



Prevalence of subclinical mastitis and antimicrobial resistance profiles in dairy cows from a milk collection centre in Tualag, Ecuador

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Abstract

Subclinical mastitis remains a major constraint to dairy productivity in smallholder systems, however, Ecuador-specific evidence describing pathogen profiles and antimicrobial susceptibility patterns is still limited. This cross-sectional study estimated cow-level prevalence of subclinical mastitis using the California Mastitis Test (CMT) and characterised bacterial isolates and their antimicrobial susceptibility among dairy cows supplying a milk collection centre in Tualag, Ecuador. Seventy clinically healthy lactating cows were screened at the quarter level using CMT, and cows with at least one CMT-positive quarter were classified as positive. Milk samples from CMT-positive cows ($n = 13$) were cultured using standard bacteriological techniques, and antimicrobial susceptibility was assessed using the Kirby-Bauer disk diffusion method. Results were classified as susceptible, intermediate, or resistant. Thirteen of 70 cows tested CMT-positive, corresponding to a cow-level prevalence of 18.6% (95% CI: 11.2–29.2). No significant differences were observed in the distribution of CMT-positive quarters or among cow age groups. *Staphylococcus aureus* was identified as the most common pathogen (38.5%), followed by other *Staphylococcus* spp. and coagulase-positive staphylococci (23.1% each). *Streptococcus uberis* and *S. dysgalactiae* were each detected in 7.7% of samples. Antimicrobial susceptibility profiles varied, particularly among staphylococcal isolates, with resistance observed to selected antimicrobial compounds. These findings confirm the presence of subclinical mastitis in Ecuadorian smallholder dairy systems and indicate that infections are predominantly associated with staphylococcal pathogens, underscoring the importance of improved milking hygiene and locally informed antimicrobial management.

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1. Introduction

Subclinical mastitis (SCM) represents a significant challenge for dairy production in Ecuador, particularly within smallholder systems, where routine mastitis screening and access to laboratory diagnostics are often limited (Amer et al. 2018; Muloi et al. 2025). Additionally, these smallholder producers frequently exhibit suboptimal herd health management practices, allowing intramammary infections to persist undetected, thereby reducing milk yield, compromising milk quality, and ultimately undermining farm profitability (Amer et al. 2018; Halasa et al. 2007). Consequently, despite the economic importance of the dairy sector in several regions of Ecuador, epidemiological evidence regarding the prevalence of SCM, associated aetiological agents, and antimicrobial susceptibility patterns in smallholder production systems remains limited. Subclinical mastitis is widely recognised as a major source of economic loss in dairy production worldwide. Recent evidence from Brazil indicates that milk production losses comprised 63.8% of total mastitis-related costs, followed by animal disposal (29.5%) (Corrêa et al. 2024). Likewise, field-based assessments in Mexico have estimated mastitis-associated losses of approximately USD 0.248–1.253 per cow per day (Aparicio Roque et al. 2025). Because the subclinical form typically occurs without overt signs, infections may persist for prolonged periods unless specific screening tools are

applied, thereby contributing substantially to cumulative production losses (Cuenca-Condoy et al. 2024).

A broad range of bacterial pathogens has been associated with SCM. However, Gram-positive organisms, including *S. aureus*, coagulase-negative staphylococci (CNS), and *Streptococcus* spp., are most frequently reported across diverse dairy systems (Kerro Dego and Vidlund 2024; Touaitia et al. 2025). These pathogens differ in their epidemiology and transmission dynamics, with *S. aureus* is primarily transmitted during milking and often associated with persistent or recurrent infections. In contrast, *S. uberis* and *S. dysgalactiae* are more commonly linked to exposure to contaminated environments and are influenced by housing conditions and hygiene practices (Nogara et al. 2025). Antimicrobial resistance (AMR) has also become a key challenge in mastitis management, as it can reduce treatment efficacy and promote persistence of infection within herds. In small-scale dairy systems, antimicrobials are frequently used for mastitis treatment, sometimes in the absence of bacteriological confirmation, which may facilitate the emergence of resistant strains. Recent studies have reported substantial variability in susceptibility profiles, particularly among staphylococci, underscoring the importance of locally generated antimicrobial susceptibility data to inform rational therapeutic choices (Kumar et al. 2025; Mazreku et al. 2025). From a One Health perspective, the persistence of antimicrobial-resistant bacteria in dairy

herds may also pose risks along the food chain and to public health (Tarigan et al. 2025).

In resource-limited settings, field diagnostic tests such as the California Mastitis Test (CMT) are widely used to detect elevated somatic cell counts and to identify cows suspected of having subclinical mastitis (SCM) (Stanek et al. 2024; Tommasoni et al. 2023). However, this test does not provide information on the aetiological agent or on antimicrobial susceptibility. Consequently, it should be complemented with bacteriological culture and antimicrobial susceptibility testing in order to obtain a more robust diagnostic framework. In Ecuador, several studies have reported the presence of subclinical mastitis (SCM) and have independently described aspects of its local bacterial aetiology, highlighting the need to generate evidence that simultaneously characterises SCM prevalence, pathogen distribution, and antimicrobial susceptibility patterns, particularly within small-scale dairy production systems. Such information is essential for informing mastitis control programmes adapted to the local context and for supporting the prudent use of antimicrobials under prevailing production conditions. Thus, the current study sought to: (i) use the California Mastitis Test to estimate the prevalence of subclinical mastitis in cows; (ii) use bacteriological culture to identify the bacterial pathogens associated with CMT-positive cows; and (iii) characterize the antimicrobial susceptibility patterns of the recovered isolates in dairy cows from smallholder farms that supply a milk collection centre in Ecuador.

2. Materials and Methods

2.1. Study design and animals

A cross-sectional descriptive study was conducted at a milk collection centre, a representative of small-scale dairy production systems in Ecuador. The study population consisted of 85 lactating dairy cows regularly supplying milk to the collection centre at the time of the study. From this population, 70 cows were selected based on predefined inclusion and exclusion criteria. The sampling strategy consisted of near-exhaustive sampling of the accessible lactating cow population supplying the milk collection centre. Of the 85 cows present during the study period, 70 met the predefined inclusion criteria, representing approximately 82% of the study population. Eligible animals were lactating cows that were clinically healthy at the time of sampling and actively contributing milk to the collection centre. Cows exhibiting clinical signs of mastitis and/or evidence of systemic illness were excluded. All enrolled animals were managed under typical smallholder conditions, with hand or machine milking in accordance with routine practices within the local production system. Basic characteristics of the study population, including age distribution, breed type, production system, and milking method, were recorded during sampling and are summarised in Table 1. The dairy cows included in the study were predominantly crossbred animals typical of smallholder dairy systems in the region. Detailed production variables such as parity and stage of lactation were not consistently available for all animals supplying milk to the collection centre and therefore not included in the analysis.

2.2. Screening for subclinical mastitis

Screening for subclinical mastitis was conducted during routine milking using the California Mastitis Test (CMT) across the different udder quarters. Milk samples were placed directly into the CMT paddle wells, an equal volume of CMT reagent was added, and the mixture

was gently swirled until gel formation and changes in viscosity were observed (Tesfaye 2018). CMT reactions were interpreted using the standard scoring scale (negative, trace, 1+, 2+, and 3+). A quarter was considered positive when the CMT score was $\geq 1+$, indicating an increased somatic cell content consistent with subclinical mastitis. A cow was classified as CMT-positive when at least one quarter showed a score of $\geq 1+$ (Dingwell et al. 2003).

2.3. Bacteriological analysis

Bacteriological analysis was performed on cows presenting at least one CMT-positive quarter, and an aseptic milk sample was collected from the quarter with the highest CMT score for bacteriological culture. Prior to sampling, the teats were disinfected with 70% ethanol and the first streams of milk were discarded to reduce the risk of environmental contamination. Subsequently, approximately 10 mL of milk was collected into sterile tubes and transported to the laboratory under refrigerated conditions (4 °C), arriving within 4 hours of collection. For bacteriological culture, 10 μ L of each milk sample was inoculated onto 5% sheep blood agar and MacConkey agar (Oxoid, United Kingdom) to enable the isolation and differentiation of Gram-positive and Gram-negative bacteria associated with mastitis. The plates were incubated under aerobic conditions at 37 °C, and bacterial growth was evaluated after 24–48 h of incubation. A culture was considered positive when one or more colonies of bacteria with uniform morphology were detected, following the criteria commonly used in mastitis diagnostics by the National Mastitis Council (NMC) (Middleton et al. 2017).

Preliminary identification of isolates was based on colony morphology, haemolysis patterns on blood agar, and Gram staining. Samples showing three or more morphologically distinct bacterial colony types were considered contaminated and were excluded from aetiological interpretation (Middleton et al. 2017; Stanek et al. 2024). When two colony morphotypes were observed, the predominant type was recorded as the most probable aetiological agent. Subsequent classification to genus and, where appropriate, species level was undertaken using conventional biochemical assays. Staphylococcal isolates were characterised using the catalase test, tube coagulase test, and mannitol fermentation, enabling differentiation of *S. aureus* from other staphylococci. Streptococcal isolates were distinguished by a negative catalase reaction, together with CAMP testing, esculin hydrolysis, and haemolysis characteristics on blood agar (Stanek et al., 2024). Bacteriological procedures were quality controlled using reference strains *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, in accordance with standard microbiological quality assurance

Table 1. Characteristics of lactating dairy cows included in the study population (n = 70)			
Variable	Category	n	(%)
Age (years)	3	10	14.3
	4	12	17.1
	5	18	25.7
	6	16	22.9
	7–8	14	20.0
Breed type	Crossbred dairy cattle (Holstein $\times$ local breeds)	55	78.6
	Holstein-type	15	21.4
Milking method	Hand milking	44	62.9
	Machine milking	26	37.1

practices.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Mueller–Hinton agar (Oxoid, United Kingdom), following the methodology described by Chávez Rosero et al. (2025). Pure bacterial cultures were suspended in sterile saline and adjusted to a 0.5 McFarland turbidity standard before being evenly inoculated onto Mueller-Hinton agar plates. Antibiotic disks were applied and plates incubated under aerobic conditions at 37 °C for 18–24 h. The antimicrobial agents tested included penicillin, ampicillin, amoxicillin-clavulanic acid, tetracycline, erythromycin, gentamicin, streptomycin, enrofloxacin, and trimethoprim-sulfamethoxazole. These antimicrobials were selected because they are commonly used for mastitis treatment in veterinary practice and widely available in Ecuador. Inhibition zone diameters were measured and interpreted as susceptible, intermediate, or resistant according to CLSI interpretive standards. Quality control for susceptibility testing was performed using the same reference strains (*S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922).

2.5. Statistical analysis

Data were analysed using descriptive and inferential statistical methods. The prevalence of subclinical mastitis was estimated at the cow level, together with 95% confidence intervals (CI), calculated using the Wilson score method, which is recommended for binomial proportions when sample sizes are small or when prevalence estimates approach the limits of 0 or 1. The chi-square (χ^2) test was used to assess associations and distributions among categorical variables, including goodness-of-fit analysis to examine the distribution of CMT-positive quarters, as well as comparisons between age groups. Given the limited number of positive observations and the possibility of low cell counts, inferential results were interpreted primarily as exploratory. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). Statistical significance was evaluated at a threshold of $p < 0.05$.

3. Results

3.1. Subclinical mastitis prevalence (CMT)

Of the 70 lactating cows examined for subclinical mastitis, thirteen tested positive using the CMT, corresponding to a cow-level prevalence of 18.6% (13/70; 95% CI: 11.2–29.2) (Table 2). Considering that each cow has four udder quarters, a total of 280 quarters were examined, of which 13 tested positive, representing a quarter-level prevalence of 4.6% (13/280). The distribution of CMT-positive udder quarters varied slightly among the quarters, with the left rear quarter showing the highest proportion (30.8%), followed by the left front, right front, and right rear quarters (23.1% each) ($\chi^2 = 5.08$, $df = 4$, $p = 0.28$) (Fig. 1). With

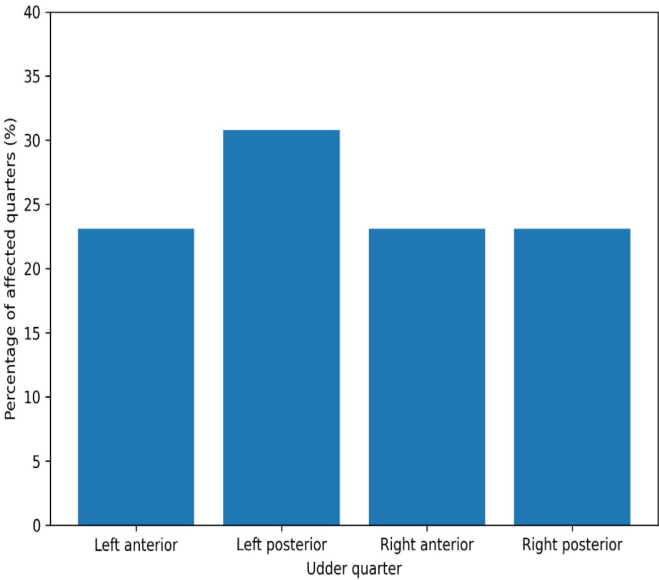


Fig. 1. Distribution of udder quarters affected by subclinical mastitis. Percentage distribution of CMT-positive quarters among the four udder quarters (n = 13)

respect to age distribution, the highest proportion of CMT-positive cases occurred in 6-year-old cows (5/13; 38.5%), followed by 5-year-old cows (4/13; 30.8%), while lower proportions were observed in 3-year-old cows (2/13; 15.4%) and in cows aged 7 and 8 years (1/13 each; 7.7%) (Fig. 2). However, due to the limited number of positive cases, these results are presented primarily as descriptive distributions to facilitate interpretation.

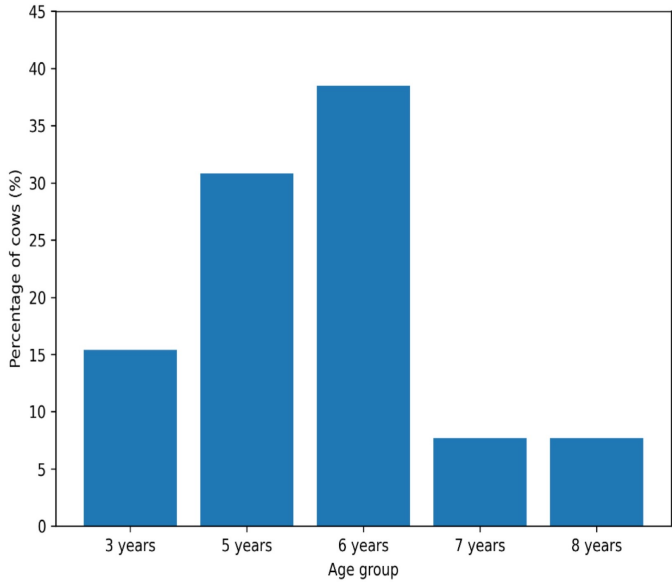


Fig. 2. Distribution of subclinical mastitis cases according to age. Percentage distribution of CMT-positive cows across age groups (n = 13)

Table 2. Prevalence of subclinical mastitis detected by the California Mastitis Test (CMT) at cow level (n = 70)			
CMT outcome	n	%	95 % CI
CMT-positive	13	18.6	11.2 - 29.2
CMT-negative	57	81.4	70.8 - 88.8
Total	70	100.0	--
Note: Cow-level prevalence was calculated as the proportion of cows with ≥1 CMT-positive quarter. The 95% confidence interval (CI) for the prevalence estimate was calculated using the Wilson method			

3.2. Bacterial isolates associated with subclinical mastitis

Bacteriological culture of milk samples collected from CMT-positive udder quarters yielded 13 isolates, corresponding to one isolate per positive quarter. *S. aureus* was the most frequently recovered pathogen, accounting for 38.5% (5/13) of the isolates. Coagulase-negative staphylococci (CNS) and unidentified *Staphylococcus* spp. each

represented 23.1% (3/13) of the isolates. The isolates reported as *Staphylococcus* spp. could not be reliably identified to the species level using the conventional phenotypic and biochemical methods applied. Coagulase-negative staphylococci represents a heterogeneous group of opportunistic mastitis pathogens commonly implicated in subclinical intramammary infections. In contrast, *S. uberis* and *S. dysgalactiae* were detected less frequently, each representing 7.7% (1/13) of the isolates (Fig. 3). Furthermore, the distribution of bacterial isolates among the age groups of CMT-positive cows showed greater pathogen diversity in cows aged 5 and 6 years, in which *S. aureus* predominated, followed by CNS and other *Staphylococcus* isolates (Fig. 4). In contrast, cows aged 3 and 7–8 years presented fewer isolates and exhibited lower pathogen diversity.

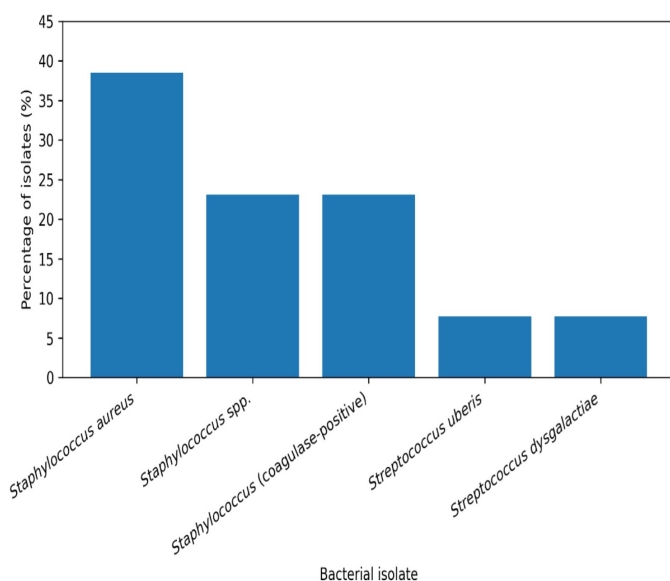


Fig. 3. Distribution of bacterial isolates recovered from cows with subclinical mastitis tested positive using the California Mastitis Test (CMT) (n = 13)

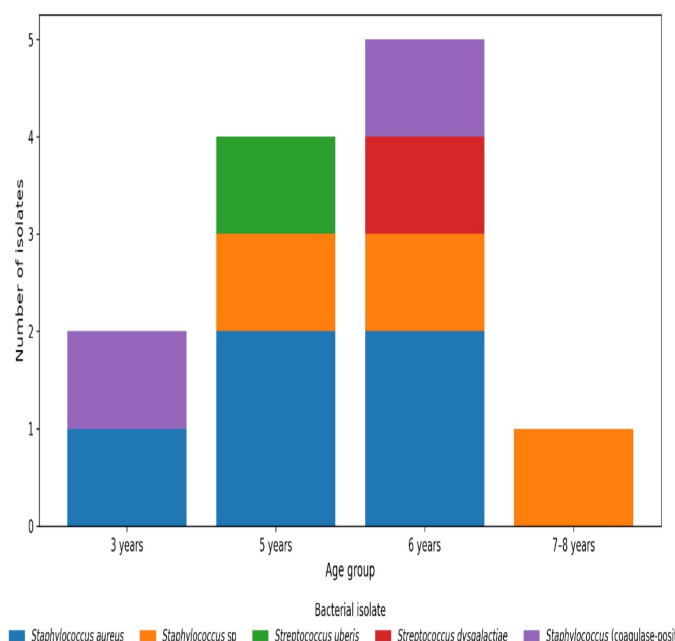


Fig. 4. Distribution of bacterial isolates according to age group

3.3 Antimicrobial susceptibility patterns

The distribution of antimicrobial susceptibility (i.e., susceptible, intermediate, resistant) among the 13 isolates recovered from CMT-positive quarters varied across bacterial groups. *S. aureus* isolates (n = 5) were predominantly susceptible to most of the agents tested, with only a few results showing intermediate or resistant antimicrobial responses. In contrast, *Staphylococcus* spp. isolates (n = 3) showed a higher proportion of resistant outcomes, suggesting reduced susceptibility to certain antimicrobials within this group. Coagulase-negative staphylococci (CNS; n = 3) exhibited more variable and agent-dependent susceptibility profiles. Among the streptococcal isolates, *S. uberis* (n = 1) demonstrated susceptible to intermediate responses, whereas *S. dysgalactiae* (n = 1) yielded resistant responses, although interpretation is limited by the small number of isolates (Fig. 5).

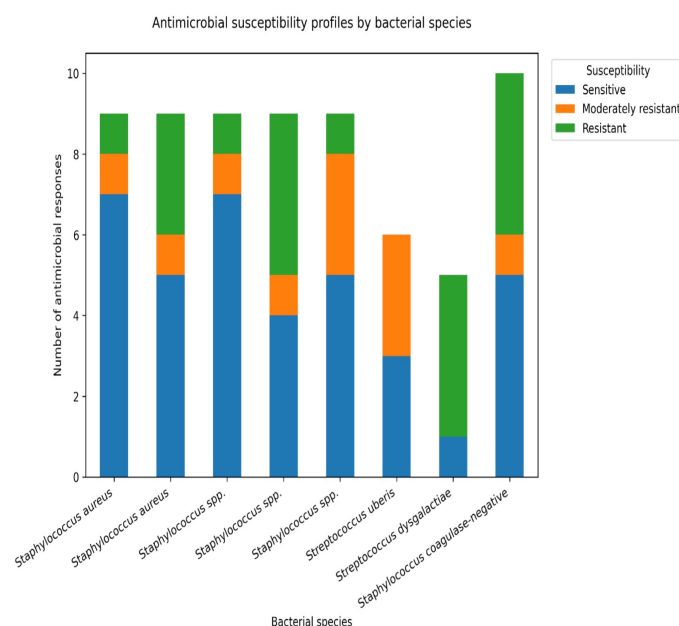


Fig. 5. Antimicrobial susceptibility profiles of bacterial species isolated from CMT-positive cows (Stacked bars represent the number of antimicrobial responses classified as susceptible, moderately resistant, or resistant for each bacterial species)

Evaluation by individual antimicrobial agents demonstrated variability among species. Both *S. aureus* and *Staphylococcus* spp. showed susceptibility to several compounds, including amoxicillin-clavulanic acid, cephalexin, gentamicin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline. However, both groups exhibited resistance to the β -lactam antibiotic cloxacillin. *S. uberis* displayed susceptible to intermediate resistance to most of the antimicrobials evaluated, although resistance to cephalexin was observed. By contrast, *S. dysgalactiae* displayed resistance to several antimicrobials, except trimethoprim-sulfamethoxazole. Likewise, coagulase-negative *Staphylococcus* isolates showed susceptibility to several antimicrobials, but resistance to cloxacillin and trimethoprim-sulfamethoxazole was detected (Fig. 6). Given the limited number of isolates for some bacterial species, particularly the streptococcal isolates (n = 1 each), the observed resistance patterns should be interpreted with caution, as these proportions may be unstable and may not reflect broader population-level trends in antimicrobial resistance.

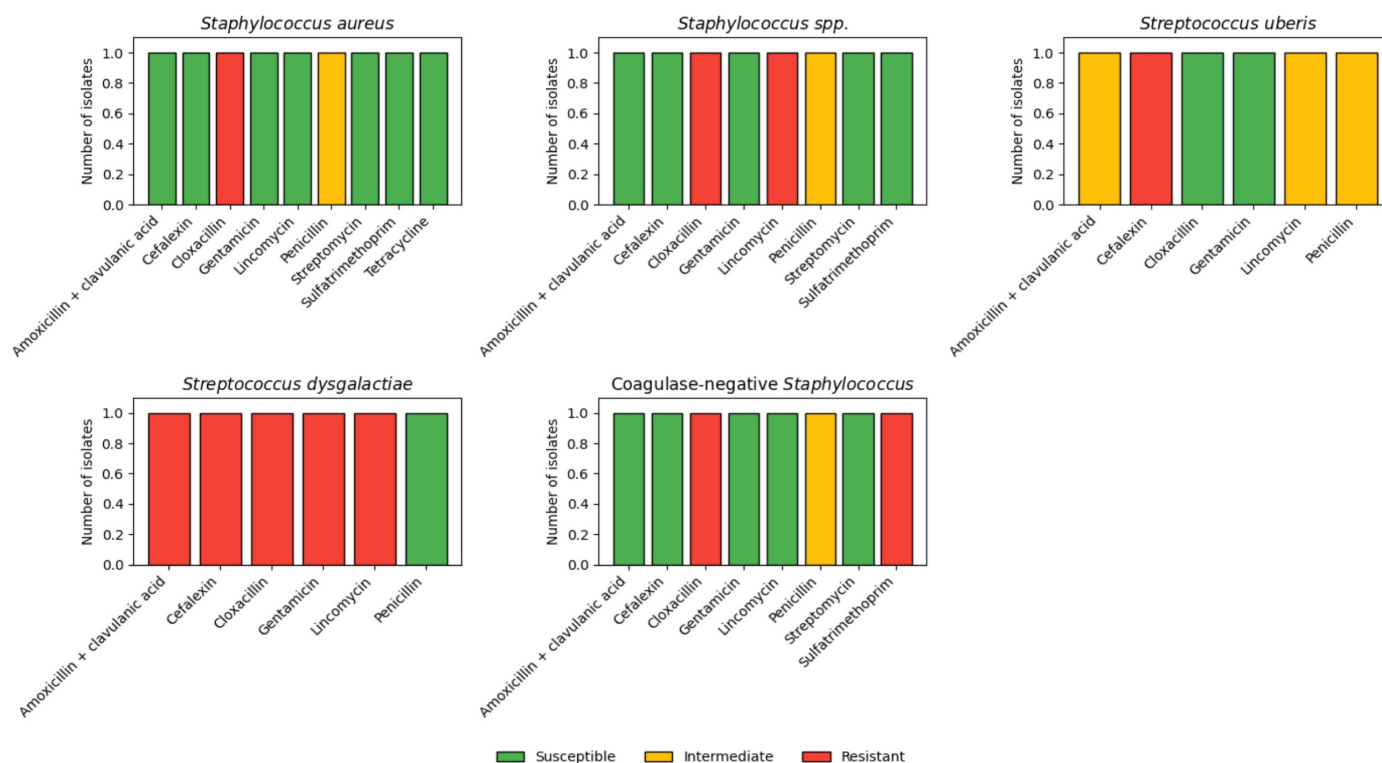


Fig. 6. Antimicrobial susceptibility profiles of Gram-positive bacterial isolates. Bar charts show the number of isolates classified as susceptible (green), intermediate (yellow), or resistant (red) to each tested antimicrobial agent for *Staphylococcus aureus*, *Staphylococcus spp.*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and coagulase-negative *Staphylococcus*

4. Discussion

This study provides integrated evidence on subclinical mastitis in a small-scale milk collection setting in Ecuador by combining cow-level CMT screening with bacteriological identification and antimicrobial susceptibility profiling. The observed cow-level prevalence of subclinical mastitis (18.6%) indicates that a considerable proportion of clinically healthy lactating cows may harbour intramammary infections without overt clinical signs. Although moderate in magnitude, this prevalence is consistent with estimates reported in comparable smallholder dairy systems, where subclinical mastitis frequently remains under-recognised due to limited routine diagnostic screening (Iraguha et al. 2026; Maya-Delgado et al. 2025). In this context, even a moderate prevalence of SCM may have cumulative implications for milk yield, processing quality, and overall production efficiency, given the tendency of persistent intramammary infections to sustain elevated somatic cell counts (Gonçalves et al. 2023; Silva et al. 2026). Nonetheless, the present study did not quantify milk yield or measure somatic cell counts beyond CMT-based screening. Accordingly, any potential production consequences should be interpreted as inferences supported by existing evidence, rather than as direct outcomes demonstrated within this investigation.

Overall, the distribution of CMT-positive quarters was relatively homogeneous across the udder quarters. In contrast to intensive dairy production systems, where rear quarters are often reported to be more frequently affected due to higher milk production or greater anatomical exposure, the homogeneity observed in this study may reflect similar exposure patterns across all quarters under smallholder management conditions (Silva et al. 2021; Zhang et al. 2016). However, as this study did not collect detailed information on milking hygiene practices, teat preparation, or housing conditions, management-related explanations

for this pattern remain speculative and should therefore be interpreted with caution. Although the majority of CMT-positive cows were within the 5–6-year age category, the relatively small number of positive animals and the uneven distribution of cows across age groups limit the strength of age-related interpretations. Previous studies have suggested that mature cows may accumulate greater exposure to mastitis pathogens over successive lactations (Gantner et al. 2023; Koçyiğit et al. 2016; Mureithi and Njuguna 2016). However, the absence of statistically significant differences observed among age groups suggests that age alone should not be considered a strong determinant of infection risk in this population.

Bacteriological analysis revealed a predominance of staphylococcal isolates, with *S. aureus* being the most frequently recovered pathogen. These findings are consistent with previous epidemiological studies that identify *S. aureus* and other staphylococci as dominant agents of subclinical mastitis in diverse dairy production systems, particularly in those where transmission during milking facilitates the spread of infection within herds (Dyson et al. 2022; Sağlam Gülmez et al. 2017). However, it is important to note that bacteriological culture in this study was performed exclusively in cows with subclinical mastitis, which may underestimate the overall diversity of mastitis-causing pathogens present in the herd, as some infections with low bacterial loads or at early stages could remain undetected in culture. The relatively low proportion of streptococcal isolates, including *S. uberis* and *S. dysgalactiae*, may suggest a comparatively smaller contribution of environmental mastitis pathogens. Nevertheless, it is important to consider that the occurrence of these microorganisms can be influenced by multiple factors, such as bedding management, environmental contamination, the timing of sample collection, and the diagnostic sensitivity of bacteriological culture (Kabelitz et al. 2021; Teotia et al.

2025). In addition, previous antimicrobial exposure at the farm level may also influence bacterial recovery rates, although this information was not available in the present study. Analysis of pathogen distribution across different age groups suggested greater isolate diversity among cows aged 5–6 years. However, this pattern is likely explained by the higher number of CMT-positive cows within these categories rather than by a true age-related predisposition to specific pathogens. Similar caution has been recommended in studies with a limited number of isolates, where apparent differences may arise from sampling variability rather than biological effects (Zaman Faruk et al. 2025).

Antimicrobial susceptibility testing demonstrated heterogeneous responses across bacterial species and antimicrobial compounds. In particular, staphylococcal isolates showed variable resistance patterns, which is consistent with reports documenting the increasing occurrence of antimicrobial resistance in dairy production systems (Kang et al. 2024; Mbindyo et al. 2021). However, future studies involving larger sample sizes and multiple herds would be required to more accurately characterise antimicrobial resistance trends in Ecuadorian dairy systems. From a broader perspective, antimicrobial resistance in mastitis pathogens has implications beyond herd health, as resistant bacteria may persist within dairy production environments and potentially disseminate through animal, environmental, and food-chain interfaces. These concerns are increasingly framed within the One Health concept, which emphasises the interconnected nature of antimicrobial resistance across human, animal, and environmental systems (Dhital et al. 2026; Elbehiry and Marzouk 2025).

Overall, this study provides preliminary evidence on the occurrence of subclinical mastitis, the bacterial pathogens associated with CMT-positive udder quarters, and the corresponding antimicrobial susceptibility patterns in a milk collection centre supplied by smallholder dairy producers in Ecuador. Although the data are not intended to establish a definitive epidemiological baseline, they provide initial descriptive evidence that may support future large-scale investigations aimed at strengthening mastitis control efforts, with particular emphasis on pathogen management and the implementation of evidence-based prudent antimicrobial use. In this context, several limitations should be acknowledged. The study was conducted with a relatively small sample size and within a single milk collection centre, which limits the generalisability of the findings. Furthermore, bacterial identification relied on conventional phenotypic methods, and antimicrobial susceptibility testing was performed using disc diffusion without molecular confirmation of resistance determinants. Consequently, the results should be interpreted as preliminary descriptive observations rather than as representative estimates of regional epidemiological patterns.

5. Conclusions

Subclinical mastitis was detected in 18.6% of lactating cows in the studied milk collection system, indicating that intramammary infections may remain largely unnoticed in smallholder dairy herds. *Staphylococcus* spp., particularly *S. aureus*, were the predominant pathogens identified, suggesting that contagious transmission during milking may play an important role in the epidemiology of mastitis in this production context. The variability observed in antimicrobial susceptibility patterns highlights the importance of bacteriological diagnosis and evidence-based antimicrobial use in mastitis

management. Although the findings should be interpreted cautiously due to the limited sample size, they provide preliminary insights into mastitis pathogens and antimicrobial susceptibility patterns in Ecuadorian smallholder dairy systems.

Declarations

Funding: This research received no external funding

Conflict of interest: The authors declare that they have no conflict of interest

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Ethics approval: Ethical review/approval was not required for this observational study according to Universidad Técnica de Ambato policy, as sampling was non-invasive and conducted during routine milking with owner consent, ensuring minimal stress or discomfort to the animals. No experimental treatments or interventions were applied beyond standard herd management procedures. All procedures involving animals were conducted in accordance with the ethical principles for animal research and followed national and institutional guidelines for the care and use of animals in research

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