UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Posgrados

Identificación de resistencia antimicrobiana por MIC de microorganismos obtenidos en muestras de leche de vacas positivas para mastitis clínica y subclínica en el Ecuador

Tesis en torno a una hipótesis o problema de investigación y su contrastación.

Claudia Tamara Mejía Ortega

Verónica Barragan Ph.D.
Patricio Rojas Silva M.D., Ph.D.
Jorge Ron Román Ph.D.
Directores de Trabajo de Titulación

Trabajo de titulación de posgrado presentado como requisito
para la obtención del título de Magíster en Microbiología

Quito, 17 de enero 2025

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE POSGRADOS

HOJA DE APROBACIÓN DE TRABAJO DE TITULACIÓN

Identificación de resistencia antimicrobiana por MIC de microorganismos obtenidos en muestras de leche de vacas positivas para mastitis clínica y subclínica en el Ecuador

Claudia Tamara Mejía Ortega

Nombre del director del Programa:	Patricio Rojas Silva
Título académico:	M.D., Ph.D.
Director del programa de:	Maestría en Microbiología

Nombre del Decano del colegio Académico:	Carlos Valle
Título académico:	Ph.D.
Decano del Colegio:	COCIBA

Nombre del Decano del Colegio de Posgrados:	Darío Niebieskikwiat
Título académico:	Ph.D.

Quito, 17 de enero de 2025

© DERECHOS DE AUTOR

Por medio del presente documento certifico que he leído todas las Políticas y Manuales de la Universidad San Francisco de Quito USFQ, incluyendo la Política de Propiedad Intelectual USFQ, y estoy de acuerdo con su contenido, por lo que los derechos de propiedad intelectual del presente trabajo quedan sujetos a lo dispuesto en esas Políticas.

Asimismo, autorizo a la USFQ para que realice la digitalización y publicación de este trabajo en el repositorio virtual, de conformidad a lo dispuesto en la Ley Orgánica de Educación Superior del Ecuador.

Nombre del estudiante:	Claudia Tamara Mejía Ortega
Código de estudiante:	00331879
C.I.:	1723242671
Lugar y fecha:	Quito, 17 de enero de 2025.

ACLARACIÓN PARA PUBLICACIÓN

Nota: El presente trabajo, en su totalidad o cualquiera de sus partes, no debe ser considerado como una publicación, incluso a pesar de estar disponible sin restricciones a través de un repositorio institucional. Esta declaración se alinea con las prácticas y recomendaciones presentadas por el Committee on Publication Ethics COPE descritas por Barbour et al. (2017) Discussion document on best practice for issues around theses publishing, disponible en http://bit.ly/COPETheses.

UNPUBLISHED DOCUMENT

Note: The following graduation project is available through Universidad San Francisco de Quito USFQ institutional repository. Nonetheless, this project – in whole or in part – should not be considered a publication. This statement follows the recommendations presented by the Committee on Publication Ethics COPE described by Barbour et al. (2017) Discussion document on best practice for issues around thesis publishing available on http://bit.ly/COPETheses.

DEDICATORIA

Dedicado para mi ángel en el cielo, quién me acompañó y apoyo hasta su último día de vida. Segundo David Mejía, mi padre. Cumpliste tu misión conmigo papito, aunque no estes aquí para verlo.

AGRADECIMIENTOS

A la Universidad de San Francisco de Quito, al instituto de Microbiología, a la Academia de Investigación y Enseñanza Superior (ARES) de Bélgica por la beca y la Universidad de las Fuerzas Armadas ESPE por su apoyo por parte del proyecto BruTryp para que se pueda realizar esta investigación como becaria y al Grupo de Investigación en Sanidad Animal y Humana (GISAH).

A todos los docentes e investigadores que conocí estos dos años y conforman el Instituto de Microbiología, especialmente a Patricio, Verónica y Paúl mis tutores por su apoyo, su guía y orientación constante durante el trabajo realizado. Cristina, Pablito y Renato por ser excelentes docentes y amigos en tiempos difíciles. Y toda la corte 2022 de la Maestría en Microbiología en especial a mis amigos más cercanos y que fuera de los laboratorios también eran el mejor equipo, que bueno fue encontrarlos: Esteban R., es MejOr amigo y apoyo emocional gracias por siempre estar, Karina B. la mejor profesora, Erick C. nuestro chelopt, Vicky N., mi gran apoyo y Cristopher P. el tóxico más querido.

A Jorge Ron Román mi tutor, Sarah Martin, Claude Saegerman increíbles científicos, personas y amigos, su apoyo incondicional en tiempos muy duros y guía durante todo este tiempo hicieron que valga la pena seguir el camino de la ciencia y tener un grupo que no abandona ni en lo profesional ni personal.

Cristina Cholota, gran profesional y persona, amiga y compañía incondicional para guiarme en la tesis o para hablar de la vida. Gracias a técnicas, tesistas y pasantes del Laboratorio de Biotecnología Animal, este gran equipo de los laboratorios de Biotecnología que pusieron su apoyo, consejos y gestión para que pueda resolver las trabas que a veces surgían.

A mi mamita y a mi familia.

Gracias Javier Ortiz, amor de mi vida, mi lugar seguro y mayor sostén cuando todo parecía estar mal.

RESUMEN

La mastitis bovina, causada por infecciones bacterianas y la resistencia antimicrobiana (RAM) como resultado del uso extensivo de antibióticos para mejorar la producción y el tratamiento de enfermedades en animales en período seco, es una preocupación importante para la industria lechera. La identificación de los agentes causales y la evaluación de sus perfiles de resistencia son esenciales para proporcionar una línea base sobre la RAM y para comprender el impacto de la industria lechera con una perspectiva de "Una sola salud". Se aislaron cepas Gram positivas de muestras de leche bovina con mastitis, se identificaron utilizando microbiología convencional y secuenciación del gen *16S rRNA* con los cebadores 27F y 1492R. Para determinar la susceptibilidad a los antimicrobianos, se determinó la concentración mínima inhibitoria (MIC) en paneles Sensititre. Se identificaron 21 cepas pertenecientes a *Staphylococcus aureus* (n = 11), *Staphylococcus chromogenes* (n = 3), *Staphylococcus devriesei* (n = 1), *Enterococcus faecium* (n = 3) y *Enterococcus faecalis* (n = 3). Los ensayos de MIC mostraron que el 62% (13/21) de las cepas bacterianas exhibieron resistencia a los antibióticos probados, mientras que 38% (8/21) de los antibióticos resultaron ser efectivos para controlar la mastitis causada por *Staphylococcus* spp. y *Enterococcus* spp. *in vitro*. El 72% de *S. aureus* fueron resistentes a la ampicilina, el 63,6% a la penicilina y una cepa de *S. aureus fue* resistente a la meticilina. Además, *S. chromogenes* fueron resistentes a la penicilina, mientras que *E. faecalis* mostró resistencia a quinupristina/dalfopristina, rifampicina y tetraciclinas. Un análisis general mostró que las cepas resistentes aisladas de muestras de leche bovina con mastitis positiva fueron más resistentes a betalactamicos (47,5%), seguidas de la tetraciclina y la estreptogramina (13% cada una) y las fluoroquinolonas (10%). Las cepas resistentes recuperadas en este estudio brindan una oportunidad para comprender el papel de la industria láctea en el desarrollo de la RAM, el potencial zoonótico debido al consumo de leche cruda y

derivados contaminados, y la necesidad de que los productores presten mayor atención a la mastitis para mejorar la seguridad alimentaria y sus beneficios económicos, así como, que las entidades de control brinden herramientas para combatir los desafíos de la industria láctea y mejorar la salud pública en la región.

Key words: MIC, RAM, industria láctea, *S. aureus*, mastitis bovina, Una Salud.

.

ABSTRACT

Bovine mastitis, caused by bacterial infections and presence of antibiotic-resistant strains resulting from the extensive use of antibiotics to enhance production and treatment of diseases in dry-period animals is a major concern for the dairy industry. Identification of the causative agents and evaluation of their resistance profiles are essential to provide a baseline of knowledge on AMR in mastitis and to understand the impact on the dairy industry with a "One-Health" perspective. Gram-positive strains were isolated from mastitis-positive bovine milk samples, identified using conventional microbiology and *16S rRNA gene* sequencing with primers 27F and 1492R. To determine antimicrobial susceptibility, the minimum inhibitory concentration (MIC) was determined on Sensititre panels. Twenty-one strains were identified as *Staphylococcus aureus* (n=11), *Staphylococcus chromogenes* (n=3), *Staphylococcus devriesei* (n=1), *Enterococcus faecium* (n=3), and *Enterococcus faecalis* (n=3). Determination of resistance by MIC showed that 62% (13/21) of the bacterial strain exhibited resistance to the antibiotics tested, while 38% (8/21) of the antibiotics were found to be effective in controlling mastitis caused by *Staphylococcus* spp. and *Enterococcus* spp. *in vitro.* Among the strains, 72% of *S. aureus* were resistant to ampicillin, 63.6% to penicillin, and one was a methicillin-resistant *S. aureus* strain. In addition, isolates such as *S. chromogenes* were resistant to penicillin, while *E. faecalis* showed resistance to quinupristin/dalfopristin, rifampicin, and tetracyclines. A general analysis showed that *Staphylococcus* spp. and *Enterococcus* spp. isolated from mastitis bovine milk samples were most resistant to the beta-lactam (47.5%), followed by tetracycline and streptogramin (13% each) and fluoroquinolones (10%). The resistant strains recovered in this study provide an opportunity to understand the role of the dairy industry in the development of AMR, the zoonotic potential due to the consumption of raw milk and contaminated derivatives, and the need for farmers to pay greater attention to mastitis to improve food security

and its economic benefits, as well as control entities providing tools to combat the challenges of the dairy industry and improve public health in the region.

Key words: MIC, AMR, Dairy industry, bovine mastitis, One Health.

CONTENT INDEX

INDEX OF TABLE

INDEX OF FIGURE

PART 1: LITERATURE REVIEW

Introduction

The agricultural and livestock sector represents 7.57% of Ecuador's PBI, and the dairy industry contributes 0.34% of that total PIB (MAG, 2024). Data from Instituto Nacional de Estadística y Censos (INEC) indicate that milk production for 2023 reached 5.7 million liters per day, consolidating Pichincha, Cotopaxi, Manabí, Chimborazo, Tungurahua, Carchi, Azuay and Santo Domingo de los Tsáchilas as the provinces with the highest production (INEC, 2024). Although 51% corresponds to the formal industry (urban and rural) made up of large agricultural production units (APU), 49% of the informal industry covers 64% of the rural sector, which bases its family economy on subsistence agricultural and livestock production in small APUs, giving us a picture of the reality of the sector in our country (CFN, 2024). Ecuador deployed a nationwide program, managing to be declared by World Organization for Animal Health (WOAH) as foot and mouth disease country free since 2015 (MAG, 2015), however there are other diseases that affect cattle and still have high prevalence such as mastitis.

Mastitis

Mastitis is an inflammation of mammary tissue with a multifactorial origin, caused by mechanical or chemical injuries, or infectious processes due to microorganisms that colonized the mammary glands duct system (Kibebew, 2017;Cheng & Han, 2020). The severity of the disease is classified into two types: clinical mastitis (CM), where noticeable health deterioration in the animal is observed, and subclinical mastitis (SCM), which is less apparent but associated with changes in production and milk quality (Zhang et al., 2022; de Souza et al., 2024).

Economic and productive losses

Economic losses due to mastitis in the dairy industry include reduced milk production, discarded dairy products, treatment costs, and the sacrifice of affected animals. A study

conducted in the United States estimate these costs, finding that reduced milk production due to mastitis leads to approximately $163 in losses, with an average of $326 lost per affected animal (Liang et al., 2017). In Africa, mastitis prevalence reaches 30%, with Ethiopia showing the highest incidence, estimating annual losses of $147 per cow positive to mastitis (Hogeveen et al., 2019). In Brazil, the cost of losses due to mastitis is estimated around $112 per affected animal and Colombia is around $70 - $100 per decrease in production, treatment and animal slaughter (Guimarães et al., 2017; Romero et al., 2018; Hurtado-Prieto & Cucunubo-Santos, 2023; de Souza et al., 2024).

Diagnostics tests

California Mastitis Test (CMT) is a qualitative method and Somatic Cell Count (SCC) is a quantitative test to predict intramammary infections for the diagnosis of subclinical mastitis and give an insight into the milk quality of cattle (Hoque et al., 2015). CMT reagent is an anionic detergent, this detergent breaks the membranes of somatic cells (macrophages, neutrophils) releasing the intracellular contents (DNA), which denature and form aggregates. The viscous gel is proportional to the SCC and the severity can be estimated in ranges from 1 to 3, being 3 the most severe (Poutrel & Rainard, 1981).

SCC is a quantitative method that considers the number of somatic cells per milliliter of milk present in an individual mammary quarter. An SCC value ≤90 000 cells/mL is considered as a negative threshold for mastitis testing because it is normal for the presence of somatic cells in the udder (Sharma et al., 2011). But if the value is >200 000 cells/mL, it is considered positive and the increase is directly related to the severity of the infection (Alhussien & Dang, 2018; Cheng & Han, 2020; Zhang et al., 2022).

Risk Factor

Feeding and temperature influence milk production, animals in warm conditions store nutrients and spend more energy for thermal regulation, the breed of the cow and the number of calvings is related to the development of infections. Farm management conditions are important, a technician farm will be able to perform mechanical milking, have manure management, good milking practices and divided areas for dry animals to minimize the risk of disease transmission (Liu et al., 2019).

Etiological agents

Many microorganisms have been described as important pathogens involved in the etiology of contagious mastitis, such as *Streptococcus agalactiae, S. uberis*, *S. dysgalactiae, Staphylococcus aureus*, *Mycoplasma bovis* and *Corynebacterium bovis*, and others such as *E. coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and coagulase-negative Staphylococci (SCN) and *Enterococcus* spp. are named as environmental mastitis pathogens (Li et al., 2023). Other Gram-negative bacteria, coliforms and fungi are related to contamination or poor hygiene practices and inadequate cleaning in the milking system (El-Sayed et al., 2017). Contagious pathogens are capable of colonizing and caused inflammation on mammary glands in the milking process, whilst, environmental or opportunistic bacterial are present on the habitat of the animals but, they can take advantage of an animal with low defenses and avoid the immune system response to colonize. (Rifatbegović et al., 2024).

Intramammary infection

Mastitis develops through three main stages: invasion, infection, and inflammation (Ruegg, 2017). Invasion occurs when pathogens, whether contagious or environmental, enter the udder canal and reach the mammary alveoli. Infection occurs when the animal's immune system is weakened, and its immune response is insufficient to control invading microorganisms. The udder duct, lined with keratin, acts as the first line physical barrier to

prevent the entry of pathogens and leakage of milk. This keratin layer not only has a structural function, but also contains compounds, such as fatty acids with bacteriostatic and bactericidal properties, which weaken the membrane of microorganisms, making them more vulnerable to osmotic pressure (Ezzat Alnakip et al., 2014).

If microorganisms manage to cross this barrier, the innate immune response (IIR) is activated. IIR mobilizes cells such as nuclear polymorphs (PMN), neutrophils, macrophages and dendritic cells. It also utilizes humoral defense mechanisms, such as the release of cytokines and reactive oxygen species (ROS) (Stanek et al., 2024).

Macrophages and dendritic cells have pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which interact with characteristic structures of microorganisms called pathogen-associated molecular patterns (PAMPs). This process increases the production of proinflammatory cytokines and triggers the acute phase response (PAR), which includes ubiquitination. If the animal controls the infection, the immune system restores homeostasis. But if inflammation persists, the adaptive immune response is activated, involving T and B lymphocytes. These cells interact with antigen-presenting cells (APCs) to initiate cytotoxicity mediated by interleukins (IL) and tumor necrosis factor alpha (TNF-α). This mechanism generates the antibodies necessary to eliminate pathogens and establish immunological memory, preparing the organism to respond rapidly to future infections (Haxhiaj et al., 2022).

Antimicrobial mechanisms of action

Since the discovery of penicillin in 1928, antibiotics have been used to treat infections in animals and humans. Antibiotics are bactericidal (with lethal action) and bacteriostatic (limiting development) compounds. At the molecular level, their mechanisms of action range from inhibition of synthesis to damage of membrane integrity, inhibition of nucleic acids

synthesis, as well as inhibition of protein synthesis and control of enzymatic activity from metabolism (Calvo & Martínez-Martínez, 2009).

Inhibition of cell wall synthesis

The bacterial cell wall is mainly composed of peptidoglycan, a structure formed by repeats of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), linked by tetrapeptide chains that confer rigidity and resistance (Suárez & Gudiol, 2009). Beta-lactams, such as penicillins, cephalosporins, carbapenemics and monobactams, are bactericidal agents and act as inhibitors of cell wall synthesis by taking advantage of their beta-lactam ring to compete with pentapeptides for binding to the transpeptidase enzyme or penicillin-binding protein (PBP) (Bush & Bradford, 2016). Due to their higher affinity for PBP, beta-lactams prevent the formation of tetrapeptide cross-links, which are necessary to stabilize peptidoglycan. In addition, these antibiotics can activate endolysin enzymes, which break the bonds between NAM and NAG molecules, weakening the integrity of the cell wall, leading to its lysis and death. (Suárez & Gudiol, 2009). These molecules are effective against Gram-positive bacteria, such as *S. aureus*, but in the case of *Enterococcus* spp., they only exert a bacteriostatic action (Calvo & Martínez-Martínez, 2009).

Inhibition of protein synthesis

Antibiotics that inhibit protein synthesis act specifically on the bacterial 30S and 50S ribosomal subunits at the translation stage. In the 30S subunit, aminoglycosides bind irreversibly, causing errors in the reading of messenger RNA (mRNA) and blocking the formation of the initiation complex, whereas tetracyclines bind reversibly, prevent the binding of transfer RNA (tRNA) to the A site and prevent the incorporation of new amino acids (Lin et al., 2018). On the other hand, at the 50S subunit, macrolides and lincosamides bind irreversibly and disrupt aminoacyl tRNA binding at the A or P sites, preventing nascent chain elongation;

chloramphenicol inhibits peptidyl transferase activity, which is key to peptide bond formation; and oxazolidinones block the formation of the initiation complex by preventing the correct association of the 30S and 50S subunits (Wilson, 2014). These actions disrupt the synthesis of essential bacterial proteins, which stops growth or causes cell death.

Inhibition of nucleic acid synthesis

Inhibition in nucleic acid synthesis is based on the disruption of essential processes such as DNA replication and transcription. Rifampicin, for example, binds to the beta subunit of bacterial DNA-dependent RNA polymerase, blocking the initiation of messenger and ribosomal RNA synthesis. On the other hand, quinolones act by inhibiting DNA gyrase (topoisomerase II) and topoisomerase IV enzymes, essential for DNA negative supercoiling and unlinking newly synthesized DNA, which prevents replication and repair process (Baran et al., 2023). In addition, some antibiotics cause direct damage to DNA: metronidazole generates free radicals that oxidize genetic material, while nitrofurantoin produces toxic compounds after being metabolized, affecting DNA and other bacterial processes. It has also been observed that activation of the SOS repair system may contribute to further damage, enhancing the bactericidal effect (Calvo & Martínez-Martínez, 2009).

Disruption of osmotic to the membrane integrity

External membrane of Gram-negative bacteria consists internally of phospholipids and externally of lipopolysaccharides (LPS). LPS has three components antigen O, core and lipid A, this lipid has two divalent cations, Ca_2+ and Mg_2+ that bind and stabilize the phosphates molecules (Aguayo et al., 2016). Polymyxin and colistin have affinity for the phosphates of the lipid A region of LPS, the cationic diaminobutyric acid (Dab) residues of colistin binds with the anionic LPS molecules, by displacing Ca_2+ and Mg_2+ from the external cell membrane,

leading to permeability changes in the cell envelope, inhibition of the endotoxin activity, break the osmotic resistance, and leakage of cell contents (El-Sayed Ahmed et al., 2020).

Antimicrobial resistance

Antimicrobial resistance (AMR) is a phenomenon that occurs naturally among bacteria; however, the indiscriminate use by anthropogenic activity and the selective pressure of antibiotics has accelerated and facilitated the exchange of acquired antibiotic resistance genes (ARGs) (de Souza et al., 2024). Mobile genetic elements (MGEs), such as plasmids, transposons, integrons, and gene cassettes are crucial to horizontal genes transfer (HGT) and promote recombination and genetic exchange through processes such as conjugation, transduction, and transformation of resistance genes between cells (Partridge et al., 2018). Others resistance mechanisms include decreased membrane permeability, target alteration, inactivating enzymes and efflux mechanisms.

The ‘Golden Age of Antibiotics’ was a success in reducing mortality associated with various infectious diseases. However, the abuse of antibiotics in the medical, veterinary and agricultural industries promoted the development and proliferation of resistant strains. By the 1970s, the introduction of more advanced drugs, such as fifth-generation cephalosporins, was necessary to combat infections caused by resistant nosocomial strains (Fernandes et al., 2013).

‘ESKAPE’ is a group of multi-resistant microorganisms emerged, which includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp. These pathogens represent a significant threat to public health due to their ability to evade antimicrobial treatments (Baran et al., 2023). For example, Enterobacterial species are considered a critical risk due to resistance genes such as extended spectrum β-lactamases (ESBL) and vancomycin resistance (Dahmen et

al., 2013). Also, *Staphylococcus* spp. with their resistance to most β-lactams and their ability to form biofilms represents a challenge even to last-line drugs such as linezolid (Silva et al., 2023).

Staphylococcus spp. antimicrobial resistance strategies

S. aureus is described as the most common bacteria isolated in mastitis. These Gram-positive cocci produce PBP1 and PBP2, which have different roles in the synthesis of peptidoglycan and are essential for cell survival (Silva et al., 2023). Resistance of this bacterium occurred in waves, first with the production of inactivating enzymes like penicillinase (PC1) encoded by the *blaZ* gene, which, due to its high affinity for penicillins, hydrolyzed its beta-lactam ring, thus inactivating the drug (Bush & Bradford, 2020). The second wave of resistance came against methicillin, a synthetic drug that introduced the first resistant strains in 1960. The production of an alternative PBP, PBP2a, which does not bind to the beta-lactam drug and can continue peptidoglycan synthesis despite the presence of antibiotic, is the defining resistance mechanism of methicillin-resistant *Staphylococcus aureus* (MRSA) and is encoded by the *mecA* and *mecC* genes, which are found on a mobile genetic element in the bacterial chromosome of most MRSA strains (Larsen et al., 2022).

In staphylococci, overexpression of efflux systems such as the *TetA* (*TetA* K and *TetA* L) efflux pumps assists the exchange of a proton by a tetracycline molecule against a concentration gradient to expel the drug. Another strategy relies on target site modification, for example mutations in topoisomerase that reduce the binding affinity of fluoroquinolones, as well as the *Fus*B and *FusC* mechanisms that help the elongation factor EF-G have a conformational change despite the presence of fusidic acid and can be released to bind to aa-tRNAs and continue translation (Foster, 2017). The staphylococcal biofilm is a microbial community of cells that form a matrix with their own extracellular polymeric substances (Otto, 2018). This matrix provides a 1000-fold increased tolerance to antibiotics by having persistent

cells that are indifferent to the mechanisms of action of antimicrobials, by having reduced drug penetration and by increasing gene expression and acquisition through intercellular communication (quorum sensing) (Guo et al., 2022).

Mechanisms of multi-resistance of *Enterococcus* spp.

E. faecalis and *E. faecium* have a combination of natural and acquired resistance. *E. faecalis* has a PBP5 with modifications in the regions that interact with the β-lactam ring, which confers low affinity for ampicillin and intrinsic resistance to cephalosporins and clindamycin (Miller et al., 2014). It has acquired resistance to vancomycin mediated by genes such as *vanA* and *vanB*, which alter the structure of the peptidoglycan precursor and prevent the binding. The *erm* genes induce resistance by ribosomal modification and the *msrA* genes of plasmid origin generate efflux pumps that obtain the energy necessary to pump macrolides (such as erythromycin and azithromycin) and streptogramins B out of the bacterial cell through the hydrolysis of ATP. The production of modifying enzymes such as acetyltransferases (AAC), nucleotidyltransferases (ANT), and phosphotransferases (APH) inactivate the binding of aminoglycosides to the ribosome (Torres & Cercenado, 2010).

Public health and antibiotics use

Antibiotics such as beta-lactams and cephalosporins are commonly used to treat mastitis, particularly during the dry period, with the aim of preserving milk production, but inadequate management of withdrawal times has increased antibiotic residues (Sachi et al., 2019). The presence of antibiotic residues above safe levels in meat and milk represents a risk to human health, due to potential allergic reactions, dysbiosis from food ingested, and the development of resistant bacteria (Padol et al., 2015;Olatoye et al., 2016). In recent decades, outbreaks associated with the consumption of raw milk contaminated with zoonotic agents such as *S. aureus, M. bovis*, *Brucella* spp., *Campylobacter* spp., *Listeria monocytogenes*, fungi,

viruses, and parasites have been reported and mainly of them are considering like etiological pathogens in mastitis (Kapoor et al., 2023; Holzhauer & Wennink, 2023).

The World Health Organization (WHO) and the World Organization for Animal Health (WHOA) have proposed strategic objectives with a "One Health" approach, aiming that zoonoses and AMR stop been one of the biggest public health problems of the 21st century through surveillance, research and mitigation efforts (Kasimanickam et al., 2021;Murray et al., 2022).

Antimicrobial susceptibility tests

Phenotypic assays are used to test the susceptibility of an isolate *in vitro* against antimicrobial drugs and determining the resistant pattern. These tests are characterized by being reproducible and the standards are chosen depending on two manuals, The Clinical and Laboratory Standards Institute, (CLSI) and European Committee of Antimicrobial Susceptibility Testing (EUCAST), which details all the different procedures to follow in case of disc diffusion method or Minimum Inhibitory Concentration (MIC) to obtain quantitative results.

The conditions of the inoculum used, and the Muller Hinton medium are based on standards, as well as the use of ATCC quality control strains to validate the assays. Results are interpreted based on clinical breakpoints defined for each antimicrobial. The measure in millimeter is for disc diffusion and concentration µg/mL is for MIC assays classifying strains as susceptible, intermediate, or resistant (Kadeřábková et al., 2024). Resistance gene amplification by PCR (polymerase chain reaction) or whole genome sequencing (WGS) complements phenotypic studies for characterization and mechanisms of AMR-associated genes (Galhano et al., 2021).

The determination of these profiles in cattle strains in Ecuador could contribute to the development of specific strategies to combat AMR and reduce the risks associated with it.

PART 2: SCIENTIFIC RESEARCH

Antimicrobial resistance in microorganisms isolated from cow's positive for mastitis

Introduction

Bovine mastitis is a localized inflammation of the mammary glands, usually associated with bacterial infections during milking. The most studied etiological microorganisms (contagious and environmental) in cases of mastitis have been *Staphylococcus aureus, Streptococcus agalactiae, S. uberis, Mycoplasma bovis, E. coli* and coagulase-negative staphylococci (SCN) (El-Sayed et al., 2017). The disease represents a significant economic loss on dairy industry worldwide (Ruegg, 2017; Cheng & Han, 2020; Rifatbegović et al., 2024) and continues to have high prevalences in many Latin American countries exceeding 40% on farms in Colombia and Brazil, with estimates losses of between $70 - $100 per decrease in production, treatment and slaughter of the diseased animal (Guimarães et al., 2017; Romero et al., 2018; Hurtado-Prieto & Cucunubo-Santos, 2023).

Ecuador is the 5th largest producer of milk in South America and Pichincha is the main dairy-producing province in the country. There are two main milk production systems, intensive and extensive, which is an important activity by small-scale producers as a means of subsistence (Aguilar & Álvarez, 2019). In APUs, milking represents a critical point for bacterial contamination of the animal's udder, favoring the spread of animal diseases and damaging the economic sustainability of raw milk production (Aguilar & Álvarez, 2019). The most prevalent disease reported in these APUs in different provinces of Ecuador has been bovine mastitis (Amer et al., 2018; Aguilar & Álvarez, 2019; Avellán et al., 2019; Ibarra Rosero et al., 2022).

Studies conducted in the province of Los Rios - Ecuador determined that mastitis losses range from $ 96 per cow/year (Sánchez et al., 2020).

The “One Health” approach understands the link between animal, human and environmental health. The extensive use of therapeutic antibiotics in the cattle industry, as growth promoters and prophylactics in cases of intramammary use in the dry-off period of animals with mastitis, have contributed to the emergence of beta-lactam antibiotic resistant pathogens and antibiotic residues in milk that endanger the consumer (Sachi et al., 2019; Garcia et al., 2019; Matailo et al., 2023). Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *E. faecium* (VRE) and Enterobacterales resistant to carbapenemics and third-generation cephalosporins are on the WHO list of priority pathogens because of their ability to cause serious infections in humans (WHO, 2024). Considering that these bacteria are part of the causative agents of bovine mastitis and that there is a potential for zoonotic transmission from cattle and contaminated feed known as “from farm to fork” (Chen & Wu, 2021; Bag et al., 2022; Shekhar, 2024). The study of mastitis in the context of One Health highlights the importance of identifying cattle milk bacteria and antimicrobial susceptibility profiles to understand the current state of the dairy industry, and to contribute with strategies in animal welfare, food safety and public health.

The aim of this study was to identify the bacterial agents responsible for bovine mastitis in a farm with intensive and artisanal milking, and to evaluate the antimicrobial susceptibility of these strains. We are confident that our results will provide a baseline of knowledge on AMR in dairy production.

Materials and methods

Bacterial recovery

Samples were obtained from a cryovial bank stored at -80°C at the Animal Biotechnology Laboratory of the Universidad de las Fuerzas Armadas ESPE. These bacterial isolates were recovered from milk samples of cattle diagnosed with mastitis (CM and SCM) from farms in Manabí, Salinas de Guaranda and Hacienda "El Prado" (IASA-ESPE). A total of 73 strains preserved at -80°C in glycerol cryovials and Microbank™ bead cryovials, characterized as suspected *Staphylococcus* sp. by culture and Gram staining methods during an undergraduate study, were processed for this study. These samples were cultured in Merck® Tryptic Soy Broth (TSB) and incubated for 48 hours with shaking at 220 rpm. Recovered colonies were recultured on BD Nutrient Agar (AN) Difco™ solid medium at 37 °C for up to 24 hours (Vrieling et al., 2015).

For Gram staining of the isolates recovered, a drop of saline solution was applied to the glass slide, and a sterile loop was used to transfer a single colony for fixation. In the case of liquid media, a drop was placed directly on the slide. The Gram staining procedure followed the protocol from the Microbiology Institute of Universidad San Francisco (IM), using crystal violet and iodine for 1 minute, followed by 10 seconds with decolorizing solution, and safranin as a counterstain for 30 second. Samples with no growth and bacteria with a different morphotype from that of Gram-positive cocci, indicating contamination, were discarded in this study. All data have been processed and are available at **Appendix A**.

Strains recovered with the Gram-positive cocci morphotype (n = 21) were processed using identification algorithms through specific media and enzymatic tests. Gram-positive cocci were inoculated onto Mannitol salt agar (MAN) (BD BBL™), DNase agar (BD Difco™) and Bile Esculin (BE) (BD BBL™) mediums. A positive (+) value was considered when the enzyme reaction was present in the differential media. Positive MAN (+) values were assigned to mannitol fermentation, which changes the pH of the medium from red to yellow, negative

MAN (-) values were assigned to red colonies that grew and did not ferment mannitol, and MAN (+/-) values were assigned to incomplete fermentation, DNAase (+) for the formation of the transparent halo on bacterial growth, and BE positive (+) was assigned to esculin hydrolysis and reaction with ferric ions, which gives a brown-black halo on the medium (Swan, 1954).

Catalase tests were performed on all isolates using a drop of 3% hydrogen peroxide, and a (+) value was assigned when O_2 release occurred (Chester, 1979). The coagulase test was applied to the positive isolates of the previous test, a positive value (+) was assigned to clot formation by the conversion of soluble fibrinogen into insoluble fibrin from rabbit plasma by the enzyme thrombin (Sperber & Tatini, 1975).

The isolates selected for the study were cultured in AN medium, and then with a sterile loop a colony of each strain were recovered and inoculated in 5 mL of TSB liquid medium to be incubated at 37°C for 24 hours for the subsequent DNA extraction step.

DNA extraction from bacterial strains

DNA extraction was performed following the protocol of the commercial kit Wizard® Genomic DNA Purification (Promega) with modification on DNA rehydration. To obtain the cellular pellet of Gram-positive bacteria, 1 mL of culture was centrifuged in sterilized 1.5 mL Eppendorf tubes at 13,000 rpm for 3 minutes, discarding the supernatant. This procedure was replicated in triplicate and 120 µL of lysozyme was applied to incubated for 40 minutes. Cellular lysis was carried out using the buffer provided in the kit, with samples incubated at 80°C for 5 minutes and then cooled to room temperature before adding RNase solution, followed by incubation at 37°C for 30 minutes with shaking at 200 rpm. To precipitate proteins, a precipitation solution was added, mixed, and kept on ice for 5 minutes before centrifugation at 13,000 rpm for 4 minutes. DNA precipitation was performed using isopropanol and 70% molecular-grade ethanol was applied to wash it, using inversion movements. The tubes were

allowed to air dry for 20 minutes. Finally, 50 µL of rehydration solution was added to each DNA tube, and they were left at 4°C overnight. The DNA samples were stored at -20°C until use.

The DNA concentration was quantified using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™), and the integrity of the DNA was assessed by electrophoresis on a 0.8% agarose gel at 100V for 30 minutes. The bands were visualized using the ChemiDoc™ imaging system (BioRad). Samples with concentrations higher than 25 ng/µL were diluted for the required PCR condition see ***Appendix C***.

Molecular identification of *16S rRNA gene*

For molecular identification of the isolates, the *16S rRNA gene* sequence of the V1-V9 region of approximately 1500 bp was amplified by PCR with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), following a protocol developed by Frank et al. (2008) with modifications. The PCR reaction was carried out in a total volume of 25 µL, including 1 ng/µL of sample DNA in 24 µL of Master Mix (Invitrogen), with final concentrations of 1.5 mM $MgCl_2$, 0.8 mM dNTPs (deoxynucleotide triphosphates), 0.4 µM of each primer, and 0.5 units of *Platinum Taq* DNA polymerase (Invitrogen). The PCR conditions in the Pro-Flex™ 3 x 32-well PCR System were: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 1.45 minutes, with a final extension of 10 minutes (Frank et al., 2008). Observation of the amplicons was done on a 1.5% agarose gel see ***Appendix D***. The PCR products were sent to Macrogen® (Korea) for Sanger sequencing.

The obtained sequences forward and reverse (n = 42) were cleaned and assembled using MacVector software (https://macvector.com/). Consensus sequences were aligned and

compared with sequences from databases available in GenBank using BLAST (Basic Local Alignment Search Tool) to determine the percentages of identity of homologous sequences.

Alignment was completed using the ClustalW (http://www.clustal.org/) tool integrated in MEGAX. For phylogenetic analysis, a maximum likelihood dendrogram with 1000 bootstrap was built using MEGAX software (http://www.megasoftware.net).

The algorithm was established with our 21 consensus sequences and 22 high homology sequences available in GenBank from different geographical locations (we used sequences with percentages > 98%). A Gram-negative *E. coli* bacterium isolated in a mastitis study was included in the alignment (Yalcin et al., 2024).

Antimicrobial susceptibility testing

For the *in vitro* antimicrobial susceptibility analysis, the criteria established by the CLSI were followed (CLSI, 2024). Inoculation of 21 strains was standardized to OD600 = 0.063 in sterile 0.85% w/v saline solution. A sterile cotton-tipped applicator with the suspension was spread on the surface of Mueller Hinton agar (MH) (BD Difco™) using the Kirby-Bauer disk diffusion method spread four times in an overlapping direction. Antibiotic discs were placed on the agar plate to be incubated at 37°C for 20 hours (Kadeřábková et al., 2024).

The antibiotics discs used for the antibiogram were ampicillin (AMP, 10 µg), vancomycin (VAN, 30 µg), erythromycin (E, 15 µg), cefoxitin (FOX, 30 µg), tetracycline (TET, 30 µg), penicillin (P, 10 µg), nitrofurantoin (F/M, 300 µg) and linezolid (LZD, 10 µg). The halo diameters for the discs from MH medium were measured in millimeters and characterized as susceptible, intermediate or resistant based on breakpoints provided by CLSI M100 (https://em100.edaptivedocs.net/Login.aspx) and VET01S manuals (http://vet01s.edaptivedocs.net/Login.aspx). *S. aureus* ATCC 25923 was used as internal quality control. The strains that exhibited intermediate susceptibility and/or resistance

phenotypes in the antibiogram tests (n = 17) were analyzed by determining the MIC quantitative method.

MIC assays

Seventy strains were cultured onto Mueller Hinton agar plates and incubated at 37°C for 24 hours to obtain overnight culture and isolated colonies. Sensititre™ panels (Thermo Fisher) was used for Gram-positive bacteria (GPALL1F), following the kit specifications for inoculation. This panel contains 21 antibiotics belonging to 14 families or pharmacological classes: beta-lactams [ampicillin (AMP), penicillin (P), oxacillin (OXA+), cefoxitin (FOXS)], glycopeptides [vancomycin (VAN)], lipopeptides [daptomycin (DAP)], macrolides [erythromycin (ERY)], lincosamides [clindamycin (CLI)], fluoroquinolones [ciprofloxacin (CIP), levofloxacin (LEVO), moxifloxacin (MXF)], aminoglycosides [gentamicin (GEN), streptomycin (STR)], oxazolidinones [linezolid (LZD)], tetracyclines [tetracycline (TET)], phenicols [chloramphenicol (CHL)], nitrofurans [nitrofurantoin (NIT)], sulfonamides [trimethoprim/sulfamethoxazole (SXT)], streptogramins [quinupristin/dalfopristin (SYN)] and rifamycin [rifampin (RIF)]. Colonies were collected with a sterile loop and inoculated into tubes containing sterile demineralized water until a turbidity comparable to the 0.5 McFarland standard (OD 600nm = 0.063) was obtained (Kadeřábková et al., 2024).

A volume of 30 µL of the suspension was added to a tube containing 11 mL cation-adjusted Mueller Hinton broth and homogenized by inversion. After that, 50 µL of the mixture were dispensed into 96 well-plates of the Sensititre panel. Panels were incubated at 37°C for 20 hours and the read was following the Sensititre Plate Worksheet (Yalcin et al., 2024). Panels with slow-growing strains were incubated for up to 24 hours. *S. aureus* ATCC 25923 were used as control.

Results

Staphylococcus spp. are the major Gram-positive cocci responsible for bovine mastitis.

A recovery rate of 28.76% (21/73) was obtained. The 21 isolates recovered obtained the Gram-positive cocci staining morphotype considered for the study, as detailed in the bacterial recovery part of the Materials and methods, 9 strains were identified for the Pichincha farms (I), 3 strains for Manabí (15, 18 and 19 I) and 9 for Salinas (G) strains. Identification revealed the presence of *S. aureus* with positive coagulase and DNase test in 52.38% (11/21) of the samples. *Staphylococcu*s spp. with a negative coagulase test (CNS) in 19.05% (4/21) and *Enterococcus* spp. with a negative catalase test and growth on BE culture medium in 28.57% (6/21) of the isolates. The results of growth on selective/differential media and biochemical tests are shown in **Table 1**.

The amplification and sequencing of the 16S rRNA gene of approximately 1500 bp resulted in the identification of isolates corresponded to *Staphylococcus aureus* (n=11), *Staphylococcus chromogenes* (n=3), *Staphylococcus devriesei* (n=1), *Enterococcus faecium* (n=3), and *Enterococcus faecalis* (n=3). All the sequences obtained got a homology percent mayor than 98%. Homology percentages for each sequence and accession numbers for bacterial sequences from the GenBank database are shown in **Table 1.**

Table 1 *Phenotypic and molecular identification of Gram-positive cocci isolates.*

			Tests to determine phenotype					Genetic identification		
n°	Code	Microorganisms	MAN	CAT	BE	COG	DNase HCl 1N	% homology	GenBank accession alignment sequences	GenBank accession dendrogram
N	2I	*S.aureus*	+	+		+	+	99,91	CP103860.1	MN889346.1
2	3I	*S.aureus*	+	+		+	+	100	CP103860.1	MW033903.1
3	4I	*S. devriesei*	-	+		-		99,41	MH127803.1	MH127803.1
4	7I	*S. chromogenes*	+/-	+		-		99,23	OR142912.1	FJ389197.1
5	12I	*S.aureus*	+	+		+	+	99,22	MK809241.1	MZ208977.1

6	13I	*S.aureus*	+	+		+	+	99,79	MN889346.1	MN650918.1
7	14I	*S.aureus*	+	+		+	+	99,93	KF495200.1	MF424800.1
8	15M	*S.aureus*	+	+		+	+	99,64	JN084557.1	MZ169417.1
9	16I	*S.aureus*	+	+		+	+	98,98	MK405715.1	MH496644.1
10	17I	*S.aureus*	+	+		+	+	99,78	CP150756.1	OR770531.1
11	18M	*S.aureus*	+	+		+	+	100	CP150756.1	MT272781.1
12	19M	*S.aureus*	+	+		+	+	99,93	CP150756.1	MF424452.1
13	1G	*S. chromogenes*	-	+		-		99,71	OR142912.1	OR142912.1
14	11G	*S. chromogenes*	-	+		-		99,93	OR142907.1	KJ783392.1
15	20G	*E. faecium*	+/-	-	+			100	CP130861.1	LC317309.1
16	24G	*E. faecalis*	+/-	-	+			99,93	ON974825.1	MN379675.1
17	28G	*E. faecium*	+/-	-	+			99,93	MF424452.1	PP218401.1
18	29G	*S.aureus*	+	+		+	+	99,85	JN084557.1	JN084557.1
19	38G	*E. faecium*	+/-	-	+			99,86	CP130861.1	OR793051.1
20	41G	*E. faecalis*	+/-	-	+			100	MF369841.1	MG694634.1
21	47G	*E. faecalis*	+/-	-	+			99,86	PQ395783.1	MF369992.1
22		*E. coli*								OR889630.1

Note. MAN: mannitol salt agar; Positive (+) MAN was for mannitol fermentation, which changes the pH of the medium and turns from red to yellow; MAN negative (-) not ferment mannitol. MAN (+/-) incomplete mannitol fermentation; BE: Bilis esculin agar; Positive (+) BE was assigned to the esculin hydrolysis and the reaction with ferric ions that gave a brown-black halo; DNase: DNase agar test, Positive (+) was assigned when precipitation of the polymerized DNA was produced by applying hydrochloric acid and a clear zone was evident; CAT: catalase test, Positive (+) was assigned to the release of O_2 upon contact with hydrogen peroxide and (-) when there was no reaction; COG: coagulase test; (+) positive was the assay to coagulum formation of rabbit plasma in a tube by the action of the coagulase enzyme; % homology: the relationship between this study sequences and GenBank database.

The analysis obtained using the maximum likelihood with the evolutionary model Jukes-Cantor had the highest log likelihood value (-4310.71), indicating a good fit between the observed data and the assumed evolutionary model. Forty-four nucleotide sequences were analyzed with a total of 1417 positions. Maximum likelihood dendrogram with 1000 bootstrap based on the alignment of the 44 sequences is shown in **Figure 1**.

The clustering of bacterial species identified from their *16S rRNA* gene sequences belongs to two specific groups of bacteria separated into two clades: *Staphylococcus* sp. and *Enterococcus* sp. The outgroup of the tree indicates that the partial *16S rRNA* gene sequence of *E. coli* has the greatest evolutionary distance with respect to the other bacterial groups.

The isolates from all 3 farms had high bootstrap support values, indicating high confidence in the phylogenetic relationship between these isolates. The bootstrap support value of 100 between *E. faecium* and *E. faecalis* reflected high reliability in the clustering of these sequences.

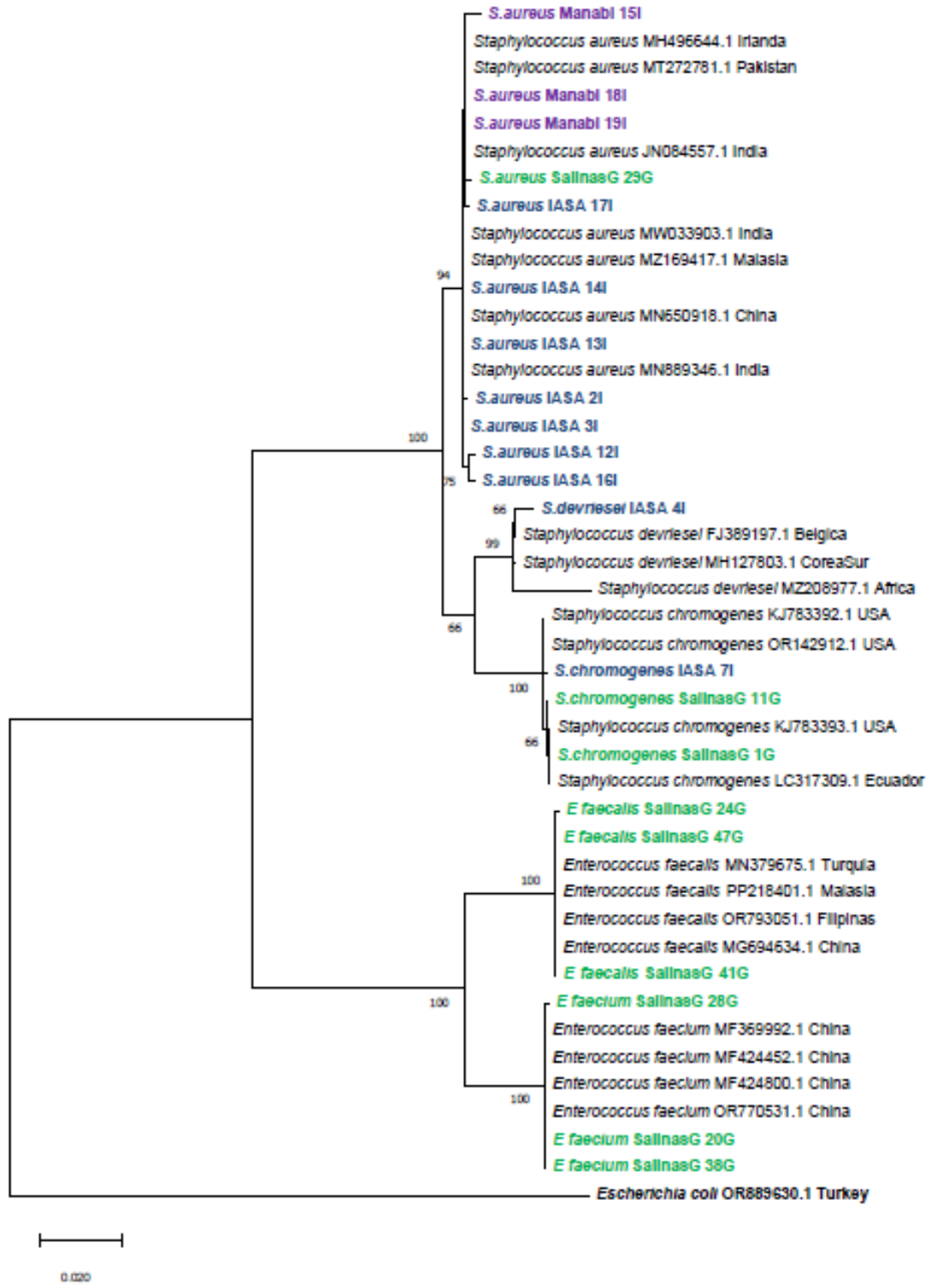


Figure 1 Identification of bacteria based on the phylogenetic distance of a *16S rRNA* gene sequence fragment recovered for antimicrobial susceptibility study. The evolutionary history of 21 isolated was inferred using Maximum Likelihood method and Jukes-Cantor model.

Antimicrobial susceptibility testing of Gram-positive cocci causing bovine mastitis

Of the total 21 strains identified in the study, 17 strains showed an intermediate phenotype or resistance to at least one antibiotic by antibiogram test. The information of the antibiograms with the measurement in millimeters from halos of the 21 strains and MIC values in µg/mL of the 17 strains is presented in **Appendix B**. The results of the interpretation of the MIC values of the 17 strains are shown below in the **Table 2**. Strains with resistance to 3 or more antibiotic families were considered multidrug resistant.

Table 2 *MIC interpretation of 21 antibiotics from Staphylococcus sp. and Enterococcus sp. strains of this study.*

		MIC BREACKPOINTS (µg/mL)																				
MICROORGANISM		AMP	P	VA	TE	LZD	DAP	NITRO	TGC	CHL	LEVO	RIF	SYN	STR	CIP	E	CLI	DTEST	FOXS	OXA+	GEN	SXT	MXF
S.aureus	2I	R	R	S	S	S	S	S	S	I	S	S	S		S	S	S	-	S	S	S	S	S
S.aureus	3I	R	R	S	S	S	S	S	S	I	S	S	S		S	I	S	-	S	S	S	S	S
S. devriesei	4I	S	S	S	S	S	S	S	S	S	I	S	S		S	S	S	-	S	S	S	S	S
S.aureus	12I	R	R	R	R	NS	NS	I	NS	I	S	R	R		S	I	R	++	R	R	S	R	R
S.aureus	13I	R	R	S	S	S	S	S	S	S	S	S	S		S	S	S	-	S	S	S	S	S
S.aureus	15I	S	S	S	S	S	S	S	S	S	S	S	S		S	S	S	-	S	S	S	S	S
S.aureus	16I	R	R	S	S	S	S	S	S	S	S	S	S		S	S	S	-	S	S	S	S	S
S.aureus	17I	R	R	S	S	S	S	S	S	S	S	S	S		S	S	S	-	S	S	S	S	S
S.aureus	18I	S	S	S	S	NS	S	S	S	I	I	S	S		S	I	S	-	S	S	I	S	S
S.aureus	19I	R	S	S	S	S	S	S	S	S	S	S	S		S	S	S	-	S	S	S	S	I
S. chromogenes	1G	S	R	S	S	S	S	S	S	S	S	S	S		S	S	S	-	S	S	S	S	S
S. chromogenes	11G	S	R	S	S	S	S	S	S	S	S	S	R		S	S	S	-	S	S	S	R	S
E. faecium	20G	S	S	S	S	S	NS	I	S	S	S	S	S	S	S	I							
E. faecalis	24G	S	S	S	R	S	S	S	S	I	I	R	R	S	I	I							
S.aureus	29G	R	R	S	R	S	S	S	S	S	R	S	S	NS	R	S	S	-	S	S	S	S	R
E. faecalis	41G	S	S	S	R	S	S	S	S	S	I	R	R	S	I	S							
E. faecalis	47G	S	S	S	R	S	S	S	S	S	I	S	R	S	S	S							
S. aureus ATCC 25923		S	S	S	S	S	S	S	S	I	S	S	S		S	S	S	-	S	S	S	S	S

Note. The assignment of resistance (R), intermedial (I), susceptible (S) and non-susceptible (NS) was based on CLSI breakpoints parameters and Sensititre GPALL1F Worksheet. A total of 21 measures antibiotics were reported: Ampicillin (AMP), penicillin (P), vancomycin (VA), tetracycline (TE), linezolid (LZD), daptomycin (DAP), nitrofurantoin (NITRO), tigecycline (TGC), chloramphenicol (CHL), levofloxacin (LEVO), rifampin (RIF), quinupristin/dalfopristin (SYN), streptomycin (STR), ciprofloxacin (CIP), cefoxitin (FOXS), oxacillin (OXA+), gentamicin (GEN), moxifloxacin (MXF), trimethoprim/sulfamethoxazole (SXT), clindamycin (CLI) and erythromycin (E). DTEST (++) indicated inducible clindamycin resistance from E to CLI. Blank cells have no interpretation criteria.

From the data obtained in MIC assays, it is reported that the bacteria had resistance profiles for 62% (13/21) of the antibiotics tested. The antibiotics (LZD, DAP, NITRO, TGC, CHL, STR, E and GEN) were shown to be effective in 38% (8/21) by *in vitro* assays for the control of mastitis caused by *Staphylococcus* spp. and *Enterococcus* spp. A general analysis determined that bacteria isolated from mastitis-positive cattle milk samples are most resistant to the beta-lactam 47.5% (19/40), tetracycline and streptogramin 13% (5/40) each one and fluoroquinolones 10% (4/40).

S. aureus (strain 12I) showed resistance to multiple drugs in MIC assays, including beta-lactams, glycopeptides, tetracycline, quinupristin/dalfopristin, streptomycin, moxifloxacin, trimethoprim/sulfamethoxazole, lincosamides and rifampin. Intermediate resistance for chloramphenicol, nitrofurantoin and erythromycin with positive D-test. And sensibility by gentamicin, levofloxacin and ciprofloxacin. *S. aureus* (strain 29G) was multiresistant to beta-lactams, tetracyclines and fluoroquinolones, while a single strain of *S. chromogenes* (11G) was multiresistant to beta-lactams, streptogramin and sulfonamide.

S. devriesei (strain 4I) showed broad susceptibility, except for levofloxacin, which showed intermediate resistance and *S. chromogenes* strain (1G) showed broad antibiotic sensitivity, however it showed resistance to penicillin.

Enterococcus faecalis strains were the strains with the highest resistance profile in contrast to *E. faecium* which was susceptible to all 21 antibiotics in the sensititre panel. *E. faecalis* shows congruence to intrinsic resistance. High levels of resistance to tetracycline and rifampicin are observed, in addition to streptogramins. They were intermediate to fluoroquinolones.

Discussion

The identification of Gram-positive bacteria in cases of mastitis is common, and several studies have been described where these microorganisms are contagious pathogens among cattle such as *Streptococcus* spp., *Staphylococcus* spp. and *Enterococcus* spp. (Stanek et al., 2024). *S. aureus* was the most frequently found microorganism in cases of mastitis in farms in Manabí and Quito (IASA) from this study, coinciding with phenotypic and genotypic studies in other provinces of Ecuador, such as Carchi and El Oro, in cases of clinical and subclinical mastitis in cattle (Amer et al., 2018;Ibarra Rosero et al., 2022).

In multiple studies, CNS are microorganisms associated with subclinical infections in cattle, which have a significant impact on milk production (Huang et al., 2000; Holzhauer & Wennink, 2023;Sigudu et al., 2024). *S. chromogenes* and *S. devriesei* strains identified in IASA and Salinas de Guaranda farms were in lower proportion, these CNS are considered native udder microbiota and environmental sources, but they could play an infectious potential in dysbiosis processes contributing to *S. aureus* colonization and increasing the risk of causing IMI in cases of clinical and subclinical mastitis (Wald et al., 2019). Finding *S. devreisei* is closely related to *S. haemolyticus*, another microorganism described as.common in studies mastitis (Supré et al., 2010).

In this study, *Enterococcus* spp. accounted for 28.57% of the recovered strains, with *E. faecalis* and *E. faecium* being the species identified. A study in Brazil identified *E. faecalis* (19.4%) and *E. faecium* (15.1%) in clinical mastitis cases, consistent with the data presented (F. F. Guimarães et al., 2024). *Enterococci* are part of the intestinal microbiota of humans and animals, but when found in the extra-enteric environment, they become opportunistic pathogens that are a source of contamination in livestock pens, animal feed and water sources, hence they are used as fecal indicator bacteria (FIB) (Byappanahalli et al., 2012;Shahveh et al., 2022). These species are environmental pathogens that cause inflammation in the udder if the animal is undergoing a process of infectious mastitis (Kim et al., 2022; Liu et al., 2024).

Antimicrobial resistance is a phenomenon that has occurred in nature, however the use of antibiotics in the livestock industry as growth and reproductive enhancers in farms animals

has generated selective pressure for bacteria to transfer genes at accelerated rates (Low et al., 2021;Zhang et al., 2021).

Therapy to treat mastitis caused by *Staphylococcus* spp. and *Enterococcus* spp. is based on specific medication with intramammary application in the affected quarter and dry systemic therapy with broad spectrum antibiotics (Kapoor et al., 2023). The most used antibiotics for treatment of mastitis contain formulations with various beta-lactams including penicillin and cephalosporins, residues of these antibiotics have been detected in milk above the permitted levels in several countries including Ecuador (Fejzić et al., 2015; Sachi et al., 2019; Matailo et al., 2023). In this study, greater resistance is reported for the beta-lactam family, there are circumstances such as non-compliance with withdrawal time, extended treatment and poor dosage of the same, so that selective pressure is exerted and conditions for the development of resistant strains are given.

S. aureus strain (12I) was multiresistant to several antibiotics, including methicillin, stands out. MRSA is resistant through the acquisition of a single genetic element, the staphylococcal cassette chromosome *mec* (*SCCmec*). This strain was also found to be resistant to beta-lactams, which is common in case of LA-MRSA (Chen & Wu, 2021), and this is known to occur through the acquisition of the *blaZ* gene and *mecA* gene encoding the beta-lactamase enzyme, *vanA* operon that prevents vancomycin binding and the activity of *erm*, *rpoB*, *tet*, and efflux pump genes for resistance to macrolides, lincosamides and streptogramin, rifampicin and tetracycline (Vestergaard et al., 2019). These data are important for intervention in occupational LA-MRSA infections involving zoonotic preoccupation if the livestock workers or veterinarians has wounds or a disease that weakens his immune system and makes him more vulnerable to pathogens (Chen & Wu, 2021).

Although bovine mastitis caused by *Enterococcus* spp. is less frequent compared to other Gram-positive bacteria (Klimienė et al., 2011), it remains a concern due to its intrinsic resistance to aminoglycosides and cephalosporins, as well as its role in the spread of antimicrobial resistance through the acquisition and transfer of critical resistance genes, such as vancomycin (Miller et al., 2014). Furthermore, studies suggest that the antibiotic resistance observed in *Staphylococcus* spp. and *Enterococcus* spp. is linked to their ability to form biofilms, which facilitates their persistence in the

mammary gland, contributing to recurrent infections and therapeutic failures in mastitis cases (Gomes et al., 2016). The presence of antimicrobial-resistant *Enterococcus* is increasingly recognized as an emerging problem in food-producing animals, raising concerns about public health risks in the One Health framework (Cagnoli et al., 2024).

Of the *Enterococcus* spp. isolates, *E. faecalis* was the species that presented AMR to quinupristin/dalfopristin, rifampin and tetracycline antibiotics. Studies in China have reported that *E. faecalis* is the most prevalent bacterium in bovine mastitis and shows high resistance to tetracyclines and erythromycin. (Yang et al., 2019; Liu et al., 2024). Studies in Poland are closer to our results, as they report resistance to the same antibiotics with the addition of resistance to nitrofurantoin in *E. faecium* (Rózańska et al., 2019). Without considering an infection by these etiological agents, the use of antibiotics to promote animal growth or reproduction continues to be applied (Low et al., 2021).

The prevalence of mastitis worldwide has decreased but has not been completely eradicated. In Ecuador, studies have been carried out for the disease, finding prevalences of 35 % in Carchi, 40 % in Azuay, 52.7 % in Los Ríos and up to 80 % for the subclinical type in Zamora Chinchipe (Amer et al., 2018; Sánchez et al., 2020). The actual prevalence of mastitis in this study could not be estimated, due to the low recovery of strains from the biobank. Factors related to long-term storage can cause irreversible cell damage mainly in the cell wall. As well as culture growth considerations may mean that cells are no longer viable when recovered (Guo et al., 2020). Despite this, we managed to recover bacteria to get an overview of the causative agents of mastitis on these farms.

The dairy industry in Ecuador is strong with and annual production of 5.7 million of liters, the highlander supplies more than 70% of the national production of raw milk. In 2022, 0.41 K tons of milk and dairy products were exported for a value of $1.34 million, with Colombia, USA and Peru being the main consumers (INEC, 2024). Producers must therefore strike a balance between caring for the health of the herd, maintaining food security and minimizing the environmental impact of the industry's own waste. Mastitis in Ecuadorian livestock is a challenge; there are intrinsic and extrinsic factors associated with it. The most related to AMR of the causal agents is found in the extensive use of antibiotics for treatment and prophylaxis of drying animals. In these animals, long-acting antibiotics are used, and in

many cases the withdrawal time is not respected, causing the milk to present residues related to the loss of food safety and increasing the possibility of the development of AMR, directly affecting public health.

Since 2016, Ecuadorian authorities have generated manuals for producers to obtain GAP certification and obtain an economic incentive per liter of milk, but these campaigns have stalled since the pandemic and efforts to resume them have been delayed (AGROCALIDAD, 2016). Despite the efforts of the control agencies, with programs, it has not been possible to unify them throughout the territory, adding to problems such as easy access to intramammary products without prescription by a veterinarian and the application of treatments without knowing the pathogen. Mastitis has become a disease that is poorly treated and costly for producers because selling a liter of milk at 0.31 cents does not cover the cost of $100 per year to treat this disease. (CFN, 2024).

Without improved farming practices, microbiological identification of the causative agent and routine antimicrobial susceptibility studies in veterinary diagnostic laboratories or implementation of commercial vaccines (Huilca-Ibarra et al., 2022;Vidlund et al., 2024). Mastitis will continue as a neglected disease affecting the beef cattle industry and contributing to the development of antibiotic resistance with implications for public health and delaying AMR and zoonosis mitigation strategies in the context of One Health.

CONCLUSIONS

The finding demonstrates a high prevalence of multidrug-resistant strains, particularly in *S. aureus* and *Enterococcus faecalis*, underscoring the critical need for ongoing vigilance in clinical and veterinary settings.

The identification of an MRSA strain in bovine mastitis-positive samples emphasizes the importance of continuous monitoring of animals, milk and raw dairy products, due to the infection risk posed to workers. This zoonotic potential should be assessed by veterinarians to enhance occupational safety.

The intrinsic resistance of *Enterococcus* spp. and their potential to transfer resistance to other pathogens highlight the urgency of control measures, including the rational use of antimicrobials and surveillance programs aligned with the "One Health" philosophy.

It is essential to report bovine mastitis cases to national control authorities, adopt microbiological identification for diagnosis, and determine antimicrobial susceptibility profiles to ensure effective treatment and mitigate the development of more resistant strains in Ecuador's livestock sector.

REFERENCES

AGROCALIDAD. (2016). *Guía de buenas prácticas pecuarias en ganadería de leche para pequeños productores*. Agencia de regulación y control fito y zoosanitario.

Aguayo, A., Mella, S., Riedel, G., Bello, H., Domínguez, M., & González-Rocha, G. (2016). Colistin in the post-antibiotic era. *Revista Chilena de Infectologia*, *33*(2), 166–176. https://doi.org/10.4067/S0716-10182016000200006

Aguilar, F., & Álvarez, C. (2019). *Mastitis Bovina: Vol. Vol.1* (K. Lozano, Ed.; 1rst ed.). Editorial UTMACH.

Alhussien, M. N., & Dang, A. K. (2018). Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. In *Veterinary World* (Vol. 11, Issue 5, pp. 562–577). Veterinary World. https://doi.org/10.14202/vetworld.2018.562-577

Amer, S., Gálvez, F. L. A., Fukuda, Y., Tada, C., Jimenez, I. L., Valle, W. F. M., & Nakai, Y. (2018). Prevalence and etiology of mastitis in dairy cattle in El Oro Province, Ecuador. *Journal of Veterinary Medical Science*, *80*(6), 861–868. https://doi.org/10.1292/jvms.17-0504

Avellán, R., Zambrano, M., Dela Cruz, C., Delgado, M., Rezabala, P., & Macías Yandri. (2019). Prevalencia de mastitis subclínica en el ganado bovino, mediante la prueba de California Mastitis Test, en el cantón Rocafuerte de la provincia Manabí, Ecuador. *Revista Amazónica Ciencia y Tecnologia*, *8*(1), 62–70. http://revistas.proeditio.com/revistamozonica

Bag, M. A. S., Arif, M., Riaz, S., Khan, M. S. R., Islam, M. S., Punom, S. A., Ali, M. W., Begum, F., Islam, M. S., Rahman, M. T., & Hassan, J. (2022). Antimicrobial Resistance, Virulence Profiles, and Public Health Significance of Enterococcus faecalis Isolated from Clinical Mastitis of Cattle in Bangladesh. *BioMed Research International*, *2022*. https://doi.org/10.1155/2022/8101866

Baran, A., Kwiatkowska, A., & Potocki, L. (2023). Antibiotics and Bacterial Resistance—A Short Story of an Endless Arms Race. In *International Journal of Molecular Sciences* (Vol. 24, Issue 6). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/ijms24065777

Bush, K., & Bradford, P. A. (2016). β-lactams and β-lactamase inhibitors: An overview. *Cold Spring Harbor Perspectives in Medicine*, *6*(8). https://doi.org/10.1101/cshperspect.a025247

Bush, K., & Bradford, P. A. (2020). Epidemiology of β-lactamase-producing pathogens. In *Clinical Microbiology Reviews* (Vol. 33, Issue 2). American Society for Microbiology. https://doi.org/10.1128/CMR.00047-19

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., & Harwood, V. J. (2012). Enterococci in the Environment. *Microbiology and Molecular Biology Reviews*, *76*(4), 685–706. https://doi.org/10.1128/mmbr.00023-12

Cagnoli, G., Di Paolo, A., Bertelloni, F., Salvucci, S., Buccioni, A., Marzoni Fecia di Cossato, M., & Ebani, V. V. (2024). Occurrence of Antimicrobial-Resistant Enterococcus spp. in Healthy Chickens Never Exposed to Antimicrobial Agents in Central Italy. *Antibiotics*, *13*(5). https://doi.org/10.3390/antibiotics13050417

Calvo, J., & Martínez-Martínez, L. (2009). Mecanismos de acción de los antimicrobianos. *Enfermedades Infecciosas y Microbiologia Clinica*, *27*(1), 44–52. https://doi.org/10.1016/j.eimc.2008.11.001

CFN. (2024). *Ficha Sectorial Ganadería Subgerencia de Análisis de Productos y Servicios*.

Chen, C., & Wu, F. (2021). Livestock-associated methicillin-resistant Staphylococcus aureus (LA-MRSA) colonisation and infection among livestock workers and veterinarians: A systematic review and meta-analysis. *Occupational and Environmental Medicine*, *78*(7), 465–471. https://doi.org/10.1136/oemed-2020-106418

Cheng, W. N., & Han, S. G. (2020). Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — A review. In *Asian-Australasian Journal of Animal Sciences* (Vol. 33, Issue 11, pp. 1699–1713). Asian-Australasian Association of Animal Production Societies. https://doi.org/10.5713/ajas.20.0156

Chester, B. (1979). Semiquantitative Catalase Test as an Aid in Identification of Oxidative and Nonsaccharolytic Gram-Negative. Bacteria. In *JOURNAL OF CLINICAL MICROBIOLOGY*.

CLSI. (2024). *Performance Standards for Antimicrobial Susceptibility Testing* (34th ed). Clinical and Laboratory Standards Institute. https://clsi.org/about/blog/critical-standards-added-to-clsi-s-micro-free/

de Souza, M. M. S., Dubenczuk, F. C., Melo, D. A., Holmström, T. C. N., Mendes, M. B., Reinoso, E. B., Coelho, S. M. O., & Coelho, I. S. (2024). Antimicrobial therapy approaches in the mastitis control driven by one health insights. In *Revista Brasileira de Medicina Veterinaria* (Vol. 46). Society of Veterinary Medicine of the State of Rio de Janeiro. https://doi.org/10.29374/2527-2179.bjvm002624

El-Sayed, A., Awad, W., Abdou, N. E., & Castañeda Vázquez, H. (2017). Molecular biological tools applied for identification of mastitis causing pathogens. In *International Journal of Veterinary Science and Medicine* (Vol. 5, Issue 2, pp. 89–97). Faculty of Veterinary Medicine, Cairo University. https://doi.org/10.1016/j.ijvsm.2017.08.002

El-Sayed Ahmed, M. A. E. G., Zhong, L. L., Shen, C., Yang, Y., Doi, Y., & Tian, G. B. (2020). Colistin and its role in the Era of antibiotic resistance: an extended review (2000–2019). In *Emerging Microbes and Infections* (Vol. 9, Issue 1, pp. 868–885). Taylor and Francis Ltd. https://doi.org/10.1080/22221751.2020.1754133

Ezzat Alnakip, M., Quintela-Baluja, M., Böhme, K., Fernández-No, I., Caamaño-Antelo, S., Calo-Mata, P., & Barros-Velázquez, J. (2014). The Immunology of Mammary Gland of Dairy Ruminants between Healthy and Inflammatory Conditions. *Journal of Veterinary Medicine*, *2014*, 1–31. https://doi.org/10.1155/2014/659801

Fejzić, N., Begagić, M., Šerić-Haračić, S., & Smajlović, M. (2015). Beta lactam antibiotics residues in cow's milk: Comparison of efficacy of three screening tests used in bosnia and herzegovina. *Bosnian Journal of Basic Medical Sciences*, *14*(3). https://doi.org/10.17305/bjbms.2014.3.109

Fernandes, R., Amador, P., & Prudêncio, C. (2013). β-Lactams: Chemical structure, mode of action and mechanisms of resistance. In *Reviews and Research in Medical Microbiology* (Vol. 24, Issue 1, pp. 7–17). Lippincott Williams and Wilkins. https://doi.org/10.1097/MRM.0b013e3283587727

Foster, T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. In *FEMS Microbiology Reviews* (Vol. 41, Issue 3, pp. 430–449). Oxford University Press. https://doi.org/10.1093/femsre/fux007

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., & Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology*, *74*(8), 2461–2470. https://doi.org/10.1128/AEM.02272-07

Galhano, B. S. P., Ferrari, R. G., Panzenhagen, P., de Jesus, A. C. S., & Conte-Junior, C. A. (2021). Antimicrobial resistance gene detection methods for bacteria in animal-based foods: A brief review of highlights and advantages. In *Microorganisms* (Vol. 9, Issue 5). MDPI AG. https://doi.org/10.3390/microorganisms9050923

Garcia, S. N., Osburn, B. I., & Cullor, J. S. (2019). A one health perspective on dairy production and dairy food safety. *One Health*, *7*, 100086. https://doi.org/10.1016/J.ONEHLT.2019.100086

Gomes, F., Saavedra, M. J., & Henriques, M. (2016). Bovine mastitis disease/pathogenicity: evidence of the potential role of microbial biofilms. In *Pathogens and disease* (Vol. 74, Issue 3). https://doi.org/10.1093/femspd/ftw006

Guimarães, F. F., Moraes, G. N., Joaquim, S. F., Guerra, S. T., Dalanezi, F. M., Mioni, M. S. R., Medeiros, F. M. H., Lucheis, S. B., Possebon, F. S., Pantoja, J. C. F., Ribeiro, M. G., Rall, V. L. M., Hernandes, R. T., Leite, D. S., & Langoni, H. (2024). Identification of Enterococcus spp. by MALDI-TOF mass spectrometry isolated from clinical mastitis and bulk tank milk samples. *BMC Veterinary Research*, *20*(1). https://doi.org/10.1186/s12917-024-04217-2

Guimarães, J. L. B., Brito, M. A. V. P., Lange, C. C., Silva, M. R., Ribeiro, J. B., Mendonça, L. C., Mendonça, J. F. M., & Souza, G. N. (2017). Estimate of the economic impact of mastitis: A case study in a Holstein dairy herd under tropical conditions. *Preventive Veterinary Medicine*, *142*, 46–50. https://doi.org/10.1016/j.prevetmed.2017.04.011

Guo, H., Tong, Y., Cheng, J., Abbas, Z., Li, Z., Wang, J., Zhou, Y., Si, D., & Zhang, R. (2022). Biofilm and Small Colony Variants—An Update on Staphylococcus aureus Strategies toward Drug Resistance. In *International Journal of Molecular Sciences* (Vol. 23, Issue 3). MDPI. https://doi.org/10.3390/ijms23031241

Guo, N., Wei, Q., & Xu, Y. (2020). Optimization of cryopreservation of pathogenic microbial strains. In *Journal of Biosafety and Biosecurity* (Vol. 2, Issue 2, pp. 66–70). KeAi Communications Co. https://doi.org/10.1016/j.jobb.2020.11.003

Haxhiaj, K., Wishart, D. S., & Ametaj, B. N. (2022). Mastitis: What It Is, Current Diagnostics, and the Potential of Metabolomics to Identify New Predictive Biomarkers. In *Dairy* (Vol. 3, Issue 4, pp. 722–746). MDPI. https://doi.org/10.3390/dairy3040050

Hogeveen, H., Steeneveld, W., & Wolf, C. A. (2019). Production Diseases Reduce the Efficiency of Dairy Production: A Review of the Results, Methods, and Approaches Regarding the Economics of Mastitis. *Annual Review of Resource Economics*, *11*. https://doi.org/10.1146/annurev-resource-100518-093954

Holzhauer, M., & Wennink, G. J. (2023). Zoonotic risks of pathogens from dairy cattle and their milk-borne transmission. In *Journal of Dairy Research* (Vol. 90, Issue 4, pp. 325–331). Cambridge University Press. https://doi.org/10.1017/S0022029923000730

Hoque, M. N., Das, Z. C., Talukder, A. K., Alam, M. S., & Rahman, A. N. M. A. (2015). Different screening tests and milk somatic cell count for the prevalence of subclinical bovine mastitis in Bangladesh. *Tropical Animal Health and Production*, *47*(1), 79–86. https://doi.org/10.1007/s11250-014-0688-0

Huang, L., Islam Aqib, A., Gu, X., Huang, C., Qu, W., Copyright, fvets, Wang, K., Cha, J., Liu, K., Deng, J., Yang, B., Xu, H., Wang, J., & Zhang, L. (2000). *The prevalence of bovine mastitis-associated Staphylococcus aureus in China and its antimicrobial resistance rate: A meta-analysis*. https://www.cnki.net/

Huilca-Ibarra, M. P., Vasco-Julio, D., Ledesma, Y., Guerrero-Freire, S., Zurita, J., Castillejo, P., Barceló Blasco, F., Yanez, L., Changoluisa, D., Echeverría, G., Bastidas-Caldes, C., & Waard, J. H. de. (2022). High Prevalence of Prototheca bovis Infection in Dairy Cattle with Chronic Mastitis in Ecuador. *Veterinary Sciences*, *9*(12). https://doi.org/10.3390/vetsci9120659

Hurtado-Prieto, D. C., & Cucunubo-Santos, L. G. (2023). Prevalencia de mastitis y caracterización productiva en pequeños productores de Simijaca y Tenjo (Cundinamarca), Colombia. *Revista Colombiana de Ciencia Animal - RECIA*, *15*(2), e980. https://doi.org/10.24188/recia.v15.n2.2023.980

Ibarra Rosero, E. M., Ormaza Montenegro, D. J., & Rueda Abad, R. J. (2022). Mastitis bovina en el cantón Montufar – Carchi. Prevalencia, agente causal y factores de riesgo. *AXIOMA*, *1*(26), 5–10. https://doi.org/10.26621/ra.v1i26.735

INEC. (2024). *Encuesta de Superficie y Producción Agropecuaria Continua (ESPAC): Boletín técnico*.

Kadeřábková, N., Mahmood, A. J. S., & Mavridou, D. A. I. (2024). Antibiotic susceptibility testing using minimum inhibitory concentration (MIC) assays. *Npj Antimicrobials and Resistance*, *2*(1), 37. https://doi.org/10.1038/s44259-024-00051-6

Kapoor, S., Goel, A. D., & Jain, V. (2023). Milk-borne diseases through the lens of one health. In *Frontiers in Microbiology* (Vol. 14). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2023.1041051

Kasimanickam, V., Kasimanickam, M., & Kasimanickam, R. (2021). Antibiotics Use in Food Animal Production: Escalation of Antimicrobial Resistance: Where Are We Now in Combating AMR? In *Medical sciences (Basel, Switzerland)* (Vol. 9, Issue 1). NLM (Medline). https://doi.org/10.3390/medsci9010014

Kibebew, K. (2017). *Bovine Mastitis: A Review of Causes and Epidemiological Point of View*. *7*(2). www.iiste.org

Kim, H. J., Youn, H. Y., Kang, H. J., Moon, J. S., Jang, Y. S., Song, K. Y., & Seo, K. H. (2022). Prevalence and Virulence Characteristics of Enterococcus faecalis and Enterococcus faecium in Bovine Mastitis Milk Compared to Bovine Normal Raw Milk in South Korea. *Animals*, *12*(11). https://doi.org/10.3390/ani12111407

Klimienė, I., Ružauskas, M., Špakauskas, V., Mockeliūnas, R., Pereckienė, A., & Butrimaitė-Ambrozevičienė, Č. (2011). Prevalence of Gram positive bacteria in cow mastitis and their susceptibility to beta-lactam antibiotics. *Veterinarija Ir Zootechnika*.

Larsen, J., Raisen, C. L., Ba, X., Sadgrove, N. J., Padilla-González, G. F., Simmonds, M. S. J., Loncaric, I., Kerschner, H., Apfalter, P., Hartl, R., Deplano, A., Vandendriessche, S., Černá Bolfíková, B., Hulva, P., Arendrup, M. C., Hare, R. K., Barnadas, C., Stegger, M., Sieber, R. N., … Larsen, A. R. (2022). Emergence of methicillin resistance predates the clinical use of antibiotics. *Nature*, *602*(7895), 135–141. https://doi.org/10.1038/s41586-021-04265-w

Liang, D., Arnold, L. M., Stowe, C. J., Harmon, R. J., & Bewley, J. M. (2017). Estimating US dairy clinical disease costs with a stochastic simulation model. *Journal of Dairy Science*, *100*(2), 1472–1486. https://doi.org/10.3168/JDS.2016-11565

Lin, J., Zhou, D., Steitz, T. A., Polikanov, Y. S., & Gagnon, M. G. (2018). Ribosome-Targeting Antibiotics: Modes of Action, Mechanisms of Resistance, and Implications for Drug Design. In *Annual Review of Biochemistry* (Vol. 87, pp. 451–478). Annual Reviews Inc. https://doi.org/10.1146/annurev-biochem-062917-011942

Liu, J., Li, L., Chen, X., Lu, Y., & Wang, D. (2019). Effects of heat stress on body temperature, milk production, and reproduction in dairy cows: A novel idea for monitoring and evaluation of heat stress — A review. *Asian-Australasian Journal of Animal Sciences*, *32*(9), 1332–1339. https://doi.org/10.5713/ajas.18.0743

Liu, J., Liang, Z., Zhongla, M., Wang, H., Sun, X., Zheng, J., Yang, F., & Ding, X. (2024). Prevalence and Molecular Characteristics of Enterococci Isolated from Clinical Bovine Mastitis Cases in Ningxia. *Infection and Drug Resistance*, *17*, 2121–2129. https://doi.org/10.2147/IDR.S461587

Low, C. X., Tan, L. T. H., Mutalib, N. S. A., Pusparajah, P., Goh, B. H., Chan, K. G., Letchumanan, V., & Lee, L. H. (2021). Unveiling the impact of antibiotics and alternative methods for animal husbandry: A review. In *Antibiotics* (Vol. 10, Issue 5). MDPI AG. https://doi.org/10.3390/antibiotics10050578

MAG. (2015). *Ecuador declarado país libre de fiebre aftosa*. Ministerio de Agricultura y Ganadería.

MAG. (2024). Informe ejecutivo de rendición de cuentas, periodo: enero - diciembre 2023. In *Ministerio de Agricultura y Ganadería.*

Matailo, S. R., Zaruma, J. A., & Moncada, R. E. (2023). Determinación de antibióticos en leche cruda bovina en el centro de acopio de la parroquia Ludo, cantón Sígsig. *Anatomía Digital*, *6*(3.1), 74–91. https://doi.org/10.33262/anatomiadigital.v6i3.1.2658

Miller, W. R., Munita, J. M., & Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. In *Expert Review of Anti-Infective Therapy* (Vol. 12, Issue 10, pp. 1221–1236). Expert Reviews Ltd. https://doi.org/10.1586/14787210.2014.956092

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B. H., Kumaran, E. A. P., McManigal, B., … Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*, *399*(10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0

Olatoye, I. O., Daniel, O. F., & Ishola, S. A. (2016). Screening of antibiotics and chemical analysis of penicillin residue in fresh milk and traditional dairy products in Oyo state, Nigeria. *Veterinary World*, *9*(9), 948–954. https://doi.org/10.14202/vetworld.2016.948-954

Otto, M. (2018). Staphylococcal Biofilms. *Microbiology Spectrum*, *6*(4). https://doi.org/10.1128/microbiolspec.gpp3-0023-2018

Padol, A. R., Malapure, C. D., Domple, V. D., & Kamdi, B. P. (2015). Occurrence, Public Health Implications and Detection of Antibacterial Drug Residues in Cow Milk. *Society for Environment and Development, (India)*. http://www.sedindia.org

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). *Mobile Genetic Elements Associated with Antimicrobial Resistance*. https://doi.org/10

Poutrel, B., & Rainard, P. (1981). California Mastitis Test Guide of Selective Dry Cow Therapy. *Journal of Dairy Science*, *64*(2). https://doi.org/10.3168/jds.S0022-0302(81)82560-X

Rifatbegović, M., Nicholas, R. A. J., Mutevelić, T., Hadžiomerović, M., & Maksimović, Z. (2024). Pathogens Associated with Bovine Mastitis: The Experience of Bosnia and Herzegovina. *Veterinary Sciences*, *11*(2). https://doi.org/10.3390/vetsci11020063

Romero, J., Benavides, E., & Meza, C. (2018). Assessing financial impacts of subclinical mastitis on Colombian Dairy Farms. *Frontiers in Veterinary Science*, *5*(NOV). https://doi.org/10.3389/fvets.2018.00273

Rózańska, H., Lewtak-Piłat, A., Kubajka, M., & Weiner, M. (2019). Occurrence of enterococci in mastitic cow's milk and their antimicrobial resistance. *Journal of Veterinary Research (Poland)*, *63*(1), 93–97. https://doi.org/10.2478/jvetres-2019-0014

Ruegg, P. L. (2017). A 100-Year Review: Mastitis detection, management, and prevention. *Journal of Dairy Science*, *100*(12), 10381–10397. https://doi.org/10.3168/jds.2017-13023

Sachi, S., Ferdous, J., Sikder, M. H., & Azizul Karim Hussani, S. M. (2019). Antibiotic residues in milk: Past, present, and future. In *Journal of Advanced Veterinary and Animal Research* (Vol. 6, Issue 3, pp. 315–332). Network for the Veterinarians of Bangladesh. https://doi.org/10.5455/javar.2019.f350

Sánchez, S., Sánchez, I., Gómez, J., & Loor, J. (2020). Mastitis subclínica en hatos lecheros medianos del Cantón Babahoyo provincia de los Ríos. *Journal of Science and Research*. https://doi.org/https://zenodo.org/records/4428818

Shahveh, M., Tajbakhsh, E., Momtaz, H., & Rranjbar, R. (2022). Molecular Characterization of Enterococcus faecalis and Enterococcus faecium Isolated from a Meat Source in Shahrekord Local Markets, Iran. *Archives of Razi Institute*, 1387–1396. https://doi.org/10.32592/ari.2023.78.4.1387

Sharma, N., Sing, N., & Bhadwal, M. (2011). Relationship of Somatic Cell Count and Mastitis: An Overview. *Asian-Aust. J. Anim. Sci.*, *Vol. 24*, *No. 3*, 429–438.

Shekhar, C. (2024). One Health approach to sustainable dairy production, dairy food safety and security. *The Microbiology, Pathogenesis and Zoonosis of Milk Borne Diseases: Milk Hygiene in Veterinary and Public Health*, 421–441. https://doi.org/10.1016/B978-0-443-13805-8.00008-9

Sigudu, T. T., Oguttu, J. W., & Qekwana, D. N. (2024). Antimicrobial Resistance of Staphylococcus spp. from Human Specimens Submitted to Diagnostic Laboratories in South Africa, 2012–2017. *Microorganisms*, *12*(9), 1862. https://doi.org/10.3390/microorganisms12091862

Silva, A. T. F., Gonçalves, J. L., Dantas, S. T. A., Rall, V. L. M., de Oliveira, P. R. F., dos Santos, M. V., Peixoto, R. de M., & Mota, R. A. (2023). Genetic and Phenotypic Characterization of Subclinical Mastitis-Causing Multidrug-Resistant Staphylococcus aureus. *Antibiotics*, *12*(9). https://doi.org/10.3390/antibiotics12091353

Sperber, W. H., & Tatini, S. R. (1975). Interpretation of the Tube Coagulase Test for Identification of Staphylococcus aureus. *Applied Microbiology*, *29*(4), 502–505.

Stanek, P., Żółkiewski, P., & Januś, E. (2024). A Review on Mastitis in Dairy Cows Research: Current Status and Future Perspectives. In *Agriculture (Switzerland)* (Vol. 14, Issue 8). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/agriculture14081292

Suárez, C., & Gudiol, F. (2009). Beta-lactam antibiotics. *Enfermedades Infecciosas y Microbiologia Clinica*, *27*(2), 116–129. https://doi.org/10.1016/j.eimc.2008.12.001

Supré, K., De Vliegher, S., Cleenwerck, I., Engelbeen, K., Van Trappen, S., Piepers, S., Sampimon, O. C., Zadoks, R. N., De Vos, P., & Haesebrouck, F. (2010). Staphylococcus devriesei sp. nov., isolated from teat apices and milk of dairy cows. *International*

Journal of Systematic and Evolutionary Microbiology, *60*(12), 2739–2744. https://doi.org/10.1099/ijs.0.015982-0

Swan, A. (1954). The use of a bile-esculin medium and of maxted's technique of lancefield grouping in the identification of Enterococci (Group D Streptococci). In *J. clin. Path* (Vol. 7).

Torres, C., & Cercenado, E. (2010). Interpretive reading of the antibiogram in gram positive COCCI. *Enfermedades Infecciosas y Microbiologia Clinica*, *28*(8), 541–553. https://doi.org/10.1016/j.eimc.2010.02.003

Vestergaard, M., Frees, D., & Ingmer, H. (2019). Antibiotic Resistance and the MRSA Problem. *Microbiology Spectrum*, *7*(2). https://doi.org/10.1128/microbiolspec.gpp3-0057-2018

Vidlund, J., Gelalcha, B. D., Gillespie, B. E., Agga, G. E., Schneider, L., Swanson, S. M., Frady, K. D., & Kerro Dego, O. (2024). Efficacy of novel staphylococcal surface associated protein vaccines against Staphylococcus aureus and non-aureus staphylococcal mastitis in dairy cows. *Vaccine*, *42*(6). https://doi.org/10.1016/j.vaccine.2024.01.067

Vrieling, M., Koymans, K. J., Heesterbeek, D. A. C., Aerts, P. C., Rutten, V. P. M. G., De Haas, C. J. C., Van Kessel, K. P. M., Koets, A. P., Nijland, R., Van Strijp, J. A. G., & Projan, S. J. (2015). *Bovine Staphylococcus aureus Secretes the Leukocidin LukMF= To Kill Migrating Neutrophils through CCR1*. https://doi.org/10.1128/mBio.00335-15

Wald, R., Hess, C., Urbantke, V., Wittek, T., & Baumgartner, M. (2019). Characterization of staphylococcus species isolated from bovine quarter milk samples. *Animals*, *9*(5). https://doi.org/10.3390/ani9050200

WHO. (2024). *WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance.* . CC BY-NC-SA 3.0 IGO.

Wilson, D. N. (2014). Ribosome-targeting antibiotics and mechanisms of bacterial resistance. In *Nature Reviews Microbiology* (Vol. 12, Issue 1, pp. 35–48). https://doi.org/10.1038/nrmicro3155

Yalcin, S., Ozgen, A., & Simsir, M. (2024). Molecular characteristics and antimicrobial susceptibility profiles of bovine mastitis agents in western Türkiye. *Journal of Veterinary Science*, *25*(5). https://doi.org/10.4142/jvs.24032

Yang, F., Zhang, S., Shang, X., Wang, X., Yan, Z., Li, H., & Li, J. (2019). Short communication: Antimicrobial resistance and virulence genes of Enterococcus faecalis isolated from subclinical bovine mastitis cases in China. *Journal of Dairy Science*, *102*(1), 140–144. https://doi.org/10.3168/jds.2018-14576

Zhang, J., Li, W., Tang, Y., Liu, X., Zhang, H., Zhou, Y., Wang, Y., Xiao, W., & Ying, Y. (2022). Testing Two Somatic Cell Count Cutoff Values for Bovine Subclinical Mastitis Detection Based on Milk Microbiota and Peripheral Blood Leukocyte Transcriptome Profile. *Animals*, *12*(13). https://doi.org/10.3390/ani12131694

Zhang, Y., Lu, J., Yan, Y., Liu, J., & Wang, M. (2021). Antibiotic residues in cattle and sheep meat and human exposure assessment in southern Xinjiang, China. *Food Science and Nutrition*, *9*(11), 6152–6161. https://doi.org/10.1002/fsn3.2568

INDEX OF APPENDIX

SUPPLEMENTARY MATERIAL

Appendix A *Table of results of strain recovery from cryovials stored at -80°C*

	BACTERIAL STRAIN BANK SALINAS								BACTERIAL STRAIN BANK IASA / MANABI						
n°	Criovial	Farm	Code	TSB 24h	Gram	AN 24h	Selected	n°	Criovial	Farm	Code	TSB 24h	Gram	AN 24h	Selected
1	glycerol	SalinasG	10sarf rosa	growth	BG+/CG+	CG+	**this study**	55	bead	IASA	1	growth	BG+		
2	glycerol	SalinasG	9 sarf	ngrowth				56	bead	IASA	2	growth	CG+	CG+	**this study**
3	glycerol	SalinasG	7 sarf	growth	BG+/CG+	BG+		57	bead	IASA	3	growth	CG+	CG+	**this study**
4	glycerol	SalinasG	14 sarf (2)	massive				58	bead	IASA	4	growth	CG+	CG+	**this study**
5	glycerol	SalinasG	14 sarf (1)	massive				59	bead	IASA	5	growth	CG+/CB		
6	glycerol	SalinasG	17sarf	massive				60	bead	IASA	6	growth	CG+/CB		
7	glycerol	SalinasG	20sarf	massive				61	bead	IASA	7	growth	BG+	CG+	**this study**
8	glycerol	SalinasG	22sarf	massive				62	bead	IASA	8	growth	CG+/CB		
9	glycerol	SalinasG	28sarf	massive				63	bead	IASA	9	growth	CG+/CB		
10	glycerol	SalinasG	32sarf	massive				64	bead	IASA	10	growth	CG+/CB		
11	glycerol	SalinasG	34sarf	growth	CG+	CG+	**this study**	65	bead	IASA	11	growth	CG+/BG+		
12	glycerol	SalinasG	29sarf	growth	BG+/CG+			66	bead	IASA	12	growth	CG+	CG+	**this study**
13	glycerol	SalinasG	29sarf (2)	growth	BG+			67	bead	IASA	13	growth	CG+/CB	CG+	**this study**
14	glycerol	SalinasG	49sarf	growth	BG+			68	bead	IASA	14	growth	CG+/CB	CG+	**this study**
15	glycerol	SalinasG	10sarf	massive	BG+/BG-			69	bead	Manabi	15 (3M)	growth	CG+	CG+	**this study**
16	glycerol	SalinasG	9sarf (1)	massive	BG+/CG+			70	bead	IASA	16	growth	CG+	CG+	**this study**
17	glycerol	SalinasG	9sarf (2)	massive	BG+			71	bead	IASA	17	growth	CG+/CB	CG+	**this study**
18	glycerol	SalinasG	10sarf (8)	massive	BG+			72	bead	Manabi	18(1M)	growth	CG+	CG+	**this study**
19	glycerol	SalinasG	16sarf	growth	BG+			73	bead	Manabi	19 (2M)	growth	CG+	CG+	**this study**
20	glycerol	SalinasG	26sarf	growth	BG+/CG+	CG+	**this study**								
21	glycerol	SalinasG	10STC	ngrowth											
22	glycerol	SalinasG	19STC	growth	BG+										
23	glycerol	SalinasG	29STC	ngrowth											
24	glycerol	SalinasG	4STC	growth	BG+/CG+	CG+	**this study**								
25	glycerol	SalinasG	9STC	growth	BG+										
26	glycerol	SalinasG	10STC*	growth	BG+										
27	glycerol	SalinasG	10STC2	growth	BG+										
28	glycerol	SalinasG	18STC2	growth	CG+	CG+	**this study**								
29	glycerol	SalinasG	34STC	growth	CG+	CG+	**this study**								
30	glycerol	SalinasG	13AS	ngrowth											
31	glycerol	SalinasG	26AS	massive	BG+										
32	glycerol	SalinasG	28AS	growth	BG+/BG-										
33	glycerol	SalinasG	32AS	massive	BG+										
34	glycerol	SalinasG	12STC	massive	BG+										
35	glycerol	SalinasG	1STC1	ngrowth											
36	glycerol	SalinasG	1STC2	massive	BG+										
37	glycerol	SalinasG	1STC3	massive	BG+										
38	glycerol	SalinasG	181STC3	growth	BG+/CG+	CG+	**this study**								
39	glycerol	SalinasG	6AS1	massive											
40	glycerol	SalinasG	7AS1	ngrowth											
41	glycerol	SalinasG	9AS1	growth	CG+	CG+	**this study**								
42	glycerol	SalinasG	17AS1	growth	BG+										
43	glycerol	SalinasG	6AS2	massive	BG+										
44	glycerol	SalinasG	17AS2	growth	BG+										
45	glycerol	SalinasG	7AS3	growth	BG-										
46	glycerol	SalinasG	17AS3	growth	BG+										
47	glycerol	SalinasG	4EMB	growth	CG+	CG+	**this study**								
48	glycerol	SalinasG	38EMB	ngrowth											
49	glycerol	SalinasG	17 8+H EMB	growth	BG+										
50	glycerol	SalinasG	7sarf rosa	growth	BG+										
51	glycerol	SalinasG	7sarf *	growth	BG+										
52	glycerol	SalinasG	7sarf (8)	massive	BG+										
53	glycerol	SalinasG	7AS2	growth	BG+										
54	glycerol	SalinasG	9AS3	massive	BG+/BG-										

Appendix B *Table with halo measurement values in antibiograms and interpretation of concentrations for MIC determination.*

		ANTIBIOGRAM HALO (mm)								MIC BREACKPOINTS (µg/mL)																				
MICROORGANISM		AMP10	P10	FOX30	VA30	E15	TE30	LZD10	F/M300	AMP	P	FOXS	VA	TET	LZD	NITRO	E	CLI	DTEST	TGC	CHL	OXA+	LEVO	GEN	RIF	SYN	CIP	SXT	STR	MXF	DAP
S.aureus	2I		13*	28	13	22	20	14	20	1	0,5	<=6	0,5	<=2	4	<=32	0,5	<=0,5	-	0,12	16	0,5	<=0,25	<=2	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S.aureus	3I		12*	25	16	24	20	16	18	1	1	<=6	1	<=2	2	<=32	1	<=0,5	-	0,12	16	1	1	<=2	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S. devriesei	4I		29	30	36	22	23	23	20	<=0,12	<0,06	<=6	0,5	<=2	2	<=32	<=0,25	<=0,5	-	0,25	8	<=0,25	2	<=2	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S. chromogenes	7I		39	30	29	29	25	25	22																						
S.aureus	12I		11*	21	15	18	11	6	6	>8	>8	>6	>32	>16	>8	>64	>4	>2	++	>0,5	>16	>4	1	<=2	4	>4	<=1	>4/76		4	>4
S.aureus	13I		13*	24	15	21	19	23	20	2	2	<=6	1	<=2	2	<=32	0,5	<=0,5	-	0,12	8	0,5	<=0,25	<=2	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S.aureus	14I		29	25	12	22	19	22	17																						
S.aureus	15I		29	23	14	20	17	21	17	<=0,12	0,12	<=6	1	<=2	2	<=32	0,5	<=0,5	-	0,12	8	0,5	<=0,25	<=2	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S.aureus	16I		11*	25	14	20	17	25	17	2	2	<=6	1	<=2	<=1	<=32	0,5	<=0,5	-	0,12	8	<=0,25	<=0,25	<=2	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S.aureus	17I		12*	25	15	24	20	14	17	4	2	<=6	1	<=2	4	<=32	<=0,25	<=0,5	-	0,25	4	<=0,25	<=0,25	<=2	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S.aureus	18I		26	21	15	19	19	12	15	0,25	0,12	<=6	2	<=2	8	<=32	1	<=0,5	-	0,25	16	1	2	8	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S.aureus	19I		25	29	19	25	22	15	21	0,5	0,12	<=6	1	<=2	4	<=32	<=0,25	<=0,5	-	0,25	8	<=0,25	<=0,25	<=2	<=0,5	1	<=1	<0,5/9,5		1	<=0,5
S. chromogenes	1G		10*	27	15	22	19	20	17	<=0,12	0,25	<=6	1	<=2	2	<=32	<=0,25	<=0,5	-	0,12	4	0,5	<=0,25	4	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5
S. chromogenes	11G		12*	25	16	26	12	14	18	<=0,12	0,25	<=6	1	4	4	<=32	<=0,25	<=0,5	-	0,12	8	0,5	1	<=2	<=0,5	>4	<=1	>4/76		<=0,25	1
E. faecium	20G	23	14		14	20	25	22	11	1	2		1	<=2	2	>64	1		-		8		0,5		<=0,5	<=0,5	<=1	<=1000			
E. faecalis	24G	27	16		13	21	7	21	23	0,5	2		2	>16	2	<=32	1		-	0,12	16		2		>4	>4	2	<=1000			<=0,5
E. faecium	28G	21	30		19	28	30	27	26																						
S.aureus	29G		12*	31	18	22	9	26	19	0,5	1	<=6	0,5	>16	<=1	<=32	0,5	<=0,5	-	0,12	8	<=0,25	>4	<=2	<=0,5	<=0,5	>2	<0,5/9,5	>1000	>4	1
E. faecium	38G	32	29		18	30	31	26	27																						
E. faecalis	41G	32	16		14	23	6	20	28	0,5	2		1	>16	2	<=32	0,5		-	0,25	8		2		>4	>4	2	<=1000			<=0,5
E. faecalis	47G	29	14		17	22	6	25	22	1	1		1	>16	<=1	<=32	0,5		-	0,12	8		2		2	>4	<=1	<=1000			1
S. aureus ATCC 25923			28	27	17	23	24	26	18	0,25	0,12	<=6	1	<=2	4	<=32	0,5	<=0,5	-	0,25	8	0,5	0,5	4	<=0,5	<=0,5	<=1	<0,5/9,5		<=0,25	<=0,5

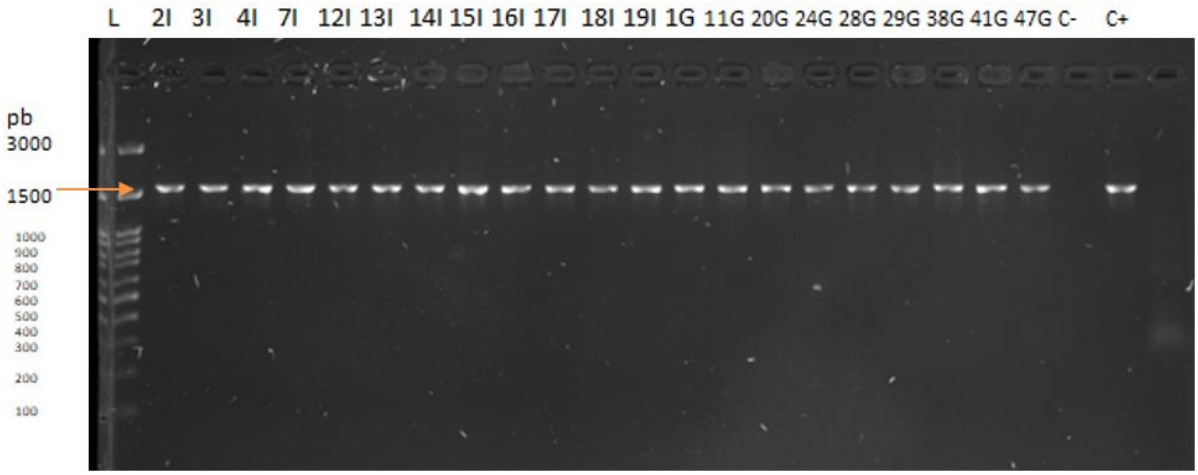
Note. (*) Positive Penicillin Disk Zone-Edge Test for β-Lactamase Detection. GEN500 and STR1000 have no interpretive criteria to *Staphylococcus* species. Daptomycin criteria is only for *E. faecalis.* The values found in the gray cells show a phenotypic resistance or intermedial profile in disk diffusion assays. Blank cells have no interpretation criteria.

Appendix C Table with DNA concentration values and 260/280 ratio values of *Staphylococcus* sp. and *Enterococcus* sp. samples with the Multiskan reader.

Code	Microorganism	DNA (ug/mL)	260/280	260/230
2I	*S.aureus*	260,4	1,997	1,781
3I	*S.aureus*	322,3	1,932	2,059
4I	*S. devriesei*	226,9	1,906	1,551
7I	*S. chromogenes*	61,2	2,083	
12I	*S.aureus*	472	1,832	2,426
13I	*S.aureus*	319,9	1,913	2,648
14I	*S.aureus*	187,1	1,964	2,83
15I	*S.aureus*	85,31	1,891	3,88
16I	*S.aureus*	247,8	1,884	2,03
17I	*S.aureus*	26,4	2,398	
18I	*S.aureus*	106,1	2,149	
19I	*S.aureus*	17,6	2,423	
1G	*S. chromogenes*	309	2,065	
11G	*S. chromogenes*	118,6	1,818	
20G	*E. faecium*	125,7	2,113	
24G	*E. faecalis*	483,5	1,907	2,22
28G	*E. faecium*	12,9	2,251	
29G	*S.aureus*	602,6	2,146	
38G	*E. faecium*	300,8	1,865	2,687
41G	*E. faecalis*	45,4	2,014	1,789
47G	*E. faecalis*	6,8	1,634	

Note. The values of 260/230 of the equipment are not reported due to equipment failures in the reading of the plate.

Appendix D Amplification of the ribosomal 16S *rRNA gene* of approximately 1500 bp using universal primers 27 and 1492R.



Note. A 3000 base pair molecular weight marker was used, and the 1.5% electrophoresis gel was stained with SYBR Safe DNA. The electrophoresis conditions were one hour at 120 Volts. The arrow indicates the approximate 1500 base pair fragment for the *16S rRNA gene* amplicon of all samples. A master mix tube with water was used as a negative control (lane C-) and as a positive control (lane C+) a *S. aureus* sample previously characterized in other studies.