

RESEARCH ARTICLE

Bacteriological and molecular characterization of mastitis-associated pathogens and milk quality in ewes and goats in Al-Kut, Iraq



Hayder Dawood Saleem¹ , Samih Riyadh Faisa² , Sammah Naeem Ghazi² , Zeid Muhsen Alsadoon³ and Anfal Jabbar Taher¹

1. Department of Medical Laboratory Techniques, Al-Manara College for Medical Sciences, University of Manara, Iraq.

2. Department of Pathological Analysis, Al-Manara College for Medical Sciences, University of Manara, Iraq.

3. Department of Microbiology, College of Veterinary Medicine, University of Wasit, Wasit, Iraq.

ABSTRACT

Background and Aim: Mastitis is an important health and production problem in small ruminants because it can impair udder health, milk yield, and milk quality. Integrated information on mastitis-associated bacterial pathogens and milk quality characteristics in sheep and goats in Al-Kut, Iraq, remains limited. This study aimed to determine the occurrence of California Mastitis Test (CMT)-positive milk samples in ewes and goats, assess N-acetyl- β -D-glucosaminidase (NAGase) reaction intensity, characterize associated bacterial pathogens using bacteriological and molecular methods, and evaluate selected milk quality parameters.

Materials and Methods: A cross-sectional study was conducted in Al-Kut, Wasit Province, Iraq, from February to August 2025. Milk samples were collected from 300 lactating small ruminants comprising 200 ewes and 100 goats aged 2–8 years. Samples were screened using CMT and a semi-quantitative NAGase colorimetric assay. CMT-positive samples underwent bacteriological culture and biochemical identification. Selected *Staphylococcus aureus* and *Escherichia coli* isolates were confirmed by polymerase chain reaction targeting the *femA* and *phoA* genes, respectively. Milk pH, electrical conductivity, protein, and fat contents were also evaluated.

Results: Overall, 145/300 (48.3%) samples were CMT-positive, with a higher proportion in ewes (113/200; 56.5%) than goats (32/100; 32.0%). Light NAGase reactions predominated among CMT-positive samples in both ewes (53/113; 46.9%) and goats (23/32; 71.9%). Among the 145 CMT-positive samples, 138 (95.2%) were culture-positive, with mixed bacterial infections accounting for 86/138 (62.3%). Among individual bacterial findings, *S. aureus* was most frequent (16.9%), followed by *E. coli* (13.5%), coagulase-negative staphylococci (8.9%), and *Streptococcus uberis* (1.6%). Five *S. aureus* and five *E. coli* isolates selected for molecular confirmation yielded the expected *femA* (647 bp) and *phoA* (903 bp) amplicons, respectively. Electrical conductivity differed significantly between species and across age groups, whereas protein content showed a significant species effect. pH and fat content showed no significant effects in the revised analysis.

Conclusion: CMT-positive mastitis-related findings were more frequent in ewes than goats in the examined population, with mixed bacterial infections predominating among culture-positive samples. The detection of major mastitis-associated bacteria and molecular confirmation of selected *S. aureus* and *E. coli* isolates highlight the importance of bacteriological surveillance, early detection, and improved milking hygiene for mastitis control in small-ruminant farms in Al-Kut.

Keywords: bacteriological characterization, California Mastitis Test, *Escherichia coli*, mastitis, milk quality, molecular diagnosis, small ruminants, *Staphylococcus aureus*.

INTRODUCTION

Small ruminants, particularly sheep and goats, play an important role in food security and rural livelihoods through milk and meat production. Maintaining animal health is therefore essential for sustaining the productivity and economic viability of small-ruminant production systems. Among the major health challenges affecting milk-producing animals, mastitis is a key disease characterized by inflammation of the mammary gland caused by physical, chemical, or microbial factors [1]. Microbial mastitis can induce substantial physiological and biochemical alterations in milk, adversely affecting milk yield, composition, processing quality, and food safety [2]. Mastitis occurs in both clinical and subclinical forms. Subclinical mastitis is particularly important because affected animals

Corresponding Author: Hayder Dawood Saleem

E-mail: hayderdawood@uomanara.edu.iq

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Co-authors: SRF: Samih2.Riyadh2@gmail.com; SNG: samahnaeem025@uomanara.edu.iq; ZMA: zaidalsadoon@uowasit.edu.iq; AJT: ajtbio69@gmail.com

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may exhibit no overt clinical signs while serving as persistent reservoirs of infection within the herd [3].

Bacterial pathogens are major causative agents of mastitis in sheep and goats. *Staphylococcus aureus*, *Escherichia coli*, coagulase-negative staphylococci (CoNS), and *Streptococcus uberis* are among the bacterial organisms frequently associated with the disease [3, 4]. Accurate detection and characterization of mastitis-associated infections require complementary diagnostic approaches. These include clinical examination; indirect screening methods such as the California Mastitis Test (CMT) and N-acetyl- β -D-glucosaminidase (NAGase) assay; conventional bacteriological culture; and molecular techniques such as polymerase chain reaction (PCR) to confirm bacterial identity [5].

Although researchers have investigated mastitis in small ruminants in some regions of Iraq, including Mosul and Diwaniyah [6, 7], important gaps remain in understanding mastitis-associated findings in Al-Kut City, Wasit Governorate. Previous investigations have largely focused on individual components of the disease, including histopathological alterations or characterization of specific bacterial pathogens. Consequently, integrated data simultaneously incorporating indirect mastitis screening, semi-quantitative assessment of NAGase reactions, bacteriological isolation and identification, molecular confirmation of selected pathogens, and evaluation of physicochemical milk quality parameters in both ewes and goats remain limited. In particular, locally generated evidence comparing CMT-positive findings and milk quality characteristics between these two small-ruminant species and across age groups is needed. Such information is relevant in Al-Kut, where small ruminants are predominantly maintained under traditional smallholder grazing systems characterized by variable nutritional resources and hygienic conditions. Establishing a local baseline of mastitis-related findings and associated bacterial profiles may therefore provide useful evidence for improving diagnostic approaches, flock-health management, and milk quality monitoring in this production setting.

Therefore, this study aimed to determine the proportion of CMT-positive milk samples among ewes and goats in Al-Kut City and to characterize the associated mastitis-related findings using complementary diagnostic approaches. Specifically, the study aimed to assess NAGase reactions using a semi-quantitative colorimetric assay; isolate and identify bacterial pathogens from CMT-positive milk samples using conventional bacteriological and biochemical methods; molecularly confirm selected *S. aureus* and *E. coli* isolates using the *femA* and *phoA* gene markers, respectively; and evaluate selected milk quality parameters, including pH, electrical conductivity, protein content, and fat content, between ewes and goats across different age groups. The study further hypothesized that the proportion of CMT-positive samples, bacterial pathogen profiles, and selected milk quality parameters would differ between the examined animal species and age groups.

MATERIALS AND METHODS

Ethical approval

Ethical approval for this study was obtained from the Research Ethics Committee of the Faculty of Science, University of Al-Manara, Iraq (Approval No. UOMANARA.AN.2025.11; dated January 25, 2025), before the commencement of the study. The approved study involved clinical examination of lactating ewes and goats and aseptic collection of milk samples for mastitis screening, bacteriological examination, molecular confirmation of selected bacterial isolates, and milk quality analysis.

Study period and location

The study was conducted from February to August 2025 in different areas of Al-Kut City, Wasit Governorate, Iraq. Animals were recruited from smallholder farms in the study area. Laboratory examination of the collected milk samples was performed at the microbiology laboratory of the Faculty of Health and Medical Technologies, University of Al-Manara.

Study design

This cross-sectional study investigated CMT-positive mastitis-related findings and associated bacterial pathogens in lactating ewes and goats using complementary screening, conventional bacteriological, biochemical, and molecular diagnostic approaches. The study population comprised animals available at smallholder farms during the study period that met the eligibility criterion of being lactating. Each milk sample represented an individual animal. CMT and NAGase were used for screening, while bacteriological culture was used to characterize bacterial isolates. Selected bacterial isolates were subsequently confirmed by molecular methods. Selected physicochemical characteristics of milk were also evaluated.

Animals and sample collection

A total of 300 milk samples were collected from lactating ewes (*Ovis aries*) and goats (*Capra hircus*) aged 2–8 years, comprising 200 samples from ewes and 100 from goats. Animals were selected from smallholder farms in different areas of Al-Kut City according to the accessibility of eligible lactating animals during the study period. The animals were predominantly managed under traditional grazing-based systems rather than intensive or standardized farm-management conditions. They relied primarily on natural grazing, with relatively limited nutritional resources associated with poor pasture conditions, and housing and sanitary practices were basic.

Clinical examination was performed before milk collection. Clinical mastitis was defined according to the criteria described in [8] based on observable abnormalities of the udder or milk, including udder swelling, pain or discomfort during milking, and abnormal milk appearance; systemic signs were also recorded when present. Animals without overt clinical signs but with a positive CMT reaction were considered CMT-positive and suspected of having subclinical mastitis. Trace CMT reactions were considered positive. NAGase was used as an additional semi-quantitative screening parameter and was not used independently to define mastitis. The CMT and NAGase results were not considered independent confirmatory tests of bacterial infection. Bacteriological culture was subsequently performed to characterize bacterial isolates.

Each milk sample represented one individual lactating animal. Age was included as an analytical variable, and animals were categorized into three age groups: 2–4, 4–6, and 6–8 years.

Milk sample preparation

Milk samples were collected aseptically following established guidelines [9]. Before collection, the udder and teats were cleaned with warm water and dried with disposable towels, then the teats were disinfected with 70% ethyl alcohol. The first streams of milk were discarded, and approximately 10–15 mL of milk was aseptically collected from each animal into a sterile tube. Each sample represented milk from an individual lactating animal rather than a pooled or quarter-milk sample. Samples were appropriately labeled, immediately refrigerated at 4°C, and transported within 6 h of collection to the microbiology laboratory of the Faculty of Health and Medical Technologies, University of Al-Manara, for subsequent analyses.

Chemical tests

California Mastitis Test: Milk samples were screened using CMT following the established procedure and the manufacturer's instructions (Hardy Diagnostics, USA) [7, 8]. Equal volumes of milk and CMT reagent were mixed, and the resulting reactions were evaluated according to gel formation and viscosity. Reactions were categorized as negative, trace, weak positive (+1), moderate positive (+2), or strong positive (+3). Gel formation was interpreted as an indirect indication of increased somatic cell content in milk.

N-acetyl- β -D-glucosaminidase activity: NAGase was assessed using a colorimetric procedure based on the enzymatic release of p-nitrophenol, with minor modifications to the previously described method [10]. Milk samples were subjected to the NAGase reaction under the specified assay conditions, and the resulting yellow color was visually compared with the blank control. Because the assay was used as a screening procedure without spectrophotometric quantification or a standard curve, results were interpreted semi-quantitatively by color intensity as light yellow (+), medium yellow (++), or intense yellow (+++). Accordingly, NAGase results were expressed as categorical reaction intensities rather than enzymatic activity units. NAGase was used as an additional indicator of mammary epithelial cell damage and intramammary inflammation and was interpreted in conjunction with CMT and bacteriological culture.

Isolation and identification of bacteria

All 145 CMT-positive milk samples, all of which also exhibited positive NAGase color reactions (ranging from light to intense yellow intensity), were subjected to bacteriological culture on 5% sheep blood-enriched blood agar and MacConkey agar (Oxoid, UK). Cultures were incubated aerobically at 37°C for 24–48 h. Following incubation, three to five representative colonies were selected from each culture plate based on distinct colony morphology. Colonies exhibiting different morphological characteristics within the same milk sample were processed separately. Selected colonies were purified by subculture and subjected to Gram-staining and appropriate biochemical tests for bacterial identification. Blood agar, nutrient agar, and MacConkey agar were used during bacterial isolation and characterization following the method described previously [11].

Species-level identification was performed using API Staph for staphylococci, API 20E for *E. coli*, and API Strep for streptococci according to the manufacturer's instructions. Identification was based on the biochemical reaction profiles generated using the respective API systems.

A mixed bacterial infection was defined as the isolation of two or more different bacterial species from the same milk sample during bacteriological culture. Samples yielding only one bacterial species were classified as single-species infections. The proportion of mixed bacterial infections was calculated using the number of culture-positive milk samples as the denominator rather than the total number of bacterial isolates.

Molecular confirmation of bacterial isolates

Following bacterial culture and phenotypic identification, 15 presumptive staphylococcal isolates and five *E. coli* isolates were recovered. Of the 15 presumptive staphylococcal isolates, 10 were identified as *S. aureus* and five as CoNS. Five *S. aureus* and five *E. coli* isolates were selected from the culture-confirmed isolates for molecular confirmation by PCR.

Genomic DNA was extracted from the selected isolates using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. Briefly, bacterial cells were processed using the kit protocol for cell lysis and genomic DNA release, followed by purification of the extracted DNA. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (DeNovix Inc., Wilmington, USA) by measuring absorbance at 260 and 280 nm before PCR amplification. DNA preparations displaying an A_{260}/A_{280} absorbance ratio within the prespecified acceptable range of 1.8 to 2.0 (measured via NanoDrop spectrophotometry) were utilized for subsequent PCR amplification. DNA samples of acceptable purity were subsequently used for PCR.

The *femA* gene was used as a molecular identification marker for *S. aureus*, whereas the *phoA* gene was used for *E. coli*. The expected amplicon sizes were 647 and 903 bp, respectively. Thus, PCR was used to molecularly confirm a selected subset of the phenotypically identified isolates rather than the entire collection of bacterial isolates [12]. The primer sequences, target genes, and expected amplicon sizes are presented in Table 1.

Table 1: Primers used for PCR amplification of target genes.

Target organism	Target gene	Primer sequence (5'–3')	Amplicon size (bp)
<i>S. aureus</i>	<i>femA</i>	F: AACAGCTAAAGAGTTTGGTGCC	647
		R: CATCACGATCAGCAAAAGCT	
<i>E. coli</i>	<i>phoA</i>	F: GTGACAAAAGCCGGACACCATAAATGCCT	903
		R: TACTACTGTCATTACGTTGCGGATTGGCGT	

F = Forward primer, R = Reverse primer, PCR = Polymerase chain reaction.

PCR reactions were prepared using GoTaq® Green Master Mix (Promega, USA) in a final reaction volume of 25 µL. Amplification was performed using a T100™ Thermal Cycler (Bio-Rad, USA). Thermal cycling consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, as described in [13]. A final extension was performed at 72°C for 5 min. Standard reference strains were included as positive controls in each PCR run (*Staphylococcus aureus* ATCC 25923 for the *femA* gene and *Escherichia coli* ATCC 25922 for the *phoA* gene), whereas reaction master mix containing nuclease-free water in place of template DNA served as the non-template negative control. Appropriate PCR controls were included in each run to monitor amplification performance and potential contamination. PCR products were separated by agarose gel electrophoresis and visualized under ultraviolet illumination.

Milk quality analysis

Milk physicochemical characteristics, including pH, electrical conductivity, protein content, and fat content, were measured using a LαKtαN 700 milk analyzer (HiYi Company, China). Samples were analyzed after arrival at the laboratory following refrigerated transportation at 4°C within 6 h of collection. Before analysis, samples were gently homogenized according to the manufacturer's operating instructions. The analyzer was checked and calibrated according to the manufacturer's recommended procedure before sample analysis. Each sample was analyzed in triplicate, and the mean of the three technical measurements was used for statistical analysis.

Statistical analysis

Data were analyzed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). Categorical variables, including CMT status and bacterial isolation, were analyzed using the Chi-square test or Fisher's exact test, as appropriate. Logistic regression was used to assess associations between mastitis-related categorical outcomes and explanatory variables, including animal species and age group, where applicable. Continuous milk quality parameters were summarized as mean ± standard deviation and analyzed using a factorial model incorporating animal species, age group, and their interaction. Where farm-level clustering was applicable, farm was included as a random effect in a mixed-effects model.

Technical triplicate measurements from each sample were averaged before statistical analysis. The normality of continuous variables was assessed before parametric analyses. Effect estimates, 95% confidence intervals, and exact two-sided p-values were reported. Statistical significance was set at $p < 0.05$ [14].

RESULTS

CMT-positive findings

Among the 300 milk samples examined, 145 (48.3%) were CMT-positive, comprising 113/200 (56.5%) ewe samples and 32/100 (32.0%) goat samples. Clinical signs were assessed before sample collection, and CMT-positive animals without overt clinical signs were considered suspected cases of subclinical mastitis. As shown in Table 2, CMT reactions among positive samples ranged from trace to strong positive (+3). The strong positive (+3) reaction accounted for the highest proportion of CMT-positive goat samples (40.6%), whereas the weak positive (+1) reaction was the most frequent among CMT-positive ewe samples (33.6%).

Table 2: Distribution of CMT results and CMT scores in ewes and goats in Al-Kut City.

CMT result	Ewe milk, n	Ewe milk (%)	Goat milk, n	Goat milk (%)
+1	38	33.6	9	28.1
+2	19	16.8	10	31.3
+3	34	30.1	13	40.6
Trace	22	19.5	—	—
CMT-positive	113	56.5	32	32.0
CMT-negative	87	43.5	68	68.0

CMT = California Mastitis Test. Total samples examined = 200 ewe milk samples and 100 goat milk samples. Percentages for +1, +2, +3, and trace reactions were calculated among CMT-positive samples within each species; percentages for CMT-positive and CMT-negative results were calculated using the total number of samples examined within each species.

NAGase activity in mastitis milk samples

The NAGase reaction intensity across the tested milk samples was categorized semi-quantitatively as light (+), medium (++), or intense (+++) visual yellow reactions, as detailed in Table 3. In ewe milk samples, light yellow (+) reactions were most frequent (53/113; 46.9%), followed by medium yellow (++; 31/113; 27.4%) and intense yellow (+++; 29/113; 25.7%) reactions. In goat milk samples, light yellow reactions predominated (23/32; 71.9%), followed by medium yellow reactions (8/32; 25.0%), whereas an intense yellow reaction was detected in only one sample (1/32; 3.1%).

Table 3: Semi-quantitative NAGase reaction intensity in CMT-positive milk samples from ewes and goats.

NAGase reaction intensity	Ewe milk, n	Ewe milk (%)	Goat milk, n	Goat milk (%)
Light yellow (+)	53	46.9	23	71.9
Medium yellow (++)	31	27.4	8	25.0
Intense yellow (+++)	29	25.7	1	3.1
Total	113	100	32	100

CMT = California Mastitis Test, NAGase = N-acetyl- β -D-glucosaminidase. NAGase reactions were evaluated semi-quantitatively according to the intensity of the yellow color produced by p-nitrophenol.

Phenotypic and biochemical characterization of bacterial isolates

Bacterial isolates recovered from mastitis-associated milk samples were characterized based on colony morphology, Gram-staining characteristics, growth on selective and differential media, and biochemical reactions. The phenotypic and biochemical characteristics of the identified bacterial species are summarized in Table 4.

Fifteen presumptive staphylococcal isolates were recovered from the culture-positive milk samples. Based on biochemical identification, 10 isolates were identified as coagulase-positive *S. aureus*, whereas five were classified as CoNS. *S. aureus* isolates were characterized as Gram-positive cocci arranged in clusters and showed positive catalase, DNase, mannitol fermentation, and coagulase reactions. On mannitol salt agar, the isolates showed characteristic growth associated with mannitol fermentation. CoNS isolates were Gram-positive cocci arranged in clusters and showed positive catalase activity but negative coagulase, DNase, and mannitol fermentation reactions (Table 4).

E. coli isolates were characterized as Gram-negative rods and produced pink, lactose-fermenting colonies on MacConkey agar. The isolates were positive for indole production and methyl red reactions and exhibited characteristic reactions on triple sugar iron agar. On eosin methylene blue agar, the isolates produced a characteristic metallic-green sheen and exhibited motility on semi-solid medium.

Table 4: Phenotypic and biochemical characteristics of mastitis-associated bacterial isolates.

Characteristic	<i>Staphylococcus aureus</i> (n = 10)	<i>Escherichia coli</i> (n = 5)	CoNS (n = 5)	<i>Streptococcus uberis</i> (n = 5)
Gram stain	+	–	+	+
Cellular morphology	Gram-positive cocci in clusters	Gram-negative rods	Gram-positive cocci in clusters	Gram-positive cocci in pairs and chains
Growth condition	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Catalase	+	+	+	–
DNase	+	–	–	–
Mannitol fermentation	+	–	–	–
Coagulase	+	–	–	–
Selective/differential medium	Mannitol salt agar (+)	EMB agar, metallic-green sheen (+)	–	–

CoNS = Coagulase-negative staphylococci, DNase = Deoxyribonuclease, EMB = Eosin methylene blue. + = Positive reaction, – = Negative reaction.

S. uberis isolates were characterized as Gram-positive cocci arranged in pairs and chains. The isolates produced small, translucent colonies on CO₂-supplemented blood agar and showed hemolytic activity. They were negative for catalase, coagulase, DNase, and mannitol fermentation reactions. Identification of the bacterial isolates was subsequently confirmed using the corresponding API identification systems.

A total of 145 CMT-positive milk samples, comprising 113 ewe and 32 goat samples, were subjected to bacteriological culture. Mixed bacterial infections accounted for 86/145 (59.3%) culture-positive samples. Among the individual bacterial findings presented in Figure 1, *S. aureus* was the most frequently identified (16.9%), followed by *E. coli* (13.5%) and CoNS (8.9%), whereas *S. uberis* was identified at the lowest frequency (1.6%).

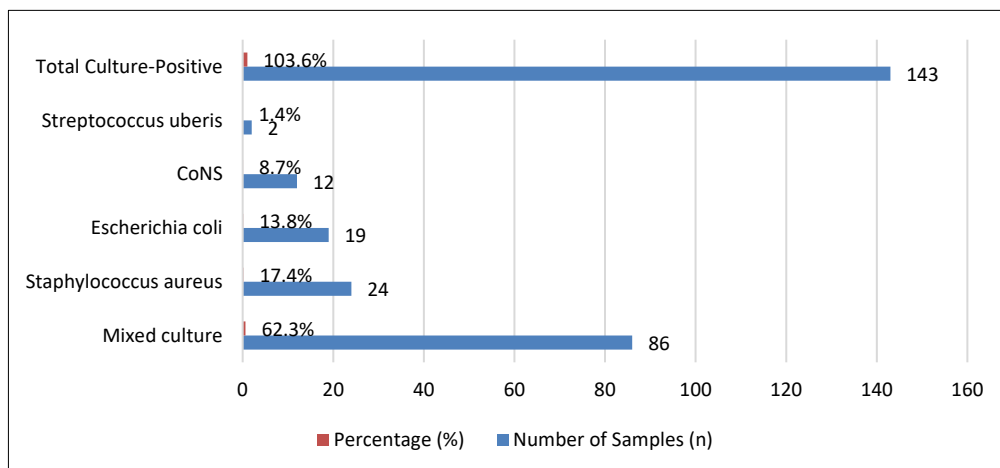


Figure 1: Distribution of bacterial isolation categories recovered from CMT-positive milk samples Distribution (%) of bacterial isolates recovered from CMT-positive milk samples from ewes and goats. CMT = California mastitis test.

Molecular confirmation of bacterial isolates

PCR was performed to molecularly confirm selected *S. aureus* and *E. coli* isolates following phenotypic and biochemical identification. Amplification of the *femA* gene yielded the expected 647-bp amplicon in the five selected *S. aureus* isolates (Figure 2). Lane M represents the 100-bp DNA ladder, lanes 1–5 represent the positive *S. aureus* isolates showing the expected 647-bp amplicon, and NC represents the negative control.

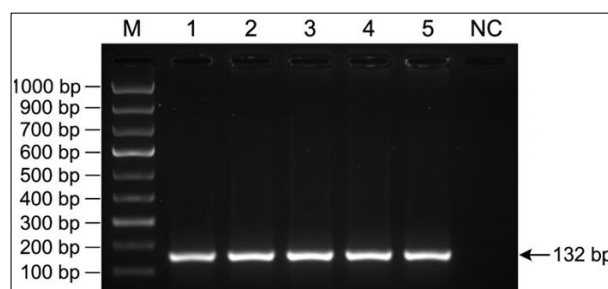


Figure 2: Agarose gel electrophoresis showing PCR product amplification of the *femA* gene (132 bp) for the molecular identification of *Staphylococcus aureus* isolates recovered from CMT-positive milk samples. Lane M: 100-bp DNA ladder; Lanes 1–5: Representative positive *Staphylococcus aureus* field isolates exhibiting the target 132-bp band; Lane NC: Negative control (nuclease-free water). CMT = California mastitis test; PCR = Polymerase chain reaction.

Similarly, amplification of the *phoA* gene yielded the expected 903-bp amplicon in the five selected *E. coli* isolates (Figure 3). Lane M represents the 100-bp DNA ladder, lanes 1–5 represent the positive *E. coli* isolates showing the expected 903-bp amplicon, and NC represents the negative control. These amplification results molecularly confirmed the identities of the selected phenotypically and biochemically identified *S. aureus* and *E. coli* isolates.

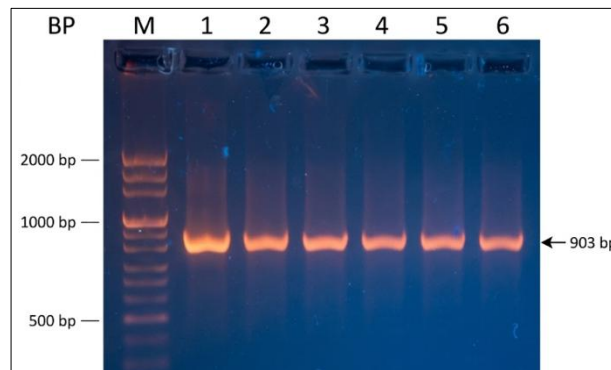


Figure 3: Agarose gel electrophoresis showing PCR product amplification of the *phoA* gene (903 bp) for the molecular identification of *Escherichia coli* isolates recovered from CMT-positive milk samples. Lane M: 100-bp DNA ladder; Lanes 1–6: Representative positive *E. coli* field isolates displaying the characteristic 903-bp amplicon; Lane NC: Negative control (nuclease-free water). CMT = California mastitis test; PCR = Polymerase chain reaction.

Milk quality parameters

Milk quality parameters varied between ewes and goats across the examined age groups (Table 5). pH values ranged from 7.50 to 7.62, with no significant effects of animal species ($p = 0.684$), age group ($p = 0.742$), or species $\times$ age group interaction ($p = 0.816$).

Table 5: Milk quality parameters in ewes and goats according to age group.

Age group (years)	Milk quality parameter	Ewe, mean $\pm$ SD (95% CI)	Goat, mean $\pm$ SD (95% CI)	Species p-value	Age p-value	Species $\times$ age p-value
2–4 (Ewe n = 50; Goat n = 20)	pH	7.52 $\pm$ 0.13 (7.48–7.56)	7.61 $\pm$ 0.12 (7.55–7.67)	0.684	0.742	0.816
	EC (mS/cm)	12.56 $\pm$ 0.17 (12.51–12.61)	9.71 $\pm$ 0.32 (9.56–9.86)	<0.001	<0.001	<0.001
	Protein (%)	3.87 $\pm$ 0.23 (3.80–3.94)	3.10 $\pm$ 0.24 (2.99–3.21)	0.018	0.537	0.031
	Fat (%)	4.44 $\pm$ 0.33 (4.35–4.53)	4.37 $\pm$ 0.16 (4.30–4.44)	0.761	0.824	0.913
4–6 (Ewe n = 90; Goat n = 35)	pH	7.61 $\pm$ 0.18 (7.57–7.65)	7.50 $\pm$ 0.21 (7.43–7.57)	0.684	0.742	0.816
	EC (mS/cm)	12.51 $\pm$ 0.29 (12.45–12.57)	9.10 $\pm$ 0.50 (8.93–9.27)	<0.001	<0.001	<0.001
	Protein (%)	3.82 $\pm$ 0.17 (3.78–3.86)	3.84 $\pm$ 0.18 (3.78–3.90)	0.018	0.537	0.031
	Fat (%)	4.44 $\pm$ 0.25 (4.39–4.49)	4.31 $\pm$ 0.24 (4.23–4.39)	0.761	0.824	0.913
6–8 (Ewe n = 60; Goat n = 45)	pH	7.62 $\pm$ 0.15 (7.58–7.66)	7.55 $\pm$ 0.25 (7.47–7.63)	0.684	0.742	0.816
	EC (mS/cm)	7.70 $\pm$ 0.23 (7.64–7.76)	9.81 $\pm$ 0.33 (9.71–9.91)	<0.001	<0.001	<0.001
	Protein (%)	3.91 $\pm$ 0.33 (3.82–4.00)	4.13 $\pm$ 0.25 (4.05–4.21)	0.018	0.537	0.031
	Fat (%)	4.31 $\pm$ 0.22 (4.25–4.37)	4.32 $\pm$ 0.18 (4.27–4.37)	0.761	0.824	0.913

CI = Confidence interval, EC = Electrical conductivity, SD = Standard deviation. Values are presented as mean $\pm$ SD with 95% CI. Data were analyzed using a factorial model including animal species, age group, and the species $\times$ age group interaction. The reported p-values represent the main effects of species and age group and their interaction and are not separate comparisons within individual age groups. Statistical significance was set at $p < 0.05$.

Electrical conductivity showed significant effects of animal species, age group, and their interaction ($p < 0.001$ for all effects). Protein content showed a significant species effect ($p = 0.018$) and species $\times$ age group

interaction ($p = 0.031$), whereas the age group effect was not significant ($p = 0.537$). Fat content showed no significant effects of species ($p = 0.761$), age group ($p = 0.824$), or their interaction ($p = 0.913$).

Overall, electrical conductivity differed significantly according to species and age group, whereas protein content differed significantly according to species and showed a significant species $\times$ age group interaction. No significant effects were observed for pH or fat content. These results represent comparisons among the examined animals and do not constitute direct comparisons with a predefined healthy control group.

DISCUSSION

CMT-positive findings and species-related differences

The present study identified a higher proportion of CMT-positive milk samples in ewes than in goats within the examined population. This finding demonstrates a species-related difference in CMT-positive mastitis-associated findings; however, the cross-sectional study design does not permit identification of the biological, environmental, or management factors responsible for this difference [15]. Mastitis is an economically important disease of dairy sheep and goats because it adversely affects milk production, milk quality, and farm profitability [16]. Therefore, the higher proportion of CMT-positive findings observed among ewes warrants consideration within the local production context.

Differences in susceptibility to mastitis between small-ruminant species may be influenced by multiple host- and management-related factors. Udder characteristics, immune responses, nutritional status, housing conditions, milking hygiene, bedding cleanliness, udder preparation, and exposure to contaminated environmental surfaces may influence the occurrence and transmission of mastitis-associated pathogens [17]. However, the present study did not directly measure these factors. Consequently, the higher proportion of CMT-positive samples among ewes should not be attributed to any specific biological or management factor on the basis of the present data.

CMT is widely used as an indirect screening method for detecting mammary inflammation. In the present study, CMT reactions ranged from trace to +3, with the +3 category accounting for the highest proportion among CMT-positive goat samples, whereas +1 reactions predominated among CMT-positive ewe samples. Higher CMT scores are generally associated with increased somatic cell concentrations and greater mammary inflammatory responses [18]. Previous studies have also described the characteristic increase in viscosity and gel formation associated with stronger CMT reactions [19, 20]. These physical changes reflect the interaction between the CMT reagent and milk cellular components and form the basis for semi-quantitative interpretation of the test [21]. However, because the present study did not directly measure somatic cell count, CMT reaction intensity should be interpreted as a screening indicator rather than a quantitative measure of inflammatory severity.

NAGase activity

NAGase has been investigated as an indicator of mammary epithelial integrity and inflammatory changes during mastitis [22]. Increased NAGase activity in milk has been associated with cellular damage and the release of lysosomal enzymes from mammary epithelial cells and leukocytes during inflammatory processes [22, 23]. In the present study, all examined CMT-positive samples produced measurable semi-quantitative NAGase color reactions, although the distribution of reaction intensities differed between ewes and goats. Light yellow reactions predominated in both species, particularly among goats, whereas intense reactions were less frequent.

Previous investigations have reported increased milk NAGase activity in animals with clinical or subclinical mastitis, supporting its potential utility as an adjunct indicator of udder health [24]. Nevertheless, the present assay was interpreted visually and semi-quantitatively, without spectrophotometric measurement or a standard curve. Accordingly, differences in color intensity cannot be directly translated into quantitative enzymatic activity or severity of mammary tissue damage. NAGase serves as a sensitive lysosomal biomarker associated with mammary epithelial cellular damage and inflammation during intramammary infection [25]. NAGase findings in this study should therefore be interpreted together with CMT and bacteriological results rather than as an independent diagnostic endpoint. The use of complementary indirect screening procedures may improve the identification of animals requiring further diagnostic evaluation [26].

Phenotypic and biochemical characterization of bacterial isolates

Bacteriological findings showed that mastitis-associated milk samples contained both Gram-positive and Gram-negative bacterial species, highlighting the heterogeneous bacterial profile associated with mammary

infections. Mixed bacterial infections represented 59.3% of culture-positive milk samples. Similar polymicrobial findings have been reported under field conditions [27]. Exposure to multiple bacterial sources within the animal environment and during milking has been proposed as one factor contributing to mixed bacterial recovery [28].

The high proportion of mixed bacterial infections observed in the present study suggests that multiple bacterial species were frequently recovered from the same CMT-positive milk sample. Similar observations have been reported in other mastitis investigations [29]. However, comparisons of mixed-infection frequencies among studies should be made cautiously because reported proportions can be influenced by differences in animal populations, sampling procedures, geographical conditions, mastitis definitions, culture methods, and criteria used to classify bacterial isolates [30–32]. Inadequate milking hygiene, contaminated housing or bedding, and repeated environmental exposure may contribute to polymicrobial contamination or infection [33]; however, these factors were not quantitatively evaluated in the present study and therefore cannot be considered established causes of the observed findings.

Among the individual bacterial findings, *S. aureus* was the most frequently identified species. Ten of the 15 presumptive staphylococcal isolates were identified as coagulase-positive *S. aureus*, whereas the remaining five were classified as CoNS [34]. The *S. aureus* isolates exhibited biochemical characteristics consistent with their phenotypic identification, including positive catalase, DNase, mannitol fermentation, and coagulase reactions [35]. *S. aureus* is recognized as an important mastitis-associated pathogen in ruminants because of its capacity to colonize mammary tissue and persist within infected mammary glands [36–38]. The remaining CoNS isolates showed a distinct biochemical profile characterized by catalase positivity and negative coagulase reactions [39].

E. coli was the second most frequently identified bacterial species. Its phenotypic and biochemical characteristics included lactose fermentation on MacConkey agar, a metallic-green sheen on eosin methylene blue agar, positive indole and methyl red reactions, and characteristic API 20E profiles. *E. coli* is commonly regarded as an environmentally associated mastitis pathogen and may gain access to the mammary gland following exposure to contaminated surroundings. *S. uberis* was detected less frequently in the present study. Nevertheless, its identification further demonstrates that multiple bacterial taxa were associated with CMT-positive milk samples in the examined population [40].

Molecular confirmation of selected bacterial isolates

Molecular analysis further confirmed the identities of selected phenotypically and biochemically characterized isolates. PCR amplification of the *femA* and *phoA* genes generated the expected amplicons for the selected *S. aureus* and *E. coli* isolates, respectively [41, 42]. The agreement between the phenotypic identification and target gene amplification supports the usefulness of combining conventional bacteriological approaches with molecular confirmation when characterizing selected bacterial isolates.

PCR provides a targeted approach for confirming bacterial identity and can complement conventional culture and biochemical characterization [43, 44]. However, in the present study, molecular confirmation was performed on only five *S. aureus* and five *E. coli* isolates. Therefore, these results confirm the selected isolates rather than characterizing the complete bacterial population recovered from the milk samples. Consequently, these findings should not be extrapolated to all bacterial isolates obtained in this study.

Milk quality parameters

The present study demonstrated differences in selected milk quality parameters between ewes and goats and among the examined age groups. Electrical conductivity showed significant effects of species, age group, and their interaction, whereas protein content showed a significant species effect and species × age group interaction. In contrast, pH and fat content showed no significant effects of species, age group, or their interaction.

Electrical conductivity of milk can vary with alterations in ionic composition and mammary gland permeability associated with inflammatory processes [45–47]. Nevertheless, the present study did not include a predefined healthy control group and did not directly measure somatic cell count. Therefore, the observed differences in electrical conductivity cannot be interpreted as changes caused specifically by mastitis. Instead, they represent variation among the examined animals according to species and age group.

Protein content also differed significantly according to species and showed a species × age group interaction. Previous studies have linked changes in mammary gland physiology and milk composition to inflammatory processes [48, 49]. However, because the current study lacked a healthy comparison group, the observed protein differences cannot be attributed directly to mastitis. Similarly, the absence of significant effects for pH and fat content indicates that these parameters did not vary significantly according to the factors included in the present

statistical model. Previous studies have shown that milk composition can vary with mammary health status and other physiological and management-related factors [50, 51]. Accordingly, the present findings should be regarded as descriptive characteristics of the examined CMT-positive population rather than evidence of mastitis-induced physicochemical changes.

Study limitations

This study has several limitations. First, animals were recruited from accessible smallholder farms rather than through a population-based sampling design; therefore, the findings should not be generalized to all small-ruminant populations in Iraq. Second, the study did not include a predefined healthy control group, limiting direct comparisons of milk quality parameters between healthy and CMT-positive animals. Third, somatic cell count was not measured, and clinical and suspected subclinical cases were not analyzed as separate outcome groups. Fourth, the study did not quantitatively assess farm-level, nutritional, environmental, housing, and milking hygiene characteristics and therefore could not evaluate them as potential risk factors. Fifth, antimicrobial susceptibility testing was not performed, and the antimicrobial resistance profiles of the recovered bacterial isolates remain unknown. Finally, molecular confirmation was performed only on selected *S. aureus* and *E. coli* isolates rather than on the complete bacterial collection.

Future studies should incorporate population-based sampling strategies, predefined healthy comparison groups, direct somatic cell count measurements, and antimicrobial susceptibility testing. Molecular characterization of a larger proportion of recovered isolates would provide a more comprehensive understanding of the bacterial population associated with mastitis. Analytical studies incorporating farm-level, environmental, nutritional, housing, and milking-management variables would also help identify factors associated with CMT-positive findings and bacterial infections in small-ruminant production systems.

CONCLUSION

This study demonstrated a higher proportion of CMT-positive milk samples among ewes than goats in the examined small-ruminant population in Al-Kut City, Iraq. Bacteriological examination identified several mastitis-associated bacterial species, with mixed bacterial infections accounting for a substantial proportion of culture-positive samples. Among the individual bacterial findings, *S. aureus* was the most frequently identified pathogen, followed by *E. coli*, CoNS, and *S. uberis*. Molecular amplification of the *femA* and *phoA* genes confirmed the identities of selected *S. aureus* and *E. coli* isolates, respectively. Milk quality analysis also showed significant variation in electrical conductivity and protein content by species and/or age-related factors, whereas pH and fat content did not show significant effects in the revised analysis.

The findings provide useful local evidence on CMT-positive mastitis-associated findings, bacterial profiles, and selected milk quality characteristics in small-ruminant herds managed under smallholder conditions. Routine udder examination, CMT-based screening, appropriate milking hygiene, and timely bacteriological investigation of suspected cases may support earlier detection and improved mastitis control practices. Where resources permit, molecular confirmation of selected isolates can complement conventional bacteriological identification and strengthen diagnostic confidence.

A major strength of this study was its multimodal diagnostic approach, combining CMT screening, semi-quantitative NAGase assessment, conventional bacteriological culture, biochemical identification, and molecular confirmation of selected bacterial isolates. The inclusion of both ewes and goats enabled species-level comparison within the same geographical setting, while age group analysis provided additional information on variation among the examined animals. The study also incorporated physicochemical milk quality measurements, thereby providing a broader assessment of mastitis-associated findings beyond pathogen detection alone. In addition, the combination of phenotypic and molecular approaches strengthened the identification of selected *S. aureus* and *E. coli* isolates and generated locally relevant baseline information for small-ruminant production systems in Al-Kut.

Overall, the study provides integrated bacteriological, molecular, and milk quality evidence on mastitis-associated findings in ewes and goats in Al-Kut City. The predominance of CMT-positive findings in ewes, the frequent occurrence of mixed bacterial infections, and the recovery of important mastitis-associated pathogens highlight the need for continued surveillance and improved udder health management in local small-ruminant systems. Although the present findings should not be generalized beyond the study population, they provide a useful foundation for future epidemiological, antimicrobial resistance, and molecular investigations aimed at strengthening mastitis prevention and control in sheep and goats.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

During the preparation of this manuscript, the authors used Some AI tools for solely to improve language clarity and readability. The authors thoroughly reviewed, verified, and edited all output, and take full responsibility for the integrity, accuracy, and original content of the published work.

AUTHORS' CONTRIBUTIONS

HDS: Conceptualization, methodology, formal analysis, investigation, visualization, supervision, writing – original draft, and writing – review and editing. SRF, SNG, ZMA, and AJT: Methodology and investigation. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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