and milk serum amyloid A (M-SAA), have been proposed[3]. Caths, small proteins of the innate immune response produced in epithelial and mucosal tissues, exhibit manifold functions including direct antimicrobial activity and chemotactic roles[12]. Cath concentrations in milk rise substantially in animals with IMIs caused by various pathogens[3]. Hp, an Acute Phase Protein (APP) composed of two α (16 to 23 kDa) and two β subunits (35 to 40 kDa), displays antibacterial and immunomodulatory activities[13]. In cattle, milk Hp correlates positively with the presence, severity, and recovery from mastitis[14,15]. Proteomic analyses indicated specific increases in milk Hp levels associated with intramammary infections in dairy goats[16], prompting further evaluation as a diagnostic marker. M-SAA is another positive APP[17–19]. It is a well-studied subclinical mastitis biomarker in dairy cows[18,20,21] but requires further validation for specificity across ruminant species[3]. The enzyme N-acetyl-β-d-glucosaminidase (NAGase) has also emerged as a promising candidate in cows due to its strong correlation with milk SCC and relative ease of measurement[22]. In goats, studies have shown that NAGase activity is significantly higher in infected udder halves compared to non-infected ones[23,24]. Lysozyme (LZ), a glycoprotein with antibacterial properties against Gram-positive bacteria, also shows increased activity in milk during mastitis, particularly in clinical cases, making it a potential candidate biomarker[23].

Previous studies clearly report that diagnostic tools like SCC, the CMT, or milk conductivity perform differently depending on the goat lactation stage[4,25,26]. Therefore, to obtain reliable information on their diagnostic performance, potential mastitis biomarkers should also be assessed with a stage-specific approach. This study provides the first comprehensive, combined evaluation of Cath, Hp, M-SAA, NAGase, LZ, and SCC in association with BC across lactation stages to assess their potential as mastitis markers in dairy goat milk.

Results

Milk biomarkers

Of the 630 half-udder milk samples collected from the same 105 goats in the three lactation stages, BC-positive samples were 115 (18.25%), corresponding to 26 (12.38%), 53 (25.24%), and 36 (17.14%) positive samples in early, mid, and late lactation, respectively (Table 1).

The proportion of BC-positive samples was higher in mid compared to early and late lactation ($p<0.01$) (Table 2). *S. caprae*-positive samples were more prevalent in mid than in late lactation and in early than in late lactation, NASM-positive samples in mid and late than early lactation, and other bacterial species in mid than late and early lactation ($p<0.01$; Table 2). The prevalence of NASM-positive samples was affected by parity ($p=0.01$; Table 2). No effect appeared on the prevalence of *S. aureus*-positive samples.

Figure 1 illustrates the distribution of milk BC results according to three lactation stages, early, mid, and late. Figure 1A reports the distribution of all BC-positive samples in the three lactation stages according to the bacterial pathogen, while Fig. 1B reports the distribution according to the species of NASM.

Productive performance and udder somatic cell count

Sample sizes for the three udder-health categories were highly unequal across time points. In early lactation 75 goats had both udder halves showing no signs of bacterial growth, 20 goats had a positive bacterial count in only one udder (SING), and 3 goats had a positive bacterial count in both udder halves (DOUB). In mid lactation 59 goats were classified as HEAL, 30 goats as SING, and 9 as DOUB, whereas in late lactation 71 goats were HEAL, 23 as SING and 4 as DOUB. Milk Yield (MY) and protein content were affected by parity ($p=0.02$ and 0.07, respectively) whereas MY, butterfat and protein were affected by farm ($p=0.01$; Table 3). HEAL goats had a tendency toward higher protein than DOUB goats throughout lactation ($p<0.1$) and had lower butterfat than SING goats in early lactation ($p<0.01$; Table 3). No effect appeared on the udder-SCC (U-SCC).

Milk biomarkers

Potential milk biomarkers were assessed by western Immunoblotting for Cath and Hp (Fig. 2, original images in Supplementary File 1), by ELISA for M-SAA, and by enzyme activity assay for NAGase and LZ. H-SCC was measured by the fluoro-opto-electronic method.

Compared to BC-negative samples, BC-positive samples had a higher half-udder SCC (H-SCC) in early and mid-lactation ($p<0.05$), a greater predicted probability of Cath-positive samples in early and mid-lactation ($p<0.01$) and a greater predicted probability of Hp-positive samples in mid-lactation ($p<0.01$). No effect appeared on NAGase and M-SAA (Table 4).

Based on the AUC obtained by ROC analysis, the diagnostic power for detecting a BC-positive sample (Table 5) was poor in early and fair in mid lactation for H-SCC (0.692 and 0.706, respectively), fair in early and poor in mid lactation for Cath (0.726 and 0.615, respectively), poor in mid lactation for NAGase (0.635), and poor for LZ, measured only in early lactation (0.620). The discrimination ability for BC-positive and BC-negative samples was lost in late lactation. Cath was the only marker with an AUC > 0.5 at this stage.

Based on contingency analysis (Table 6) an SCC threshold > 270,000 cells/mL provided the highest likelihood ratio (LR = 3.18) in early lactation, followed by > 500,000 (LR = 2.86) and > 1,000,000 (LR = 2.38). In mid-

Species	All	%	Early	%	Mid	%	Late	%
S. caprae	31	4.92	12	5.71	13	6.19	6	2.86
S. equorum	24	3.81	1	0.48	14	6.67	9	4.29
S. lentus	6	0.95	-	-	1	0.48	5	2.38
S. simulans	4	0.63	1	0.48	2	0.95	1	0.48
S. xylosus	4	0.63	-	-	3	1.43	1	0.48
S. chromogenes	3	0.48	1	0.48	1	0.48	1	0.48
S. gallinarum	2	0.32	-	-	-	-	2	0.95
S. haemolyticus	1	0.16	1	0.48	-	-	-	-
S. croceilyticus	1	0.16	1	0.48	-	-	-	-
S. petrasii	1	0.16	1	0.48	-	-	-	-
Total non-aureus staph	7	12.22	18	8.57	34	16.19	25	11.90
Corynebacterium stationis	12	1.90	-	-	11	5.24	1	0.47
S. aureus	8	1.27	3	1.43	2	0.95	3	1.43
Serratia spp.	7	1.11	2	0.95	2	0.95	3	1.43
Mannheimia spp.	2	0.32	1	0.48	1	0.48	-	-
Acinetobacter spp.	1	0.16	1	0.48	-	-	-	-
Aerococcus viridans	1	0.16	-	-	-	-	1	0.48
Lactococcus lactis	1	0.16	-	-	1	0.48	-	-
Streptococcus dysgalactiae	1	0.16	-	-	1	0.48	-	-
Unidentified	5	0.79	1	0.48	1	0.48	3	1.43
Total positive	115	18.25	26	12.38	53	25.24	36	17.14
Total negative	515	81.75	184	87.62	159	75.71	174	82.86
Total samples	630	100.00	210	100.00	210	100.00	210	100.00

Table 1. Bacteriological culture results. The table reports the detail of the bacteriological culture results obtained on goat half-udder milk samples considering the whole sample set (all) and separately for early, mid, and late lactation.

Variable [1]	Lactation phase[2]			SEM[3]	*P*-value		
	Early	Mid	Late		Time	Parity	Farm
Positive bacterial culture	0.125 A	0.246 B	0.142 A	0.027	< 0.01	0.05	0.18
Negative bacterial culture	0.876 A	0.753 B	0.858 A	0.030	< 0.01	0.06	0.23
S. aureus	0.014	0.010	0.014	0.008	0.56	0.90	0.31
S. caprae	0.057 A	0.064 B	0.025 C	0.017	0.04	0.28	0.64
Non-*aureus* staphylococci and mammaliicocci	0.034 A	0.101 B	0.080 B	0.021	< 0.01	0.01	0.53
Mannheimia haemolytica and *Serratia marcescens*	0.014	0.014	0.014	0.008	1.00	0.85	0.21
Other	0.005 A	0.058 B	0.010 A	0.016	0.01	0.95	0.29

Table 2. Estimated probabilities (LS means) of positive bacterial culture and intramammary infection by bacterial species, obtained from the GLIMMIX model adjusted for time, parity and farm. Values are referred to the half-udders of Saanen and Alpine dairy goats having 1 to 6 parity and raised in 2 commercial dairy farms sampled across three phases of the lactation cycle. [1]Positive and negative bacterial culture status was defined according to the details provided in materials and methods. [2]Probabilities at each time point are the Least Squares Means from the statistical model. Comparisons between time points are indicated with different letters for $p < 0.01$. Letters appear when the p-value for the time effect is significant only. [3]Standard error: largest standard error for the fixed effects.

lactation, an SCC threshold > 1,000,000 cells/mL had the highest LR (3.31), followed by > 500,000 (LR = 1.92) and > 270,000 (LR = 1.59), reflecting the need for higher SCC thresholds as lactation progresses. In late lactation, none of the selected thresholds was significantly associated with BC positivity. In early lactation, Cath exhibited the highest likelihood ratio (LR = 10.33), while in mid-lactation, Cath and Hp had similar LR (6.59 and 6.67, respectively). In late lactation Cath displayed the highest LR, but the association with a positive BC remained below the statistical significance threshold.

Discussion

This study presents a comprehensive evaluation of the milk SCC and a panel of potential milk biomarkers, Cath, Hp, M-SAA, NAGase, and LZ, as diagnostic tools for detecting IMI in dairy goats across lactation stages.

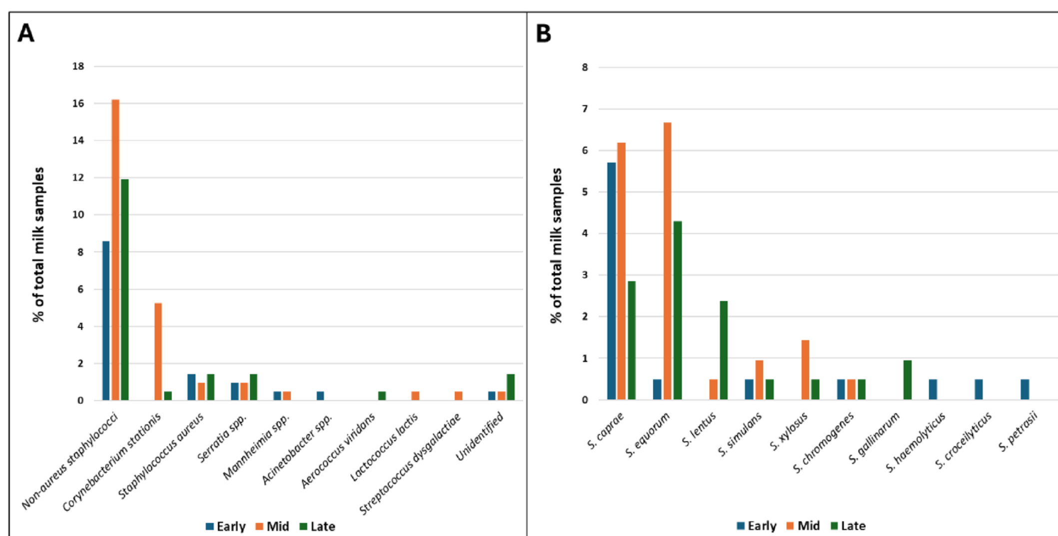

Fig. 1. Distribution of bacteriological culture results according to the lactation stage. (**A**) Percentage of total milk samples with a positive bacteriological culture according to the pathogen and lactation stage (early, mid, late). (**B**) Percentage of total milk samples with a positive bacteriological culture for non-aureus staphylococci (NASM) according to the species and lactation stage (early, mid, late).

Variable[1] (unit)	UH[2]	Lactation phase[3]			SEM[4]	*P*-value				
		Early	Mid	Late		UH[4]	Time	UH x time	Parity	Farm
Milk yield	HEAL	3.32	2.97	2.10	0.1363	0.40	< 0.01	0.77	0.02	< 0.01
(Kg/d)	SING	3.33	3.17	2.20	0.2689					
	DOUB	4.05	3.29	2.08	0.6861					
Butterfat	HEAL	3.80 A	3.11	3.64	0.087	0.13	< 0.01	0.10	0.33	0.01
(g/100 g)	SING	4.29B	3.06	3.70	0.155					
	DOUB	3.62	3.16	3.46	0.400					
Protein	HEAL	3.23	2.98	4.17	0.045	0.09	< 0.01	0.93	0.07	< 0.01
(g/100 g)	SING	3.18	2.96	4.12	0.078					
	DOUB	3.02	2.84	3.85	0.196					
U-SCC	HEAL	806	833	2698	376	0.80	0.43	0.57	0.68	1.00
(cells/mLx10^{-3})	SING	921	1163	1645	689					
	DOUB	425	1622	1310	1608					

Table 3. Productive performances and somatic cell count of Saanen and Alpine dairy goats. Udders were categorized as healthy (HEAL), or with one (SING) or both (DOUB) udder halves with a positive bacterial count across three phases of the lactation cycle. Udder health categorization was determined by the BC result on each half-udder collected at the respective lactation stage. [1]U-SCC: udder somatic cell count. [2]UH: Udder health, HEAL: goats having both udder halves showing no signs of bacterial growth, SING: goats having a positive bacterial count in only one udder half, DOUB: goats having a positive bacterial count in both udder halves. Udder health categorization was determined by the bacteriological culture result on each half-udder collected at the respective lactation stage. [3]Comparisons at each time point are indicated with different letters for the udder health × time interaction (UH × time; A/B is $p < 0.01$). [4]Standard error: largest standard error for the fixed effects.

Our results highlight the known complexity of mastitis diagnosis in goats, revealing stage-specific variations in potential biomarker performance and reinforcing the need for refined approaches to udder health monitoring in this dairy species.

The bacteriological profile of IMIs in this study is in line with the prominent role of NASM in goat mastitis, particularly concerning *S. caprae* as the predominant causal agents of chronic, subclinical mastitis[25]. The low prevalence of major pathogens suggests generally good mammary gland health in the two herds, further highlighting the need for sensitive biomarkers to detect chronic, low-grade infections, as BC is limited by low sensitivity and cost for routine screening. Productive performance data revealed an impact of mastitis coherent with the minor pathogen prevalence, with healthy goats tending to have higher protein content than those

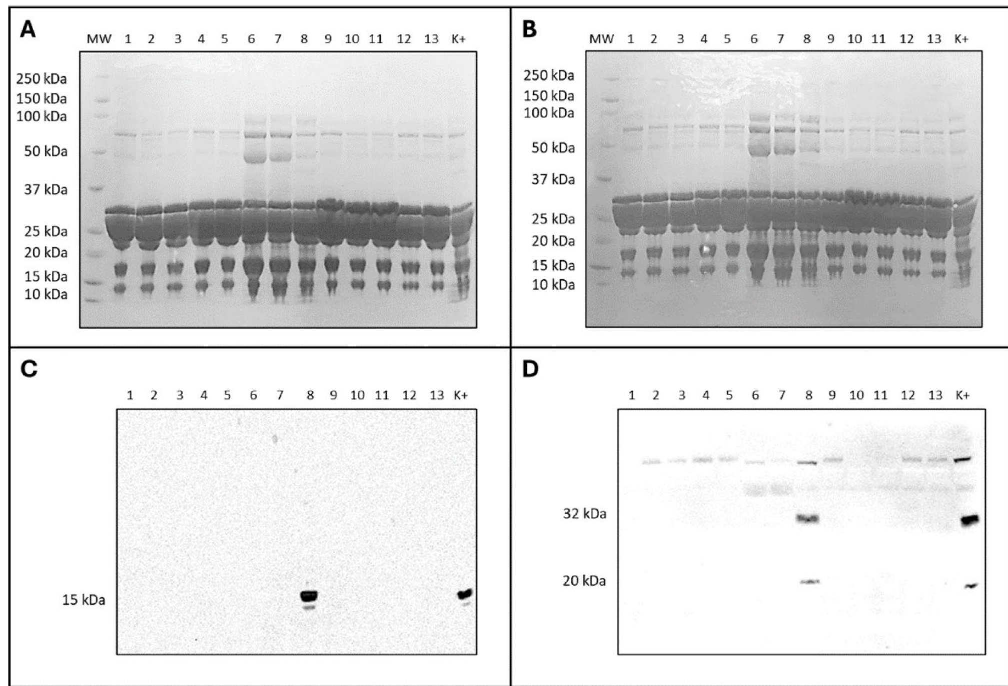

Fig. 2. Representative images of the total protein staining and western immunoblotting reactivity for cathelicidins (**A**, **C**), and haptoglobin (**B**, **D**). Bio-Rad all blue molecular weight (MW) standards (**A**, **B**) and estimated MW of Cath (**C**) and Hp (**D**) are indicated on the left. K + is the positive control milk sample loaded in all the gels, lane 8 corresponds to a positive milk sample, while all the other lanes correspond to different negative samples. In view of some non-specific reactivity observed at higher MW (**D**), only reactive bands at the expected MW (32 + 20 kDa) were considered as a positive result for Hp. An equal volume of milk (1 µL) was loaded in each lane.

Variable[1] (unit)	BC	Lactation phase[2]			SEM[3]	*P*-value				
		Early	Mid	Late		BC	Time	BC x time	Parity	Farm
H-SCC[1]	Negative	277 a	585 a	1029	160	0.10	< 0.01	0.01	0.22	0.44
(cells/mLx10^{-3})	Positive	621 b	906 b	821						
Cathelicidin	Negative	0.012 A	0.012 A	0.221	0.096	< 0.01	< 0.01	< 0.01	0.68	0.71
(Probability)	Positive	0.288 B	0.124 B	0.313						
Haptoglobin	Negative	0.092	0.020 A	0.066	0.048	0.01	0.04	0.05	0.72	0.61
(Probability)	Positive	0.205	0.170 B	0.083						
NAGase[1]	Negative	17.48	9.11	32.92	5.577	0.50	< 0.01	0.12	0.51	0.74
(µM)	Positive	27.31	12.12	26.75						
Serum amyloid A	Negative	12.23			7.188	0.33			0.85	0.32
(µg/mL)	Positive	20.00								
Lysozyme	Negative	190.2			12.42	0.64			0.05	0.31
(U/mL)	Positive	194.9								

Table 4. Milk biomarkers. Relationships of the different measured variables with bacteriological culture -negative and -positive half-udder milk of Saanen and Alpine dairy goats across three phases of the lactation cycle. [1]H-SCC: half-udder somatic cell count, NAGase: N-acetyl-β-D-glucosaminidase. [2]Comparisons at each time point are indicated with different letters for the bacteriology × time interaction (BACT × time, A/B is $p < 0.01$, a/b is $p < 0.05$). [3]Standard error: largest standard error for the fixed effects.

with both udder halves affected ($p < 0.1$), and lower butterfat than single udder affected goats in early lactation ($p < 0.01$). There were no significant effects on milk yield or U-SCC, in contrast with the findings in dairy cattle (14). However, a confounding effect might be the slight discrepancy between sample collection time and DHI record measurement time. DHI-derived MY, fat, protein and udder SCC (U-SCC) were recorded on whole-udder milk and were used only as production context, not for direct comparison with half-udder potential biomarkers.

Variable	Lactation phase		
	Early	Mid	Late
Half-udder somatic cell count	0.692	0.706	0.495
Cathelicidin	0.726	0.615	0.572
Haptoglobin	0.577	0.599	0.500
NAGase[1]	0.593	0.635	0.448
Milk serum amyloid A	0.587		
Lysozyme	0.620		

Table 5. Diagnostic power of milk biomarkers. The table reports the ability of milk biomarkers to detect a positive bacterial culture in half-udders of Saanen and Alpine dairy goats across three phases of the lactation cycle, based on the area under the receiver operator characteristics curve. [1]N-acetyl-β-D-glucosaminidase.

H-SCC confirmed its diagnostic utility in early and mid-lactation, with AUC values of 0.692 and 0.706, respectively, although failing to discriminate BC-positive samples in late lactation. Contingency analysis indicated that to maintain diagnostic relevance across lactation stages SCC thresholds have to be increased, reflecting the known rise in SCC in goat milk during lactation[4,5] and its association with neutrophils peaking in late lactation, in relation with an involution response as well as a possible role in clearance of IMI before the next lactation[4,5]. H-SCC measured in half-udder samples collected for bacteriology were lower than DHI values; however, these better reflect CMT results, as DHI measurements are carried out on the milk from the whole udder collected in one milking session.

Cath emerged as the best performing potential biomarker, particularly in early lactation, with the highest AUC value, a very high likelihood ratio, and high specificity. As with the SCC, diagnostic performance declined in mid-lactation and lost significance in late lactation. The marked association of Cath positivity with BC positivity is consistent with its role as antimicrobial and immune defense protein released specifically in milk in response to microbial stimuli[12] and is in line with previous research on other dairy ruminants[3,16,27,28]. In late lactation, the loss in performance might be linked to the higher percentage of neutrophils and to the high Cath abundance in this cell type[16,29]. As these cells increase naturally in late lactation as an adaptive response to mammary involution, this can potentially confound Cath specificity for IMI[4,30]. On the other hand, as Cath originates also from epithelial cells as a specific response to infection, it is a more reliable indicator of IMI-specific inflammation than purely neutrophilic markers[18,31,32]. In line with this, Cath values of BC-positive samples tended to be higher than those of BC-negative samples also in late lactation, while the same was not true for H-SCC.

Hp also showed diagnostic potential, particularly in mid-lactation, consistent with its role as an acute-phase protein (APP)[13,15,31] and in line with previous proteomics studies by our group in goat milk[16]. However, its lower sensitivity limits its standalone utility. The association with BC was not significant in late lactation also for this marker, and the ROC AUC values indicated poor diagnostic performance across phases. The near-significant bacteriology × phase interaction ($p = 0.0524$) suggests dynamic responses, but low sensitivity in late lactation limits diagnostic utility, possibly as a result of non-IMI related inflammatory responses such as increased blood/milk barrier permeability due to tissue remodeling. Some interest is provided by its higher value in BC-positive than BC-negative samples also in late lactation. Future improvements may be allowed by the optimization of antibody reactivity or implementation in other immunoassay formats, such as competitive or capture ELISA.

NAGase exhibited good diagnostic potential in early lactation according to contingency analysis. However, its association with BC was not significant in mid and late lactation and ROC AUC values indicated limited standalone utility. The phase-specific decline ($p < 0.0001$ for phase effect) also reflects its tight correlation with SCC and neutrophils[23]; NAGase values in BC-positive samples were lower than those of BC-negative samples in late lactation, as observed for neutrophils.

M-SAA assessment was technically limited by matrix effects, preventing reliable diagnostic conclusions. The high physiological M-SAA levels were combined with low technical reproducibility and poor dilution linearity by ELISA, possibly due to its amyloid nature and propensity for aggregation at elevated levels, as observed in other species[33]. Notably, the high M-SAA levels and their lack of correlation with IMI were indicated previously by proteomics studies on late lactation goat milk[16].

LZ lacked significant correlation with SCC or BC ($p > 0.05$), suggesting species-specific limitations in goats, potentially due to higher physiological levels compared to other dairy ruminants, lower sensitivity to IMI, or physiological differences with other ruminants, despite its antibacterial role[23].

BC, an imperfect gold standard for subclinical IMI due to its low sensitivity, might be particularly unreliable in late lactation goat milk. The high neutrophil concentration and the associated antimicrobial activity may create a less permissive environment to bacterial growth, reducing BC sensitivity and increasing false-negative results. This hypothesis is supported by several observations in late lactation: (i) the least-squares mean SCC of BC-negative samples tended to be higher than that of BC-positive samples, (ii) BC-positive samples were significantly reduced in late compared to mid-lactation, and (iii) apparent positivity to *S. caprae*, a prevalent IMI agent in goats, decreased significantly with lactation progress. Furthermore, these findings align with recent reports of reduced bacterial counts associated with elevated SCC[34]. In the context of diagnostic standards, potential molecular approaches, such as PCR or metagenomics, may also present significant challenges. Specifically, they amplify non-viable bacterial DNA and do not provide a direct measure of the active microbial load, which is a crucial distinction for diagnosing active infection. Finally, their targeted nature does not enable

	All samples (whole lactation)						Early lactation						Mid lactation						Late lactation					
	>270 [a]	>500	>1000	Cath	Hp	NAG	>270 [a]	>500	>1000	Cath	Hp	NAG	>270 [a]	>500	>1000	Cath	Hp	NAG	>270 [a]	>500	>1000	Cath	Hp	NAG
P value[b]	<0.0001	<0.0001	0.0001	<0.0001	0.0002	0.0073	<0.0001	0.0034	NS	<0.0001	0.02	0.0038	0.0001	0.0001	<0.0001	<0.0001	<0.0001	NS	NS	NS	NS	NS	NS	NS
LR[c]	1.58	1.58	1.75	**2.86**	2.60	1.52	3.18	2.86	2.38	**10.33**	3.07	7.31	1.59	1.92	3.31	6.59	**6.67**	1.46	1.10	1.03	0.94	**1.48**	1.01	0.93
Se[c]	**0.74**	0.57	0.34	0.39	0.21	0.38	**0.62**	0.38	0.19	0.50	0.23	0.12	**0.79**	0.63	0.40	0.29	0.25	0.50	**0.81**	0.65	0.35	0.45	0.15	0.38
Sp[c]	0.53	0.64	0.81	0.87	**0.92**	0.75	0.80	0.87	0.92	0.95	0.93	**0.98**	0.51	0.68	0.88	0.95	**0.96**	0.66	0.27	0.37	0.62	0.70	**0.85**	0.60
PPV[c]	0.25	0.25	0.27	**0.37**	0.35	0.25	0.30	0.29	0.25	**0.59**	0.30	0.50	0.36	0.40	0.54	**0.68**	**0.68**	0.34	0.16	0.15	0.14	**0.20**	0.13	0.14
NPV[c]	**0.91**	0.88	0.85	0.87	0.84	0.85	**0.94**	0.91	0.89	0.93	0.90	0.89	**0.87**	0.84	0.81	0.81	0.80	0.79	**0.89**	0.86	0.85	0.88	0.87	0.85

Table 6. Results of the contingency analysis. Contingency analysis for a positive bacteriological culture (BC) result for any microorganism *versus* three SCC threshold levels (>270,000, >500,000 and >1,000,000 cells per mL of milk), cathelicidin (Cath), haptoglobin (Hp) and NAGase (NAG). The data have been assessed considering all the sample set (whole lactation) and samples collected in the three lactation stages separately (early, mid, and late lactation). The highest values for each parameter are indicated in bold. [a]SCC threshold expressed in cells x 10^3/mL of milk. [b]Based on the Fisher's exact test. [c]Based on the Wilson-Brown method: *LR* Likelihood ratio, *Se* Sensitivity, *Sp* Specificity, *PPV* Positive predictive value, *NPV* Negative predictive value. The highest values for each parameter are indicated in bold.

the discrimination of positive from contaminated samples, typically based on recognized CFU thresholds and colony morphology, which is crucial for environmental pathogens[35].

The strengths of our study include the longitudinal design across lactation stages, the analysis at the half-udder level, and the robust statistical approach, incorporating ROC and contingency analyses to evaluate the potential biomarker performance. Our results suggest that Cath positivity and H-SCC, using lactation-stage specific thresholds, show promise for detecting subclinical IMI in early and mid-lactation, potentially improving udder health monitoring in dairy goats. Combining these approaches might enhance diagnostic sensitivity. Furthermore, these immune markers could provide value for future research studies on mammary gland immunity. These potential biomarkers can serve as indicators for the local immune response, enabling researchers to accurately monitor the kinetics of inflammation and recovery during natural and experimental infections, evaluate novel preventive and therapeutic measures, and investigate the specific pathophysiology of mastitis in small ruminants. These findings support further validation in larger, diverse goat populations and development of quantitative, field-applicable assays.

As limitations, we note that our study was carried out on two herds with good udder health where we detected predominantly minor pathogens. Sample sizes for the three udder-health categories were highly unequal across time points, the very small number of DOUB cases at each time point results in large standard errors of the adjusted (modelled) means and consequently in unstable estimates after adjustment. Accordingly, apparent unexpected patterns involving the DOUB group should be interpreted cautiously and may reflect imprecision rather than true biological effects. The inclusion of only two cosmopolitan breeds limits generalizability to broader goat populations or clinical cases, and biomarker-pathogen associations were not assessed due to statistical power issues. Breed effects could not be separated from farm effects, limiting breed-specific conclusions. Cath and Hp were assessed by western blot, which is less robust for absolute quantification than dedicated quantitative immunoassays. Future research should refine IMI detection methodologies, including duplicate and repeated sampling to strengthen the reliability of IMI detection by bacteriological culture, validate these potential biomarkers in larger, diverse goat populations, explore their interactions with management variables and specific pathogens, and develop cost-effective, field-applicable assays to address late-lactation challenges, enhancing mastitis control in dairy goats.

In conclusion, while SCC and BC remain essential, Cath and Hp, combined with phase-specific SCC thresholds, have the potential to improve diagnostic value in early and mid-lactation, enhancing subclinical IMI diagnosis and supporting sustainable farming practices. As a further, more immediate potential application, these potential biomarkers could provide a valuable contribution to research studies on goat mammary gland health.

Methods

Animal management, sample collection, data recordings and experimental design

The sample size was determined through power analysis using the formula provided by Cross and Wayne[36]. Assuming a 95% confidence level and a 5% margin of error, a suitable sample number was calculated to be 340. IMI prevalence was estimated from published work[29] and retrospective IMI prevalence data from our laboratory. The model incorporated an estimated sensitivity of 60% for BC. The final sample set used for the analysis included 630 samples resulting from 210 half-udder milk samples collected from 105 goats during 3 phases of the lactation cycle. Milk samples were obtained from two commercial dairy farms located in northern Italy from April 11, 2023, to November 22, 2023, in the context of routine milking procedures. Parity distribution ranged from 1 to 6: 13 goats were at the 1 st lactation (12.38%), 46 at the 2nd lactation (43.81%), 32 at the 3rd lactation (30.48%), 4 at the 4th lactation (3.81%), 8 at the 5th lactation (7.62%), and 2 at the 6th lactation (1.90%). All goats were pregnant in the late lactation phase. Goats kidded between February and March, were housed in freestall barns with deep straw bedding and were milked twice daily while lactating. In early (39 ± 13 days in milk, DIM), mid (136 ± 12 DIM) and late lactation (269 ± 12 DIM), sterile milk samples were collected from each half-udder before the morning milking, as described previously[29]. Briefly, teats were cleaned and disinfected using single-use towels embedded with chlorhexidine, foremilk was stripped, and milk was aseptically collected into sterile vials. A visual and physical examination of the udder and foremilk was performed at the time of sample collection. Clinical signs of severe mastitis were observed only in two goats that were excluded from the study considering the focus on subclinical mastitis. The samples were refrigerated and immediately transported to the diagnostic laboratory where BC was carried out, H-SCC was measured, and two 1 mL aliquots of milk were frozen at −20 °C for potential biomarker assessment. Furthermore, individual MY, butterfat, protein and udder SCC (U-SCC) of the goats included in the experiment were extracted from the dairy herd improvement (DHI) records provided by the Regional Farmers' Associations during the morning milking at 2 ± 7 d from sample collection, obtained by Fourier Transform Infrared (FTIR) spectroscopy and somatic cell counting by flow cytometry as part of the DHI records.

Bacteriological culture

BC was performed according to the National Mastitis Council guidelines[35]. Ten μL of half-udder milk were spread onto quarter sections of blood agar and incubated at 37 °C for 24 h. The following day, a visual inspection was performed, recording the number and appearance of colonies for each sample, and the plate was incubated again for an additional 24 h. Milk was categorized into negative, contaminated, or positive, considering a threshold of 100 CFU/mL (one colony) for *S. aureus* and 1000 CFU/mL (ten colonies of the same morphology) for other pathogens. Plates with more than two colony morphologies were considered contaminated. Species identification was carried out by MALDI-TOF MS as described previously[8].

Half-udder somatic cell count

H-SCC was measured by the opto-fluo-cytometric method with a Bentley Somacount 150™ (Bentley Instrument, USA).

Milk cathelicidin and haptoglobin assessment by western immunoblotting

Frozen milk samples were thawed within one month from collection and used to assess potential biomarkers. Milk Cath and Hp were determined by western immunoblotting after denaturing sodium dodecyl-sulphate gel electrophoresis (SDS-PAGE) as described previously[37], with minor modifications. Multiple antibody candidates were tested, and the selected antibodies were retained as they provided the most specific signal with lowest non-specific background in this matrix, particularly after optimization of the anti-bovine Hp antibody on a subset of goat milk samples. In brief, 5 µL of milk sample were mixed with 45 µL of Laemmli buffer (Sigma-Aldrich), heated at 95 °C for 3 min, and then centrifuged at 10,000 rpm for 1 min. Ten µL of each sample were then loaded into precast Mini-PROTEAN TGX Gels (Bio-Rad) with Precision Plus Protein All Blue Standards (Bio-Rad) or MagicMark XP markers (Thermo Fisher Scientific) included in the first well of each gel. In this way, the same amount of milk was loaded in each lane enabling standardization of the procedure, making it independent from physiological and pathological fluctuations in protein concentration, maintaining a consistent analytical approach in all assays, and reflecting a biological comparison. Electrophoresis was performed at room temperature for approximately 40 min using a constant voltage of 180 V in 1X Tris/Glycine/SDS Buffer (Bio-Rad) running buffer. Following denaturing SDS-PAGE, the proteins were transferred onto a Mini-Size nitrocellulose (Bio-Rad) using the Trans-Blot Turbo (Bio-Rad). The transfer was carried out at a voltage of 25 V for 6 min in 1X transfer buffer (Bio-Rad). All membranes were subjected to reversible protein staining with Ponceau S solution (Sigma-Aldrich) to check for protein separation and protein transfer quality. Subsequently, the membrane was blocked with EveryBlot Blocking Buffer (Bio-Rad) for 15 min at room temperature in agitation. Following blocking, the membrane was incubated with the primary antibody specific to the target protein of interest: 1 µg/mL anti-cathelicidin mAb[38] or 1:50,000 Rabbit anti-bovine Hp K-49[39] in EveryBlot blocking buffer. After one hour of incubation at room temperature in agitation, the membrane was washed five times for 3 min with TBS-Tween 20 0.1% (TBS-T). Subsequently, the membrane was incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Invitrogen) for 30 min at room temperature. The membrane was washed five times for 3 min with TBS-T. The membrane was finally incubated for 5 min with Clarity Western ECL Substrate (Bio-Rad) and the chemiluminescent signal was acquired with an IBright 1500 (Thermo Fisher Scientific). Western blots were scored as positive/negative based on the presence of a visible band at the expected molecular weights of 15 kDa for Cath and 32 + 20 kDa for Hp, respectively. Blots were first scored by one researcher and independently re-checked by a second researcher, blinded to bacteriological results. Agreement between the two scorings was 98%, and discrepancies were resolved by joint review. Thus, Cath and Hp results are interpreted as qualitative/semi-quantitative evidence of presence rather than absolute concentration.

Assessment of milk serum amyloid A by enzyme-linked immunosorbent assay (ELISA)

M-SAA concentration was measured with the Milk serum amyloid A Mast ID ELISA kit (Bentley Instruments, Chaska, MN, USA) only on the early lactation sample. In fact, upon completing the first two assay plates according to the manufacturer's instructions, most of the measured values were above the highest standard of the calibration curve. Therefore, all milk samples were diluted 1:50 and reassessed. Additional dilution checks did not show acceptable linearity/parallelism with the standard curve, indicating relevant matrix effects and preventing reliable absolute quantification. M-SAA measurement was performed in duplicate on all BC-positive samples, all samples negative to BC but with SCC > 270.000 cells x 10^3/mL, and an equivalent number of samples negative to BC and with SCC < 270.000 cells x 10^3/mL. Intra-assay reproducibility was compromised by matrix effects, as evidenced by lack of dilution linearity.

Assessment of N-acetyl-β-D-glucosaminidase activity by enzymatic assay

NAGase activity was measured as described previously[22]. In brief, milk was centrifuged at 10,000 x g for 30 min, and the serum was collected. Subsequently, a standard curve was prepared using 4-methylumbelliferon (4-MeU, Merck) starting with a concentration of 200 µM. In a 96-well dark-bottom plate, 10 µL of either the samples or standard curve was added. To each well, 40 µL of 4-methyl umbelliferyl-N-acetyl-beta-D-glucosaminide (MUAG, Merck) substrate was added. The plate was then incubated at 25 °C for 15 min in the dark, and the reaction was quenched by adding 150 µL of glycine-titriplex III buffer per well. Finally, fluorescence was measured at 355 nm excitation and 460 nm emission on a fluorimeter (Ascent, Thermo Labsystem, FL, USA).

Assessment of lysozyme activity by enzymatic assay

LZ activity was measured with the EnzCheck Lysozyme Assay Kit (Thermo Fisher Scientific), using a fluorimeter at 494 nm excitation and 518 nm emission (Ascent, Thermo Labsystem FL, USA). The results were expressed as Units. For each plate, a standard curve was prepared within a range of 8–500 U/mL. In view of the clear lack of association with BC results in this sample subset, measurements were carried out only for early lactation.

Data analysis

The population considered for data analysis included only animals from which milk had been collected from both half udders on all three sampling occasions and included 37 Saanen goats (parity 2.95 ± 1.78) farmed in Bergamo, Italy, and 68 Alpine goats (parity 2.37 ± 0.48) farmed in Piacenza, Italy. Half-udders were classified as positive or negative based on BC results and used to assess the diagnostic power of milk analytes for milk BC. Furthermore, goat udders were classified as healthy (HEAL), affected by one (SING) or two BC-positive halves (DOUB) and used to assess the effect of mammary gland BC on production parameters of the individual goats.

The data were analyzed using SAS software, version 9.4 (SAS Inst. Inc., Cary, NC, USA), and are presented in graphs and tables as the least squares means and pooled standard error for individual means over time. Data expressed as dichotomous variables (prevalence of positive BC, bacteriology, Cath, Hp) are presented as the predicted probability of detecting a positive value (i.e., event = 1). Data underwent ANOVA testing using a mixed model (Glimmix Procedure, SAS Inst. Inc.). For the prevalence of positive BC and bacteriology, the statistical model included the fixed effects of parity, farm, and time (Early, Mid and Late lactation) and assumed the individual goat and the individual half-udder nested within the goat as random effects. For MY, butterfat, protein and U-SCC, the statistical model included the fixed effects of udder health (UH; HEAL; SING; DOUB), parity, farm, time and the first-order interaction effect of UH × time and assumed the individual goat as a random effect. For M-SAA and LZ the statistical model included the fixed effects of BC (BACT; positive and negative), parity and farm, while for H-SCC, NAGase, Cath and Hp, the statistical model also included the fixed effect of time and the first-order interaction effect of BACT × time, and assumed the individual goat and the individual half-udder nested within the goat as random effects. The analyses were conducted using four covariance structures—autoregressive, unstructured, antedependence, and spatial power—with their heterogeneous counterparts. These were ranked according to their Akaike information criteria, with the one having the lowest criterion being chosen for final statistical analysis[40]. The distribution of residuals was visually assessed. The pairwise comparison was performed using the least significant difference test with the Tukey adjustment for multiple comparisons. Significance was declared for $p \leq 0.05$, and differences for $p \leq 0.1$ were discussed in the context of tendencies. To evaluate the predictive ability of the different potential biomarkers for detecting positive BC, receiver operating characteristic (ROC) curves were constructed for each biomarker using logistic regression models (Logistic procedure, SAS Inst. Inc.). The BACT was assumed as an outcome variable and each biomarker was tested as an independent predictor. Separate models were fitted for each time using the BY statement to account for potential differences over the lactation phases. The area under the ROC curve (AUC) was estimated for each model to assess predictive performance, and pairwise comparisons of AUC values across different biomarkers were conducted using the ROCCONTRAST statement. The results were classified based on conventional thresholds of $0.9 \leq AUC$ as excellent, $0.8 \leq AUC < 0.9$ as considerable, $0.7 \leq AUC < 0.8$ as fair, $0.6 \leq AUC < 0.7$ as poor, and $0.5 \leq AUC < 0.6$ as fail[41]. Contingency analysis was carried out with GraphPad Prism 10 by the two-sided Fisher's exact test at a 95% confidence interval, with odds ratios (OR) calculated via the Baptista-Pike method and sensitivity, specificity, likelihood ratio (LR), positive predictive value (PPV), and negative predictive value (NPV) determined using the Wilson-Brown method.

Data availability

The data used for this study can be provided by the corresponding author (MFA) upon request.

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Author contributions
Conceptualization: M.F.A.; Methodology: M.F.A., A.M., V.B., P.M., R.P.; Data curation: M.F.A, F.S., M.M.; Formal analysis: F.S., S.F.P., M.M., M.P., L.F.P.; M.M.V.; Visualization: M.F.A., M.M.; Resources: M.F.A, R.P; Writing—original draft preparation: M.F.A, F.S.; Writing—review and editing: all authors; Supervision: M.F.A.; Project administration: M.F.A, R.P.; All authors contributed to the manuscript and approved the submitted version of the manuscript for publication.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
The milk used for this study was collected during regular milking procedures from dairy goats. No specific activities were carried out on the animals for the purposes of the study but collecting milk from each half-udder in separate tubes at the scheduled milking time. Animals were farmed and kept in respect of all the guidelines and regulations under the supervision of the farmers and the farm veterinarian. Milking procedures were conducted by trained personnel in accordance with best practices for animal welfare and veterinary care. No experimental interventions or invasive procedures were performed on the animals for the purpose of this research. Therefore, ethical committee authorization was not requested. All methods were performed in accordance with the relevant guidelines and regulations and followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. The farmers provided their informed consent to collect the samples for the purposes of this study.

Consent to publish

The farmers agreed to use the data for publication without disclosure of the farm identification information.

Additional information

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