Research Article

Multidrug-Resistant *Staphylococcus* spp. in Dairy Cattle: An Assessment of Antimicrobial Pressure and Public Health Risk within the One Health Framework

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Abstract | Food safety is a cornerstone of public health, particularly in agricultural systems where the quality and safety of primary production directly affect human consumption. In the province of Guayas, Ecuador, a microbiological study was conducted on dairy cows in the canton of Daule, an area of high agricultural activity with extensive livestock systems and manual milking. The objective was to detect subclinical mastitis using the California Mastitis Test (CMT) and to characterise the antimicrobial resistance of *Staphylococcus* spp. isolated from bovine milk. Seventy-five samples were collected in accordance with bioethical principles and processed following standardised microbiological protocols: pre-enrichment in Soy Tripticase Broth with NaCl and pyruvate, inoculation on blood agar, Gram staining, catalase test, and disk diffusion antibiogram (Kirby-Bauer), using veterinary antibiotics (gentamicin, tetracycline, penicillin, cephalexin, and amoxicillin + clavulanic acid). Inhibition halos were interpreted according to CLSI breakpoints. Statistical analysis revealed significant differences in sensitivity profiles ($\chi^2 = 645.24$; $p < 0.001$), while the multiple resistance index (MAR) showed critical values (≥0.6) in all antibiotics evaluated, particularly penicillin (0.679) and tetracycline (0.667), reflecting sustained antimicrobial pressure. Hierarchical cluster analysis identified therapeutic groupings and functional redundancies, showing the proximity between β-lactams and the separation of gentamicin and tetracycline due to their mechanisms of action. The results indicate the possible circulation of resistance genes such as blaZ, mecA, tetM, and tetO, with relevant zoonotic implications. The study highlights the need to strengthen local microbiological surveillance, rationalise the use of antimicrobials with pharmacodynamic criteria, and adopt the One Health approach to contain the spread of resistance, preserve therapeutic efficacy, and protect public health in clinical and agricultural settings.

Keywords | Antibiotics, Cattle farming, Antimicrobial resistance, Health management, Antimicrobial sensitivity

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INTRODUCTION

Livestock farming is a fundamental pillar of economic and food security for millions of families in tropical regions of Asia, Africa and Latin America. It is particularly important in Ecuador, where the dairy industry accounts for around 4% of agri-food GDP and provides direct employment for over a million people (Medina *et al.*, 2024; Moreno *et al*., 2023; Segovia, 2023). In view of the advance of the agricultural frontier, experts such as Parra *et al*. (2019) have pointed out the urgent need to adopt sustainable approaches that boost productivity without compromising ecological stability or the living conditions of small producers.

Milk is an essential food in the human diet due to its water-soluble vitamin content, especially B vitamins, which makes it a key nutritional resource globally (Ionita, 2022). In this context, tropical grazing-based production systems are emerging as a sustainable and efficient alternative for Latin America, optimising the use of natural resources and contributing to animal welfare (López-Vigoa *et al*., 2017; Pezo, 2019). Milk productivity is conditioned by multiple physiological and management factors, such as the age of the animal, the number of lactations, the quality of the diet and the general health status, all of which directly affect the quantity and quality of the final product (Patiño-Burbano *et al*., 2023; DANE, 2013).

First-calf heifers produce less milk, reaching their peak capacity during their second and third calving. From then on, production decreases by approximately 5% with each cycle. In addition, climatic conditions affect milk yield; in tropical and subtropical areas, production is higher during the rainy season, while in colder climates it decreases during winter (Muñoz *et al*., 2022).

Antimicrobial resistance (AMR) is a growing threat to public and animal health, especially in dairy production systems, where milk acts as a vehicle for the transmission of resistant microorganisms (WHO, 2021; Espinosa Castaño *et al*., 2019). Bovine mastitis, one of the most prevalent diseases in dairy cattle, is frequently caused by bacteria of the genus *Staphylococcus* spp., including *S. aureus* and is usually treated with β-lactam antibiotics, macrolides and aminoglycosides (Pellegrino *et al*., 2011; García Alarcón *et al*., 2025). However, the indiscriminate therapeutic and prophylactic use of these drugs has favoured the selection of multi-resistant strains, making treatment difficult and increasing the risk of antimicrobial residues in milk (Walsh, 2018; Rahman *et al*., 2021).

In Ecuador, recent studies have shown the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in raw milk samples, as well as multidrug-resistant strains in cattle with subclinical mastitis (Escobar *et al*., 2022). In addition, the country has a National Plan for the Prevention and Control of AMR, led by the National Institute for Public Health Research (INSPI), which has implemented surveillance in sentinel hospitals and regional laboratories to monitor resistance genes such as blaCTX-M, mecA and mcr-1 (INSPI, 2022; Ministry of Public Health (MPH), 2019).

In Peru, a worrying prevalence of subclinical mastitis in dairy cows has been revealed, reflected in high somatic cell counts that compromise milk quality and animal welfare (Alvarado *et al*., 2019). Most alarming is the advance of bacterial resistance: Strains of *Staphylococcus* spp., the main causative agents of this disease, have shown high levels of resistance to commonly used antibiotics such as amoxicillin and penicillin, reaching percentages of 92% and 96% in certain regions. This trend is not unique to Peru in Brazil, 100% resistance to penicillin has been documented in similar isolates, posing a serious health alert for the region (Dias *et al*., 2010).

The inappropriate use of antibiotics in livestock farming, especially without prior sensitivity testing, has led to the emergence of resistant bacteria such as *Staphylococcus* spp., affecting both animal and human health. This situation is exacerbated by diseases such as subclinical mastitis, which is difficult to detect but very common in dairy systems, causing economic losses on farms and compromising milk quality (García *et al*., 2025; Vayas, 2021). In Ecuador, recent studies reveal that the excessive use of antimicrobials and a lack of knowledge about preventive practices exacerbate this situation (Puga and Mendieta, 2025). It is therefore essential to implement control programmes that promote the responsible use of antibiotics, accompanied by training for farmers and operators to strengthen good milking practices and hygienic herd management (Patiño-Burbano *et al*., 2023; Agrocalidad, 2022).

This study on antimicrobial resistance in dairy farms highlights the urgent need to improve hygiene conditions through effective sanitary practices and to implement preventive strategies that reduce the systematic use of conventional antibiotics (Kaasch *et al*., 2024).

The main objective of the research was to evaluate the resistance profile of *Staphylococcus* spp. isolated from the milk of cows with subclinical mastitis, contributing to an understanding of the magnitude of the problem in dairy production systems. Furthermore, strengthening animal health is key to protecting public health, as antimicrobial residues and resistant strains in milk pose risks to consumers and food safety (Trombete and Souza, 2014; Walsh, 2018).

MATERIALS AND METHODS

Location

The province of Guayas, located in the coastal region of Ecuador between parallels 0°06′ and 3°59′ south and meridians 78°42′ and 81°00′30″ west (Montaño, 2012), is characterised by its tropical savannah climate, estuarine ecosystems, mangroves and intense agricultural activity. It borders Manabí and Los Ríos to the north, Bolívar, Chimborazo, Cañar, Azuay and Los Ríos again to the east, El Oro and the Gulf of Guayaquil to the south, and Santa Elena, Manabí and the Pacific Ocean to the west (CELIR, 2019; MAGAP, 2016). In this context, the canton of Daule stands out as a key productive hub, with an area of 462.07 km² and approximate coordinates of 1°51′37″ S and 79°58′36″ W (Barzola, 2021). Recognised as the rice capital of the country, it also has significant livestock activity based on extensive systems with low technification, with an average production of 8 litres of milk per cow per day (Llanos, 2025; Vargas, 2018).

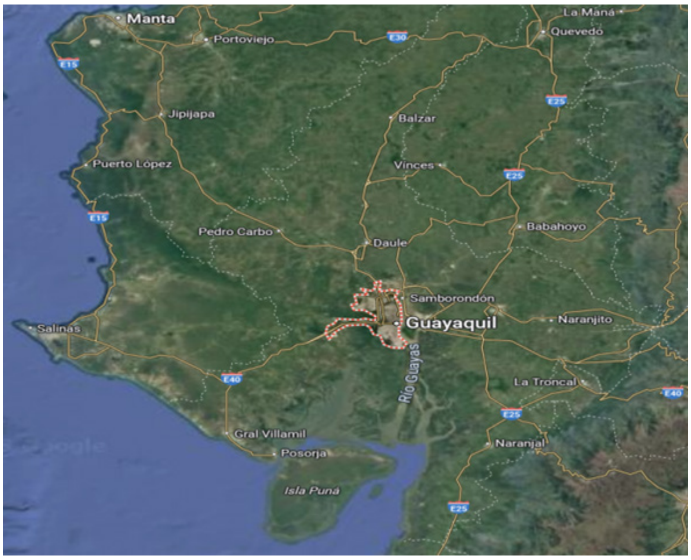

Figure 1: Political map of province Guayas.

Climate

In the province of Guayas, where the climate sets the pace for productive, social and natural life. With average temperatures ranging between 26°C and 28°C, and highs that can reach 32.6 °C in the warmest months, this fertile land receives abundant rainfall between February and March, more than 280 mm per month, while August and September are much drier, with rainfall below 15 mm (GADPG, 2021). Relative humidity remains high, between 75% and 83%, and generous sunlight, which shines for 9 to 11 hours each day, favours crops and livestock farming throughout the region (Woozberley, 2020; Montero, 2022).

Characteristics of dairy farming

Regarding the racial composition of the cattle herd, crossbred animals predominate (Holstein x Brown Swiss x Bos indicus). These animals exhibit physiological adaptations to the warm, humid climate of the coastal region and are managed under extensive free-range grazing systems, complemented by strategic low-intensity nutritional supplementation (Cabezas *et al*., 2019; Yánez *et al*., 2021). These breeds have an average milk production of 6 to 8 litres per cow per day, although this yield varies according to nutritional, health and reproductive management (Almeida *et al*., 2025). Agroclimatic conditions such as temperature, humidity and high daily solar radiation favour the development of pastures and forage crops (Ortíz, 2025; Jarquín and Aguilar, 2025). These parameters allow for the maintenance of extensive and semi-intensive livestock systems, although they also pose challenges such as heat stress and water management (Cisneros *et al*., 2024).

During the first and second thirds of lactation (0–200 days postpartum), cows face a negative energy balance, as metabolic demand exceeds nutrient intake. During this period, peak production is reached between 45 and 60 days, followed by a persistence phase where production stabilises before beginning to decline (García-Roche, 2017; Ortega, 2022). Supplementation with concentrates, mineral salts and quality forage is key to sustaining performance and facilitating the recovery of body condition (Valle, 2024; Castaño and Pérez, 2017). Manual milking is the usual method in the area, carried out in open or semi-technified pens, with basic hygiene and management routines (Bonifaz and Requelme, 2011; Terán, 2012). The microbiological quality of milk can be compromised by the use of non-potable water and poor cleaning practices, which has been documented in studies conducted on manual milking systems in Ecuador (Contero and Cachipuendo, 2021).

Bioethical considerations

Milk samples for the study were collected in accordance with fundamental bioethical principles, particularly informed consent. Each participating producer was duly informed about the objectives of the study, the procedures involved and the use of the samples collected, and gave their voluntary authorization to participate (FDA, 2023). The samples were collected using non-invasive techniques that guarantee animal welfare and under hygienic conditions that preserve the microbiological quality of the milk, in accordance with good veterinary practices (FAO, 2017). This ethical approach reinforces the research team's commitment to respecting the human and animal participants in the study (Díaz and García, 2024).

Sampling

For this study, milk samples were collected from 75 cows belonging to local production systems. Samples were taken from three representative farms in the Daule District, Guayas province: the "Huaconcito" farm (1°52′00″S 79°59′00″W), the "Don Vito" farm and the "Tres Hermanos" farm (both at 1°33′00″S 80°01′00″W), 25 milk samples from cows at each farm, for a total of 75. These samples were selected to undergo the California Mastitis Test (CMT) as a rapid diagnostic method for subclinical mastitis, a condition that affects the mammary gland without obvious clinical manifestations (Gómez *et al*., 2015). Samples were collected in the field using specific materials such as CMT test strips, EDTA-free tubes, gloves, boots, sanitary uniforms, and coolers to preserve the cold chain (Lituma, 2023).

California mastitis test (CMT)

Subclinical mastitis was detected using the CMT reagent, recognised for its effectiveness and speed in identifying changes in milk associated with mammary inflammation (Kiarie, 2024; McFadden, 2025). The CMT test was applied following protocols validated by Schalm and Noorlander (1957) and adapted by the National Mastitis Council (Kerro, 2020; Gómez-Quispe *et al.*, 2015). Before applying the test, the udder was cleaned with iodine solution and dried with disposable paper, following recommended hygiene protocols to avoid cross-contamination (Proquimia, 2023). Then, two streams of milk were extracted from each mammary quarter and mixed with 2 ml of CMT reagent on the palette, stirring to facilitate interpretation of the result (Reyes and Argüello, 2015). Once the affected quadrant was identified, the milk sample was collected in a test tube, labelled and refrigerated until analysis at the Microbiology Laboratory of the Faculty of Veterinary Medicine of the University of Guayaquil for microbiological analysis, following recommendations to ensure the viability of the pathogens present (Jurado *et al.*, 2021; Zubieta and Quiroga, 2022; Sánchez *et al.*, 2019).

Preparation of culture media (Blood Agar)

Blood agar was prepared under aseptic conditions to promote the growth of microorganisms and detect haemolysis, which is useful in the isolation of *Staphylococcus* spp. (Microbe Notes, 2022; Acharya, 2023). Following the manufacturer's recommendations, 40 g of blood agar base was dissolved in 1000 ml of distilled water and sterilised in an autoclave at 121 °C for 15 minutes. When the medium reached 45-50 °C, 5-10% human blood was added to the mixture, mixed well, avoiding the formation of bubbles, and poured into Petri dishes under direct flame to avoid contamination. It was left to solidify for 40 minutes and then refrigerated until further use (Universidad Continenta, 2023; UNAM, 2019).

Pre-enrichment procedure for samples

Before being seeded in blood agar, milk samples underwent a pre-enrichment process in Tryptic Soy Broth (TSB) medium supplemented with 7.5% NaCl and 1% sodium pyruvate, designed to promote the slightly selective growth of halotolerant bacteria and increase their concentration to detectable levels, in a 1:10 ratio. This step is essential to improve the sensitivity of the culture, as it allows the recovery of stressed or low-abundance cells of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* (CoNS) (Thurmond *et al.*, 1989; Ding *et al.*, 2017). Pre-enrichment significantly increases the recovery of pathogens such as *Staphylococcus aureus* in raw milk samples, thus optimising the effectiveness of conventional microbiological methods (Romero, 2020; Castañeda *et al.*, 2025).

Sample inoculation on blood agar

Two millilitres of the pre-enriched culture were transferred to Petri dishes containing blood agar using a 1000-microlitre pipette under aseptic conditions to prevent cross-contamination, following recommendations for safe handling in microbiology (Microbe Online, 2023; WHO, 2021). After plating, the samples were left to dry for 5 to 6 hours at room temperature, which promotes sample adhesion to the medium without interfering with the diffusion of haemolysins (Aryal, 2022). The plates were then divided into quadrants with a previously disinfected inoculation loop between each sample, ensuring traceability and avoiding interference between cultures (ASM, 2016). The plates were labelled and placed in an incubator at 37°C for 24 hours, the optimum temperature for the growth of mesophilic bacteria such as *Streptococcus* spp. and *Staphylococcus aureus* (ATCC, 2025; UNAM, 2025). Samples that showed growth with β-haemolysis were subjected to Gram staining for morphological characterisation and classification according to their cell wall, following standard protocols for bacterial identification (Microbe Notes, 2022; Aryal, 2022).

Gram staining and catalase test

After 24 hours of incubation at 37°C, haemolysis was examined on blood agar plates, observing transparent halos around some colonies, characteristic of β-haemolysis, associated with bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus* (Microbe Notes, 2022). Gram staining was used to characterise the microscopic morphology (Monteagudo *et al.*, 2021; Acharya, 2023). The catalase test was used to confirm the presence of *Staphylococcus* spp. and differentiate it from *Streptococcus* spp., as both are Gram-positive bacteria but differ in their enzymatic activity. A drop of hydrogen peroxide (3% H_2O_2) was placed on an isolated colony on a microscope slide, and immediate bubble formation was observed, indicating a positive reaction due to the presence of the catalase enzyme, characteristic of *Staphylococcus* spp. (Hardy Diagnostics, 2024; Microbe Online, 2022).

Preparation of mueller-hilton agar

Mueller-Hinton agar was prepared following standardised protocols to ensure its effectiveness in antimicrobial susceptibility testing. Thirty-eight grams of the dehydrated medium were weighed and dissolved in 1,000 ml of distilled water, heating to boiling point to ensure complete dissolution (Microbe Online, 2022). The mixture was then autoclaved at 121 °C for 15 minutes, in accordance with CLSI and FDA recommendations for culture media

(Vásquez *et al*., 2023; Thermo Fisher Scientific, 2024). Once cooled to room temperature, the medium was poured into sterile Petri dishes under aseptic conditions, using a direct flame as a barrier against contaminants (Sanders, 2012). The plates were allowed to solidify for 20 minutes and placed upside down to prevent condensation from forming on the surface of the agar, which could interfere with the distribution of microorganisms and affect the interpretation of the results (Sanders, 2012; Aryal, 2022).

Antibiogram

The samples were seeded in a sterile work area using Petri dishes previously prepared with Mueller-Hinton agar, the medium recommended by the CLSI for antimicrobial susceptibility testing by the disc diffusion method (Mueller and Hinton, 1941; Bauer *et al*., 1966). One hundred microlitres of a bacterial solution dissolved in 0.85% sterile saline solution, adjusted to standard turbidity (0.5 McFarland), were inoculated into each plate, ensuring homogeneous distribution using a sterile swab extension technique (CLSI, 2020). The antibiogram was tested with antibiotic disks of gentamicin (10 µg), tetracycline (30 µg), penicillin (10 units), cephalexin (30 µg), amoxicillin + clavulanic acid (20/10 µg), which were placed equidistantly on the surface of the agar, respecting a minimum distance of 24 mm between them and 15 mm from the edge, according to the Kirby-Bauer protocol (Hudzicki, 2009). The plates were labelled and grouped in batches of six units for traceability and incubated at 37°C for 18 hours in an inverted position, optimal conditions for bacterial growth and the formation of inhibition zones (Thermo Fisher Scientific, 2024; Microbe Notes, 2022).

Reading the inhibition halos

The antibiogram was interpreted using the disc diffusion method (Kirby-Bauer), a technique standardised by the World Health Organisation and the Clinical and Laboratory Standards Institute (Rapoport, 2021; Hudzicki, 2009) to assess antimicrobial sensitivity. *Staphylococcus* spp. strains were classified as sensitive (S), intermediate (I) or resistant (R) by comparing the diameters of the inhibition halos with the breakpoints established by the CLSI (2020). A zone diameter of ≥15 mm was considered sensitive for gentamicin, ≥19 mm for tetracycline, ≥29 mm for penicillin, and ≥20 mm for amoxicillin with clavulanic acid. For cephalexin, CLSI and EUCAST have not established breakpoints. These values are essential for guiding antimicrobial treatment, as noted by Baroja *et al*. (2021), and should be updated regularly due to the evolution of resistance, according to Buldain *et al*. (2023). In addition, the use of media such as Mueller-Hinton agar and the standardisation of the inoculum (0.5 McFarland) are essential requirements to ensure the reproducibility and reliability of the results (Mueller and Hinton, 1941; Sader *et al*., 2024).

Data analysis

The data were organised in long format, considering, for each antibiotic evaluated, the number of isolates classified according to sensitivity criteria: sensitive (S), intermediate (I) or resistant (R). Absolute and relative frequencies were calculated and visually represented using stacked bar charts to describe the phenotypic profile of antimicrobial susceptibility. To evaluate statistical differences in the distribution of susceptibility categories among different antibiotics, Pearson's chi-square test was applied to a contingency matrix (antibiotic × classification) (Agresti, 2018). Additionally, the Multi-Resistance Index (MAR) was calculated for each antibiotic, defined as the ratio between the number of resistant isolates and the total number of tests performed. A reference value of 0.2 was considered a critical threshold for identifying antibiotics associated with high antimicrobial pressure, as proposed by Krumperman (1983). Finally, a hierarchical clustering analysis was applied using Euclidean distances and Ward's linkage method to identify clusters of antibiotics based on the similarity of their sensitivity profiles (Murtagh and Legendre, 2014). All analyses were performed in R software (version 4.3.2) using the tidyverse, ggplot2, dendextend, and readxl packages.

Calculation of the multiple antibiotic resistance index (MAR)

To evaluate antimicrobial pressure in bacterial isolates, the Multiple Antibiotic Resistance Index (MAR) was calculated using the following formula:

$$MAR=a/b$$

Where a corresponds to the number of antibiotics to which an isolate was resistant and b to the total number of antibiotics evaluated for that isolate. This index was calculated for each antibiotic considering the proportion of resistant isolates among the total number of strains analysed. A MAR index ≥ 0.2 was considered a critical threshold value, suggesting exposure to environments with high or indiscriminate use of antimicrobials (Krumperman, 1983).

RESULTS AND DISCUSSION

Figure 2 shows the percentage distribution of bacterial isolates against five antibiotics, classified into three categories according to their sensitivity profile: Sensitive (S), Intermediate (I) and Resistant (R). A worrying pattern of high resistance was observed in several compounds, notably amoxicillin + clavulanic acid, penicillin and tetracycline, all with resistance rates above 40%. In the specific case of amoxicillin + clavulanic acid, more than 50% of the isolates were classified as resistant, while tetracycline showed a more distributed pattern, with similar proportions among the three categories. gentamicin was the only antibiotic with a predominance of the sensitive phenotype (approximately 44% of isolates), although it also presented intermediate and resistant levels, suggesting still acceptable efficacy but with signs of possible selective pressure. Amoxicillin and cephalexin were excluded from the graphical analysis due to the absence of clinical breakpoints established by CLSI/EUCAST for Staphylococcus spp. isolated from bovine milk, which prevents standardized interpretation and comparison with other international studies. In the case of cephalexin, although 94% resistance was observed (47 of 50 isolates), the results presented inconsistent inhibition zones and low reproducibility, which limits its analytical validity as a comparison parameter. Additionally, amoxicillin alone was tested and subsequently discarded, while amoxicillin-clavulanic acid was included, given that it has official interpretive criteria and more clinically stable results. This differentiation helps avoid confusion between the efficacy of the antibiotic as monotherapy and that of its combination with a β-lactamase inhibitor, ensuring greater precision in the interpretation of resistance profiles. A Chi-square test was applied to evaluate the association between the type of antibiotic and the sensitivity classification (S, I, R). The results showed a statistically significant association between these variables ($\chi^2 = 645.24$; $p < 0.001$), indicating that the sensitivity profile differs significantly depending on the antibiotic evaluated. This finding reflects that resistance is not distributed evenly among antimicrobials, and that certain compounds, such as beta-lactams, have critical levels of resistance. This situation could be associated with frequent or inappropriate use in veterinary clinical practice in the study region.

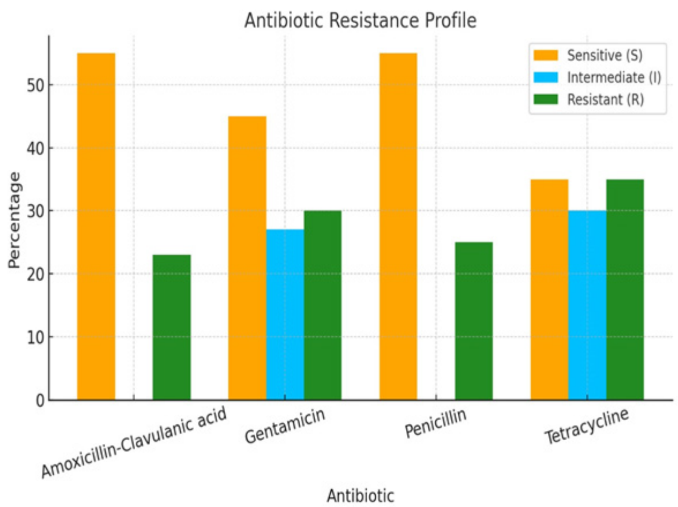

Figure 2: Antibiotic sensitivity profile. Percentage of isolates classified as sensitive (S), intermediate (I) or resistant (R).

The results show a worrying prevalence of antimicrobial resistance in *Staphylococcus* spp. especially against β-lactam antibiotics such as penicillin and amoxicillin + clavulanic acid. This pattern coincides with that reported by Castro *et al.* (2018), who documented that 47.5% of *S. aureus* isolates in Cartagena were resistant to methicillin, while *S. epidermidis* reached a resistance rate of 68.7%. From a molecular point of view, it has been described that *S. aureus* can acquire resistance through three main mechanisms: production of β-lactamases, modification of penicillin-binding proteins (PBPs), and the presence of the *mecA* gene, responsible for methicillin resistance. These mechanisms allow the bacteria to evade the action of β-lactam antibiotics, even those combined with β-lactamase inhibitors, such as clavulanic acid. The coexistence of multiple mechanisms in the same strain aggravates the clinical problem and hinders empirical treatment (Castellano and Perozo, 2010).

In contrast, gentamicin showed a more favourable profile in this study, with a higher proportion of sensitive strains. However, recent research in Venezuela reveals that up to 61.7% of coagulase-negative *Staphylococcus* strains carry gentamicin resistance genes (aac(6')/aph(2")), suggesting a latent threat to its future efficacy. Therefore, although gentamicin is still useful, its use should be strategic and supported by sensitivity testing (Castellanos *et al.*, 2016). Similar studies in Colombia also reported the presence of these genes in more than 87% of *S. epidermidis* strains isolated in neonatal units, reinforcing concerns about their regional spread (Pinilla *et al.*, 2009).

The results obtained in this research reveal high resistance of *Staphylococcus* spp. to β-lactam antibiotics, while tetracycline showed a more distributed pattern between sensitivity, intermediate resistance, and total resistance. This behaviour is partially in line with regional studies, but also presents significant contrasts with international research. Similarly, the multicentre study conducted in Colombia by De La Cadena *et al.* (2023) showed that, between 2018 and 2021, the resistance profiles of the 10 most frequent microorganisms remained relatively stable, except in the case of *Pseudomonas aeruginosa,* which showed a significant increase in resistance to piperacillin-tazobactam and carbapenems. In contrast, our research shows a more marked trend towards resistance in *Staphylococcus* spp., which could be due to differences in the use of antimicrobials in veterinary versus hospital settings.

On the other hand, Giono *et al.* (2020) identified a high prevalence of ESKAPE bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), in Mexican hospitals, reinforcing the relevance of our results by demonstrating the persistence of resistant strains in clinical and veterinary settings. However, the more balanced pattern observed for tetracycline in our sample suggests that there are still opportunities to preserve its efficacy through rational use policies.

These findings reflect the selective pressure exerted by the frequent use of antibiotics in dairy production, especially in the treatment of bovine mastitis, one of the main causes of therapeutic intervention in dairy cattle (Chávez *et al*., 2025). From an epidemiological perspective, the MAR detected in bovine milk represents not only a risk to animal health, but also a threat to public health, as it facilitates the transfer of resistance genes through the food chain. López *et al*. (2022) highlight that the indiscriminate use of antibiotics in production systems has favoured the selection of resistant strains, increasing veterinary costs, loss of productivity and the burden on human health systems. In this context, it is imperative to strengthen microbiological surveillance programmes in the agricultural sector, implement rational prescription policies in veterinary medicine and encourage research into new antimicrobial agents. The dissemination of local sensitivity profiles, as recommended by Achong-Sánchez *et al*. (2024), allows for the optimisation of empirical treatment, reduces selective pressure on existing antibiotics, and prevents the spread of resistance through animal products.

In addition, the COVID-19 pandemic exacerbated the irrational use of antimicrobials in human medicine, especially in the empirical treatment of viral respiratory infections, accelerating the spread of resistant strains in hospital and agricultural settings without updated microbiological maps (PAHO, 2021). Zumbado *et al*. (2022) point out that the lack of standardised protocols during the health emergency contributed to increased resistance, especially in intensive care units and high-density animal production systems. Overall, the findings of this study are in line with the international literature, highlighting the urgency of adopting policies for the rational use of antimicrobials, strengthening microbiological surveillance systems, and promoting translational research to anticipate and mitigate the advance of resistance (WHO, 2021; PAHO, 2021).

Analysis of the multiple antibiotic resistance (MAR) index revealed high values for all antibiotics evaluated. MAR values ranged from 0.607 for gentamicin to 0.679 for penicillin, well above the critical threshold of 0.2, represented in Figure 3 by a red dotted line. This threshold indicates frequent exposure to antibiotics in the bacterial source, associated with environments where antimicrobial use is intensive or unregulated. It can also be observed that amoxicillin + clavulanic acid, tetracycline, penicillin, and gentamicin have MAR indices above 0.6, implying high selective pressure and the possible presence of acquired resistance mechanisms. In particular, penicillin was the antibiotic with the highest MAR (0.679), followed by tetracycline (0.667).

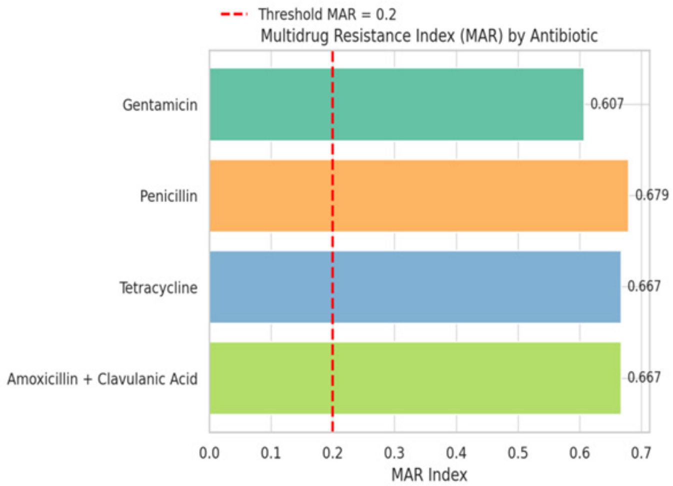

Figure 3: Multiple Antibiotic Resistance (MAR) index by antibiotic evaluated. The average MAR index values are shown for four antibiotics: Penicillin (0.679), tetracycline (0.667), amoxicillin + clavulanic acid (0.667) and gentamicin (0.607). A red reference line (---) is included, corresponding to the critical threshold MAR = 0.2, a value above which the bacteria are considered to come from an environment with high antibiotic pressure.

The high level of multidrug resistance (MDR) to penicillin and tetracycline is probably due to prolonged exposure in dairy production systems, where these antibiotics have been used for both therapeutic and prophylactic purposes. Recent studies have shown that routine use of tetracycline in cattle is associated with the persistence of resistance genes such as tetM and tetO, which can be transferred horizontally to other bacteria in the animal and human microbiome (Martínez *et al*., 2023; Vásquez *et al*., 2023).

Similarly, amoxicillin + clavulanic acid also showed a considerably high MAR (0.667), indicating its frequent use in the treatment of mammary infections and other pathologies in cattle. Although this combination seeks to counteract the action of β-lactamases, its repeated use can induce the emergence of extended-spectrum β-lactamases (ESBLs), as has been described in strains of *Klebsiella pneumoniae* and *Escherichia coli* isolated from dairy products (Singh *et al*., 2022; Ranjbar *et al*., 2020).

These high levels of MAR in bacteria from bovine milk pose a direct threat to public health, as raw milk can act as a vehicle for the transmission of resistance genes through the food chain. The FAO and OIE have warned that the use of antibiotics in animal production is one of the main factors contributing to the global spread of antimicrobial resistance (FAO, 2017; OIE, 2022). If control measures are not implemented, it is estimated that by 2050 MAR could exceed cancer in terms of mortality, with a devastating economic impact (Irfan *et al*., 2022). This behaviour is in line with recent evidence on the evolution of antimicrobial resistance, considered one of the main threats to global public health. According to Achong *et al*. (2024), a growing prevalence of multidrug-resistant (MDR), extremely drug-resistant (XDR) and pan-drug-resistant (PDR) bacteria has been observed in high-complexity hospitals in Latin America, especially in critical areas such as intensive care units.

Figure 4 shows the hierarchical cluster analysis based on Euclidean distance applied to sensitivity, resistance, and intermediation profiles of bacterial isolates from animals, allowing the identification of relevant therapeutic patterns in veterinary medicine. This statistical tool facilitates the grouping of antibiotics according to their behaviour against pathogens of clinical interest, which is essential for optimising treatment protocols and reducing antimicrobial pressure in veterinary settings (Sokal and Sneath, 1963). The proximity observed between amoxicillin + clavulanic acid and penicillin reflects their belonging to the beta-lactam family, widely used in veterinary medicine to treat respiratory, urinary and skin infections in species such as dogs, cats and cattle (Prescott, 2013). However, the resistance observed suggests the presence of beta-lactamases, enzymes that inactivate these antibiotics, a phenomenon widely reported in zoonotic bacteria such as *Escherichia coli, Klebsiella* spp. and *Staphylococcus* spp. isolated from domestic animals (Bradford *et al*., 2022; Correa *et al*., 2021).

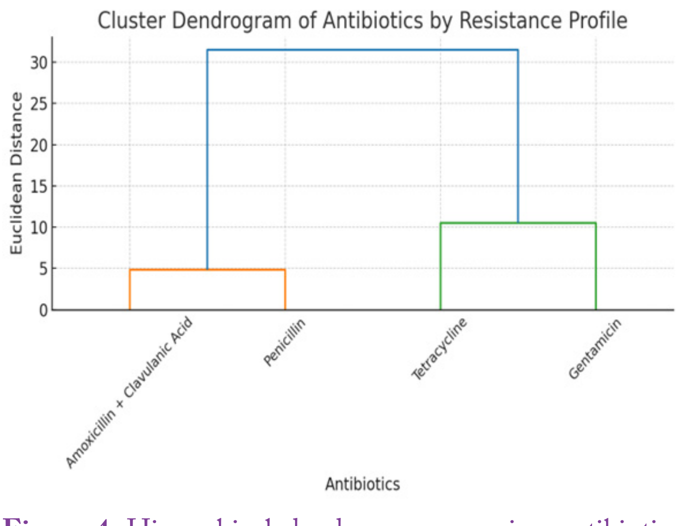

Figure 4: Hierarchical dendrogram grouping antibiotics according to their sensitivity profiles (S/I/R), using Euclidean distance and Ward's method.

Although clavulanic acid acts as an inhibitor of these enzymes, its efficacy may be compromised against extended-spectrum beta-lactamases (ESBLs), especially in environments where the indiscriminate use of antimicrobials has favoured the selection of resistant strains (Jiménez, 2021). In contrast, the independent grouping of tetracycline and gentamicin indicates marked differences in sensitivity profiles, attributable to their mechanisms of action on protein synthesis in the 30S subunit of the ribosome. These antibiotics are used in veterinary medicine to treat systemic and localised infections in production and companion animals, although their use has been regulated due to the emergence of resistance and food safety considerations (Mercer, 2022; Jara, 2024). Resistance to tetracyclines, for example, is one of the most common in the veterinary field and has been associated with the presence of tet genes in plasmids and transposons, which facilitates their horizontal spread among bacteria (Jara, 2024).

This type of analysis is particularly useful in veterinary medicine to identify therapeutic redundancies, highlight relationships between resistance profiles, and inform strategies for rotating or replacing antimicrobials. Rational antibiotic rotation in animals not only improves therapeutic efficacy but also contributes to reducing selective pressure and controlling antimicrobial resistance, a growing concern in veterinary and human public health (WHO, 2021; Laxminarayan *et al*., 2024). In addition, constant monitoring of the behaviour of strains against antibiotics commonly used in animals allows for the anticipation of emerging trends and the adjustment of therapeutic guidelines according to local epidemiology, which is key to preventing the spread of multi-resistant strains with zoonotic potential (CDC, 2022; Muñoz, 2022).

CONCLUSIONS AND RECOMMENDATIONS

The high prevalence of antimicrobial resistance observed in *Staphylococcus* spp. isolated from bovine milk, particularly against β-lactams and tetracyclines, indicates sustained antimicrobial pressure in animal production systems. The critical values of the MAR index and the statistically significant association between the type of antibiotic and the sensitivity profile confirm intensive and possibly unregulated therapeutic use in veterinary medicine.

These findings reinforce the need to consolidate microbiological surveillance systems based on local susceptibility, optimise veterinary prescribing using pharmacodynamic and pharmacokinetic criteria, and adopt integrated strategies under the one health approach. This multidisciplinary approach allows antimicrobial resistance to be addressed as an interconnected phenomenon between animal, human and environmental health, promoting coordinated interventions to preserve therapeutic efficacy and contain the spread of resistance in clinical and agricultural systems.

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NOVELTY STATEMENT

This study provides original evidence for Ecuador by (i) characterizing sensitivity phenotypes of Staphylococcus spp. isolated from bovine milk with subclinical mastitis in extensive coastal systems, (ii) quantifying antimicrobial pressure using the MAR index (with values consistently ≥ 0.6), and (iii) identifying therapeutic clusters by dendrogram (β-lactams vs. aminoglycosides/tetracyclines) that reveal functional redundancies and guide antibiotic rotation. The integration of on-farm CMT, Kirby–Bauer antibiograms with CLSI breakpoints, and multivariate analyses (χ^2 and Ward clustering) establishes an operational framework for local surveillance with direct implications for One Health strategies and rational antimicrobial prescribing in veterinary medicine.

AUTHOR'S CONTRIBUTION

IG-P: Contributed to the design of the study and literature review.
MV-L: Participated in data tabulation and style correction.
VC-V: Carried out the fieldwork and experimental analysis.
JDT-M: Collaborated in the methodological design and statistical processing.
MR-B: Contributed to the logistics and laboratory analysis.
DY-A: Corresponding author, was responsible for overall coordination, scientific supervision and final editing.
PM: Developed the advanced statistical validation and review.

Generative AI and AI-assisted technology statement

Generative AI tools were used exclusively for editorial purposes (suggestions on clarity, grammar, and APA 7 reference formatting). No scientific text, dataset, result, figure, table, or statistical analysis was generated by AI. All data processing, χ^2 calculations, MAR index estimation, and cluster analyses were performed in R (v4.3.2) and verified by the authors. AI outputs were critically reviewed and, where appropriate, adjusted by the team prior to inclusion. No AI models were used to design experiments, create scientific images, or interpret results.

Conflict of interest

The authors have declared no conflict of interest.

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