UNIVERSITI TEKNOLOGI MARA

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF ANTIBIOTIC RESISTANCE AND BIOFILM FORMATION IN *Staphylococcaceae* FAMILY ISOLATED FROM BOVINE MILK ASSOCIATED WITH SUBCLINICAL MASTITIS

INTAN NURFARZANA BINTI MOHD SAFINI

MSc

September 2026

UNIVERSITI TEKNOLOGI MARA

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF ANTIBIOTIC RESISTANCE AND BIOFILM FORMATION IN Staphylococcaceae FAMILY ISOLATED FROM BOVINE MILK ASSOCIATED WITH SUBCLINICAL MASTITIS

INTAN NURFARZANA BINTI MOHD SAFINI

Thesis submitted in fulfilment
of the requirements for the degree of
Master of Science
(Molecular Biology)

Faculty of Applied Sciences

September 2026

CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on 9 July 2026 to conduct the final examination of Intan Nurfarzana binti Mohd Safini on her Master of Science thesis entitled "Phenotypic and Genotypic Characterization of Antibiotic Resistance and Biofilm Formation in Staphylococcaceae family Isolated from Bovine Milk Associated with Subclinical Mastitis" in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiners recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

Siti Khairiyah Mohd Hatta, PhD
Associate Professor
Faculty of Applied Sciences
Universiti Teknologi MARA
(Chairman)

Ida Muryany Md Yasin, PhD
Associate Professor
Faculty of Applied Sciences
Universiti Teknologi MARA
(Internal Examiner)

Sharifah Norkhadijah Syed Ismail, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(External Examiner)

PROF DR HJH ZURAEDA IBRAHIM
Dean
Institute of Postgraduate Studies
Universiti Teknologi MARA

Date: 15 September 2026

AUTHOR'S DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Teknologi MARA. It is original and is the results of my own work, unless otherwise indicated or acknowledged as referenced work. This thesis has not been submitted to any other academic institution or non-academic institution for any degree or qualification.

I, hereby, acknowledge that I have been supplied with the Academic Rules and Regulations for Postgraduate, Universiti Teknologi MARA, regulating the conduct of my study and research.

Name of Student	:	Intan Nurfarzana binti Mohd Safini
Student ID. No.	:	2023355151
Programme	:	Master of Science (Molecular Biology) – AS753
Faculty	:	Applied Sciences
Thesis Title	:	Phenotypic and Genotypic Characterization of Antibiotic Resistance and Biofilm Formation in Staphylococcaceae Family Isolated from Bovine Milk Associated with Subclinical Mastitis
Signature of Student	:	…….....
Date	:	September 2026

ABSTRACT

Bovine mastitis caused by members of Staphylococcaceae family poses a threat to global dairy production due to antibiotic resistance and biofilm barriers, which reduce the efficacy of antibiotic therapy. This study aimed to isolate and identify members of the Staphylococcaceae family from bovine milk associated with mastitis and to determine their antibiotic resistance profiles and biofilm-forming activity. Milk samples from 70 udder quarters were collected from a dairy herd in Alor Gajah, Malacca. Initial screening utilizing Mannitol Salt Agar (MSA) and standard biochemical tests yielded 153 presumptive staphylococcal isolates, of which 78 antibiotic resistant isolates were selected for further characterization. Molecular identification and phylogenetic reconstruction were performed targeting the *sodA* gene. Antibiotic susceptibility against six antibiotics was assessed via the disk diffusion assay followed by genotypic screening of resistance genes and microtiter plate biofilm assay. Molecular analysis confirmed all 78 isolates as members of the Staphylococcaceae family comprising *Staphylococcus* spp. (n = 35), *Mammaliicoccus sciuri* (n = 5), *Macrococcus bohemicum* (n = 13) and *Macrococcus caseolyticus* (n = 25). Phenotypically, 100% (n = 78) of the isolates were resistant to penicillin, while 22% (n = 17) and 12% (n = 9) exhibited resistance to cefoxitin and tetracycline, respectively. Conversely, the isolates exhibited low resistance to erythromycin, gentamicin and sulfamethoxazole-trimethoprim. Notably, two *S. chromogenes* strains (AP622002 and BP647054) exhibited multidrug-resistance (MDR) profiles. Genotypically, the *blaZ* gene was identified in 64.1% of penicillin-resistant isolates, while 5.9% and 88.2% of cefoxitin-resistant isolates harboured *mec* resistance genes of *mecA* or *mecD*, respectively. For tetracycline, 88.9% of resistant isolates carried *tetK*, while 100% also carried *tetM*. The *dfrA* gene was detected in 33.3% of sulfamethoxazole-resistant isolates and the *aacA-aphD* gene was present in the single gentamicin-resistant isolate. Biofilm assays revealed that 38.5% of the isolates were biofilm formers, with solely *S. chromogenes* isolate (AP622005) exhibiting a strong biofilm formation activity. Furthermore, a significant positive correlation (p = 0.001) was observed between biofilm formation and antibiotic resistance activity. These findings provide a comprehensive phenotypic and genotypic characterization of bovine mastitis associated Staphylococcaceae family, offering novel insights into underreported pathogens, particularly *Macrococcus* species. Collectively, the identification of MDR profiles and presence of resistance genes alongside biofilm forming phenotypes in isolates associated with bovine mastitis underscore the risk of environmental dissemination. These insights highlight an urgent need for targeted antibiotic stewardship and implementation of alternative mastitis control strategies aligned with a One Health approach.

ACKNOWLEDGEMENT

Alhamdulillah, I am thankful to Allah SWT for His magnificence and for providing me with the strength and fortitude to complete this master's journey.

First and foremost, I would like to thank to my supervisors, Dr Norashirene Mohamad Jamil, Assoc. Prof. Dr Aziyah Abdul Aziz and Ms. Sophia Karen Bakon. Thank you for your endless guidance, ideas and advice throughout this journey with full inspiration. I would also like to extend my gratitude to Dr. Zuraifah Asrah Mohamand, Dr. Siti Khairiyah Mohd Hatta, Dr. Asyura Amdan, Dr. Nur Nadiah Md. Yusof, Madam Rafidah Rasol and Dr. Umi Marshida Abd Hamid. This milestone would not have been possible without the tremendous support and kindness showered by the people around me.

My sincere appreciation is also dedicated to my fellow MSc buddies, Iqbal Hafiz, Airin, Aqilah, Hidayah and Hassanah. And not forgetting my best friends, Hamizah, Ayu, Aliff, Najwa, Insyirah, Fatihah, Faiezatul, Maisarah, Qistina, Wana, Tasha and Sofea. To all my other dear friends who provided continuous moral support, uplifting words and prayers and encouragement whenever I needed it most, your kindness means more than words can express. I am equally grateful to post-graduate and undergraduate students who have lend me a helping hand in a way or another throughout my study, thank you so much. A debt of gratitude is also owed to Mrs. Zaiemah Sangit for her continuous assistance throughout the laboratory work.

Finally, my deepest and most heartfelt gratitude goes to my beloved family. I am profoundly grateful to my siblings, Faizah, Farahin, Fatihah, Irfan, Fadhli, Farisah and Fadhilah, for their unwavering love and encouragement. Their presence has been a pillar of strength throughout this endeavour. I would also like to extend my sincere thanks to my relatives for their prayers and moral support during my studies. Above all, I owe my greatest gratitude to my beloved parents, Mohd Safini and [illegible], for their unconditional love, endless prayers, patience and sacrifices. I also wish to dedicate this milestone to my late grandmother, whose support for my MSc's pursuit meant the world to me; this milestone is deeply cherished as a gift in her loving memory. This achievement would not have been possible without their unwavering faith in my potential and their continuous blessings.

TABLE OF CONTENTS

		Page
CONFIRMATION BY PANEL OF EXAMINERS		**ii**
AUTHOR'S DECLARATION		**iii**
ABSTRACT		**iv**
ACKNOWLEDGEMENT		**v**
TABLE OF CONTENTS		**vi**
LIST OF TABLES		**ix**
LIST OF FIGURES		**xi**
LIST OF SYMBOLS		**xiv**
LIST OF ABBREVIATIONS		**xv**
LIST OF NOMENCLATURES		**xviii**
CHAPTER 1	**INTRODUCTION**	**1**
1.1	Research Background	1
1.2	Motivation for This Work	3
1.3	Problem Statement	4
1.4	Significance of Study	5
1.5	Research Questions	6
1.6	Research Objectives	7
1.7	Thesis Scope and Limitation	7
CHAPTER 2	**LITERATURE REVIEW**	**9**
2.1	Bovine Mastitis	9
2.1.1	Clinical Mastitis	9
2.1.2	Subclinical Mastitis	11
2.2	Pathogenesis of Bovine Mastitis	12
2.3	Risk Factors of Mastitis	14
2.3.1	Pathogen Factors	14
2.3.2	Host Factors	15
2.3.3	Environmental Factors	16
2.4	The Staphylococcaceae Family in Bovine Mastitis	17
2.4.1	*Staphylococcus* spp.	17

2.4.2 *Macrococcus* spp. 20
2.4.3 Molecular Identification of Staphylococcaceae 22
2.5 Prevalence of Staphylococcaceae in Bovine Mastitis 23
2.6 Antibiotics in Treating Staphylococcaceae Family Associated with Bovine Mastitis 24
2.6.1 Mechanism of Action of Antibiotics 27
2.7 Mechanisms and Dissemination of Antibiotic Resistance in Staphylococcaceae Family Associated with Bovine Mastitis 29
2.8 Biofilm Associated with Bovine Mastitis 37

CHAPTER 3 RESEARCH METHODOLOGY 40

3.1 Materials 40
3.1.1 Bacterial Cultures and Conditions 40
3.1.2 Buffer and Solution 40
3.1.3 Chemicals 40
3.1.4 Reagents 41
3.1.5 Molecular Kit 41
3.1.6 Media 41
3.1.7 Primers 41
3.2 Methods 42
3.2.1 Study Area and Design 42
3.2.2 Sample Size Calculation of Staphylococcaceae Isolation 43
3.2.3 Sample Collection 44
3.2.4 California Mastitis Test 44
3.2.5 Isolation of *Staphylococcus* spp. from Milk Samples 46
3.2.6 Preparation of 20% Luria-Bertani Broth-Glycerol 46
3.2.7 Standard Biochemical Tests 46
3.2.8 Genomic DNA Extraction 48
3.2.9 Species Identification 49
3.2.10 Antibiotic Susceptibility Test 50
3.2.11 Interpretation of Resistance Measurement for *Staphylococcus* 50
3.2.12 Amplification of Antibiotic Resistance Genes 51
3.2.13 Biofilm Formation Assay 52
3.2.14 Phylogenetic Analysis 53
3.2.15 Data Analysis 54

CHAPTER 4 RESULTS AND DISCUSSION **55**

4.1 Detection of Bovine Milk Associated with Subclinical Mastitis 55
4.2 Isolation of *Staphylococcus* on Mannitol Salt Agar 59
4.3 Correlation of CMT Status and Number of Recovered Isolates 62
4.4 Biochemical Characterisation of *Staphylococcus* 64
4.5 Molecular Confirmation via *sodA* Gene 66
4.6 Sequence Analysis and Species Identification using BLASTn 67
4.6.1 Identification of *Staphylococcus* spp. 68
4.6.2 Identification of Other Staphylococcaceae Family 70
4.7 Phylogenetic Tree Analysis 71
4.8 Antibiotic Sensitivity Profile of Staphylococcaceae 74
4.8.1 Multidrug Resistance Profiles 84
4.9 Genotypic Characterization of Antibiotic Resistance 85
4.9.1 Relationship between Phenotypic and Genotypic Resistance in Staphylococcaceae 85
4.9.2 Screening of *blaZ* Gene That Confers Resistance to Penicillin 87
4.9.3 Screening of *mec* Genes That Confer Resistance to Cefoxitin 89
4.9.4 Screening of *aacA-aphD* Gene That Confers Resistance to Gentamicin 92
4.9.5 Screening of *tet* Genes That Confer Resistance to Tetracycline 94
4.9.6 Screening of *dfrA* Gene That Confers Resistance to Sulfamethoxazole-Trimethoprim 97
4.10 Biofilm Formation Activity of Staphylococcaceae Isolates Associated with Mastitis 99
4.10.1 Correlation between Antibiotic Resistance and Biofilm Formation 106

CHAPTER 5 CONCLUSION **110**

REFERENCES **113**

APPENDICES **151**

AUTHOR'S PROFILE **178**

LIST OF TABLES

Tables	Title	Page
Table 2.1	Prevalence of SCM Associated with *Staphylococcus* in Asia	25
Table 2.2	Standard Therapeutic Dosages, Treatment Durations and Administration Pathways for Target Antibiotic Classes in Bovines	26
Table 2.3	Antibiotic Resistance Genes, Mobile Genetic Elements and Mechanism of Actions in *Staphylococcus* spp.	31
Table 3.1	Primers for Amplification of Antibiotic Resistance Genes of *Staphylococcus* spp.	42
Table 3.2	Interpretation of California Mastitis Test (CMT) Scores Corresponding to the Reaction Observed	45
Table 3.3	Classification of Antibiotics and Interpretation of Resistance Measurement	51
Table 3.4	Primer Sequences, Target Genes, Amplicon Sizes and Cycling Conditions	52
Table 4.1	Quarter-Level CMT Score for Milk Samples Obtained from 20 Examined Lactating Bovines from a Dairy Farm in Malacca, Malaysia	56
Table 4.2	Distribution of CMT Scores Across All Milk-Yielding Quarters	58
Table 4.3	Summary of Biochemical Characteristics for Presumptive Staphylococci Isolates (n = 153)	64
Table 4.4	The Resistance Profile of Staphylococcaceae Isolates Associated with Bovine Subclinical Mastitis Against Six Antibiotics Tested	75

Table 4.5 Distribution of Phenotypic Resistance and Concordance with Resistance Genes Across Staphylococcaceae Species 86

Table 4.6 Distribution of Biofilm-Forming Strains of Staphylococcaceae (n = 78) Isolated from Bovine Milk Associated with SCM 100

Table 4.7 Biofilm-Forming Capabilities of Staphylococcaceae Isolates (n = 78) 101

Table 4.8 Association Between Antibiotic Resistance Profiles and Biofilm Formation Activity in Staphylococcaceae Isolates (n = 78) 108

LIST OF FIGURES

Figures	Title	Page
Figure 2.1	Bovine Udder is Divided into Four Quarters. Teats with Label 1 and 3 Indicate Healthy Teats. Teats with Label 2 and 4 Indicate Unhealthy Teats That Show Inflammation of Swollen and Red Teats	10
Figure 2.2	The Anatomy of the Bovine Teat	12
Figure 2.3	Biofilm Formation of *S. aureus* inside the Mammary Gland of a Bovine. (A) Entry of *S. aureus* into the Teat Orifice (B) Surface Proteins That Are Involved in the Adhesion of *S. aureus* to Epithelial Cell of Mammary Gland (C) *S. aureus* Invades the Host Epithelial Cell by Zipper Mechanisms and Remodelling of the Actin Cytoskeleton (D) Formation of Biofilm by Planktonic *S. aureus* Involving Attachment, Microcolonies Formation, Maturation and Dispersal	37
Figure 3.1	The Studied Dairy Farm Facility in Alor Gajah, Malacca. (A) Overview of Livestock Housing and Husbandry Infrastructure (B) Configuration of the Automated Mechanical Milking Parlour	43
Figure 4.1	Colony Morphologies of *Staphylococcus* spp. on MSA. (A) *S. aureus* ATCC 25923 (CoPS Control Strain) (B) *S. epidermidis* ATCC 12228 (CoNS Control Strain) (C) Representative of Presumptive CoPS Isolates (AY622018) (D) Representative of Presumptive CoNS Isolates (BP647054)	60
Figure 4.2	Distribution of Total Bacterial Colonies Recovered from Each Bovine by CMT Result	64
Figure 4.3	PCR Amplification of *sodA* Gene (480 bp) in Representative Presumptive Staphylococci Isolates. M: 100bp DNA Ladder	

(Vivantis), Lane 1: *S. haemolyticus* ATCC 29970, Lane 2 to 14: Representative of Tested Isolates (AY625098, AP625042, AP625045, AP625048, AP620080, AP620081, AP620082, BY760095, AP620079, AP620083, AY625084, AP620088, AP620089, Respectively); Lane 15: Negative Control 67

Figure 4.4 Neighbour-Joining Tree Inferred from Partial *sodA* Gene Sequences of 78 Isolates 73

Figure 4.5 Heatmap Visualization of Antibiotic Susceptibility Profiles in *Staphylococcus* spp. (n = 35) and *M. sciuri* (n = 5) Isolates Against Six Antibiotics 76

Figure 4.6 Heatmap Visualization of Antibiotic Susceptibility Profiles in *Macrococcus* spp. (n = 38) Isolates Against Six Antibiotics 77

Figure 4.7 PCR Amplification of *blaZ* Gene (173 bp) in Representative Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (positive control); Lane 2 to 14: Tested Isolates (AP622005, AP622007, AP622010, AP622016, AP622023, AP622027, AP622017, AP622034, AP610124, AP610126, AP610129, AP610132 and AP610133, Respectively); Lane 15: Negative Control 87

Figure 4.8 PCR Amplification of *mecA* Gene (147 bp) in *S. chromogenes* and *M. sciuri* Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2: *M. sciuri* Isolate AY764127; Lane 3: *S. chromogenes* Isolate AP610142; Lane 4: Negative Control 89

Figure 4.9 PCR Amplification of *mecD* Gene (867 bp) in Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2 to 14: Tested Isolates (AP625045, AP625046, AP625048, AP620077, AP620078, AP620079, AP620080, AP620081, AP620082, AP620083, AP620084, AP620085 and AP620087, Respectively); Lane 15: Negative Control 90

Figure 4.10 PCR Amplification of *aacA-aphD* Gene (227 bp) in a Resistant *M. caseolyticus* Isolate; Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *Enterococcus faecium* strain S98b (Positive Control); Lane 2: Tested Isolate (AP625042) and Lane 3: Negative Control 93

Figure 4.11 PCR Amplification of *tetK* Gene (360 bp) in Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *Enterococcus faecalis* strain E533 (Positive Control); Lane 2 to 10: Tested Isolates (AP622002, AP622010, AP622016, AP622027, BP647054, AP622005, BY760095, AP622023 and BY760105, Respectively); Lane 11: Negative Control 94

Figure 4.12 PCR Amplification of *tetM* Gene (158 bp) in Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2 to 10: Tested Isolates (AP622002, AP622010, AP622016, AP622027, BP647054, AP622005, BY760095, AP622023 and BY760105, Respectively); Lane 11: Negative Control 95

Figure 4.13 PCR Amplification of *dfrA* Gene (270 bp) in Resistant *S. chromogenes* Isolates. Lane M: 100 bp DNA Ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2 to 4: Tested Isolates (AY574001, AP622002 and BP647054, Respectively); Lane 5: Negative Control 99

LIST OF SYMBOLS

Symbols

bp	Base pair
°C	Celsius
CFU/mL	Colony Forming Units per Millilitres
ODc	Cut-off Optical Density
kb	Kilobase
µg	Microgram
µL	Microliters
µM	Micromolar
mg/mL	Milligrams per Millilitres
mL	Millilitres
m	Minute
M	Molarity

LIST OF ABBREVIATIONS

Abbreviations

AR	Antibiotic Resistance
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic Resistance Genes
AST	Antibiotic Susceptibility Testing
BLASTn	Basic Local Alignment Search Tool for nucleotides
BM	Bovine Mastitis
CLSI	Clinical and Laboratory Standards Institute
CM	Clinical Mastitis
CMT	California Mastitis Test
CN	Gentamicin
CoNS	Coagulase Negative Staphylococci
CoPS	Coagulase Positive Staphylococci
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
DNA	Deoxyribonucleic Acid
DVS	Department of Veterinary Services
E	Erythromycin
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediamine Tetra-Acetate
EFSA	European Food Safety Authority

EPS	Extracellular Polymeric Substance
FgBP	Fibrinogen-Binding Proteins
FnBP	Fibronectin-Binding Proteins
FOX	Cefoxitin
gDNA	Genomic Deoxyribonucleic Acid
HGT	Horizontal Gene Transfer
MDR	Multidrug Resistance
MGE	Mobile Genetic Element
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
MVAG	Malaysian Veterinary Antimicrobials Guidelines
NAS	Non-*aureus* Staphylococci
NCBI	National Center for Biotechnology Information
PABA	Para-Aminobenzoic Acid
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEN	Penicillin G
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid

SCC	Somatic Cell Count
SCM	Subclinical Mastitis
SEM	Standard Error Mean
SXT	Sulfamethoxazole-Trimethoprim
TAE	Tris-Acetate EDTA
TET	Tetracycline
tRNA	Transfer Ribonucleic Acid
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
VCIA	Veterinary Clinically Important Antibiotic
WOAH	World Organization for Animal Health

LIST OF NOMENCLATURES

Nomenclatures

H_2O_2	Hydrogen Peroxide
NaCl	Sodium Chloride

CHAPTER 1
INTRODUCTION

1.1 Research Background

Bovine mastitis (BM) is an intramammary infection (IMI) that imposes a significant economic burden on the global dairy industry including in Malaysia, through reduced milk yield and quality, as well as increased veterinary management costs (Cizmeci *et al.*, 2019; Cheng & Han, 2020; Stanek *et al.*, 2024). As noted by Ali *et al.* (2020), mastitis prevalence in Malaysian dairy herds is estimated at 52.6% (n = 309), highlighting the substantial local burden in dairy farming of this disease. In lactating bovines, mastitis can be divided into two categories based on clinical manifestation: clinical and subclinical mastitis. Clinical mastitis (CM) is characterized by visible symptoms and changes to the bovine's milk and its composition (Cobirka *et al.*, 2020). In contrast, subclinical mastitis (SCM) shows invisible symptoms, which makes it visually undetectable without further diagnostic testing (Haxhiaj *et al.*, 2022). Despite the absence of apparent udder inflammation, SCM can still transmit the aetiological agents from one infected bovine to another uninfected bovine (Haxhiaj *et al.*, 2022). Moreover, SCM is associated with an elevation of somatic cell counts (SCCs), reflecting underlying intramammary inflammation (Sumon *et al.*, 2020). Specifically regarding SCM, a recent local study reported that 31.4% (n = 74) of milk samples exceeded the SCC threshold of ≥ 200,000 cells per mL, indicating subclinical infection in the bovines (Saeed *et al.*, 2022). Thus, it is essential to regularly monitor the SCM in order to prevent the spread of infection within the herd and minimize the risk of zoonotic transmission (Gebremedhin *et al.*, 2022; Bedruddin, 2023).

To date, more than 140 types of aetiological agents have been documented in association with BM, including Gram-positive and -negative bacteria (Boukhalfa *et al.*, 2021). These bacteria are categorized as environmental or contagious pathogens, including staphylococci, streptococci, *Escherichia coli* and *Enterococcus* spp. (Cheng & Han, 2020; Ali *et al.*, 2021; Tomanić *et al.*, 2023). In Asia, *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) are the major pathogens associated with mastitis (Saeed *et al.*, 2022; Yang *et al.*, 2023). However, eradicating *Staphylococcus* spp. from infected herds remains challenging due to their virulence factors, resistance

in farm environments, ability to form biofilms and low response to current treatment (MacFadyen *et al.*, 2018; Boukhalfa *et al.*, 2021). Crucially, the list of potential mastitis pathogens has evolved to include *Macrococcus* spp. in veterinary infections (Carroll *et al.*, 2023). Members of this genus have been isolated from infections such as bovine mastitis, ovine abscesses and dermatitis (MacFadyen *et al.*, 2018). Despite its historical classification as a commensal organism, recent genomic studies indicate that *Macrococcus* shares virulence traits with *S. aureus* (Carroll *et al.*, 2023). Furthermore, the capacity of this genus for antibiotic resistance and biofilm formation allows these bacteria to evade host immune defences, thereby complicating control measures in dairy herds (Mnif *et al.*, 2020). Collectively, the emergence of these related genera underscores the growing threat of the Staphylococcaceae family in dairy environments.

Therapeutic intervention for BM predominantly relies on antibiotic classes such as β-lactams, tetracyclines, macrolides and aminoglycosides. These drugs serve as Veterinary Clinically Important Antibiotics (VCIAs) for the treatment of bacterial infections in animals (Rajamanickam *et al.*, 2020; Molineri *et al.*, 2021; Tomanić *et al.*, 2023; Książczyk *et al.*, 2026). The reliance of these antibiotics is globally recognised by the World Organisation for Animal Health (WOAH, 2025), which reported that tetracyclines and penicillins remain the predominant antibiotic classes intended for livestock use, accounting for 28% and 18% of total use, respectively. Reflecting these global practices locally, the Malaysian Veterinary Antimicrobials Guidelines (MVAG) outline several antibiotic classes for veterinary therapeutics, including penicillins, tetracyclines, aminoglycosides, cephalosporins, lincosamides, macrolides and sulfonamides. However, uncontrolled administration of antibiotics in animal husbandry has raised concerns due to development of antibiotic resistance (AR) that leads to the emergence of multidrug-resistant (MDR) bacteria (Kim & Ahn, 2022). Consequently, continuous and systematic monitoring of AR profiles is essential for an early detection of the MDR strains in BM to ensure effective mastitis management.

Current surveillance often relies solely on phenotypic testing, which observes bacterial susceptibility in the presence of antibiotics. For example, findings by Gebremedhin *et al.* (2022) and Saeed *et al.* (2022) revealed that 65% (n = 26) and 43.6% (n = 17) of *Staphylococcus* isolates were phenotypically resistant to multiple antibiotics (penicillin, ampicillin, amoxicillin, oxacillin, tetracycline, cefotaxime and erythromycin), respectively. However, relying solely on phenotypic profiles is insufficient, as it does not elucidate the underlying molecular mechanisms of resistance.

Genotypically, this resistance mechanism is driven by antibiotic-resistant genes (ARGs), which are likely acquired due to the dynamic genomic plasticity of *Staphylococcus* species (Silva *et al.*, 2020; Aslam *et al.*, 2021). Furthermore, these ARGs can spread across bacterial populations through horizontal gene transfer (HGT) facilitated by mobile genetic elements (MGEs). This mechanism amplifies the dissemination of resistant strains complicating treatment efforts even if the genes are phenotypically silent in the carrier bacteria. Thus, implementing genomic surveillance programs in animal husbandry integrated with phenotypic characterization is imperative to mitigate the risks posed by ARG dissemination.

The ability of *Staphylococcus* spp. to form biofilm poses additional challenges to mastitis control by enhancing bacterial survival within host cells (Saeed *et al.*, 2022). Biofilm formation occurs when bacterial clusters irreversibly attach to biotic or abiotic surfaces, creating a complex community of microorganisms embedded in a self-produced matrix enriched with complex extracellular polymeric substances (EPS) (Vestby *et al.*, 2020). In BM cases, biofilm formed on the udder hinders the treatment by providing a barrier to the pathogen, protecting it from the host immune defences and antibiotic treatments (Rychshanova *et al.*, 2022). This protective layer makes the infection more difficult to treat and contributes to its persistence. Consequently, monitoring and surveillance of antibiotic resistance (AR) and biofilm formation in *Staphylococcus* associated with mastitis are crucial for effective mastitis management, prudent antimicrobial administration and combating antibiotic resistance (Langhorne *et al.*, 2023).

1.2 Motivation for This Work

Bovine mastitis remains the most persistent and economically damaging disease in dairy industry, causing significant losses in milk production and quality. Globally and locally, standard herd management relies on routine cow-side diagnostic screening such as California Mastitis Test (CMT) to detect subclinical infection, alongside proper milking hygiene, environmental sanitation and dry-cow antibiotic therapy to detect, prevent and manage mastitis (Cizmeci *et al.*, 2019). Despite the implementation of these practices, the recurrence of SCM cases in local dairy herds suggests that current diagnostic and treatment strategies may be insufficient. This persistence serves as the primary motivation for this study, driving the need to investigate underlying factors

such as bacterial biofilms that may protect pathogens from standard antibiotic treatments.

Furthermore, the emergence of antibiotic resistance is an utmost concern for both veterinary and public health. The indiscriminate use of antibiotics in livestock can accelerate the emergence of multidrug-resistant bacteria, threatening food safety and consumer health. This research aims to determine the resistance profiles of Staphylococcaceae in the local setting, ensuring that data are available to guide better therapeutic decisions.

Lastly, distinguishing between *Staphylococcus* and closely related genera like *Macrococcus* using standard microbiological protocols is often challenging and frequently leads to misidentification. This limitation contributes to challenges for herd management, as misdiagnosis can lead to ineffective treatments. Therefore, this study is motivated by the need to employ molecular identification to clarify which bacterial species are associated with mastitis, ensuring that veterinarians and farmers can implement targeted therapeutic interventions.

1.3 Problem Statement

Bovine SCM remains an economic burden on the dairy industry worldwide, primarily driven by silent production losses and premature culling of infected bovines. In Asia, the estimated annual cost of mastitis failure per cow is $US121, with 2.6% to 43.1% of this deficit directly attributed to reduced milk production (Khasanah *et al.*, 2021). This issue is relevant to the local dairy industry, where SCM prevalence rate of 31.4% (n = 72) was recently reported on the East Coast of Malaysia (Saeed *et al.*, 2022). A major challenge in controlling SCM is the absence of visible symptoms; the udder appears normal, rendering early detection difficult and often allowing the infection to progress to a chronic stage before it is identified.

The establishment of this SCM is governed by several factors, including host, environmental and pathogens factors (Cheng & Han, 2020; Dego, 2020; Stanek *et al.*, 2024). Host determinants such as older bovine and unfavourable teat morphology elevate susceptibility (Stanek *et al.*, 2024). This vulnerability is exacerbated by poor farm management practices, including improper ventilation, inadequate milking hygiene and contaminated bedding, all of which may facilitate the rapid colonization of the teat canal by environmental and contagious bacteria (Cheng & Han, 2020).

Consequent to these exposures, members of Staphylococcaceae family have increasingly emerged as predominant pathogens associated with local SCM, posing a threat due to the virulence factors that enhance bacterial persistence.

In Malaysia, *Staphylococcus* spp. stand out as primary aetiological agents, contributing to infections ranging from 16.5% to 55% (Krishnamoorthy *et al.*, 2021). Generally, most global and local mastitis studies focus exclusively on *S. aureus* and CoNS. This narrow focus creates a critical epidemiological blind spot. Emerging evidence suggests that closely related genera such as *Macrococcus* are frequently misidentified due to phenotypic similarity. Consequently, the epidemiology, resistance patterns and virulence potential of non-*aureus* Staphylococcaceae in Malaysian dairy herds remain largely unknown, leading to an underestimation of their threat and the failure of treatments.

Furthermore, BM treatment relies heavily on antibiotic administration, driving to an emergence of AR. In Malaysia, the MVAG recommends that the antibiotics may be administered through intramammary or intramuscular infusion. A major concern is the dissemination of resistant strains due to the acquisition of MGEs carrying ARGs within Staphylococcaceae, which is still insufficiently reported in local settings. To make matters worse, biofilm-forming Staphylococcaceae is the most concerning bacteria related to the SCM, a significant factor in the persistence of SCM. The biofilm formation enhanced resistance to antibiotics, creating substantial challenges in developing targeted interventions to manage and control mastitis infections effectively. Therefore, the absence of integrated phenotypic-genotypic surveillance of Staphylococcaceae associated with SCM in Malaysia prevents accurate diagnosis, appropriate therapy selection and the development of effective resistance control strategies.

1.4 Significance of Study

This study provides a novel contribution to the understanding of the aetiological agents of bovine SCM in Malaysia, specifically highlighting the emergence of the *Macrococcus* genus. Overcoming the limitations of traditional phenotypic misidentification, this research establishes critical baseline epidemiological data on non-*aureus* Staphylococcaceae. By identifying these species at the molecular level, this

work addresses a significant knowledge gap, redefining which bacterial species are recognized as persistent threats in local dairy environments.

Furthermore, this study bridges a research gap in local AR surveillance by determining the resistance genes underlying the resistance profiles of the isolated Staphylococcaceae family. The baseline data obtained from this study will provide practitioners with precise, evidence-based guidance for targeted therapeutic interventions. As a result, this genomic insight facilitates the development of prudent antibiotic stewardship programs, directly reducing treatment failures and mitigating the excessive use of broad-spectrum antibiotics in dairy herds.

Additionally, by characterizing the biofilm-forming capabilities of these isolates, this study elucidates mechanism of bacterial persistence in bovine mastitis that compromises the antibiotic efficacy. For dairy farmers, understanding the correlation between biofilm formation capacity and AR profile enables the implementation of improved herd management practices and the development of alternative, anti-biofilm mastitis control strategies tailored for chronic infections.

Ultimately, these findings will enhance local monitoring and disease surveillance by providing data to farmers and the Department of Veterinary Services (DVS) to facilitate the targeted and effective management of infected bovines. Consequently, this study will provide valuable insight into the One Health approach to AR transmission, recognizing that antibiotic-resistant pathogens in dairy cattle can impact consumers and public health through the food chain and shared agricultural environments. By addressing these interconnected risks, this research contributes to broader efforts to ensure food safety, support healthy lives and promote well-being for all ages.

1.5 Research Questions

The research questions of this study are as follows:

a) What are the species in Staphylococcaceae family isolated from bovine milk associated with subclinical mastitis in the studied herd?

b) What are the phenotypic antibiotic resistance profiles and underlying resistance gene profiles of the isolated Staphylococcaceae isolates?

c) What is the biofilm-forming ability of the antibiotic-resistant Staphylococcaceae isolates?

1.6 Research Objectives

The research objectives of this study are as follows:

a) To isolate and identify Staphylococcaceae family from bovine milk associated with subclinical mastitis
b) To determine the phenotypic antibiotic resistance profiles and screen for the presence of antibiotic resistance genes among selected-resistant isolates
c) To evaluate the ability of the resistant Staphylococcaceae isolates to form biofilm

1.7 Thesis Scope and Limitation

This study focused on the isolation and characterization of Staphylococcaceae family from bovine milk associated with mastitis. The research was conducted at a dairy farm located in Alor Gajah, Malacca. Sampling was carried out between August and October 2023. The biological samples consisted of bovine milk confirmed as mastitis-positive based on qualitative screening using the California Mastitis Test (CMT). Methodologically, the study encompassed the phenotypic isolation of bacteria using Mannitol Salt Agar (MSA), followed by preliminary identification via Gram staining, catalase and coagulase tests. Molecular confirmation was performed targeting the *sodA* gene to definitively distinguish *Staphylococcus* species from closely related genera such as *Macrococcoides* and *Macrococcus*. Subsequent analysis focused on evaluating antibiotic susceptibility patterns using the disc diffusion method and characterizing the specific resistance genes associated with the observed resistance profiles. Additionally, the study investigated the virulence potential of the isolates by assessing biofilm formation capabilities using the microtiter plate assay. Finally, the research examined the correlation between biofilm phenotypes and the antibiotic resistance profiles of the recovered isolates.

This study is limited to the isolation and identification of Staphylococcaceae obtained from a single sampling location in Malacca. This geographic constraint reduces the representativeness of the observed prevalence rates, antibiotic resistance profiles and biofilm-forming capabilities to other dairy herds across the state or Malaysia. Additionally, the sampling framework employed a cross-sectional design, which precludes the assessment of seasonal variations or longitudinal trends in pathogen dynamics. This temporal constrains restricts the ability to fully evaluate the effectiveness of good dairy herd management practices. Lastly, the observation of biofilm formation was limited to phenotypic methods. Without molecular confirmation of specific virulence factors, it is hard to fully understand the mechanisms of bacterial persistence or to determine the most effective antibiotic treatment.

CHAPTER 2
LITERATURE REVIEW

2.1 Bovine Mastitis

Bovine mastitis (BM) is considered a zoonotic disease driven by pathogens that can be transmitted between bovines and humans. It poses a substantial threat to the global dairy industry. This infection not only leads to a decrease in bovine milk production but also adversely affects milk quality by diminishing essential nutrient content, increasing somatic cell counts and elevating bacterial levels (Cheng & Han, 2020). Consequently, the economic impact of BM extends beyond reduced milk yield to encompass compromised product quality, posing significant challenges to dairy producers worldwide. Singh (2022) emphasized that when milk yield experiences a decrease of 10% to 30%, this provides insight into the economic losses of bovine mastitis and financial implications within the agricultural sector.

Mastitis is primarily caused by bacterial invasion of the mammary gland (Chadi *et al.*, 2022). The gland is particularly susceptible to infection because milk serves as a nutrient-rich substrate, containing protein, fat, lactose and mineral salts that are favourable for bacterial growth (Grima *et al.*, 2021). Consequently, bacterial proliferation degrades the milk composition and triggers an inflammatory response, characterized by an elevation in somatic cell count (SCC) that can reach up to over 200,000 cells per mL (Khasanah *et al.*, 2021). The increment of SCC in milk indicates that bovines are infected with intramammary infection, which is directly correlated with reduced milk yield and compromised udder health (Kirkeby *et al.*, 2021).

BM is primarily classified into two forms: clinical and subclinical. This classification is based on the presence or absence of visible clinical signs and symptoms (Negash, 2023).

2.1.1 Clinical Mastitis

Clinical mastitis (CM) is classified based on visible signs of bacterial infection on the udders and changes in milk (Zaatout, 2022). The clinical signs of CM include swelling, warmth and redness of the udder (as in Figure 2.1). These inflammatory

changes may be cross-contaminated and affect adjacent quarters (Ajose *et al.*, 2022). The milk produced may also be discoloured, flaky, clotted or watery (Kour *et al.*, 2023). Due to the visible signs, it allows detection at the early stage and enables prompt intervention and treatment of the infected bovines. The pathogens responsible for this condition include *Streptococcus uberis*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa* and pyogenic bacteria (Argaw, 2016; Cheng & Han, 2020).

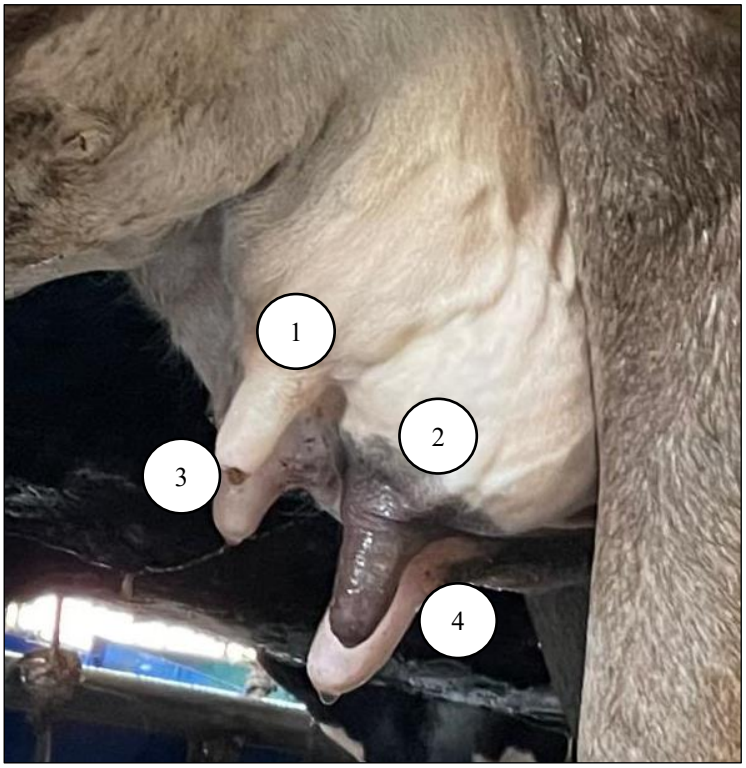


Figure 2.1 Bovine Udder is Divided into Four Quarters. Teats with Label 1 and 3 Indicate Healthy Teats. Teats with Label 2 and 4 Indicate Unhealthy Teats That Show Inflammation of Swollen and Red Teats

CM is categorized into per-acute, acute, sub-acute and chronic mastitis. These categories depend on the severity of symptoms that can lead to systemic complications (fever, depression or weight loss) (Cheng & Han, 2020). Per-acute mastitis is characterized by a sudden and severe onset of symptoms such as swollen udder, depression and high fever that may result in fatality (Egyedy & Ametaj, 2022). In comparison, acute mastitis is less severe than per-acute mastitis and presents with milder symptoms, while sub-acute mastitis is a common form of CM type that manifests with minimal symptoms of mammary gland infection (Dego, 2020). Conversely, chronic mastitis involves long-term, persistent inflammation of the cow's udder that

frequently necessitates culling decisions for affected bovines (Egyedy & Ametaj, 2022).

2.1.2 Subclinical Mastitis

Subclinical mastitis (SCM) is an asymptomatic condition of bovine intramammary gland resulting from bacterial infections (Abed *et al.*, 2021). The common aetiological agents reported to be associated with SCM are *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS), *Streptococcus agalactiae, Escherichia coli* and *Enterococcus* spp. (Argaw, 2016; Abed *et al.*, 2021). Given that the udder, teats and milk of affected bovines retain a normal appearance and exhibit no visible abnormalities (Khasapane *et al.*, 2023), identifying the disease presents a diagnostic challenge. Consequently, diagnosis relies on indirect methods such as somatic cell count (SCC) or California Mastitis Test (CMT).

SCC is a test that measures the total number of immune cells, consisting of polymorphonuclear neutrophils (PMNs), macrophages and lymphocytes in milk (Halasa & Kirkeby, 2020). The distribution of these immune cells depends on the inflammatory status of the udder. In healthy bovines, milk typically maintains a low-level SCC containing fewer than < 100,000 cells per mL (Stanek *et al.*, 2024). However, an elevation to the threshold of ≥ 200,000 cells per mL is indicative of SCM, confirming infection, even without visible signs (Ruegg, 2017; Kirsanova *et al.*, 2019). According to Ramuada *et al.* (2024), determining laboratory SCC values relies on flow cytometry, a quantitative methodology that demands advanced machines and qualified personnel, limiting its applicability for on-farm diagnostic.

Conversely, CMT is a detergent-based cow-side test, to detect early-stage SCM in bovines (Sangwa *et al.*, 2025). It is a rapid and qualitative test that induces changes in viscosity and colour when the CMT reagent is mixed with milk, indicating elevated somatic cell levels associated with SCM (Johri *et al.*, 2023). Sodium or potassium lauryl sulfate, the anionic surfactant in the CMT reagent, facilitates the lysis of somatic cells. This process releases cellular deoxyribonucleic acid (DNA), causing the mixture to thicken and form a viscous gel (Olatoye *et al.*, 2018; Rust *et al.*, 2023). Furthermore, the reagent contains bromothymol blue, a pH-sensitive indicator that changes the initial colour of blue to yellow in acidic milk, blue to dark purple in alkaline milk (Plummer & Plummer, 2012; Modh *et al.*, 2018). This colorimetric change occurs because milk

affected by SCM exhibits an elevated pH, rising from the normal range of 6.5 to 6.9 to approximately pH 7.1 due to intramammary infection (Johri *et al.*, 2023).

SCM causes more losses than CM, estimated to be 15 to 40 times more prevalent than CM (Cheng & Han, 2020; Satbige & Mammani, 2021; Chen *et al.*, 2022). The high prevalence rate of SCM is often associated with a limited adoption of routine screening and lack of awareness regarding preventive and control practices among dairy farmers. Since SCM lacks visible symptoms, it frequently goes untreated, allowing pathogens to establish chronic infections and form biofilms within the udder. To understand how these infections persist, it is necessary to examine the pathogenesis of the disease.

2.2 Pathogenesis of Bovine Mastitis

The primary route of entry for pathogens into the mammary gland is via the teat orifice, as illustrated in Figure 2.2. However, there are rare cases where udder infections can occur secondarily via systemic infection (Vidlund *et al.*, 2022). For instance, *Mycoplasma bovis* can enter the bloodstream and spread to the udder from other body parts (Vidlund *et al.*, 2022).

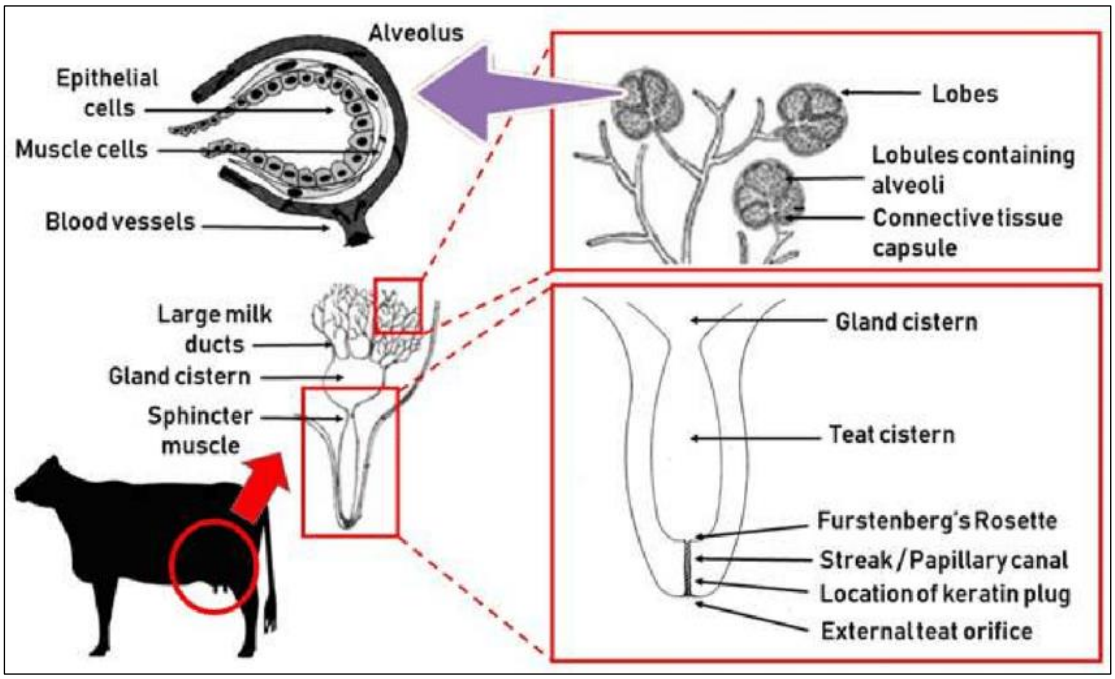


Figure 2.2 The Anatomy of the Bovine Teat
Source: Khan *et al.* (2020)

The streak canal acts as the first line of defence against mastitis pathogens. The integrity of this barrier is mediated by the sphincter muscles and elastic fibers surrounding the streak canal (Fox *et al.*, 2001; Haxhiaj *et al.*, 2022). The contraction of these sphincter muscles tightly seals the teat canal, preventing bacterial invasion (Fox *et al.*, 2001). In addition, Furstenberg's Rosette plays a role in preventing the backflow of milk when the udder is not in lactation. The presence of keratin in the streak canal serve as an additional physical barrier to bovines (Vidlund *et al.*, 2022). Keratin enhances the innate defence mechanisms of the udder by harbouring several antibacterial fatty acids, such as myristic, lauric, palmitoleic and linoleic acids (Côté-Gravel & Malouin, 2019). However, disruption of keratin layers by environmental factors creates an opening for pathogens to enter the streak canal. Once the streak canal is breached, mastitis agents can adhere, multiply and release the virulence factors that trigger the host immune response.

Pathogens use their virulence factors to adhere to the host's epithelial cells on the cell surface and evade the host's immune system. For example, in *S. aureus* the virulence factors reported in assisting the bacterial adherence include surface proteins, fibronectin-binding proteins (FnBP), fibrinogen-binding proteins (FgBP), collagen-binding proteins (Cna) and clumping factors (ClfA/B) (Touaitia *et al.*, 2025). Upon interaction with the host's cell surface, these pathogens modulate the host's actin cytoskeleton to facilitate internalization into the cell's cytoplasm, effectively allowing the bacteria to hide intracellularly.

As mastitis pathogens interact with the host, they release exotoxins and hydrolytic enzymes to facilitate tissue invasion (Li *et al.*, 2026). The secretion of hemolysins and exoenzymes degrades the epithelial lining of the cistern, ducts and alveoli, directly compromising milk synthesis. Moreover, these bacterial byproducts trigger the host immune response to release chemoattractants that attract polymorphonuclear neutrophils (PMNs) and monocytes to the site of inflammation (Haxhiaj *et al.*, 2022; Vidlund *et al.*, 2022). The PMNs engulf and digest the pathogens effectively via phagocytosis and apoptosis. However, shedding of the dead epithelial cells and leukocytes into the milk results in an elevated SCC and reduced milk quality. Consequently, a high SCC serves as a primary indicator of compromised udder health (Moschovas *et al.*, 2023).

Despite the host's defense mechanisms, pathogens have evolved evasion strategies to evade phagocytosis. In *Staphylococcus*, the extracellular FgBP and

leukotoxin subunit are typically implicated. The secretion of extracellular FgBP protects the pathogens from recognition and phagocytosis by host cells (Kuipers *et al.*, 2016). This evasion occurs because extracellular FgBP binds to fibrinogen, inhibiting complement activation and allowing the bacteria to avoid host immune responses. Additionally, Vidlund *et al.* (2022) found that the production of leukotoxin subunit (LukM) by *S. aureus* lyses the PMNs. Hence, by targeting and destroying these immune cells, *S. aureus* can impair the host immune response and establish a successful infection.

2.3 Risk Factors of Mastitis

BM is a multifactorial disease, where the risk of infection and udder health are determined by several factors, including pathogens, host and environmental factors (Cheng & Han, 2020; Teotia *et al.*, 2025). If the SCM is not effectively addressed, it may proceed to CM (Argaw, 2016). Therefore, clarifying the factors is essential for minimizing the risk and impact of BM.

2.3.1 Pathogen Factors

BM is primarily caused by microbial pathogens, with bacteria being the most prevalent etiological agents. Based on their primary reservoir and mode of transmission, these pathogens are broadly categorized into two distinct groups: contagious and environmental (Abebe *et al.*, 2016; Teotia *et al.*, 2025).

Contagious pathogens are adapted to survive within the host's mammary gland. They are typically associated with more persistent cases that may require specialised management and treatment, as the bacteria can spread from cow-to-cow in the herd through direct contact, especially during milking. The primary bacteria associated with contagious mastitis include *S. aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp., *Corynebacterium bovis* and *Streptococcus dysgalactiae* (Abebe *et al.*, 2016; Cheng & Han, 2020; Cobirka *et al.*, 2020). To control these contagious pathogens, it is essential to minimize the contact between the infected and uninfected bovines. Consequently, preventive measures include the hygienic maintenance of milking equipment, post-milking teat disinfection, strategic culling and the implementation of dry cow therapy (Smith & Hogan, 1993; Teotia *et al.*, 2025).

Conversely, environmental mastitis is caused by opportunistic pathogens present in the bovine's immediate surroundings, particularly in bedding, manure and housing areas (Kibebew, 2017; Teotia *et al.*, 2025). Unlike contagious mastitis, these infections are often acute and associated with CM, rather than SCM. Common environmental pathogens include coagulase negative staphylococci (CoNS), *Streptococcus uberis, Klebsiella* spp. and *Pseudomonas* spp. (Ashraf & Imran, 2020). Additionally, *Escherichia coli* is the most prevalent bacterial species causing environmental mastitis due to its ability to survive in various environmental niches and its potential to contaminate the udder during the milking process, contaminated bedding, or exposure to contaminated water sources (Kibebew, 2017; Ashraf & Imran, 2020). The control relies on mitigating teat-end exposure to environmental pathogens and enhancing the host's resistance to IMI through antibiotic intervention and vaccination (Smith & Hogan, 1993; Cheng & Han, 2020).

2.3.2 Host Factors

Genetic predisposition is associated with the susceptibility or resistance of bovines to mastitis infections (Miles & Huson, 2021). According to Cheng and Han (2020), anatomical structure of the udder, including teat placement, size and shape, can affect the susceptibility to mastitis. The study reviewed that large funnel-shaped teats and blind quarters may be more prone to infections, especially if milking equipment is not correctly adjusted or if milking procedures are not carefully executed. The specific shape of the teat can also be one factor that leads to improper milking processes, such as incomplete milking, leaving residual milk in the udder. This residual milk provides a favourable medium for bacterial growth, increasing the risk of BM. The pendulous or elongated udder to the ground can also increase the likelihood of contamination with environmental pathogens (Sharma *et al.*, 2017). Hence, bovines may become the factors for BM.

In addition, age of bovines may also influence the occurrence of mastitis. The first lactation heifers are more vulnerable to mastitis due to the stress of transitioning from pregnancy to milking (Koch *et al.*, 2016). This vulnerability is increased by their immune system trying to adapt to new environmental pathogens (Nitz *et al.*, 2020; Fesseha *et al.*, 2021). As the heifers are still adapting to the condition of the milking parlour, milking routine and overall management practices on the farm, their immune

system function may be weakened, making them more susceptible to bacterial infections (Dahl *et al.*, 2020). Meanwhile, older bovines may have a higher risk of acquiring bacterial infections since frequent milking may lead to their teat canal's widening or permanent opening (Cheng & Han, 2020). Furthermore, the irreversible damage caused by previous inflammations in older bovines can also lead to tissue permeability changes (Cheng & Han, 2020).

Additionally, breed selection for high milk yielding bovines has been associated with mastitis susceptibility among dairy cattle (Girma & Tamir, 2022). Notably, high-yielding breeds, such as purebred Holstein-Friesians, have demonstrated higher genetic vulnerability to intramammary infection compared to medium-yielding breeds such as Jersey (Teotia *et al.*, 2025). This increased susceptibility may result from selective breeding focused primarily on high milk production traits, which may influence their immune response and udder defense mechanisms, leading to immunocompromised condition, particularly during peak lactation. In contrast, lower producing native breed, such as Rendena, generally are less prone to infection (Girma & Tamir, 2022; Teotia *et al.*, 2025). This breed type retained highly effective physical defences against mastitis pathogens, reflecting that genetic susceptibility to BM is directly correlated with selection for high volumetric production yields.

2.3.3 Environmental Factors

Environmental factors such as cleanliness of milking equipment, proper bedding and good housing system practices are essential to prevent BM (Kibebew, 2017; Cheng & Han, 2020). The contaminated milk equipment or improper cleaning can harbour mastitis causing pathogens, which can subsequently transferred to the teat canal during milk harvesting. Consequently, emphasising proper milking hygiene and equipment sanitation significantly reduce the risk of mastitis. A study by Qiu *et al.* (2022) correlates with the data from Freu *et al.* (2023), which demonstrated that inadequate ventilation and dirty or wet bedding create hot temperatures and humid conditions that favour bacterial growth, hence increasing the mastitis risk. Moreover, overcrowded conditions can also lead to stress and increased physical contact between bovines (Cheng & Han, 2020). This condition facilitates the spread of IMIs within their herds.

2.4 The Staphylococcaceae Family in Bovine Mastitis

The family Staphylococcaceae, within the order Bacillales, the class Bacilli and the phylum Firmicutes, encompasses a diverse group of Gram-positive cocci. The family name is derived from the Greek term *staphylé,* which denotes a bunch of grapes, while *kokkos* means berry, referring to their characteristic arrangement in irregular, grape-like clusters. Members of this family are generally non-sporing, non-motile, catalase-positive and facultative anaerobes, distinguishing them from the catalase-negative *Streptococcaceae*. There are several genera included in this family, which are *Staphylococcus*, *Macrococcus*, *Salinicoccus*, *Jeotgalicoccus* and *Nosocomiicoccus,* with a closely related *Jeotgalicoccus pinnipedialis* (Rosenberg *et al.*, 2014). However, only *Staphylococcus* and *Macrococcus* are commonly associated with bovine mastitis, with *Nosocomiicoccus* being rarely implicated.

2.4.1 *Staphylococcus* spp.

In 1880, Alexander Ogston first introduced the term *Staphylococcus* to describe the group of micrococci responsible for inflammation and suppuration (Ogston, 1882). Four years later, in 1884, Friedrich Julius Rosenbach further differentiated the genus based on colony colour, distinguishing the golden-coloured *S. aureus* and white-coloured *Staphylococcus albus* (now classified as *Staphylococcus epidermidis*) (Rosenbach, 1884). Then, after two years, the genus of *Staphylococcus* was split from *Micrococcus* (Flügge & Flügge, 1896).

The genus *Staphylococcus* represents the most significant group of bacterial pathogens associated with both clinical and subclinical BM. Genetically, these bacteria are characterized by a low guanine-cytosine content, typically ranging from 30 to 40% (Foster & Geoghegan, 2015; Gherardi *et al.*, 2018). Morphologically, the cells are spherical with a diameter of about 0.5 to 1.0 μm (Foster, 1996). They are halotolerant, capable of surviving in high salt concentration (up to 10% sodium chloride (NaCl)).

Staphylococci are ubiquitous inhabitants of the dairy environment, acting as both contagious and environmental pathogens depending on the species (Vargová *et al.*, 2023; Dego & Vidlund, 2024). They primarily reside as commensals on the udder skin, teat surface and within the streak canal. However, they possess the capabilities of

pathogenic bacteria, once they breach the host's defences, proliferate and colonize the epithelial lining by expressing their virulence factors.

Taxonomically, there are more than 60 recognized species within this genus (Dego & Vidlund, 2024). However, recent phylogenetic studies using 16S rRNA gene sequence similarity and overall genomic indices have updated this taxonomy. This taxonomic revision led to the transfer of five species (*Staphylococcus sciuri*, *Staphylococcus fleurettii*, *Staphylococcus lentus*, *Staphylococcus stepanovicii* and *Staphylococcus vitulinus*) to the newly created genus *Mammaliicoccus* (Dego & Vidlund, 2024). Despite this reclassification, the remaining *Staphylococcus* species are divided into two main groups based on the production of the coagulase enzyme: Coagulase Positive Staphylococci (CoPS) and Coagulase Negative Staphylococci (CoNS).

2.4.1.1 Coagulase Positive Staphylococci

Currently, there are nine species of coagulase positive staphylococci (CoPS) that have been identified in veterinary medicine. The identified species include *S. aureus*, *S. pseudintermedius*, *S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini* and *S. cornubiensis* (González-Martín *et al.*, 2020). Additionally, *S. agnetis* is classified as coagulase variable, appearing as either CoPS or CoNS (González-Martín *et al.*, 2020). As a group, CoPS have evolved multiple mechanisms of resistance (including the production of enzymes, modification of cell wall factors and secretion of virulence proteins) against the host's immune response, contributing to their ability to cause persistent and recurrent infections (Wójcik-Bojek *et al.*, 2022). The ability of CoPS to form biofilm further contributes to their virulence, as it extends their survivability in the host and environments and poses challenges for treatment (Torlak *et al.*, 2017; Gajewska & Chajęcka-Wierzchowska, 2020).

Within this group, *S. aureus* reported as a major pathogen associated with SCM in bovine, buffalo, sheep, goats and horses (Saglam *et al.*, 2017; Dego & Vidlund, 2024). It is a contagious pathogen, persisting primarily within the infected mammary gland rather than the environment. Infections caused by this bacterium typically manifest as chronic SCM, characterized by persistently elevated SCC and sustained reduction in milk yield. Additionally, *S. aureus* poses a public health risk as the causative agent of foodborne disease (González-Martín *et al.*, 2020). The risk is driven

by the production of heat-stable staphylococcal enterotoxin, which can persist in bulk tank milk and raw dairy products, serving as vectors for transmission to humans (González-Martín *et al.*, 2020; Howden *et al.*, 2023; Yu *et al.*, 2025).

The epidemiological impact of other CoPS on bovines varies significantly by species. For instance, *S. intermedius*, *S. pseudintermedius* and *S. coagulans* are predominantly recognized as pathogens responsible for various infections in companion animals (dogs and cats) but they can be found in sporadic cases of bovine mastitis. Furthermore, while *S. hyicus* is well-known as an aetiological agent of disease in swine, it can also act as an opportunistic pathogen in bovine mastitis, similar to the coagulase-variable *S. agnetis*.

2.4.1.2 Coagulase Negative Staphylococci

Coagulase negative staphylococci (CoNS), which are increasingly referred to as non-*aureus* staphylococci (NAS) in modern veterinary literature, are a group of bacteria commonly found on the skin and mucous membranes of humans and animals (Dego, 2020; Marincola *et al.*, 2021). They inhabit various ecological niches, including bedding materials, nostrils, teat canal and udder skin. In recent years, there has been a noticeable increase in the frequency of CoNS being isolated from bovines with mastitis (Bhavana & Chaitanya, 2022), making them the most frequently reported isolates in many dairy herds. They are commonly considered emerging pathogens associated with BM in many countries when they persist within the mammary gland (Phophi *et al.*, 2019). This persistence triggers an inflammatory response within the udder, leading to an elevation of milk somatic cell counts (SCC) (Kirkeby *et al.*, 2021). The common CoNS species causing intramammary infections include *S. haemolyticus, S. chromogenes*, *S. epidermidis*, *S. saprophyticus*, *S. simulans*, *S. hominis*, *S. warneri*, *S. capitis* and *S. xylosus* (El-Seedy *et al.*, 2017; Monday & Hosu, 2022). However, the epidemiological distribution, spread mechanisms and reservoirs of these bacteria vary significantly based on environmental, management and host factors. Therefore, it is crucial to determine each species' natural habitat to distinguish between environmental and host-adapted pathogens and to design effective mastitis control measures. For example, while *S. epidermidis* typically colonizes the teat apices of dairy cows and healthy human skin, *S. simulans* is predominantly isolated directly from the milk of cows with active mastitis.

CoNS are clinically significant because some species are related to human pathogens. Furthermore, they often exhibit higher antibiotic resistance than *S. aureus*, which poses a significant challenge in treating the infections they cause (Kibebew, 2017). Previous studies have shown that CoNS are increasingly demonstrating resistance against penicillin, tetracycline, lincomycin and streptomycin (Srednik *et al.*, 2017; Garbacz *et al.*, 2021; Gebremariam *et al.*, 2022). In CoNS, antibiotic resistance is mainly acquired through genetic mutation or the uptake of antibiotic resistance genes (ARGs) carried by mobile genetic elements (MGEs) (Heilmann *et al.*, 2019). Also, CoNS are capable of forming biofilms similar to *S. aureus* (Águila-Arcos *et al.*, 2017). This attribute can extend their infection duration and lower their sensitivity against antibiotics.

Among these diverse species, *S. chromogenes* is the most prevalent species isolated from mastitic milk (Petzer *et al.*, 2022). Previous studies reported that this strain is an opportunistic invader residing on teat skin and teat tips. This strain also readily colonizes the udder skin of heifers during calving and is frequently isolated from the milk of primiparous cows during their first lactation (Dego & Vidlund, 2024). Consequently, it is the predominant cause of mastitis in heifers during early lactation, reflecting its strong adaptation to the bovine udder.

2.4.2 *Micrococcus* spp.

In 1916, Alice Evans first isolated the organism from raw milk and initially classified it as *Micrococcus caseolyticus* based on its morphology and carbohydrate-decomposition characteristics. In 1975, this genus was reclassified as *Staphylococcus caseolyticus*. This reclassification is due to cell wall composition and lower guanine-cytosine content, which distinguishes it from the high guanine-cytosine genus *Micrococcus* (Schleifer & Kloos, 1975). However, in 1998, extensive phylogenetic analysis based on 16S rRNA sequencing revealed that the species formed a distinct evolutionary lineage. Consequently, it was reclassified into a new genus of *Macrococcus*, designating it as *Macrococcus caseolyticus* (Kloos *et al.*, 1998).

Although closely related to *Staphylococcus*, *Macrococcus* species possess distinct phenotypic and genotypic characteristics. Genetically, this genus is characterized by a higher guanine-cytosine content of 38 to 45% (Götz *et al.*, 2006). Morphologically, the cells are spherical with a diameter of about 1.1 to 2.5 μm (Kloos

et al., 1998). Additionally, it differs from the genus *Staphylococcus* due to the absence of teichoic acids in its cell wall composition, variations in cytochrome and menaquinone patterns and susceptibility to lysostaphin, erythromycin, bacitracin and furazolidone (Götz *et al.*, 2006; Nuñez, 2014). These bacteria are frequently isolated from various animal sources, with a significant presence in bovine milk and on the skin of dairy cattle (Rosenberg *et al.*, 2014). Primarily, *Macrococcus* spp. were considered as avirulent commensals. However, the species are now recognized as emerging opportunistic pathogens in both veterinary and human clinical settings (Carroll *et al.*, 2023). Their clinical relevance in veterinary medicine is increasingly acknowledged not only due to their capacity to invade the mammary gland and cause mastitis, but also because they serve as significant reservoirs for ARGs (Carroll *et al.*, 2023). Notably, *Macrococcus* species have been identified as carriers of novel methicillin-resistance determinants (such as *mecB* and *mecD*), posing a severe risk of horizontal gene transfer to major contagious pathogens (Schwendener *et al.*, 2017). Currently, the genus comprises 12 recognized species and two subspecies, most of which are commensals isolated from animal skin and bovine milk (Madhaiyan *et al.*, 2020). The species include *Macrococcus bovicus*, *Macrococcus carouselicus*, *Macrococcus equipercicus, Macrococcus lamae*, *Macrococcus canis*, *Macrococcus brunensis*, *Macrococcus hajekii*, *Macrococcus bohemicus*, *Macrococcus goetzii*, *Macrococcus epidermidis*, *Macrococcus armenti* and *Macrococcus caseolyticus* (Carroll *et al.*, 2023).

2.4.2.1 Macrococcus caseolyticus

M. caseolyticus was first found in bovine milk by Evans. The species was defined by its potent proteolytic activity, specifically the rapid peptonization of milk. Consequently, the name ‘caseolyticus’ denotes the characteristic of casein-dissolving. In previous studies, *M. caseolyticus* was isolated from the skin of diverse hosts, including livestock, marine mammals and meat products (Kloos *et al.*, 1998; Gómez-Sanz *et al.*, 2015). In the context of bovine mastitis (BM), *M. caseolyticus* is highly clinically relevant. It has transitioned from being viewed solely as a harmless environmental microbe to an opportunistic mastitis-causing pathogen capable of elevating somatic cell counts (SCC) in infected quarters (MacFadyen *et al.*, 2018). Furthermore, its potent caseolytic properties can negatively impact raw milk quality and dairy processing. Beyond mastitis, *M. caseolyticus* has been linked to ovine

abscesses, canine dermatitis, otitis and even systemic infections in broiler chickens, underscoring its broader veterinary relevance (Foster & Paterson, 2020). A genomic study shows a heterogeneous population with diverse sequence types and virulence-associated genes (hemolysins, biofilm factors and exotoxins), suggesting the genus can acquire and disseminate antimicrobial resistance and virulence traits (Zhang *et al.*, 2022). Collectively, these findings support the re-classification of *Macrococcus* from benign commensals to clinically important opportunistic agents in livestock and companion-animal health.

2.4.2.2 Macrococcus bohemicus

M. bohemicus was first discovered by Mašlaňová *et al.* (2018) isolated from a human's knee wound. Subsequently, another strain of *M. bohemicum* was identified in 2017, recovered from bovine milk in the Republic of Ireland (Bello *et al.*, 2023). The recovery of this species from bovine milk strongly suggests its potential role as an emerging intramammary pathogen. Although its specific epidemiological impact on udder health requires further investigation, its detection in dairy environments highlights the continuous evolution of opportunistic mastitis-causing agents. Recently, a phylogenomic study by Bello *et al.* (2023) proposed splitting the genus *Macrococcus* into two distinct genera based on core protein analysis and conserved signature indels. Consequently, several species, including *M. caseolyticus* and *M. bohemicus*, were reclassified into the new genus *Macrococcoides* (for example, *Macrococcoides bohemicum*). However, as this reclassification is recent, the established nomenclature *Macrococcus* remains prevalent in current literature.

2.4.3 Molecular Identification of Staphylococcaceae

Historically, phenotypic assays such as coagulase test and 16S rRNA sequencing have served as the gold standards for staphylococcal classification. However, recent comparative genomic studies highlight significant limitations in these conventional methods. Specifically, the 16S rRNA gene is highly conserved, frequently exhibiting > 97% sequence similarity among closely related *Staphylococcus* species and emerging taxa like *Macrococcus* (Ghebremedhin *et al.*, 2008; Schiffer & Ehrmann, 2025). This high degree of conservation results in a lack of sufficient discriminatory

power for accurate, species-level differentiation (Johnson *et al.*, 2019). Furthermore, reliance on 16S rRNA is often compromised by the presence of poor-quality entries in public databases, further diminishing its diagnostic reliability (Johnson *et al.*, 2019). Consequently, when molecular limitations are compounded by the variable expression of biochemical characteristics in commercial phenotypic tests, isolates are frequently misidentified, thereby masking the true epidemiological dynamics of mastitis pathogens.

To overcome these diagnostic challenges, researchers increasingly target the *sodA* gene, which encodes the manganese-dependent superoxide dismutase. Genomic evaluations show that the *sodA* gene evolves faster than the highly conserved 16S rRNA gene, giving it far greater sequence divergence among closely related staphylococcal species (Poyart *et al.*, 2001). This higher genetic variability allows for the construction of more robust phylogenetic trees that accurately reflect species-level relationships, resolving the taxonomic overlaps that 16S rRNA cannot (Heikens *et al.*, 2005). In practice, *sodA* sequencing has proven effective in correcting misclassifications originally made by traditional phenotypic methods (Sivadon *et al.*, 2004). Given its superior genetic variability, better database quality and proven diagnostic accuracy, *sodA* serves as a highly reliable and preferred molecular marker for identifying members of the Staphylococcaceae family.

2.5 Prevalence of Staphylococcaceae in Bovine Mastitis

The Staphylococcaceae family has emerged as the most prevalent SCM pathogen (Pumipuntu *et al.*, 2019). However, prevalence of the Staphylococcaceae family in SCM varies across different regions and individual herds. Factors such as management practices, milking hygiene and environmental conditions can influence the prevalence and persistence of these pathogens (Abebe *et al.*, 2023).

In Asia and Europe, *S. aureus* and CoNS cause economic losses in reduced milk production, increased veterinary costs and quality issues with milk and milk products (Shaheen & Tantary, 2015; Dejyong *et al.*, 2022). Controlling *Staphylococcus*-associated mastitis poses a significant challenge due to the complexity of the disease, such as bacterial transmission, virulence factors and pathogenicity of bacteria. Therefore, emphasizing the pathogen's spread among herds in a particular area is

essential. Prevalence studies on SCM associated with *Staphylococcus* spp. in Asia are tabulated in Table 2.1.

Staphylococcus spp. is the most common cause of SCM in many areas of Asia. The range of SCM prevalence across these studies varies significantly, from 28.6% to 84.5%. Notably, the economic impact of SCM is emphasized, indicating incurred losses due to reductions in milk quality and quantity. Hence, new targeted interventions are needed to address the challenges posed by SCM in the dairy industry. Furthermore, understanding the regional variations is crucial for implementing effective control measures and improving overall udder health in Asian dairy farming environments.

2.6 Antibiotics in Treating Staphylococcaceae Family Associated with Bovine Mastitis

Antibiotics are a diverse group of chemical compounds that act as either bacteriostatic (inhibiting the bacterial growth) or bactericidal (killing bacteria directly) agents (Manyi-Loh *et al.*, 2018). In the livestock industry, particularly in bovine management, these agents are critical for both the treatment and prevention of mastitis (Ajose *et al.*, 2022). It is estimated that approximately 60% to 70% of all antibiotics administered to bovines are specifically used for BM treatment (Ajose *et al.*, 2022). To ensure food safety and mitigate the antibiotic resistance, farm management and antibiotic usage must strictly adhere to prudent use principles. Locally, Malaysian dairy farms are required to comply with the Malaysian Veterinary Antimicrobials Guidelines (MVAG) (2021) to eliminate the risk of antibiotic residues from entering the consumer food supply chain.

Administration of antibiotics can be achieved through intramammary infusion, topical and oral routes. The uptake of antibiotics in BM treatments depends on the type of bacteria causing the infection and the bacteria's sensitivity to antibiotics. Examples of antibiotics used to treat intramammary infections are penicillin, tetracycline, ampicillin, oxacillin, erythromycin, cefazolin, pirlimycin, sulphonamides, aminoglycosides, clindamycin, ceftiofur and tobramycin (Rajamanickam *et al.*, 2020; Molineri *et al.*, 2021; Tomanić *et al.*, 2023). While numerous antibiotic classes are available for veterinary use, this study specifically focuses on six antibiotics: gentamicin, penicillin, cefoxitin, erythromycin, tetracycline and sulfamethoxazole-trimethoprim.

Table 2.1
Prevalence of SCM Associated with *Staphylococcus* in Asia

Asian country (Region)	**Prevalence of SCM (%)**	**Prevalence of *Staphylococcus* spp. associated with SCM (%)**	**References**
Malaysia (Selangor and Johor)	81.7% (n = 49/60)	*S. aureus* (28.6%, n = 36/126)	(Othman & Bahaman, 2005)
Malaysia (Selangor)	70% to 100% (n = 7 /10 to 10/10)	*Staphylococcus* spp. (55%, n = 17/30)	(Marimuthu *et al.*, 2014)
Thailand (Central and northeast Thailand)	59.0% (n = 132/224)	CoPS (14.0%, n =32/229)	(Pumipuntu *et al.*, 2019)
		CoNS (86.0%, n =121/229)	
Malaysia (Pahang, Perak, Selangor, Negeri Sembilan, Johor)	38.3% (n = 744/1945)	Non-*aureus Staphylococcus* (NAS) (39.5%, n = 233/588)	(Ali *et al.*, 2020)
		S. aureus (13.1%, n = 77/588)	
China	75.8% (n = 874/1153)	*S. aureus* (11.10%, n = 128/1020)	(Song *et al.*, 2020)
		CoNS (27.9%, n = 322/1020)	
Thailand (Udon Thani)	82.1% (n = 214/261)	*Staphylococcus* spp. (59.4%, n =155/261)	(Todtong *et al.*, 2021)
Malaysia (Kelantan and Pahang)	31.4% (n = 72/235)	*S. aureus* (16.5%, n = 39/235)	(Saeed *et al.*, 2022)
Thailand (Sakon Nakhon)	76.2% (n = 64/84)	*S. epidermidis* (38.1%, n = 32/84)	(Camsing *et al.*, 2024)

These antibiotics were selected based on the differences in their mode of actions and implementations in Malaysian dairy industry guided by the MVAG. The clinical administration and standard reference dosage for these antibiotics are detailed in Table 2.2. Importantly, these selected antibiotics are recognized by the World Organisation for Animal Health (WOAH) List of Antimicrobial Agents of Veterinary Importance and classified as Veterinary Critically Important Antimicrobial Agents (VCIA). This dual alignment highlights their high usage and emphasising the urgent need for local resistance monitoring. Additionally, despite their historical efficacy against Staphylococcaceae, there are increasing reports of resistance against these specific antibiotics in recent local and global studies. This rising threat is largely driven by the extensive use or mismanagement of antibiotic treatments in BM, which has led to the emergence of antibiotic-resistant bacteria (ARB). Therefore, to fully understand the AR in Staphylococcaceae, it is essential to understand the mechanism of action of antibiotics that target the bacterial cell. Additionally, despite their historical efficacy against Staphylococcaceae, there are increasing reports of resistance against these specific drugs in recent local and global studies. This rising threat is largely driven by the extensive use or mismanagement of antibiotic treatments in BM, which has led to the emergence of ARB. Therefore, to fully understand the AR in Staphylococcaceae, it is essential to understand the mechanism of action of antibiotics that target the bacterial cell.

Table 2.2
Standard Therapeutic Dosages, Treatment Durations and Administration Pathways for Target Antibiotic Classes in Bovines

Antibiotic class	**Antibiotic**	**Standard Dosage**	**Duration**	**Primary Therapy Route**
Aminoglycoside	Gentamicin	20 to 40 mg/kg	5 days	Oral
β-lactam	Cefoxitin	7 mg/kg	5 days	Oral or intramammary
β-lactam	Penicillin	20000 units/kg	N/A	Intramuscular or intravenous
Macrolide	Erythromycin	300 to 600 mg	12 hours	Intramammary infusion
Tetracycline	Tetracycline	10 to 20 mg/kg	5 days	Oral
Sulfonamides	Sulfamethoxazole-trimethoprim	50 to 100 mg/kg	5 days	Oral

Note: N/A; not applicable. Source: (MVAG, 2021)

2.6.1 Mechanism of Action of Antibiotics

Antibiotics act differently on bacterial structure to inhibit or kill pathogens. The mechanisms of action include inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, disruption of cell membrane function and inhibition of metabolic pathways.

2.6.1.1 Inhibition of Cell Wall Synthesis

The bacterial cell wall is made up of peptidoglycan that consists of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which is cross-linked by a pentapeptide chain. The antibiotic classes having this action include β-lactams and glycopeptides.

β-lactams antibiotics exert their bactericidal effect by targeting penicillin-binding proteins (PBPs), the enzymes responsible for cross-linking peptidoglycan strands. The antibiotic functions by acylating the active site of transpeptidase enzymes. This irreversible binding prevents the formation of peptide cross-links, effectively halting the final stage of cell wall assembly. This disruption of the terminal transpeptidation process compromises the structural integrity of the cell wall, ultimately leading to bacterial lysis and cell death (Page, 2012).

Glycopeptides inhibit cell wall synthesis by binding with high affinity to the D-Alanyl-D-Alanine (D-Ala-D-Ala) terminus of the growing peptide chain. This binding creates a steric hindrance that prevents transpeptidases from accessing the substrate, effectively halting the elongation and cross-linking of the peptidoglycan chain (Wang *et al.*, 2018).

2.6.1.2 Inhibition of Protein Synthesis

Bacterial protein synthesis is mediated by the 70S ribosome, which consists of a 30S and a 50S subunit. Aminoglycosides and tetracyclines bind to the 30S subunit, thereby interfering with messenger ribonucleic acid (mRNA) translation. Conversely, chloramphenicol, macrolides and oxazolidinones bind to the 50S subunit, where they inhibit peptide elongation.

Aminoglycoside inhibits the protein synthesis by binding at the A-site of the 30S ribosomal subunit. This interaction induces conformational change that promotes the misreading of mRNA codons during aminoacyl-transfer ribonucleic acid (tRNA) selection. Consequently, the production of aberrant, mistranslated proteins compromises the cell membrane integrity, thereby causing bacterial cell death (Krause *et al.*, 2016).

Tetracycline enters the bacterial cell via passive diffusion through porin channels. Once inside, tetracycline binds reversibly to the 30S subunit by preventing tRNA binding to the mRNA-ribosome complex (Uddin *et al.*, 2021).

Macrolides bind reversibly to the 23S ribosomal ribonucleic acid (rRNA) component of the 50S ribosomal subunit. This interaction halts the transpeptidation or translocation of protein synthesis, resulting in the detachment of the premature polypeptide (Parnham *et al.*, 2014).

Chloramphenicol binds reversibly to the Ll6 protein of the 50S ribosomal subunit. This interaction inhibits peptidyl transferase activity, which prevents peptide bond formation. Consequently, the transfer of amino acids to the growing polypeptide chain is blocked, thereby halting protein synthesis (Uddin *et al.*, 2021).

Oxazolidinones bind reversibly to the 23S rRNA component of the 50S ribosomal subunit. This interaction halts the transpeptidation or translocation of protein synthesis, resulting in the detachment of the premature polypeptide (Papich, 2016).

2.6.1.3 Inhibition of Nucleic Acid Synthesis

Rifamycin and fluoroquinolones are responsible to inhibit the nucleic acid synthesis. Rifamycin involves the inhibition of bacterial transcription via DNA-dependent RNA polymerase. By binding to the polymerase's β-subunit, the antibiotic physically obstructs the elongation of the RNA molecule, thereby halting gene expression (Saito *et al.*, 2017).

Quinolones inhibit DNA synthesis by targeting DNA gyrase and topoisomerase IV, both of which are essential type II topoisomerase enzymes. Quinolones bind with high affinity to the gyrase A subunit, disrupting its ability to cleave and reseal DNA strands, thereby arresting cell division (Nainu *et al.*, 2021).

2.6.1.4 Disruption of Cell Membrane Function

Polymyxins exert a bactericidal effect by disrupting the integrity of the bacterial cell membrane. This antibiotic binds to the lipopolysaccharide components of the Gram-negative outer membrane, destabilizing its structure. This interaction increases membrane permeability and subsequent cell lysis (Trimble *et al.*, 2016; Reygaert, 2018; Moubareck, 2020).

2.6.1.5 Inhibition of Metabolic Pathways

Sulfamethoxazole-trimethoprim, also known as co-trimoxazole, acts synergistically by blocking sequential steps in the bacterial folate biosynthesis pathway. The sulfamethoxazole competes with para-aminobenzoic acid (PABA) for the binding site on the dihydropteroate synthase (DHPS) enzyme, thereby inhibiting the synthesis of dihydropteroate (Fischer *et al.*, 2010). Concurrently, trimethoprim acts by inhibiting the dihydrofolate reductase (DHFR) enzyme, which prevents the catalysis of tetrahydrofolate (Sehrawat *et al.*, 2023). Tetrahydrofolate is the essential cofactor required to produce thymine and purines for DNA synthesis (Hartman, 1993).

2.7 Mechanisms and Dissemination of Antibiotic Resistance in Staphylococcaceae Family Associated with Bovine Mastitis

The emergence of antibiotic resistance (AR) occurs when bacteria develop the capacity to withstand the bactericidal or bacteriostatic effects of antibiotics. AR can occur intrinsically or be acquired (Uddin *et al.*, 2021). Intrinsic resistance refers to the natural inherent structural or functional characteristics of particular bacteria that enable them to withstand specific antibiotics (Ibrahim *et al.*, 2021). Conversely, acquired resistance evolves as bacteria adapt to antibiotic pressure through a variety of mechanisms, including mutations to their chromosomal DNA and acquisition of antibiotic resistance genes (ARGs) via horizontal gene transfer (such as transformation, transduction and conjugation) (Cloeckaert *et al.*, 2017; Ibrahim *et al.*, 2021).

The rapid dissemination of ARGs is driven by mobile genetic elements (MGEs), which fundamentally contribute to genetic diversity and bacterial evolution. MGEs are DNA segments capable of moving within a genome (intra-genomes) or even

transferring between different genomes (inter-genomes) (Xie, 2021). The primary MGEs facilitating this genetic exchange include insertion sequences (IS), transposons (Tn), phages, plasmids, pathogenicity islands (PIs) and chromosome cassettes. Ultimately, this MGE-driven acquisition of new traits, including AR, virulence factors, metabolic pathways and other adaptive features, led to the emergence of highly resistant strains (Xie, 2021). To demonstrate this genetic diversity in Staphylococcaceae, Table 2.3 summarizes the primary MGEs and their corresponding acquired ARGs.

Bacteria are classified as multidrug-resistant (MDR) when they develop the ability to resist the mode of action of three or more different antibiotic classes (Pascu *et al.*, 2022). The emergence of MDR Staphylococcaceae is closely linked to the One Health approach, which recognizes that human, animal and environmental health are deeply interconnected. Specifically, the widespread administration of antibiotics across clinical, veterinary and agricultural sectors exerts a continuous selective pressure that facilitates the cross-species mobilization of these resistance determinants (Naranjo-Lucena & Slowey, 2023). Consequently, evaluating resistance genes such as *blaZ*, *mecA*, *mecD*, *tetK*, *tetM*, *dfrA* and *aacA-aphD* is essential to understand these transmission dynamics, as they present the most clinical challenges to standard antibiotic therapies.

Staphylococcaceae have developed several mechanisms to resist β-lactam antibiotics. These mechanisms include the production of β-lactamase enzyme, acquisition of alternative penicillin-binding protein (PBP2a) and mutations in PBP-encoding genes (Mlynarczyk-Bonikowska *et al.*, 2022). Among these, the most common mechanism of resistance against penicillin is enzymatic degradation driven by the acquisition of *blaZ*. The *blaZ* gene, along with its regulatory genes, *blaI* and *blaR* encodes the β-lactamase enzymes that hydrolyzes the β-lactam ring of penicillin, rendering the antibiotic inactive. Normally, penicillin binds to PBPs to inhibit the crosslinking of NAG and NAM in bacterial cell wall synthesis, leading to bacterial cell death. However, by degrading the antibiotic prematurely, β-lactamase allows peptidoglycan synthesis to proceed unimpeded. The dissemination of this resistance is facilitated by the localization of *blaZ* on plasmid (pI258 and pII147) and transposons (Tn*552*, Tn*4002* and Tn*4201*) (Mlynarczyk-Bonikowska *et al.*, 2022).

Furthermore, resistance to broader spectrum β-lactam antibiotics is driven by the acquisition of *mecA* gene (Mlynarczyk-Bonikowska *et al.*, 2022).

Table 2.3
Antibiotic Resistance Genes, Mobile Genetic Elements and Mechanism of Actions in *Staphylococcus* spp.

Antibiotic	**Antibiotic Resistance Genes (ARGs)**	**Mobile Genetic Elements (MGEs)**	**Mechanism of Antibiotic Resistance**	**References**
Aminoglycoside (Gentamicin)	*aadD*, *aphA3* and *aacA-aphD*	Tn*4001* and Tn*4003*	Enzymatic inactivation (Aminoglycoside adenyltransferase)	(Shoaib *et al.*, 2023)
Macrolides, lincosamides and streptogramin B (Erythromycin)	*ermA, ermB, ermC* and *msrA*	Tn*554*, pI258, pE194	Target site modification (rRNA methylase)	(Moawad *et al.*, 2023; Naranjo-Lucena & Slowey, 2023)
Methicillin	*mecA* and *mecC*	SCC*mec*	Target site replacement (alternative penicillin binding proteins)	(Aklilu & Ying, 2020)
Penicillin	*blaZ*, *blaI* and *blaR*	Tn*552*	Enzymatic inactivation	(Moawad *et al.*, 2023)
Tetracycline	*tetK*, *tetL* and *tet38*	pT181, pKK2187	Efflux pump	(Naranjo-Lucena & Slowey, 2023)
Tetracycline	*tetM*	Tn*916*-Tn*1545*	Ribosome protective protein	(Naranjo-Lucena & Slowey, 2023)
Sulfamethoxazole-trimethoprim (Sulfonamides)	*dfrA*, *dfrG* and *dfrK*	pSK1, pJE1, pABU1, pKKS825, pKKS627 and Tn*559*, Tn*4003*	Target replacement (Dihydrofolate reductase)	(Mlynarczyk-Bonikowska *et al.*, 2022)

When native PBPs are inactivated by these antibiotics, PBP2a functions as a surrogate transpeptidase, enabling the continuous crosslinking of the cell wall (Severin *et al.*, 2004; Shalaby *et al.*, 2020). Specifically, PBP2a catalyzes the formation of pentaglycan bridges between the L-lysine residue at position three of one peptidoglycan pentapeptide and the D-alanine residue at position four of an adjacent chain (Mlynarczyk-Bonikowska *et al.*, 2022). By maintaining this structural crosslinking, PBP2a allows continuous cell wall synthesis, thereby conferring the characteristic methicillin resistance phenotype as seen in methicillin resistant *S. aureus* (MRSA) (Fergestad *et al.*, 2020). The *mecA* gene is mobilized by the staphylococcal cassette chromosome *mec* (SCC*mec*), a specific MGE that carries the *mecA* along with its regulatory genes *mecI* and *mecR1* and cassette chromosome recombinase (*ccr*) into the bacterial chromosome (Fishovitz *et al.*, 2014; Aklilu & Ying, 2020).

Within the Staphylococcaceae family, resistance genes for the PBP2a transpeptidase are notably diverse, including eight genes that have been reported to date. The *mecA* gene has been found in *S. aureus*, *S. pseudintermedius*, *Mammalliicoccus fleuretti*, *Mammalliicoccus vitulinus*, *Mammalliicoccus sciuri* and in many other species of the genus *Staphylococcus* (Mlynarczyk-Bonikowska *et al.*, 2022; Liu, 2024). Evolutionarily, the *mecA1* gene originally identified in *M. sciuri* is considered the precursor to both the *S. aureus mecA* gene and *mecA2* gene (Mlynarczyk-Bonikowska *et al.*, 2022). Beyond *mecA*, other *mec* genes exhibit distinct species distributions. For example, *mecA2* has been found in *M. vitulinus* and *S. capitis*, *mecB* gene in *M. caseolyticus* and *S. aureus*, *mecC* gene in *S. aureus* and *M. sciuri*, *mecC1* gene in *S. xylosus*, *mecC2* gene in *S. saprophyticus* and *mecD* gene in *M. caseolyticus* (Mlynarczyk-Bonikowska *et al.*, 2022). The localization of these genes on MGEs poses a continuous threat, effectively illustrating the One Health transmission dynamic. For instance, the emergence of *mecB* and *mecD* genes, originally isolated from canine and bovines *M. caseolyticus* strains, were subsequently identified in human *S. aureus* isolates in 2018 (Mlynarczyk-Bonikowska *et al.*, 2022). Notably, the *mecB* gene was localized on plasmid, pSAWWU4229_1, that co-harboured multidrug resistance genes for aminoglycosides, macrolides and tetracyclines. This highlights the severe clinical implications of plasmid-mediated, cross-species gene transfer in the ongoing evolution of staphylococcal resistance.

Alternatively, resistance against β-lactams may also occur through non-*mec* mechanisms, which involve either overproduction of penicillinase or mutations in the

genes encoding PBP2 and PBP4 and their associated regulatory genes (Brdová *et al.*, 2024). Strains exhibiting the overproduction of penicillinase are classified as borderline oxacillin-resistant *S. aureus* (BORSA). Although these strains lack *mec* genes, they exhibit a low level of resistance to oxacillin with minimum inhibitory concentration (MIC) values ranging from one to eight mg/mL. Furthermore, the penicillinase produced by BORSA strains may act as an extended-spectrum β-lactamase (ESBL) (Nomura *et al.*, 2020). Conversely, strains exhibiting the mutation of *S. aureus* proteins are classified as modified penicillin-binding *S. aureus* (MODSA) (Brdová *et al.*, 2024). Unlike BORSA, MODSA can confer resistance to a broad spectrum of β-lactams antibiotics, including ceftobiprole, ceftriaxone, cefazolin, nafcillin, ampicillin and cefoxitin (Mlynarczyk-Bonikowska *et al.*, 2022). This resistance profile is driven by mutations in the *pbp4*, *acrB*, *gdpP* and *yjbH* genes (Brdová *et al.*, 2024). These mutated genes lead to the overproduction of PBP4, which subsequently increases the peptidoglycan crosslinking during the bacterial cell wall production (Basuino *et al.*, 2018).

Beyond cell wall disruption, Staphylococcaceae actively resist 30S ribosomal inhibitors like tetracycline. Specifically, these bacteria can mediate resistance via active efflux pumps or ribosomal protection proteins (RPPs) (Brdová *et al.*, 2024). In staphylococci, several efflux pumps categorized within the major facilitator superfamily (MFS) have been reported in the removal of tetracyclines, including TetK, TetL, Tet38, Tet42, Tet43, Tet45 and Tet63 (Mlynarczyk-Bonikowska *et al.*, 2022). Among these, TetK and TetL are the most prevalent within the Staphylococcaceae family, encoded by *tetK* and *tetL* genes, respectively (Klempt *et al.*, 2022; Mlynarczyk-Bonikowska *et al.*, 2022). These membrane-associated efflux proteins utilize the proton-motive force to continuously pump tetracycline molecules out of the bacterial cell before they can bind to the ribosomes. While Staphylococcaceae harbouring *tetK* or *tetL* gene exhibit a high level of tetracycline resistance, comparative studies indicate they remain susceptible to minocycline (Brdová *et al.*, 2024). Genetically, in staphylococci, the *tetK* gene is predominantly located on plasmids, such as pT181, pSTE2, pNS1, pKH1, pKH6, pT127 and pBC1, whereas *tetL* is found on plasmids such as pKKS825 and pKK2187 (Mlynarczyk-Bonikowska *et al.*, 2022; Schwarz *et al.*, 2014). In *M. caseolyticus*, *tetL* is notably found on plasmid *rep22* (Zhang *et al.*, 2022).

In contrast, tetracycline resistance mediated by RPPs involves direct interaction with the ribosome, which induces a conformational change that lowers the ribosome's

affinity for tetracycline. Among the 13 RPPs described to date, TetM is the most common RPP found in clinical isolates of staphylococci, streptococci and enterococci and is encoded by the *tetM* gene (Wilson *et al.*, 2020). This specific RPP acts as a guanosine triphosphatase (GTPase), conferring a broader resistance profile that encompasses both tetracycline and minocycline, thereby providing a more comprehensive resistance mechanism than *tetK*-encoded efflux pumps (Mlynarczyk-Bonikowska *et al.*, 2022). The localization of *tetM* gene on plasmids and conjugative transposons facilitates its widespread distribution across diverse bacterial species (Brdová *et al.*, 2024). Specifically, dissemination of *tetM*-mediated tetracycline resistance is largely driven by the Tn*916*-Tn*1545* family of conjugative transposons (Emaneini *et al.*, 2013; Naranjo-Lucena & Slowey, 2023).

Staphylococcaceae frequently develop aminoglycoside resistance through the acquisition of genes encoding aminoglycoside-modifying enzymes (AMEs) (Brdová *et al.*, 2024). These enzymes catalyze the modification of hydroxyl or amine groups on aminoglycoside molecules. By structurally altering the drug, AMEs reduce its binding affinity for the ribosome, effectively neutralizing its bactericidal activity. Based on their specific biochemical activity, these enzymes are categorized into three classes: aminoglycoside O-phosphotransferases (APH), aminoglycoside N-acetyltransferases (AAC), or aminoglycoside O-nucleotidyl transferases (ANT) (Brdová *et al.*, 2024). Among clinical isolates of Staphylococcaceae, the most prevalent resistance genes for AME-mediated resistance is the bifunctional *aac(6')-Ie-aph(2'')-Ia* gene, also known as *aacA-aphD* (Mlynarczyk-Bonikowska *et al.*, 2022). The integration of *aacA-aphD* on composite transposon Tn*4001* confers resistance to broad-spectrum aminoglycosides. In a study on staphylococci isolated from bovines associated with CM, strains harbouring *aacA-aphD* gene showed a high prevalence of 23% (n = 22) (Qu *et al.*, 2019). Notably, this bifunctional enzyme remains the sole determinant identified that confers gentamicin resistance in *S. aureus* (Mlynarczyk-Bonikowska *et al.*, 2022).

Additionally, staphylococci acquire single-function determinants such as the *ant(4')-Ia* and *aph(3')-IIIa* genes, known as *aadD* and *aphA3* genes, respectively (Brdová *et al.*, 2024). These aminoglycoside genes are frequently localized on insertion sequence (IS256) or within transposons like Tn*3851*, Tn*4031* and Tn*5404* (Mlynarczyk-Bonikowska *et al.*, 2022), which have been described to transfer from *Enterococcus* spp. to *S. aureus* (Weigel *et al.*, 2007; Mlynarczyk-Bonikowska *et al.*,

2022). As a result, strains harbouring these genes exhibit resistance to kanamycin, neomycin and tobramycin.

Macrolide resistance is now widespread globally, with a high percentage of staphylococci exhibiting cross-resistance to all the macrolide, lincosamides and streptogramin B (MLSB) group antibiotics (Brdová *et al.*, 2024). This broad-spectrum resistance profile arises from the structural overlap of the ribosomal binding sites targeted by these distinct antibiotic classes (Brdová *et al.*, 2024). There are three mechanisms of resistance in this class of antibiotics, which are modification of the ribosomal unit, ribosome protection or efflux pump and synthesis of inactivating enzymes (Miklasińska-Majdanik, 2021). Among these mechanisms, modification of the ribosomal target site constitutes the predominant mechanism encountered in clinical isolates (Brdová *et al.*, 2024). This modification is mediated by the adenylyl-N-methyltransferase enzyme, which is encoded by erythromycin ribosomal methylation genes (*erm* genes), most notably *ermA*, *ermB* and *ermC* (Brdová *et al.*, 2024). These enzymes catalyze the dimethylation of adenine A2058 in 23S rRNA, which prevents the antibiotics from binding to the modified ribosome. The *erm* genes can be expressed either constitutively or inducibly. In the case of constitutive expression, a complex of methylase with active mRNA is continuously synthesized, leading to resistance to all MLSB antibiotics. However, in the case of inducible, activation of an inactive mRNA is required by an inducer of antibiotics such as macrolides with 14 and 15 carbon atoms of erythromycin and azithromycin. With the inducible reaction, only then can methylase synthesis begin.

In all described *erm* genes, *ermA* and *ermC* are predominantly found in staphylococci (Miklasińska-Majdanik, 2021). The *ermB* gene, however, exhibits a higher prevalence among *Enterococcus* and *Streptococcus* species (Brdová *et al.*, 2024). Both *ermA* and *ermC* genes are commonly expressed constitutively in the presence of regulatory genes encoding leading peptides at the level of translation. For example, *ermA* is preceded by *pepL* and *pep1* gene whereas *ermC* is preceded by *pep* gene (Mlynarczyk-Bonikowska *et al.*, 2022). The differing rates at which *ermA* and *ermC* constitutive variants form can likely be attributed to their distinct localizations. The *ermA* gene is generally found on the *S. aureus* chromosome within the Tn*554* or the Tn*6072* (Mlynarczyk-Bonikowska *et al.*, 2022). In contrast, the *ermC* is primarily located on plasmids such as pE194, pT48, pE5, pJR5, pA22, pJ3356::POX7, pWBG738, or pUSA03 (Mlynarczyk-Bonikowska *et al.*, 2022). Without inducers,

translated leader peptides interact with mRNA and prevent ribosome access to their binding site, thereby impeding erm methylase transferase translation. When an inducer attaches to the ribosome, the translation of the guide peptides is inhibited and the mRNA conformation is changed, which subsequently permits the synthesis of the erm methyltransferase.

In staphylococci, macrolide resistance can also be driven by an alternative RPP mechanism mediated by *msrA* gene, which encodes the adenosine triphosphate (ATP)-binding cassette subfamily F (ABC-F) of MsrA protein (Brdová *et al.*, 2024; Miklasińska-Majdanik, 2021). This protein overlaps with the antibiotic's binding site and utilizes ATP hydrolysis to induce conformational changes that destabilize and physically displace the antibiotic molecule from the ribosome. Phenotypically, this displacement selectively confers resistance against macrolides and type B streptogramins, leaving the bacteria susceptible to lincosamides. Beyond MsrA, the ABC-F protein class encompasses other staphylococcal determinants, such as Vga and Lsa, which displace lincosamides and pleuromutilins (Vimberg *et al.*, 2020). Collectively, the utilization of these ATP-dependent RPP highlights a strategy that allows staphylococci to confer resistance against 50S ribosomal inhibitors.

Staphylococcaceae can develop resistance to metabolic inhibitors. Resistance to sulfamethoxazole-trimethoprim (co-trimoxazole) requires the concurrent evasion of two distinct steps in the folate biosynthesis pathway. This dual evasion is mediated by a mutation in the chromosomal DHPS encoding gene (*folP* gene) to reduce affinity for sulfonamides, combined with the acquisition of DHFR encoding genes (*dfrA*, *dfrD*, *dfrG* and *dfrK* gene) to confer trimethoprim resistance (Nurjadi, Zizmann, *et al.*, 2021; Brdová *et al.*, 2024). These *dfr* genes are frequently plasmid-borne, which facilitates their rapid horizontal transmission (Brdová *et al.*, 2024). A study conducted by Mlynarczyk-Bonikowska *et al.* (2022) identified that the *dfrA* gene is located in several plasmids, including pSK1, pJE1 and pABU1. Similarly, the *dfrK* gene is found residing in plasmids such as pKKS825 and pKKS627 and even in a transposon called as Tn*559* (Mlynarczyk-Bonikowska *et al.*, 2022). These mechanisms enable high-level resistance and are increasingly reported in clinical isolates (Brdová *et al.*, 2024; Shao *et al.*, 2025). Consequently, the frequent localization of these *dfr* genes on plasmid and transposons ensures the rapid and widespread dissemination of co-trimoxazole resistance across diverse staphylococcal populations.

2.8 Biofilm Associated with Bovine Mastitis

A biofilm is a community of microorganisms (such as bacteria, fungi and algae) that adhere to a surface and produce a slimy and structured extracellular matrix (Notcovich *et al.*, 2018). In BM, the Staphylococcaceae family utilizes this mechanism to persist within the mammary gland. The biofilm matrix acts as a physical barrier, reducing antibiotic penetration and lowering the metabolic activity of the encased bacteria, which facilitates survival (Zaatout, 2022). The stages of biofilm formation include adhesion, proliferation, maturation and detachment (Figure 2.3).

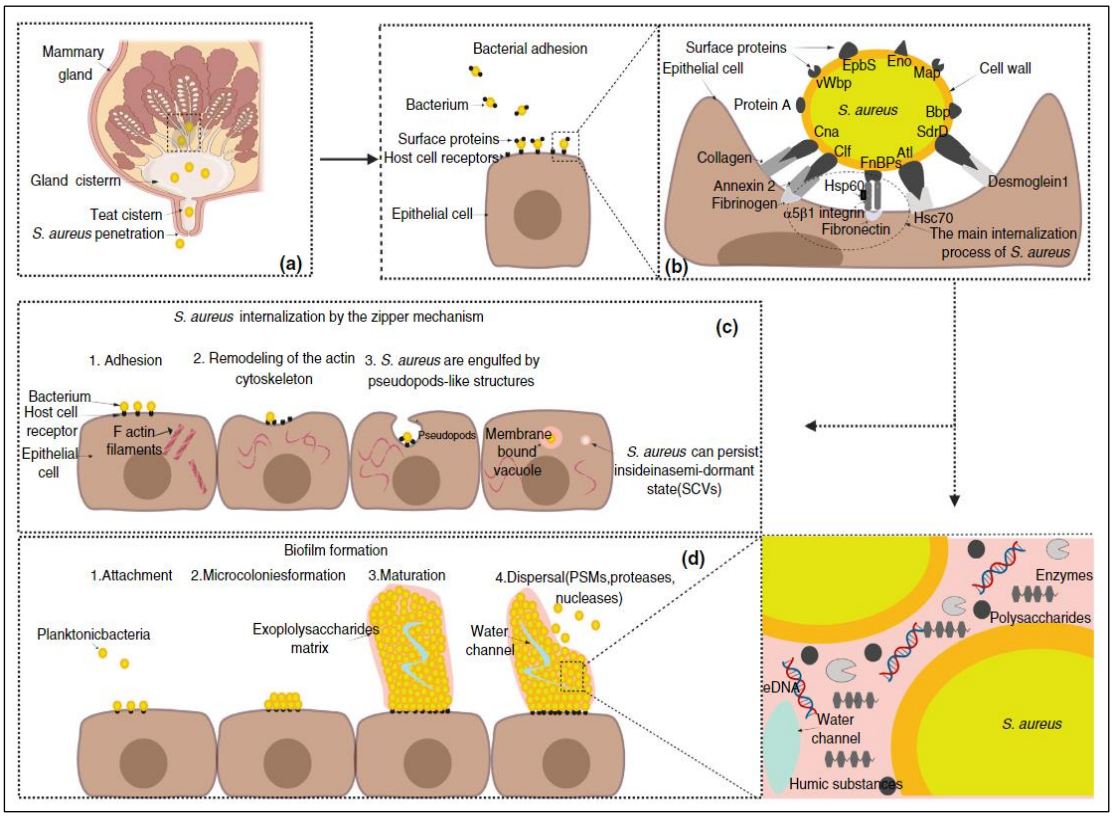


Figure 2.3 Biofilm Formation of *S. aureus* inside the Mammary Gland of a Bovine. (A) Entry of *S. aureus* into the Teat Orifice (B) Surface Proteins That Are Involved in the Adhesion of *S. aureus* to Epithelial Cell of Mammary Gland (C) *S. aureus* Invades the Host Epithelial Cell by Zipper Mechanisms and Remodelling of the Actin Cytoskeleton (D) Formation of Biofilm by Planktonic *S. aureus* Involving Attachment, Microcolonies Formation, Maturation and Dispersal
Source: Zaatout *et al.* (2020)

The process begins with adhesion or attachment, where *Staphylococcus* has contact with the host tissue. This attachment is mediated by specific interactions between microbial surface components, known as microbial surface components

recognizing adhesive matrix (MSCRAMMs), and host matrix proteins (Campos *et al.*, 2022). The MSCRAMMs include fibronectin-binding proteins (FnBPs), fibrinogen-binding proteins (FgBPs), collagen adhesin (Can) protein, serine-aspartate repeat proteins (Sdr), extracellular fibrinogen binding protein (Efb) that interact specifically with bovine mammary epithelial cells (Campos *et al.*, 2022).

Beyond anchoring pathogens to extracellular surfaces, MSCRAMMs can also facilitate host cell invasion. For instance, following the binding of staphylococcal FnBPs to host fibronectin, host $\alpha_5\beta_1$ integrin signalling promotes the cytoskeleton arrangement. This process drives the active internalization of bacteria into endosomes via a zipper-type mechanism (Medina-Estrada *et al.*, 2016). In a study demonstrated by Josse *et al.* (2017), this interaction upregulates the expression of the *fnbA* and *fnbB* genes, with the resulting protein synthesis enhancing both colonization and intracellular invasion of mammary epithelial cells. While *S. aureus* relies on these mechanisms, CoNS, like *S. epidermidis* and *S. saprophyticus*, possess alternative laminin-binding protein to adhere to laminin in the extracellular matrix of host tissues (Gomes *et al.*, 2016).

Following surface attachment and under favourable conditions, bacteria transition into the proliferation and accumulation phase (Peng *et al.*, 2022). During this stage, the sessile community synthesises an extracellular polymeric substance (EPS), also called extracellular matrix (ECM), which provides structural integrity that wraps the multiplying cells together. This protective barrier is a complex EPS matrix composed of exopolysaccharides, surface proteins and extracellular DNA (eDNA) (Oknin *et al.*, 2023).

Exopolysaccharide is the main component of the biofilm formation matrix. Its synthesis can be either dependent or independent of polysaccharide intercellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG). Within a PIA-dependent mechanism, polysaccharide intercellular adhesin (PIA) represents the most thoroughly characterized intercellular glue in staphylococcal biofilms (Zaatout, 2022). The biosynthesis of PIA is mediated by a multi-enzyme cascade encoded by the chromosomal *icaADBC* operon (Peng *et al.*, 2022). Although this mechanism enables massive accumulation of PIA within the matrix, its synthesis represents a highly energy-intensive process that demands a significant metabolic investment from the bacterial cell (Nguyen *et al.*, 2020). Conversely, biofilms can also accumulate via a PIA-independent mechanism, utilizing proteinaceous pathways entirely distinct from

exopolysaccharides (Lasa & Penadés, 2006; Nguyen *et al.*, 2020). One of the proteins discovered for biofilm formation without incorporating exopolysaccharides is biofilm-associated protein (Bap), which is encoded by *bap* gene (Peng *et al.*, 2022). Strains possessing the *bap* gene exhibit a robust capacity for cell-to-cell aggregation, substituting carbohydrate chains with a structural mesh of surface proteins and eDNA to build the matrix scaffolding.

The next stage of biofilm formation is maturation. The biofilm maturation leads to the three-dimensional mushroom or tower structure (Rather *et al.*, 2021). The formation of channels plays a role in transporting nutrients, waste removal and gas exchange to the cells in deeper biofilm layers (Peng *et al.*, 2022). The complex and mature biofilm structure allows antibiotic tolerance, making them a significant challenge in treating chronic infections.

Detachment is the last stage of biofilm formation. In survivability of biofilm, the mature and fully developed biofilm detaches itself from the surfaces by dispersing its planktonic state to establish biofilm in other locations (Moormeier & Bayles, 2017; Sharma *et al.*, 2017). Phenol-soluble modulins (PSMs) have contributed to the mechanisms of biofilm detachment in *Staphylococcus* (Zaatout *et al.*, 2020). PSMs are surfactant-like compounds that enable the degradation and solubilization of the adhesive components in the biofilm matrix, hence resulting in detachment of the cells from the biofilm colony (Grando *et al.*, 2022). On the other hand, biofilm detachment can occur by enzymatic degradation. In Staphylococcaceae, protease families are reported to mediate the biofilm dispersal, including metalloprotease aureolysin, cysteine protease and serine protease (Schilcher & Horswill, 2020). The study stated that Bap is degraded by metalloprotease aureolysin and serine protease, while FnBPs are degraded by serine protease. However, evidence of cysteine protease in biofilm detachment is yet to be discovered.

S. aureus was also found to produce thermonuclease enzymes (nuc1 and nuc2) that degrade the eDNA of the staphylococcal biofilm matrix (Schilcher & Horswill, 2020). Overexpression of the *nuc1* gene decreased biofilm formation, whereas a mutation in the *nuc1* gene increased biofilm formation (Tang *et al.*, 2011). This finding suggests that under physiological conditions, the nuclease activity in staphylococcal strains naturally suppresses or limits biofilm development. In biofilm detachment, high suppression of the *nuc1* gene is required. In contrast, the *nuc2* gene does not cooperate in the mechanisms of biofilm development.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials

The materials used in this study are listed in the following sections.

3.1.1 Bacterial Cultures and Conditions

The control cultures used in this study are *Staphylococcus aureus* ATCC 25923, *Staphylococcus haemolyticus* ATCC 29970, *Staphylococcus aureus* ATCC 33591, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* ATCC 35984, *Streptococcus agalactiae* ATCC 13813 and *Escherichia coli* ATCC 25922. These control cultures were obtained from the microbiology laboratory culture collection of the Faculty of Applied Sciences, UiTM Shah Alam.

All bacterial cultures used in this study were maintained in Tryptone Soy broth (TSB) in the universal bottle at 4 °C. For long-term storage, all cultures, including the reference strains, were kept in 20% LB broth-glycerol at -80 °C.

3.1.2 Buffer and Solution

A 10X Tris Acetate-EDTA (TAE) buffer with a pH of 8.0 (1st BASE, Asia). In order to use in gel electrophoresis, the buffer was diluted to 1X concentration by diluting 100 mL of 10X TAE buffer with 900 mL of distilled water. The buffer was stored at 20 to 25 °C, in accordance with the manufacturer's recommendations.

3.1.3 Chemicals

Chemicals used in this study include crystal violet (Sigma-Aldrich, USA), glycerol (Sigma-Aldrich, USA), hydrogen peroxide (Sigma-Aldrich, USA), ethyl alcohol (Chemiz, Malaysia), 99.9% methanol (Bendosen, Malaysia), D+Glucose Anhydrous Food Grade (Bendosen, Malaysia) and phosphate-buffered saline tablets (Dulbecco 'A') (Oxoid, UK). All the chemicals were stored at room temperature unless stated otherwise.

3.1.4 Reagents

Reagents used in this study included MyTaq Red Mix, 2x (Bioline, UK), 6X DNA Loading Dye Buffer Double Blue (Solid Biodyne, Estonia), VC 1kb and 100bp DNA ladder (Vivantis), Visafe Red Gel Stain (Vivantis) and stored in -20 °C upon purchase. Other reagent, such as California Mastitis Test reagent (Vetbiochem) was purchased and stored at room temperature.

A total of six antibiotic discs (Liofilchem, Italy) were used in this study, which are gentamicin (10 μg), tetracycline (30 μg), sulfamethoxazole-trimethoprim (25 μg), cefoxitin (30 μg), penicillin G (10 μg) and erythromycin (15 μg). The antibiotic discs were stored in -20 °C.

3.1.5 Molecular Kit

The HigherPurity™ Bacterial DNA Isolation Kit (Canvax biotech, Spain) was used for genomic DNA extraction method in this study. This genomic extraction kit was stored at room temperature.

3.1.6 Media

Microbiological media used in this study were purchased from Oxoid (Oxoid, UK) and Milipore (Merck, Germany). Preparation involved dissolving the specified weight of powder into 1 L of distilled water and mixing homogenously. Luria-Bertani (LB) broth (Miller) (Merck, Germany) was prepared using 25 g/L. The media obtained from Oxoid (UK) were prepared as follows: Mueller Hinton Agar (MHA) (38 g/L), Mueller Hinton Broth (MHB) (21 g/L), Mannitol Salt Agar (MSA) (111 g/L), Tryptone Soy Agar (TSA) (40 g/L) and Tryptone Soy Broth (TSB) (30 g/L). All media preparation was then autoclaved at 121°C for 15 minutes for sterilisation.

3.1.7 Primers

The primers used in this study are listed in Table 3.1 and were synthesised by Next Gene Scientific Sdn. Bhd. Each primer was reconstituted at 100 μM using molecular-grade water and stored at – 20 °C. The working primers were prepared at 10 μM concentration using molecular-grade water prior to use.

Table 3.1
Primers for Amplification of Antibiotic Resistance Genes of *Staphylococcus* spp.

Genes	Primer Sequence (5' to 3')	Size of Amplicon (bp)
sodA	F: 5′-CCITAYICITAYGAYGCIYTIGARCC-′3	480
	R: 5'- ARRTARTAIGCRTGYTCCCAIACRTC-3'	
blaZ	F: 5'-ACTTCAACACCTGCTGCTTTC-3'	173
	R: 5'-TGACCACTTTTATCAGCAACC-3'	
mecA	F: 5'-GTGAAGATATACCAAGTGATT-3'	147
	R: 5'-ATGCGCTATAGATTGAAAGGAT-3'	
mecD	F: 5'-TCCTTTAGCGATAGATGGTGAA-3'	867
	R: 5'-CTCCCATCTTTTCTCCATCCT -3'	
tetK	F: 5'-GTAGCGACAATAGGTAATAGT-3'	360
	R: 5'-GTAGTGACAATAAACCTCCTA-3'	
tetM	F: 5'-AGTGGAGCGATTACAGAA-3'	158
	R: 5'-CATATGTCCTGGCGTGTCTA-3'	
dfrA	F: 5' -CACTTGTAATGGCACGGAAA-3'	270
	R: 5' -CGAATGTGTATGGTGGAAAG-3'	
aacA-aphD	F: 5'-TAATCCAAGAGCAATAAGGGC-3'	227
	R: 5'-GCCACACTATCATAACCACTA-3'	

3.2 Methods

The methodology of this study is explained in the following sections.

3.2.1 Study Area and Design

Bovine milk was chosen as the sample source due to its rich nutrient content, such as proteins, carbohydrates, lipids and vitamins, which favours bacterial growth (Machado *et al.*, 2020). In addition, milk is the most reliable biological material for the isolation of mastitis-associated pathogens and is widely used in numerous studies for diagnosing intramammary infections (Zaatout *et al.*, 2020; Pangprasit *et al.*, 2023).

In the present study, raw milk samples were collected from 20 lactating bovines at a dairy farm in Alor Gajah, Malacca (2.41109° N, 102.21742° E). Sampling was conducted in August and October 2023. This specific dairy farm was selected based on

its well-established housing conditions, as shown in Figure 3.1, which include proper milking and feeding areas and herd health management practices, as well as the accessibility and cooperation granted by the farm owner. Operating as one of the region's largest high-yield milk production facilities, this farm maintains a commercial herd size of approximately 200 lactating bovines and utilizes an automated mechanical milking system for milk collection. Furthermore, the farm is officially registered under the Melaka State Department of Veterinary Services (DVS), which facilitated access to audited historical records regarding herd antibiotic administration.



Figure 3.1 The Studied Dairy Farm Facility in Alor Gajah, Malacca. (A) Overview of Livestock Housing and Husbandry Infrastructure (B) Configuration of the Automated Mechanical Milking Parlour

3.2.2 Sample Size Calculation of Staphylococcaceae Isolation

The sample size for the isolation of Staphylococcaceae potentially associated with subclinical mastitis (SCM) was determined to ensure that the study results are statistically valid, reliable and representative of the broader population. The sample size was calculated by using a formula: $N = \frac{Z^2 \times P_{exp} \times (1-P_{exp})}{d^2}$ described by Thrusfield (2005), where N = sample size, Z = 1.96, P_{exp} = expected prevalence and d = absolute precision. The calculation was based on a 95% confidence interval, a 5% margin of

error and 43.6% of the *Staphylococcus* spp. associated with SCM as reported by (Ali *et al.*, 2020). Accordingly, a total of 378 presumptive *Staphylococcus* spp. isolates were isolated from the bovine milk samples. However, to accommodate laboratory resource constrain to downstream phenotypic and molecular assays, a representative 153 isolates recovered from 20 bovines were selected for further characterization. This subsample represents approximately 40% of the initial calculated sample size, thereby supporting statistical power with resource allocation constraints.

3.2.3 Sample Collection

A total of 20 composite milk samples were aseptically collected from 20 randomly selected Jersey-Friesian crossbred bovines, following the guidelines by the National Mastitis Council (NMC, 2017). Across the 20 bovines, a total of 80 udder quarters were physically examined. The udder quarters showing visible signs of mastitis were excluded from sampling. The sample collection protocol was executed by the farm milkers; this included standard pre-milking hygiene procedures where teats were washed, dried and disinfected with 70% ethanol. To prevent environmental contamination, the first three streams of milk from each functional udder quarter were discarded. Subsequently, a volume of two mL of milk from each functional quarter was collected into a sterile 50 mL tube, following the procedure as described in the previous studies (Reyher & Dohoo, 2011; Pumipuntu *et al.*, 2019). The samples were transported in an icebox (4 °C) during transportation to the Microbiology Laboratory, Faculty of Applied Sciences, UiTM Shah Alam, within 24 hours for further analysis.

3.2.4 California Mastitis Test

The California Mastitis Test (CMT) test was performed according to the protocol described by Khasanah *et al.* (2021). Briefly, two mL of milk from each udder quarter was sampled into each sterile plastic cups and an equal volume of CMT reagent was added. The mixture was gently swirled in a circular motion and the formation of a gel or slime and any colour changes was observed for approximately five to ten seconds. The reaction results were interpreted and recorded according to McGill's standard operating procedure, as tabulated in Table 3.2. Bovines with at least one positive CMT reaction with a score of trace (T), weak positive (1), or distinct positive (2) are considered as SCM (Ramírez *et al.*, 2014; Mgonja *et al.*, 2023; Michira *et al.*, 2023).

Table 3.2
Interpretation of California Mastitis Test (CMT) Scores Corresponding to the Reaction Observed

	Score	Meaning	Description of reaction	Individual quarter sample	Somatic cell counts range (cell/mL)
	N	Negative	No gel formation was observed in the mixture.	No mastitis	0 to 200,000
	T	Trace	The mixture exhibited a slimy or gel-like consistency.	Trace of mastitis	> 200,000 to 400, 000
	1	Weak Positive	The mixture distinctly transformed into a gel state.	Mastitis	400,000 to 1,200,000
	2	Distinct Positive	The mixture thickened and immediately formed a gel.	Mastitis	1,200,000 to 5,000,000

3.2.5 Isolation of *Staphylococcus* spp. from Milk Samples

Staphylococcus isolates from milk samples were isolated according to Sahar *et al.* (2019), with slight modifications. An amount of 0.1 mL milk sample was aseptically spread onto Mannitol Salt Agar (MSA) plate and then incubated at 37°C for 24 hours.

The morphological characteristics of staphylococcal colonies, which were circular, smooth and convex, were examined. Red colonies that did not ferment mannitol were presumptively identified as CoNS, while yellow colonies that fermented mannitol were presumptively identified as *S. aureus*. To validate the results, *S. aureus* ATCC 25923 was employed as a positive control and *S. epidermidis* ATCC 35984 as a negative control.

Each presumptive *Staphylococcus* colony was subsequently purified on MSA by the dilution streaking method and the procedure was performed repeatedly until pure, isolated colonies were obtained. The pure colonies were then preserved in glycerol stock for future usage.

3.2.6 Preparation of 20% Luria-Bertani Broth-Glycerol

A 40% glycerol solution was prepared for long-term preservation of bacterial culture. Briefly, a volume of 40 mL of glycerol was mixed with 60 mL of distilled water, followed by autoclaving. An overnight culture was prepared by inoculating the isolated colony in sterile test tubes containing 5 mL of Luria-Bertani (LB) broth. The culture was incubated at 37 °C, with shaking at 220 rpm for 16 to 18 hours. For long term preservation, 500 µL of bacterial culture was mixed with 500 µL of sterile 40% glycerol in a sterile cryovial to achieve a final glycerol concentration of 20%. The glycerol stock was preserved at −80 °C until further use.

3.2.7 Standard Biochemical Tests

The standard biochemical tests conducted for the identification of *Staphylococcus* spp. in this study are explained in the following sections.

3.2.7.1 Gram Staining

Gram staining is a crucial microbiological technique to differentiate between Gram-positive and Gram-negative bacteria (Tripathi & Sapra, 2021). To validate the results, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were included as positive and negative controls, respectively.

Initially, bacterial culture was smeared on the glass slide and heat fixed. Then, the bacterial smear was flooded with crystal violet as the first stain for a minute. Subsequently, iodine was flooded on the slide as the mordant to prevent the dye from being easily removed for a minute. Then, the slide was decolorized with alcohol for 20 seconds. Finally, the slide was counterstained with safranin for a minute. In between each step, the slide was rinsed with tap water.

Upon observation under a light microscope, Gram-positive bacteria appear purple, retaining the crystal violet dye due to their thick peptidoglycan layer. In contrast, Gram-negative bacteria appeared pink as the crystal violet stain was washed off during decolorization and replaced by the safranin counterstain.

3.2.7.2 Catalase Test

The catalase test was conducted to distinguish *Staphylococcus* from *Streptococcus* by observing bubble formation resulting from the reaction of bacterial colonies with hydrogen peroxide (H_2O_2) (Reiner, 2010). Catalase enzyme neutralizes the bactericidal effects of H_2O_2 by catalysing its degradation into water and oxygen. To validate the results, *S. epidermidis* ATCC 35984 and *S. agalactiae* ATCC 13813 were used as positive and negative controls, respectively.

To perform the test, a 3% (v/v) H_2O_2 solution was prepared by diluting the 30% H_2O_2 into 90 mL of sterile distilled water. An isolated colony was then placed onto the glass slide and two to three drops of the 3% H_2O_2 solution were added to observe the bubble formation. A positive result, indicated by the formation of bubbles, is characteristic of *Staphylococcus*, whereas a negative result, indicated by the absence of bubbles, is typical of *Streptococcus* (Foster, 1996).

3.2.7.3 Coagulase Test

Coagulase tube test was conducted to differentiate coagulase-positive staphylococci (CoPS) and -negative staphylococci (CoNS). In this test, the ability of bacteria to produce coagulase enzyme converts fibrinogen in rabbit plasma into fibrin, resulting in clot formation (Giri, 2018). To validate the results, *S. aureus* ATCC 25923 was used as a positive control and *S. epidermidis* ATCC 35984 was used as a negative control.

To perform the test, an amount of 0.5 mL of the diluted rabbit plasma (BBL™ Coagulase with ethylenediamine tetra-acetate (EDTA)) was added to a sterile test tube. Two to three bacterial colonies were inoculated into the plasma and the tube was incubated at 37 °C. The samples were examined for clot formation after four hours; if negative, they were re-examined after six hours. A positive result, indicated by the formation of clot, is characteristic of CoPS, whereas a negative result, indicated by the absence of clot, is typical of CoNS.

3.2.8 Genomic DNA Extraction

Genomic DNA (gDNA) of bacterial isolates was extracted using HigherPurity™ Bacterial DNA Isolation Kit (Canvax biotech, Spain), with slight modifications to the manufacturer's instructions at the elution step. At this step, 40 µL of pre-warm nuclease-free water was added directly onto the minispin column, incubated at room temperature for five minutes and centrifuged at ≥ 6000 × g (8000 rpm) for another minute. The procedure was repeated with an additional 20 µL of pre-warm nuclease-free water to maximize the DNA yield. The gDNA was kept at −20 °C until further use.

The successful gDNA extraction was confirmed via agarose gel electrophoresis. A concentration of 1% agarose gel was prepared by dissolving 0.35 g of agarose powder in 35 mL of 1X TAE buffer and heating the mixture in a microwave until fully dissolved. Subsequently, a volume of 3.5 µL Visafe Red Gel Stain was added to the molten agarose before it was poured into a gel tray fitted with a comb. After the gel had solidified, the electrophoresis chamber was filled with 1X TAE buffer until the gel was completely submerged. A mixture of 2.5 µL gDNA and 0.5 µL DNA loading dye was then loaded into each well, with a 1kb DNA ladder included in the first well to estimate

the targeted sizes of the samples. The electrophoresis was run with the conditions of 80 V for 60 minutes and the gel was visualized under UV light using UV transilluminator (Bio-Rad, USA).

3.2.9 Species Identification

The identity of the *Staphylococcus* isolates was confirmed by polymerase chain reaction (PCR) amplification of the superoxide dismutase (*sodA*) primers: d1 (5′-CCITAYICITAYGAYGCIYTIGARCC-′3) and d2 (5′-ARRTARTAIGCRTGYTCCCAIACRTC-′3) (Abdul-Aziz *et al.*, 2015). The *sodA* gene was selected as the phylogenetic marker because it exhibits higher divergence than 16S rDNA, allowing for effective differentiation of closely related *Staphylococcus* species (Poyart *et al.*, 2001; Kosecka-Strojek *et al.*, 2019). A total volume of 25 µL PCR reaction mixture was prepared, consisting of 5 µL nuclease-free water, 12.5 µL MyTaq Red Mix, 2x (Bioline, UK), 1.25 µL of each primer and 5 µL of gDNA template. Conventional PCR was carried out using a Mastercycler (Eppendorf, Germany) under the following conditions: 95 °C initial denaturation for 3 minutes, followed by 30 cycles of 95 °C denaturation for 30 seconds, 37 °C primer annealing for 60 seconds and extension at 72 °C for 45 seconds, plus a final extension at 72°C for 10 minutes to ensure complete amplification of the target amplicons.

The successful *sodA* amplification was confirmed via agarose gel electrophoresis. A concentration of 2% agarose gel was prepared by dissolving 0.7 g of agarose powder in 35 mL of 1X TAE buffer and heating the mixture in a microwave until fully dissolved. Subsequently, a volume of 3.5 µL Visafe Red Gel Stain was added to the molten agarose before it was poured into a gel tray fitted with a comb. After the gel had solidified, the electrophoresis chamber was filled with 1X TAE buffer until the gel was completely submerged.

A volume of 2 µL of PCR products was loaded into each well, with a 100bp DNA ladder included in the first well to facilitate estimation of the amplicon sizes. A positive control strain, *S. haemolyticus* ATCC 29970, with an expected amplicon size of 480 bp, was also included. The electrophoresis was run at 70 V for 70 minutes and the gel was visualized under UV light using UV transilluminator (Bio-Rad, USA).

Following electrophoresis, the amplified PCR products were sent for Sanger sequencing. The sequenced data were aligned using BioEdit and further analyzed using

the Nucleotide Basic Local Alignment Search Tool (BLASTn) program against the GenBank database.

3.2.10 Antibiotic Susceptibility Test

The *Staphylococcus* isolates were subjected to antibiotic susceptibility testing (AST) using the Kirby-Bauer disk diffusion method, following the protocol by Clinical Laboratory Standards Institute (CLSI, 2023) that was described by Moreno *et al.* (2025). Overnight cultures were prepared in Mueller-Hinton Broth (MHB) from the fresh bacterial colonies grown on Tryptic Soy Agar (TSA) and incubated at 37 ˚C with shaking at 150 rpm for 16 to 18 hours. The cultures were subsequently diluted 1:100 in fresh MHB and incubated for approximately three hours to reach the exponential growth phase. The bacterial suspension was then adjusted to a turbidity equivalent to 0.5M McFarland standard (OD_{600} = 0.08 to 0.13), equivalent to approximately 1×10^8 CFU/mL (Daly *et al.*, 2017; Azharollah *et al.*, 2021).

The standardized inoculum was evenly lawned onto Mueller-Hinton Agar (MHA) plates. Antibiotic discs were then aseptically placed onto the inoculated agar surface. The antibiotics tested include gentamicin (10 μg), tetracycline (30 μg), sulfamethoxazole-trimethoprim (25 μg), cefoxitin (30 μg), penicillin G (10 μg) and erythromycin (15 μg). These antibiotic discs were selected based on their common use in the treatment of bovine mastitis, different antibiotic classes and their relevance in veterinary practices (Ali *et al.*, 2020; Saeed *et al.*, 2022; WOAH, 2025). The plates were subsequently incubated at 37˚C for 24 hours, after which zones of inhibition were measured. Isolates exhibiting resistance to at least three distinct antibiotic classes were classified as multidrug resistant (Pascu *et al.*, 2022). A negative control strain of *S. aureus* ATCC 25923 was employed for the AST, as it is sensitive to the majority of antibiotics (Treangen *et al.*, 2014).

3.2.11 Interpretation of Resistance Measurement for *Staphylococcus*

The diameter of the inhibition zones was measured using a calliper and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) (2023) guidelines in Table 3.3.

Table 3.3
Classification of Antibiotics and Interpretation of Resistance Measurement

Antibiotics	Class of Antibiotics	Disc Potency (μg)	Interpretive Categories and Zone Diameter Breakdown (mm)		
			Susceptible	Intermediate	Resistance
Cefoxitin	β-lactam	30	≥22 for *S. aureus* ≥25 for CoNS	-	≤21 for *S. aureus* ≤24 for CoNS
Erythromycin	Macrolides	15	≥23	14-22	≤13
Gentamicin	Aminoglycoside	10	≥15	13-14	≤12
Penicillin G	β-lactam	10	≥29	-	≤28
Sulfamethoxazole -trimethoprim	Sulphonamides	25	≥16	11-15	≤10
Tetracycline	Tetracycline	30	≥19	15-18	≤14

Source: (Clinical and Laboratory Standard Institute (CLSI), 2023)

3.2.12 Amplification of Antibiotic Resistance Genes

Screening for antibiotic resistance genes (ARGs) in Staphylococcaceae isolates was performed using primers listed in Table 3.4. Selection of the ARGs was based on prior studies identifying the resistance determinants responsible for phenotypic resistance in *Staphylococcus* spp..

A total volume of 12.5 μL of PCR reaction mixture was prepared, consisting of 3.5 μL of nuclease-free water, 6 μL of MyTaq Red Mix (2x, Bioline, UK), 0.5 μL of each primer and 2 μL of gDNA template. Conventional PCR was carried out using a Mastercycler (Eppendorf, Germany) under the following conditions: 94 °C initial denaturation for 5 min, followed by 30 cycles of 94 °C denaturation for 15 sec, primer-specific annealing temperatures for 15 sec and extension at 72 °C for 20 sec, plus a final extension at 72 °C for 5 min to ensure complete amplification of the target amplicons. The amplified PCR products were then analyzed on a 2% agarose gel in 1X TAE buffer, stained with Visafe Red Gel Stain, at 70 V for 70 minutes to confirm the presence of the targeted ARG amplicons.

Table 3.4
Primer Sequences, Target Genes, Amplicon Sizes and Cycling Conditions

Genes	Confers resistance to	Primer Sequence (5' to 3')	Size of Amplicon (bp)	Tm (°C)	Ref.
blaZ	Penicillin	F: 5'-ACTTCAACACCTGCTGCTTTC-3' R: 5'-TGACCACTTTTATCAGCAACC-3'	173	53	(Srednik *et al.*, 2017)
mecA	Cefoxitin	F: 5'-GTGAAGATATACCAAGTGATT-3' R: 5'-ATGCGCTATAGATTGAAAGGAT-3'	147	55	(Shi *et al.*, 2025)
mecD	Cefoxitin	F: 5'-TCCTTTAGCGATAGATGGTGAA-3' R: 5'-CTCCCATCTTTTCTCCATCCT -3'	867	59	(Schwendener *et al.*, 2017)
tetK	Tetracycline	F: 5'-GTAGCGACAATAGGTAATAGT-3' R: 5'-GTAGTGACAATAAACCTCCTA-3'	360	48	(Yang *et al.*, 2016)
tetM	Tetracycline	F: 5'-AGTGGAGCGATTACAGAA-3' R: 5'-CATATGTCCTGGCGTGTCTA-3'	158	48	(Yang *et al.*, 2016)
dfrA	Sulfamethoxazole-trimethoprim	F: 5' -CACTTGTAATGGCACGGAAA-3' R: 5' -CGAATGTGTATGGTGGAAAG-3'	270	57	(Shoaib *et al.*, 2023)
aaacA-aphD	Gentamicin	F: 5'-TAATCCAAGAGCAATAAGGGC-3' R: 5'-GCCACACTATCATAACCACTA-3'	227	55	(Ahmed *et al.*, 2020)

Note: Tm: annealing temperature; Ref: reference

3.2.13 Biofilm Formation Assay

Biofilm formation of the Staphylococcaceae isolates was determined using 96-well flat-bottomed, polystyrene tissue culture-treated microtiter plates following the protocol described by Stepanović *et al.* (2007). Fresh colonies of each isolate grown on TSA were suspended in 5 mL of Tryptic Soy Broth (TSB) and incubated at 37°C for 16 to 18 hours under static conditions. The bacterial inoculum was then adjusted to a turbidity equivalent to 0.5M McFarland standard (OD_{600}= 0.08 to 0.13), equivalent to approximately 1 X 10^8 CFU/mL (Daly *et al.*, 2017; Azharollah *et al.*, 2021). The standardized inoculum was subsequently diluted at 1:100 ratio in TSB supplemented with 1% (w/v) glucose.

A volume of 200 µL of the diluted inoculum was pipetted into three wells (triplicate) for each isolate. Plates were incubated at 37°C for 24 hours under static conditions to allow biofilm formation. Following incubation, the content was discarded by gentle flicking and each well was washed three times with 300 µL of sterile phosphate-buffered saline (PBS, pH 7.2). The plates were then inverted and air-dried. Adherent cells were fixed by adding 150 µL of methanol to each well and incubating

for 20 minutes. Then, the methanol was discarded and the plates were dried overnight at room temperature in an inverted position.

Biofilms were stained with 150 µL of 2% crystal violet dye for 15 minutes. The excess stain was discarded and the plates were rinsed under running tap water to remove any unbound stain. The plates were again air-dried at room temperature and the retained crystal violet was resolubilized by adding 150 µL of 95% ethanol to each well. To minimize evaporation, the plates were covered with lids and incubated at room temperature for 30 minutes without shaking. The optical density at 570 nm (OD_{570}) was measured using TECAN Infinite 200 PRO microplate reader.

A biofilm producer (OD_{pos}), *S. epidermidis* ATCC 35984 and a non-biofilm producer (OD_{neg}), *S. epidermidis* ATCC 12228, were used as positive and negative control strains, respectively (Azharollah *et al.*, 2021). All assays were performed in quadruple for each isolate and a control strain was also included to minimize errors and obtain reliable data analysis.

The biofilm-forming capacity of the isolates was evaluated using the cut-off optical density (ODc) as a standardized threshold. This threshold is commonly calculated as three standard deviations above the mean of a negative control (ODc = average OD of the negative control + (3 × ODc)). The final OD for each tested strain is calculated by subtracting the cut-off value (ODc) from the strain's average OD. Any resulting negative values are recorded as zero, whereas positive values indicate the presence of biofilm production. Based on their optical density at absorbance 570 nm (OD_{570}), isolates were classified as follows: non-biofilm producer ($OD \leq ODc$), weak biofilm ($ODc < OD_{570} \leq 2ODc$), moderate biofilm ($2ODc < OD_{570} \leq 4ODc$) and strong biofilm ($4ODc < OD_{570}$) (Stepanović *et al.*, 2007). The results were statistically validated using Student's t- test.

3.2.14 Phylogenetic Analysis

Multiple sequence alignment was performed using ClustalW in Molecular Evolutionary Genetic Analysis (MEGA12) (Kumar *et al.*, 2018). A phylogenetic tree was constructed using the Neighbour-Joining method with the Poisson model. The robustness of the tree topology was evaluated using 1,000 bootstrap replications. The tree was rooted using sequences of *Abyssicoccus albus* strain LIPI11-2-Ac043 (NR_146680.1) obtained from GenBank (Madhaiyan *et al.*, 2020).

3.2.15 Data Analysis

All data obtained was qualitatively and quantitatively analysed. The assembled data was recorded in Microsoft Excel. Statistical analyses were conducted using IBM SPSS Statistics version 20. Descriptive statistics, including frequencies, percentages, means and standard errors of the mean (SEM), were calculated to summarize the demographic and experimental data.

A non-parametric Mann-Whitney U test was employed to assess whether the number of recovered bacterial isolates differed significantly based on udder health status, as determined by the California Mastitis Test (CMT). Furthermore, a Spearman's rank correlation coefficient (r_s) was determined to evaluate the relationship between the degree of inflammatory response (CMT scores) and the total of bacterial isolates recovered.

Cohen's Kappa coefficient (κ) was subjected to assess the statistical agreement between phenotypic resistance patterns and the presence of resistance genes.

For the biofilm formation assay, optical density (OD_{570}) values were analyzed. The biofilm-forming ability of the isolates was compared to the negative control (*S. epidermidis* ATCC 12228) using a student's t-test. Isolates demonstrating a statistically significant increase in crystal violet retention ($p < 0.05$) compared to the negative control were classified as biofilm producers.

A Fisher's exact test was applied to determine whether a significant relationship exists between the categorical levels of biofilm production and the phenotypic antibiotic resistance patterns of the isolates. This analysis correlated the ordinal biofilm categories (non, weak, moderate, strong) with the discrete counts of phenotypic antibiotic resistance.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Detection of Bovine Milk Associated with Subclinical Mastitis

A total of 20 lactating bovines were sampled from a dairy farm in Malacca in August and October 2023. These bovines were initially screened for their udder appearance, representing a potential total of 80 udder quarters. Out of these 80 sampled udder quarters, nine quarters were observed to be non-secreting. This functional loss may be due to permanent tissue induration that destroys secretory alveoli and causes glandular atrophy (Dego & Vidlund, 2024). Consequently, the remaining 71 functional milk-yielding quarters were sampled and the collected milk was evaluated for subclinical mastitis (SCM) using the California Mastitis Test (CMT) reagent (Vetbiochem).

The CMT reagent comprises sodium lauryl sulfate and pH indicator, which serve as diagnostic functions when mixed with milk samples. As an amphiphilic molecule, sodium lauryl sulfate lyses the leukocyte cell membranes, releasing intracellular nuclear DNA that subsequently polymerizes to form a gelatinous matrix (Tanni *et al.*, 2021). The viscosity of this gel is directly proportional to the somatic cell concentration, serving as a qualitative indicator of inflammation severity (Peckler & Adcock, 2025). Concurrently, the pH indicator, bromocresol purple, detects pH alterations in the milk samples. While normal bovine milk is slightly acidic, mastitic milk shifts towards alkalinity due to an influx of blood bicarbonate and the reduced secretory activity of the mammary gland (Alnakip *et al.*, 2014; Nandi, 2021). Accordingly, positive reactions are characterized by both gel formation and a colour shift to deep purple. These resulting colour changes and coagulation reactions were observed and recorded, as tabulated in Table 4.1.

In this study, 56% (n = 40) of all milk-yielding quarters were detected as CMT-positive. To contextualize these findings within Malaysia, a high prevalence of SCM has consistently been reported in several local studies, although recent literature on the subject remains limited. For instance, a study conducted by Saeed *et al.* (2022) found that 31.4% (n = 74) of milk samples exhibited SCM based on the somatic cell counts (SCCs).

Table 4.1
Quarter-Level CMT Score for Milk Samples Obtained from 20 Examined Lactating Bovines from a Dairy Farm in Malacca, Malaysia

Bovine Tag	Right Rear	Right Front	Left Rear	Left Front	CMT
574	-	-	1	N	+ve
661	N	T	2	2	+ve
684	1	N	1	-	+ve
622	1	-	2	1	+ve
625	-	2	1	1	+ve
764	T	-	N	N	+ve
604	T	N	T	N	+ve
620	N	T	1	1	+ve
605	2	T	1	2	+ve
610	1	1	T	2	+ve
646	**N**	**N**	**N**	**N**	**-ve**
558	1	N	N	N	+ve
636	T	T	2	-	+ve
560	N	1	N	N	+ve
660	**N**	**N**	**N**	**N**	**-ve**
703	N	N	T	N	+ve
529	2	N	-	N	+ve
760	T	2	2	-	+ve
682	N	T	T	T	+ve
647	N	N	N	T	+ve

Note: CMT scores interpretation is based on Table 3.2. Abbreviation: (-): non-functional udder, N: negative; T: trace; 1: weak positive; 2: distinct positive. CMT categorization: (+ve): positive; (-ve): negative.

Another local study conducted in Selangor revealed a mastitis prevalence ranging from 70 to 90% across different farms using the CMT assay (Marimuthu *et al.*, 2014). Using the same method, Othman and Bahaman (2005) reported a SCM prevalence of 81.7% (n = 49). Broadening the scope to the regional level, another study reported SCM prevalence ranging from 20% to 80%, with an average of 50% across Southeast Asian countries (Bari *et al.*, 2022). In a global context, Mbindyo *et al.* (2021) reported a higher SCM prevalence of 73.1% (n = 289) on a Kenyan dairy farm. Interestingly, the quarter-level prevalence detected in the present study is closely similar to a previous study in Africa that detected 53% (n = 202) of CMT-positive samples when screened using the

same CMT method (Michira *et al.*, 2023). Collectively, these prevalence rates confirm that SCM remains a challenge across dairy-producing regions worldwide.

The high prevalence of SCM in local and global studies is possibly attributed to several factors such as host and environmental factors (Cheng & Han, 2020). Host risk factors, such as advanced parity, early or late lactation stage and breed-specific susceptibility, profoundly influence a bovine's vulnerability to infection (Khasapane *et al.*, 2023; Lestari *et al.*, 2025). Concurrently, environmental and management factors play a vital role in influencing the infection. These include a lack of hygiene practices during milking the cows and in the milking parlour, the use of contaminated equipment or contaminated water to clean the utensils, the animals and the storage containers used to store the milk (Cheng & Han, 2020; Qiu *et al.*, 2022). Ultimately, poor hygiene intensifies the presence of bacteria in the environment, facilitating bacterial colonization on bovines' streak canal. These pathogens can subsequently migrate upward into the gland cistern, particularly if the teat sphincter remains open during post-milking and between milking (Cobirka *et al.*, 2020). This continuous bacterial pressure stimulates the bovines' immune system, consequently leading to elevated SCC and a positive CMT score (Sumon *et al.*, 2020). Therefore, proper milking management and optimized environmental conditions are required to mitigate the continuous risk of infection and prevent the progression from subclinical to clinical stages. Specifically, proper milking management such as wearing clean gloves, sanitizing bovine udders and milking liners and fore-stripping initial milk streams is essential to minimize pathogens exposure.

Nevertheless, variability in prevalence exists; in which much lower quarter-level prevalence rates of 9.72% (n = 206) and 10.5% (n = 102) have been reported in recent studies conducted in Gansu Province, China and Kosovo, respectively (Wang *et al.*, 2024; Mazreku *et al.*, 2025). Similarly, a study in Ethiopia by Tezera and Ali (2021) documented a low SCM quarter-level prevalence of 14.85% (n = 218). The prevalence differences observed from these studies could be due to a few factors such as variability of geographical locations, regional farm management, animal density and environmental hygiene (Tanvi *et al.*, 2024; Wang *et al.*, 2024).

In this study, the positive CMT scores among milk-yielding quarters were distributed as 21% (n = 15) trace, 20% (n = 14) weak positive and 16% (n = 11) distinct positive, as tabulated in Table 4.2.

Table 4.2
Distribution of CMT Scores Across All Milk-Yielding Quarters

CMT Score	Frequency (n)
Negative (N)	31
Trace (T)	15
Weak positive (1)	14
Distinct positive (2)	11

Collectively, the presence of these high-scoring quarters (trace, weak and distinct positive) suggests that the sampled herd is experiencing inflammation and producing milk with a high somatic cell count (SCC) (Chowdhury *et al.*, 2025). Specifically, this elevated SCC indicates a robust immune response, driven by leukocytes migrating into the mammary gland to fight off pathogenic invasion or tissue injury. Given that this type of inflammation often occurs without visible abnormalities in the milk or udder, these high CMT scores reflect to a high prevalence of SCM within the herd. As a semi-quantitative indicator of inflammation, CMT scores exhibit a direct positive correlation with the average SCC and effectively visualize this invisible immune response (Mgonja *et al.*, 2023). Generally, the intramammary infection is established by a threshold of ≥ 200,000 cells per mL (Huang & Kusaba, 2022; Mgonja *et al.*, 2023). In this context, literature indicates that trace scores represent an early-stage inflammatory response with a slightly elevated SCC of ≥ 200,000 cells per mL. A weak positive (1) reflects a mild level of infection with SCC levels rising to ≥ 400,000 cells per mL, while a distinct positive (2) corresponds to serious mastitis infection with an estimated high SCC exceeding 1,200,000 cells per mL (Mgonja *et al.*, 2023; Sangwa *et al.*, 2025). The presence of 11 quarters at this highest level (2) confirms that this subset of quarters is experiencing severe, established inflammatory responses, which are critical targets for intervention to prevent further progression of SCM into clinical mastitis. This score reflects an elevation in SCC that reduces milk yield, compromises the quality and shelf life of raw milk and may lead to reduced renumeration from dairy processors, emphasizing the importance of effective mastitis control (Danieli *et al.*, 2025).

Any milk collected from udder quarter exhibiting a reaction of trace, weak positive or distinct positive will classify the bovine as SCM positive (Ramírez *et al*.,

2014; Mgonja *et al*., 2023). Therefore, based on the bovine level CMT status, 90% (n = 18) of the sampled Jersey-Friesian crossbreed bovines in this study were classified as having SCM, as tabulated in Table 4.1. This bovine-level SCM positive indicates a higher prevalence than those reported in previously. For instance, a study conducted by Camsing *et al.* (2024) in Thailand found that 76.2% (n = 64) of the crossbred Holstein bovines were SCM positive. In contrast, a couple of studies by Michira *et al.* (2023) and Mazreku *et al.* (2025) have reported lower bovine-level prevalence rates of 53% (n = 107) in Kenya and 22.3% (n = 54) in Kosovo, respectively. The study by Michira *et al.* (2023) assessed a mixed herd of indigenous, exotic and crossbred bovines, whereas Mazreku *et al.* (2025) evaluated predominantly the Holstein bovines (Red and Freisian). As noted by Mgonja *et al.* (2023), prevalence rates vary by breed, with a higher prevalence of 90.5% (n = 19) in Ayrshire-Friesian crossbreeds, compared to 62.3% (n = 33) in Friesians and 53.9% (n =14) in Ayrshire bovines. In another study conducted in East Java, Indonesia, Khasanah *et al.* (2021) reported a lower prevalence rate of 68.2% (n = 101) in their studied herd consisting of Holstein-Friesian crossbreed bovines. These differences may be attributed to the genetic composition of the crossbreeds, which may exhibit different levels of milk production, thereby affecting the risk of SCM (Bari *et al.*, 2022). Moreover, the variance in prevalence rates may be attributed to the differences in geographic locations and climates (Michira *et al.*, 2023). As noted by Amin *et al.* (2025), high humidity and temperature not only favour the proliferation of environmental pathogens in housing areas but may also induce heat stress, suppressing immune function and increasing susceptibility. Additionally, the widespread infection was likely facilitated by contagious transmission of mastitis pathogens between bovines during milking (Mramba & Mohamed, 2024). This risk is particularly exacerbated when infected bovines are milked before non-mastitis bovines (Mgonja *et al.*, 2023). Collectively, these findings suggest that the high SCM prevalence at the bovine level observed in this study is likely a cumulative result of environmental heat stress, milking management practices and the increased genetic susceptibility of the crossbred herd.

4.2 Isolation of *Staphylococcus* on Mannitol Salt Agar

Bacterial isolation was performed from 20 bovine milk samples, regardless of CMT status. As a result, a total of 153 presumptive *Staphylococcus* spp. isolates were

successfully obtained using Mannitol Salt Agar (MSA). Figure 4.1 depicts representative colony morphologies on MSA, comparing the reference controls (*S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228) with presumptive CoPS and CoNS isolates obtained.

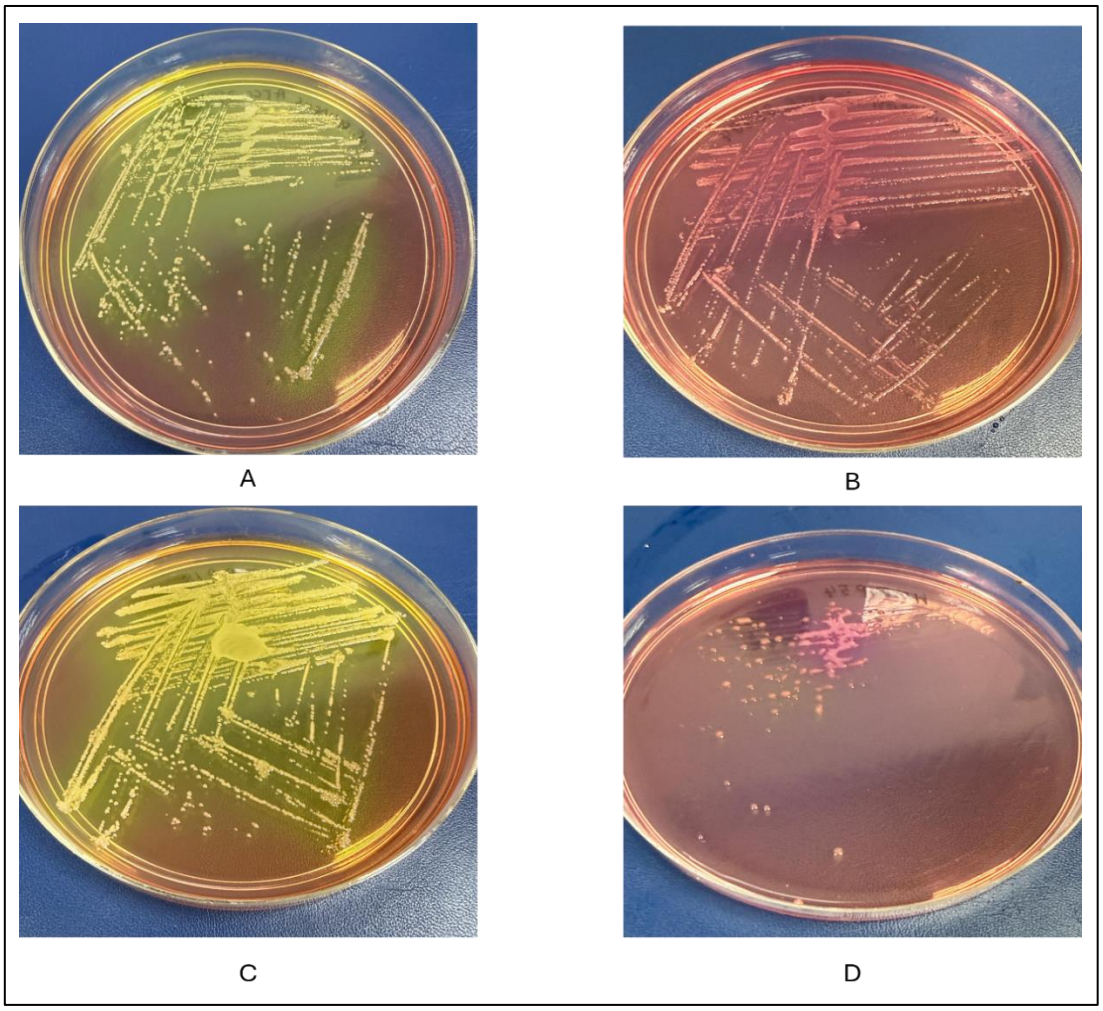


Figure 4.1 Colony Morphologies of *Staphylococcus* spp. on MSA. (A) *S. aureus* ATCC 25923 (CoPS Control Strain) (B) *S. epidermidis* ATCC 12228 (CoNS Control Strain) (C) Representative of Presumptive CoPS Isolates (AY622018) (D) Representative of Presumptive CoNS Isolates (BP647054)

To ensure the traceability of each bacterial isolate, a systematic alphanumeric nomenclature was established. This nomenclature included a first character denoting the sampling period ('A' for the first, 'B' for the second). The second character represents the colony morphology observed on MSA, with 'Y' for yellow colonies and 'P' for pink colonies. This alphabetic prefix is followed by the source animal's

numerical bovine tag and a sequential isolate number. For example, the designation AP647134 identifies the 134th isolate, which was recovered from bovine tag 647 during the first sampling period ('A') and exhibited a pink ('P') colony morphology on MSA.

MSA was chosen for isolation of *Staphylococcus* species due to its components as a selective and differential medium. Its high concentration of sodium chloride suppresses the growth of most other bacteria, allowing *Staphylococcus* to thrive (Ibrahim, 2020). Additionally, MSA contains phenol red, a pH indicator that distinguishes mannitol-fermenting bacteria from non-fermenting bacteria (Shields & Tsang, 2016). *S. aureus* is known as a mannitol fermenter, producing an acidic condition that turns the red colour of MSA into a yellow colour (Pumipuntu *et al.*, 2017). This is contradictory with CoNS, which does not ferment mannitol, resulting in the formation of red to pinkish colonies with the medium retaining its original red colour (Ayeni *et al.*, 2017). Colonies of both groups are morphologically similar, being smooth and convex; therefore, the key feature differentiating them on MSA is the colony colour change.

As mentioned above, 153 bacterial isolates were obtained and presumed as *Staphylococcus* spp.. The high recovery rate of *Staphylococcus* spp. in this study aligns with previous local studies identifying it as the predominant aetiological agent in both clinical and subclinical mastitis (Othman & Bahaman, 2005; Ali *et al.*, 2020; Saeed *et al.*, 2022). For instance, a study conducted by Marimuthu *et al.* (2014) in Selangor reported the highest prevalence of *Staphylococcus* spp. among other bacterial isolates at 55% within the studied herds. Another much recent study conducted by Saeed *et al.* (2022) in Kelantan and Pahang found 16.5% of the milk samples of subclinical bovine mastitis were harboured with *S. aureus*. Furthermore, a study conducted by Othman and Bahaman (2005) reported a prevalence of 28.6% (n = 36), which focused solely on the isolation of *S. aureus* from 60 bovine milk samples in Selangor and Johor. These findings are supported by global studies, which reported a prevalence of 55.3% (n = 52) (Mazreku *et al.*, 2025) and 39% (n = 341) *Staphylococcus* spp. (Song *et al.*, 2020) among other bacterial species in dairy herds.

Based on the colonial morphological characteristics, 84 isolates were classified as presumptive CoPS due to mannitol fermentation (yellow colonies), while 69 were classified as presumptive CoNS (pink or red colonies). A study conducted by De los Santos *et al.* (2022) demonstrated that prevalence of CoPS is higher than CoNS, with the rate of 81.2% (n = 39) and 18.8% (n = 9), respectively. Another study by Yang *et*

al. (2023), similarly reported CoPS higher than CoNS, with a prevalence of 51.8% (n = 172) and 48.2% (n = 160), respectively. CoPS, particularly *S. aureus*, are commonly associated with mastitis due to their contagious transmission and tendency to establish chronic infection (Kaczorek-Łukowska *et al.*, 2022). Their contagious nature allows them to spread readily from bovine to bovine, particularly during milking through contaminated teat cups. Meanwhile, the substantial presence of CoNS points to an environmental challenge (Ashraf & Imran, 2020). This group of bacteria is frequently isolated from the bovine teat apices, a site that has been identified as a primary reservoir for non-*aureus* staphylococci (NAS) strains (Shrimali *et al.*, 2025). These opportunistic pathogens can migrate into the gland cistern if the teat sphincter remains open post-milking or if pre-milking hygiene practice is inadequate (Traversari *et al.*, 2019). Once inside the mammary gland, these bacteria utilize various virulence factors that enable them not only to establish infection but also to persist over time (Gajewska & Chajęcka-Wierzchowska, 2020).

4.3 Correlation of CMT Status and Number of Recovered Isolates

To evaluate the relationship between SCM status and the likelihood of pathogen recovery, a Mann-Whitney U test was conducted. The results revealed no significant difference in the number of bacterial isolates recovered between 'CMT negative' and 'CMT positive' groups (p-value = 0.527). To further investigate whether the severity of the inflammatory response influenced pathogen recovery, a Spearman's rank correlation was applied across the maximum of CMT scores. Similarly, this demonstrated no significant positive correlation between increasing CMT scores and the number of recovered isolates (correlation coefficient; $r_s = 0.158$, p-value = 0.505). This lack of statistical significance is likely attributable to the small sample size of bovines (n = 20), which limits statistical power to differentiate between CMT groups. Nevertheless, this lack of significance validates the decision to culture samples from all bovines, as relying solely on CMT screening would have resulted in the oversight of bacterial isolates present during SCM.

As illustrated in Figure 4.2, all 18 bovines exhibiting a CMT positive status demonstrated a higher median of bacteria recovered (thick horizontal line), wider interquartile range (box) and greater variability (extended whiskers) compared to the negative group. However, this fluctuating pattern in the CMT-positive group revealed

no significant correlation with the categorical severity of infection. In current findings, several bovines (bovine tags 661, 574, 684, 764, 605, 529, 682 and 647) exhibited trace (T) to distinct positive (2), indicating elevated SCC levels consistent with SCM. However, milk from these quarters yielded few (< 6) or no bacterial colonies. This suggests that positive CMT status, reflective of high SCC, does not necessarily correlate with the existence of bacteria in the milk (Yazdankhah *et al.*, 2001; Stanek *et al.*, 2024). This variability may be attributed to human subjectivity during evaluation of the CMT reaction, leading to inconsistent sensitivity and prediction accuracy of SCM (Stanek *et al.*, 2024). Notably, an outlier in the CMT positive group (bovine tag 625) yielded 24 colonies. The high number of bacteria colonies likely reflects the high bacterial concentration in milk from the infected bovines. This pattern presumably possesses a greater risk for mastitis transmission to other bovines during milking (Kandeel *et al.*, 2018; Krebs *et al.*, 2023). In contrast, CMT negative screening (bovine tag 660 and 646) showed low medians with minimal bacterial recovery. Specifically, eight bacterial colonies were obtained from the milk of bovine tag 660, while no bacterial colonies were recovered from bovine tag 646. The presence of these colonies likely reflects a low bacterial load that induced only a minimal rise in SCC, remaining below the diagnostic cut-off value (≥ 200,000 cells per mL) (Huang & Kusaba, 2022; Mgonja *et al.*, 2023). This discrepancy is likely due to factor of early-stage infection (Khasapane *et al.*, 2023). At the early stages of infection, the immune response has not yet peaked, resulting in negative CMT status despite bacterial presence (Egyedy & Ametaj, 2022). Moreover, recovery of bacteria from CMT negative milk may suggest that the bacterial isolates had not adequately colonized the intramammary tissue to trigger inflammatory response (Ozbey *et al.*, 2022). Therefore, recognition of these bacterial isolates as potential mastitis pathogens is essential for maintaining healthy intramammary tissue, thereby reducing the occurrence of mastitis and the resulting adverse effects on animal health, milk quality and farm profitability.

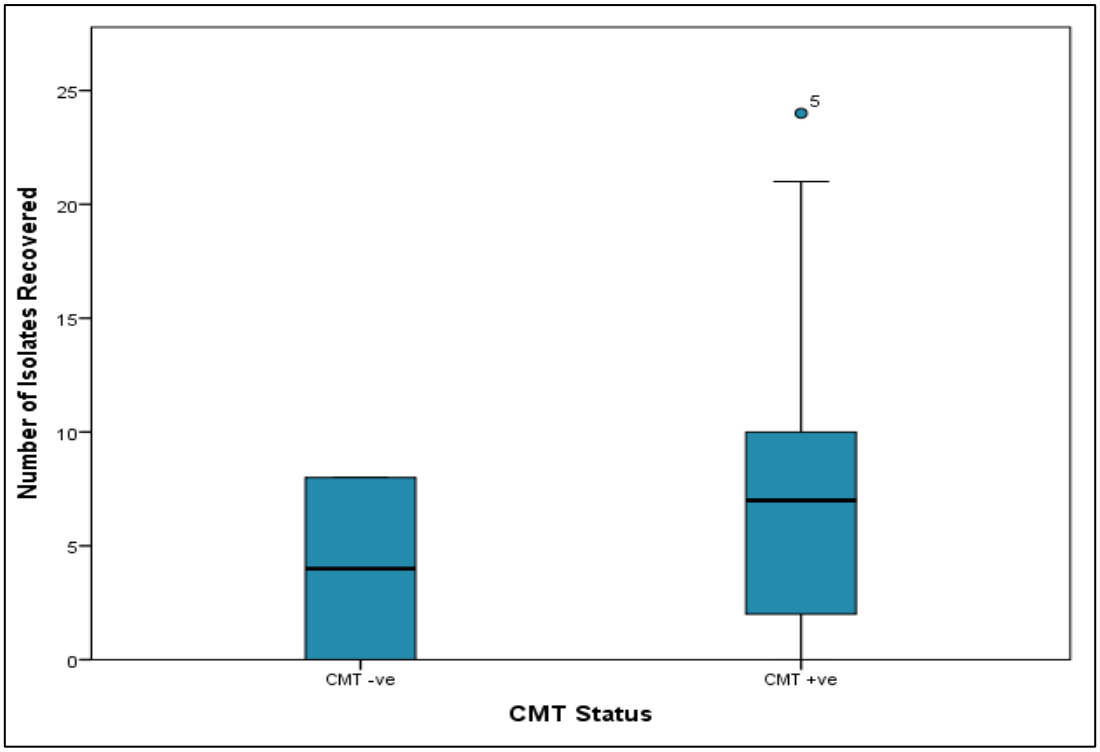


Figure 4.2 Distribution of Total Bacterial Colonies Recovered from Each Bovine by CMT Result

Notes: The boxplot displays the median (thick horizontal line) and range (whiskers) for each group. Circles (°) indicate statistical outliers, representing a specific bovine (bovine 625) with elevated bacterial counts (n = 24) that deviate from the group profile.

4.4 Biochemical Characterisation of *Staphylococcus*

The presumptive CoPS and CoNS isolates were subjected to standard biochemical tests, including Gram staining, catalase and coagulase. Table 4.3 summarizes the biochemical test results.

Table 4.3
Summary of Biochemical Characteristics for Presumptive Staphylococci Isolates (n = 153)

Biochemical Tests	Observation	Interpretation	No. of Isolates (n)	Percentage (%)
Gram staining	Purple cocci in clusters	Gram-positive	153	100
Catalase test	Bubble formation	Positive	153	100
Coagulase test	Visible clot formation	Positive (CoPS)	71	46.4
	No clot formation	Negative (CoNS)	82	53.6

For Gram staining, all presumptive *Staphylococcus* isolates appeared purple and arranged in grape-like clusters, consistent with the positive control strain, *S. aureus* ATCC 25923. A Gram-negative control, *E. coli* ATCC 25922, was also included; as expected, it stained red and exhibited a rod-shaped morphology, distinct from the coccoid bacterial isolates.

The Gram staining results confirmed the preliminary identification of the isolates as *Staphylococcus* spp. The retention of crystal violet dye by the isolate is characteristic of Gram-positive bacteria, attributable to the thick peptidoglycan layer in their cell walls, which retains the crystal violet-iodine complex (Tripathi & Sapra, 2021). This effectively differentiated the isolates from Gram-negative bacteria, such as *E. coli*, where the thinner peptidoglycan layer allows decolorization and counterstaining with safranin. Furthermore, the observation of grape-like clusters is a key morphological feature distinguishing *Staphylococcus* from other Gram-positive mastitis pathogens, such as *Streptococcus* spp., which typically appear in chains.

For catalase test, all presumptive *Staphylococcus* isolates exhibited a positive catalase reaction, determined by vigorous bubble formation upon the addition of hydrogen peroxide. This result was consistent with the positive control strain, *S. aureus* ATCC 25923. Conversely, the negative control *S. agalactiae* ATCC 13813 demonstrated no catalase activity as indicated by the absence of bubble formation, validating the reliability of the test procedure used.

The catalase test served as a differentiation method to distinguish *Staphylococcus* species from other Gram-positive cocci, particularly *Streptococcus* species, which are also common mastitis pathogens (Khatoon *et al.*, 2022). The positive reaction confirms that the isolates produce the enzyme catalase, which facilitates the breakdown of hydrogen peroxide into water and oxygen. This release of oxygen gas is visually observed as vigorous bubbling. The distinction of *Staphylococcus* is catalase-positive, while *Streptococcus* is catalase-negative, which validates the identity of the isolates and eliminates the possibility of contamination by streptococci.

A coagulase test was performed to definitively distinguish the identity of the presumptive staphylococcal isolates. This test distinguishes CoPS from CoNS, an essential distinction for evaluating potential pathogenicity. The mechanism involves the bacterial enzyme coagulase interacting with the prothrombin in the plasma to form a staphylothrombin, which converts soluble fibrinogen into insoluble fibrin, resulting in a visible clot (Giri, 2018). This ability to clot plasma is a virulence factor, often

correlating with the pathogenicity of *S. aureus*, distinguishing it from CoNS species, which typically lack this enzymatic activity (Michalik *et al.*, 2020).

Of the 84 isolates initially screened as presumptive *S. aureus* (CoPS) on MSA, only 71 isolates exhibited a positive coagulase reaction, as demonstrated by the formation of a plasma clot, which is consistent with the positive control strain, *S. aureus* ATCC 25923. While the remaining 13 isolates initially appeared positive on MSA, failed to clot plasma, indicating they were mannitol-fermenting CoNS rather than *S. aureus* and hence were reclassified as CoNS. This discrepancy between MSA screening and biochemical characterization of the coagulase test highlights the limitations of identifying *Staphylococcus* spp. isolates based solely on mannitol fermentation. This may be due to bacterial colonies possessing the metabolic pathways for mannitol fermentation that can mimic the appearance of *S. aureus* on primary culture media (Kateete *et al.*, 2010). This mimicry shows a diagnostic challenge, as it can lead to misidentification and subsequent therapeutic failure (Ayeni *et al.*, 2017). In conclusion, the reclassification these isolates have resulted in a total of 71 CoPS and 82 CoNS, consistent with their respective control strains of *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984.

4.5 Molecular Confirmation via *sodA* Gene

From the total of 153 presumptive *Staphylococcus* isolates, 78 were selected and subjected to molecular identification via partial *sodA* gene sequencing. This selection was based on the pre-initial screening of antibiotic resistance profiles showing resistance to at least one tested antibiotic (Appendix 1). The *sodA* gene was selected as phylogenetic marker because it exhibits higher divergence than 16S rRNA, allowing for effective differentiation of closely related *Staphylococcus* species (Poyart *et al.*, 2001; Kosecka-Strojek *et al.*, 2019). As shown in Figure 4.3, successful amplification was confirmed by the presence of a distinct DNA band at approximately 480 bp. The validity of PCR assay was verified by the positive control strain, *S. haemolyticus* ATCC 29970 (Lane 1), which exhibited the targeted band size, whereas the negative control (Lane 15) showed no amplification, confirming the reaction mixture was free from contamination.

In the present study, all 78 isolates were successfully amplified for *sodA* gene, confirming the presence of this target sequence within their genome. The current

findings mirrored the findings by Sivadon *et al.* (2004), who reported a 100% success rate (n = 168) in classifying *Staphylococcus* species. Therefore, this finding may verify the *sodA* gene as a reliable marker for staphylococcal identification.

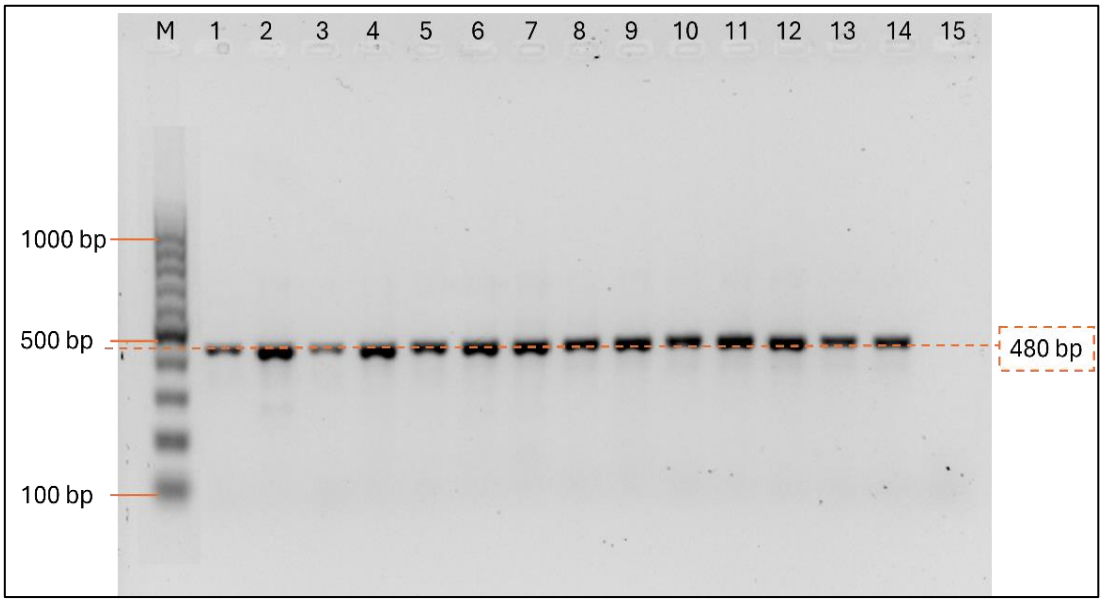


Figure 4.3 PCR Amplification of *sodA* Gene (480 bp) in Representative Presumptive Staphylococci Isolates. M: 100bp DNA Ladder (Vivantis), Lane 1: *S. haemolyticus* ATCC 29970, Lane 2 to 14: Representative of Tested Isolates (AY625098, AP625042, AP625045, AP625048, AP620080, AP620081, AP620082, BY760095, AP620079, AP620083, AY625084, AP620088, AP620089, Respectively); Lane 15: Negative Control

4.6 Sequence Analysis and Species Identification using BLASTn

Following the successful amplification of the *sodA* gene, all positive amplicons were subjected to sequencing. The obtained nucleotide sequences were trimmed and analyzed for species identification using the Basic Local Alignment Search Tool for nucleotides (BLASTn) available on the National Center for Biotechnology Information (NCBI) database. The BLASTn analysis compared the query nucleotide sequences against the reference sequences in GenBank. Species identification was established based on the highest percentage of max identity (typically ≥ 99%) and a query coverage of near 100%, accompanied by an E-value of 0.0, which indicates a statistically significant match (Alzohairy, 2024). Based on the BLASTn alignment, the sequence data definitively confirmed that all 78 isolates belong to the family Staphylococcaceae. Specifically, the family-level identification revealed three distinct genera: 51.3% (n =

40) as *Staphylococcus* spp., 16.7% (n = 13) as *Macrococcoides bohemicum* and 32.1% (n = 25) as *Macrococcus caseolyticus*. The detailed accession number and percentage identity for each isolate are summarized in Appendix 2. The high percentage identity observed (≥ 92%) across all samples validates that the *sodA* gene sequence possesses sufficient polymorphism to accurately distinguish between closely related staphylococcal species (Poyart *et al.*, 2001; Kosecka-Strojek *et al.*, 2019).

4.6.1 Identification of *Staphylococcus* spp.

Among 78 sequenced isolates, 51.3% (n = 40) were confirmed as members of the genus *Staphylococcus*. Within this genus, CoNS were dominant, comprising 95% (n = 38) of the total identified *Staphylococcus* spp., while *S. aureus* constituted only 5% (n = 2). These isolates exhibit high sequence identities between 95% to 99% with GenBank reference strains. Subsequent BLASTn analysis revealed a high degree of species diversity, identifying six distinct species within this *Staphylococcus* genus. As detailed in Appendix 2, *Staphylococcus chromogenes* was the most predominant species, accounting for 28.2% (n = 22) of the total sequenced isolates. This was followed by 9% (n = 7) identified as *Staphylococcus haemolyticus* and 6.4% (n = 5) as *Staphylococcus sciuri*. Other CoNS identified included 3.9% (n = 3) *Staphylococcus simulans* and 1.3% (n = 1) *Staphylococcus warneri*. In bovine mastitis research, species such as *S. chromogenes*, *S. haemolyticus*, *S. simulans* and *S. warneri* are increasingly referred to as non-*aureus* staphylococci (NAS), rather than CoNS. While these species were historically classified based on their negative reaction to coagulase test, the NAS terminology provides a more relevant and clearer classification of staphylococcal species associated with bovine mastitis (De Buck *et al.*, 2021). Furthermore, this reclassification reflects their emerging role as significant clinical pathogens in SCM rather than mere commensals (Ruiz-Romero & Vargas-Bello-Pérez, 2023). Since majority of these isolates are categorized as environmental pathogens, their diversity within the herd reflects the ubiquitous presence of these isolates within the dairy farm environment.

Notably, *S. chromogenes* has emerged as an opportunistic pathogen responsible for intramammary infections, particularly bovine mastitis (De los Santos *et al.*, 2022). Although reported prevalence rates vary across the literature due to differences in diagnostic protocols, the predominance of *S. chromogenes* observed in this study is

consistent with global trends. For example, in Argentina, Srednik *et al.* (2017) reported that 46.2% (n = 43) of mastitis associated isolates were identified as *S. chromogenes* via molecular targeting of *gap* gene (which encodes for glyceraldehyde-3-phosphate). Furthermore, Taponen *et al.* (2008) reported that 50% (n = 29) of the isolates in Finland were identified as *S. chromogenes* using phenotypic API Staph ID 32 tests. Similarly, a study conducted by Kim *et al.* (2019) in Korea demonstrated the highest isolation rate of 34.4% (n = 107) *S. chromogenes* among other CoNS associated with bovine mastitis. However, Waller *et al.* (2023) reported a lower prevalence of 47.1% (n = 105) *S. chromogenes* compared to 52.9% (n = 118) *S. simulans* in Sweden. The difference in the prevalence of *S. chromogenes* is likely attributed to their study design, which solely targeted the isolation of *S. chromogenes* and *S. simulans* (Waller *et al.*, 2023). Despite regional variations, these findings collectively reflect that *S. chromogenes* remains a major NAS associated with bovine mastitis.

The recovery of other CoNS species in this study correlates with other global findings. *S. haemolyticus* and *S. simulans* have been extensively cited as mastitis pathogens (Idamokoro, 2022). In addition, *S. sciuri* and *S. warneri* can also be found in bovine milk associated with mastitis, which is of particular concern given their potential as zoonotic pathogens (Ruiz-Romero & Vargas-Bello-Pérez, 2023; Ravaioli *et al.*, 2024; Zhang *et al.*, 2024). A study conducted in Korea found the presence of *S. haemolyticus*, *S. sciuri*, *S. simulans* and *S. warneri* in bovine milk (Kim *et al.*, 2019). In Iran, molecular identification targeting the *gap* gene revealed the presence of 40.7% (n = 44) *S. haemolyticus*, 15.7% (n = 17) *S. chromogenes*, 5.5% (n = 6) *S. simulans* and 10.2% (n = 11) *S. warneri* in bovine milk associated with mastitis (Hosseinzadeh & Saei, 2014). In their findings, *S. haemolyticus* and *S. chromogenes* emerged as the most frequently isolated CoNS pathogens (Hosseinzadeh & Saei, 2014). Recent findings by Getahun *et al.* (2024), further corroborate these findings, confirming that these CoNS species are common in bovine milk samples. The variation in isolation rates of CoNS species can be attributed to differences in geographical region and herd management practices (Rahman *et al.*, 2026). The dominance of CoNS over *S. aureus* observed in this herd represents an epidemiological shift with implications for herd management and therapeutic strategies due to distinct virulence factors and genetic diversity possessed by the species (Dego & Vidlund, 2024). For example, *S. chromogenes* and *S. haemolyticus* are uniquely adapted to the bovine udder as they possess virulence factors, such as the ability to form biofilms and produce α-haemolysin and protease,

which enable effective colonization of the teat canal (Beuckelaere *et al.*, 2021; Dego & Vidlund, 2024). The ability of CoNS to form biofilm poses severe therapeutic challenges, as biofilm matrices impair antibiotic penetration, rendering standard mastitis treatment ineffective and promoting chronic infection. However, the current study does not focus on the virulence genes and therefore, no information on the genetic variations of CoNS species is provided. Consequently, the diversity of species identified necessitates a targeted management strategy that addresses the presence of CoNS species.

In this current finding, *S. aureus* constituted only 2.6% (n = 2) of the total sequenced isolates. This is in contrast to the literature that reported *S. aureus* as one of the main agent of bovine mastitis, either clinical or subclinical (Touaitia *et al.*, 2025). The scarcity of *S. aureus* implies a minimized risk of chronic, biofilm-associated mastitis cases that are difficult to treat (Fidelis *et al.*, 2024). In a local study conducted in East Coast Malaysia, however, *S. aureus* was identified with a higher prevalence of 16.5% (n = 39) (Saeed *et al.*, 2022). Regionally, finding in this study mirrored the finding from neighbouring country of Thailand, where Pumipuntu *et al.* (2019) reported a lower prevalence 14% (n = 32) of CoPS compared to 86% (n = 197) of CoNS. Another study conducted by Eid *et al.* (2022) in Egypt, revealed a prevalence of 16.7% (n = 50) of *S. aureus* found in bovine milk. Variance in the prevalence rate across different studies may be driven by herd size, milking hygiene practices and awareness of disease transmission (Markos *et al.*, 2023). The low recovery of *S. aureus* suggests that contagious mastitis control measures on the farm, such as post-milking teat dipping and segregation, may be effective. Nevertheless, regular surveillance is required to prevent the re-introduction and transmission of this contagious pathogen and monitor for any emerging reservoir of chronic infections (Gonal *et al.*, 2025; Taylor & Unakal, 2025).

4.6.2 Identification of Other Staphylococcaceae Family

A substantial proportion of 48.7% (n = 38) isolates were identified as members of the *Macrococcus* genus. Specifically, 32.1% (n = 25) were classified as *Macrococcus caseolyticus*, while 21.8% (n = 13) were identified as *Macrococcoides bohemicum*, as detailed in Appendix 2. These isolates exhibit high sequence identities, ranging from 92% to 99%, with GenBank reference strains. BLASTn analysis provided species-level differentiation, classifying the isolates into two distinct species within the genus.

Although phylogenetically distinct, *M. caseolyticus* and *M. bohemicum* share a niche as common animal commensals and have recently emerged as opportunistic pathogens (Foster & Paterson, 2020; Zhang *et al.*, 2022). Their presence in this study reflects the ecological adaptation of these species to the bovine udder and the surrounding dairy environment. However, epidemiological data linking these species to bovine mastitis remains scarce. Only a limited study, such as Schwendener *et al.* (2017), has found a few *M. caseolyticus* isolated from bovine mastitis but not *M. bohemicum*. The detection of *M. caseolyticus* in this study may be linked to its ecological adaptation to dairy environments and its potent casein-hydrolyzing capabilities, which may enhance its persistence recovery from bovine milk samples (Mazhar *et al.*, 2020). In contrast, *M. bohemicum* is less frequently associated with bovine mastitis and has been predominantly reported as an opportunistic human associated pathogens, making its isolation from bovine milk relatively uncommon (Mašlaňová *et al.*, 2018). The detection of these two *Macrococcus* species in the bovine milk is noteworthy and contrasts with previous global studies, as they are relatively uncommon and are generally reported as opportunistic species, rather than predominant mastitis-associated pathogens. This discrepancy is likely attributable to the limitations of phenotypic identification in earlier studies (Ayeni *et al.*, 2017; Klempt *et al.*, 2022). Due to their phenotypic resemblance to *Staphylococcus* spp., such as being capable of growing on high salt concentrations of MSA, catalase positive and coagulase negative (Carroll *et al.*, 2023; Zhan *et al.*, 2026), *Macrococcus* isolates are frequently misidentified as CoNS when molecular confirmation is not employed. Moreover, the unexpected high prevalence of *M. bohemicum* suggests that this under-reported species may act as a silent aetiological agent of intramammary infection and an overlooked genetic reservoir for antibiotic resistance. This is due to the almost similar phenotypes of *Macrococcus* spp. to CoNS. These findings validate the necessity of employing molecular targets to overcome taxonomic uncertainty, ensuring precise identification where phenotypic methods often fail.

4.7 Phylogenetic Tree Analysis

A total of 78 partial *sodA* sequences of the isolates and an outgroup of *Abyssicoccus albus* (NR 146680.1) from GenBank were included in the Staphylococcaceae phylogenetic tree using the Neighbour-Joining method. As

illustrated in Figure 4.4, the phylogenetic tree demonstrated that all 78 isolates were clustered into well-resolved, species-specific clades. The clear separation of the *Abyssicoccus albus* outgroup confirms the appropriateness of the selected marker, evolutionary model (p-distance, Poisson) and tree-building approach for this dataset (Sivadon *et al*., 2004; Madhaiyan *et al*., 2020). The phylogenetic tree revealed two distinct lineages within the *Staphylococcus* and *Macrococcus* genera, likely validating the utility of the *sodA* gene in distinguishing between these closely related genera within the Staphylococcaceae family. This separation into major clades reflects their distinct phylogenetic positions despite shared metabolic pathways and a reported genomic homology of approximately 65% gene similarity (Mašlaňová *et al.*, 2018).

Within the *Staphylococcus* lineage, the isolates were clustered into six distinct species-specific clades. *S. chromogenes* formed a distinct and well-supported clade, which formed a monophyletic group supported by a high bootstrap value of 100%. This observation highlights the low intraspecific variability in *sodA* and reinforces the reliability of this marker for the molecular identification of these isolates (Poyart *et al.*, 2001; Sivadon *et al.*, 2004). Interestingly, *S. simulans*, *S. aureus*, S. *haemolyticus* and *S. warneri* were positioned on separate branches, further validating sufficient *sodA* divergence to distinguish these staphylococci. Despite this divergence, the results remain consistent with their relatedness observed in multi-locus and whole-genome analyses of bovine and clinical staphylococci (Naushad *et al.*, 2016; Petzer *et al.*, 2022). The close clustering of *S. aureus* and *S. simulans* is supported by a 100% bootstrap value, which is consistent with *sodA*-based phylogenies that group them within shared lineages alongside *S. haemolyticus*-related species (Ghebremedhin *et al.*, 2008). This observed relatedness is likely driven by horizontal gene transfer of the *sodA* gene from CoNS to *S. aureus* (Poyart *et al.*, 2001). Furthermore, *S. warneri* and *S. haemolyticus* demonstrated a clear phylogenetic affinity, mirroring their shared *S. epidermidis* group via conserved *sodA* region, rather than *S. aureus* proximity (Kosecka-Strojek *et al.*, 2019). This relationship highlights the complexity of species complexes and associated diagnostic challenges.

Notably, *S. sciuri* branched off independently with a bootstrap value of 100%, separated from other staphylococci. This observation is consistent with the evolution of the *S. sciuri* group, which was originally classified within the *Staphylococcus* genus but has recently been reclassified into genus *Mammaliicoccus* (as *Mammaliicoccus sciuri*) (Osada *et al.*, 2022; Zakaria *et al.*, 2026).

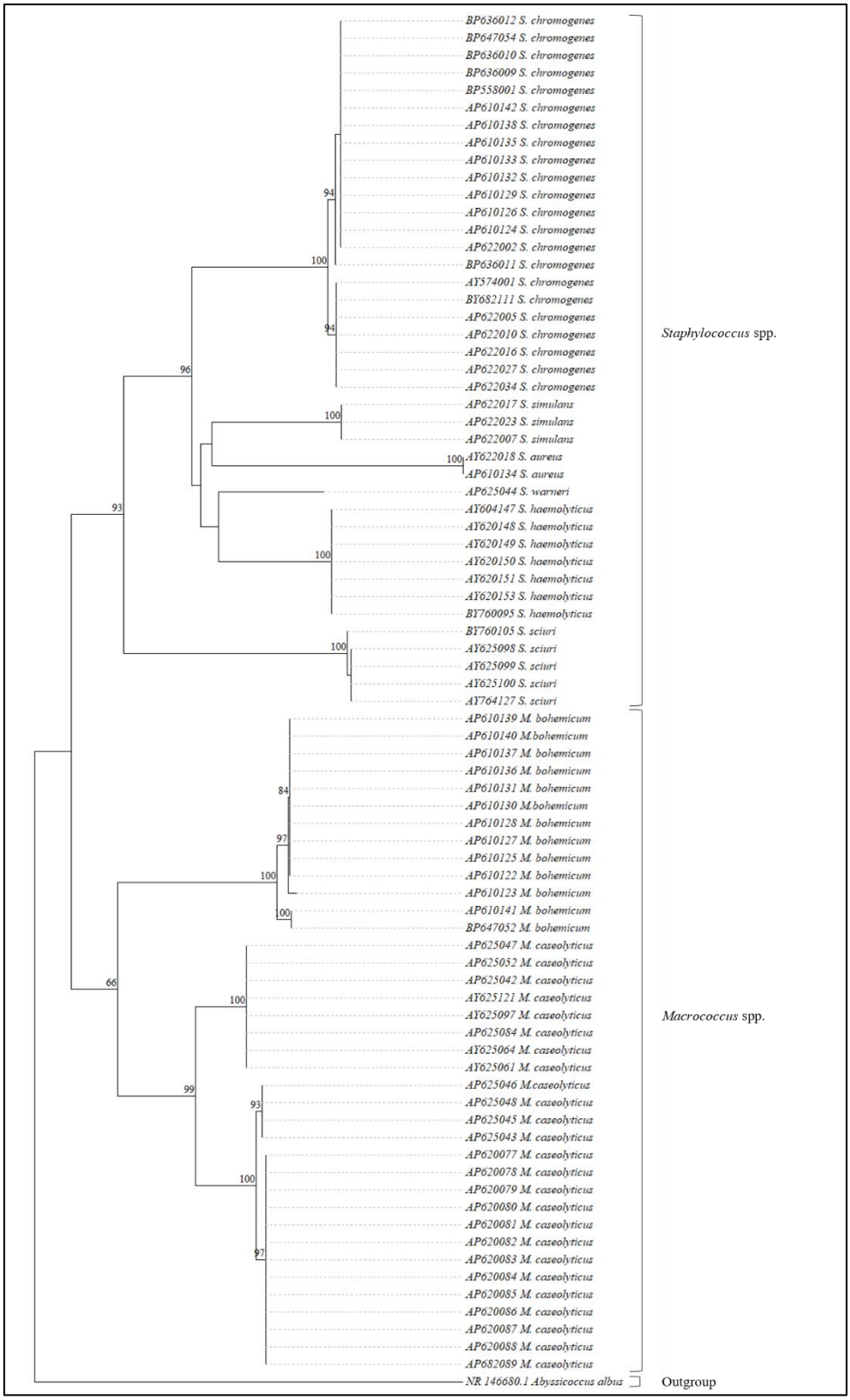


Figure 4.4 Neighbour-Joining Tree Inferred from Partial *sodA* Gene Sequences of 78 Isolates

Notes: *Abyssicoccus albus* (NR 146680.1) was used as the outgroup. The analysis was conducted in MEGA12 using the p-distance (Poisson model) with 1000 bootstrap replicates (values > 65% shown above branches). The scale bar represents 0.05 nucleotide substitution per site.

Therefore, following the reclassification, this species will be referred to as *M. sciuri*, from now on throughout this study. This reclassification is due to genomic divergence, novobiocin resistance and positive oxidative activity, as it is the sole species in the group possessing cytochrome c in its electron transport systems (Poyart *et al.*, 2001; Madhaiyan *et al.*, 2020). Furthermore, while *M. sciuri* was primarily considered as a non-pathogenic commensal, it is increasingly recognized as an opportunistic pathogen in both dairy animals and humans (Omer *et al.*, 2025; Zakaria *et al.*, 2026). Consequently, the clear separation of this clade suggests that *M. sciuri* possesses adaptive traits that allow it to persist in the dairy environment.

Within the *Macrococcus* lineage, *M. bohemicum* clades formed a strongly supported monophyletic cluster with bootstrap values ranging from 84% to 100%. Although these strains were clustered together within *M. bohemicum* clade, the presence of internal branching with bootstrap value of 100% (AP610141 and BP647052) suggests strain-level variations in partial *sodA* sequences. These variations, such as single-nucleotide polymorphisms, insertions or deletions of nucleotide bases, likely accumulated via host adaptation or microevolution (Carroll *et al.*, 2023). Meanwhile, *M. caseolyticus* was grouped in an adjacent but distinct clade, reflecting the close yet clearly differentiated relationship between these two species, as reported in comparative genomic studies (Mašlaňová *et al.*, 2018). Moreover, intraspecific branching with high bootstrap values of 93% and 100% were observed within *M. caseolyticus* strains, suggesting genomic heterogeneity. Prophage insertions and chromosome inversions may facilitates *sodA* divergence within this species (Poyart *et al.*, 2001; Moura de Sousa *et al.*, 2021). Collectively, the phylogenetic analysis based on the *sodA* gene indicates that this gene provides robust resolution at the species level for both *Macrococcus* spp. and *Staphylococcus* spp. with high bootstrap values for the major nodes supporting the overall stability of the inferred relationships (Naushad *et al.*, 2016). Consequently, these results contribute to a reliable confidence for the subsequent ecological and epidemiological interpretations based on these isolates.

4.8 Antibiotic Sensitivity Profile of Staphylococcaceae

To determine the antibiotic resistance profiles of the isolates, all 78 identified Staphylococcaceae isolates were subjected to antibiotic susceptibility testing via Kirby-Bauer disc diffusion assay against six antibiotics. The antibiotics were selected based

on their different classes that are standard therapeutic options for bovine mastitis treatment: erythromycin (E15), penicillin G (P10), cefoxitin (FOX30), tetracycline (TE30), trimethoprim-sulfamethoxazole (SXT25) and gentamicin (CN10). Isolates were classified into resistant (R), intermediate (I) or susceptible (S) according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The validity of the assay was confirmed using reference quality control strains. *S. aureus* ATCC 25923 exhibited susceptibilities to all tested antibiotics, while *S. epidermidis* ATCC 35984 displayed the expected multidrug-resistant phenotype (E15-P10-FOX30-SXT25-CN10). The antibiotic resistance profiles for each species in Staphylococcaceae are summarized in Table 4.4, while the detailed resistance profiles for each genus are illustrated in Figure 4.5 and Figure 4.6. The raw antibiotic susceptibility testing data are tabulated in Appendix 3. Representative disc diffusion assay demonstrating the zone of inhibition patterns of Staphylococcaceae isolates are provided in Appendix 4.

Table 4.4
The Resistance Profile of Staphylococcaceae Isolates Associated with Bovine Subclinical Mastitis Against Six Antibiotics Tested

Isolates	No. of Isolates (n)	No. of Isolates Resistant Against the Tested Antibiotics (%)					
		E15	P10	FOX30	TE30	SXT25	CN10
***Staphylococcus* spp.**							
S. aureus	2	0 (0)	**2 (100)**	0 (0)	0 (0)	0 (0)	0 (0)
S. chromogenes	22	0 (0)	**22 (100)**	**1 (5)**	**6 (27)**	**3 (14)**	0 (0)
S. haemolyticus	7	0 (0)	**7 (100)**	0 (0)	**1 (14)**	0 (0)	0 (0)
S. simulans	3	0 (0)	**3 (100)**	0 (0)	**1 (33)**	0 (0)	0 (0)
S. warneri	1	0 (0)	**1 (100)**	0 (0)	0 (0)	0 (0)	0 (0)
***Mammaliicoccus* spp.**							
M. sciuri	5	0 (0)	**5 (100)**	**1 (20)**	**1 (20)**	0 (0)	0 (0)
***Macrococcus* spp.**							
M. bohemicum	13	0 (0)	**13 (100)**	0 (0)	0 (0)	0 (0)	0 (0)
M. caseolyticus	25	0 (0)	**25 (100)**	**15 (60)**	0 (0)	0 (0)	**1 (4)**
Total isolates	78	0 (0)	78 (100)	17 (22)	9 (12)	3 (4)	1 (1)

Notes: No.: Number; E15: erythromycin (15 µg), P10: penicillin G (10 µg), FOX30: cefoxitin (30 µg), TE30: tetracycline (30 µg), SXT25: sulfamethoxazole-trimethoprim (25 µg), CN10: gentamicin (10 µg).

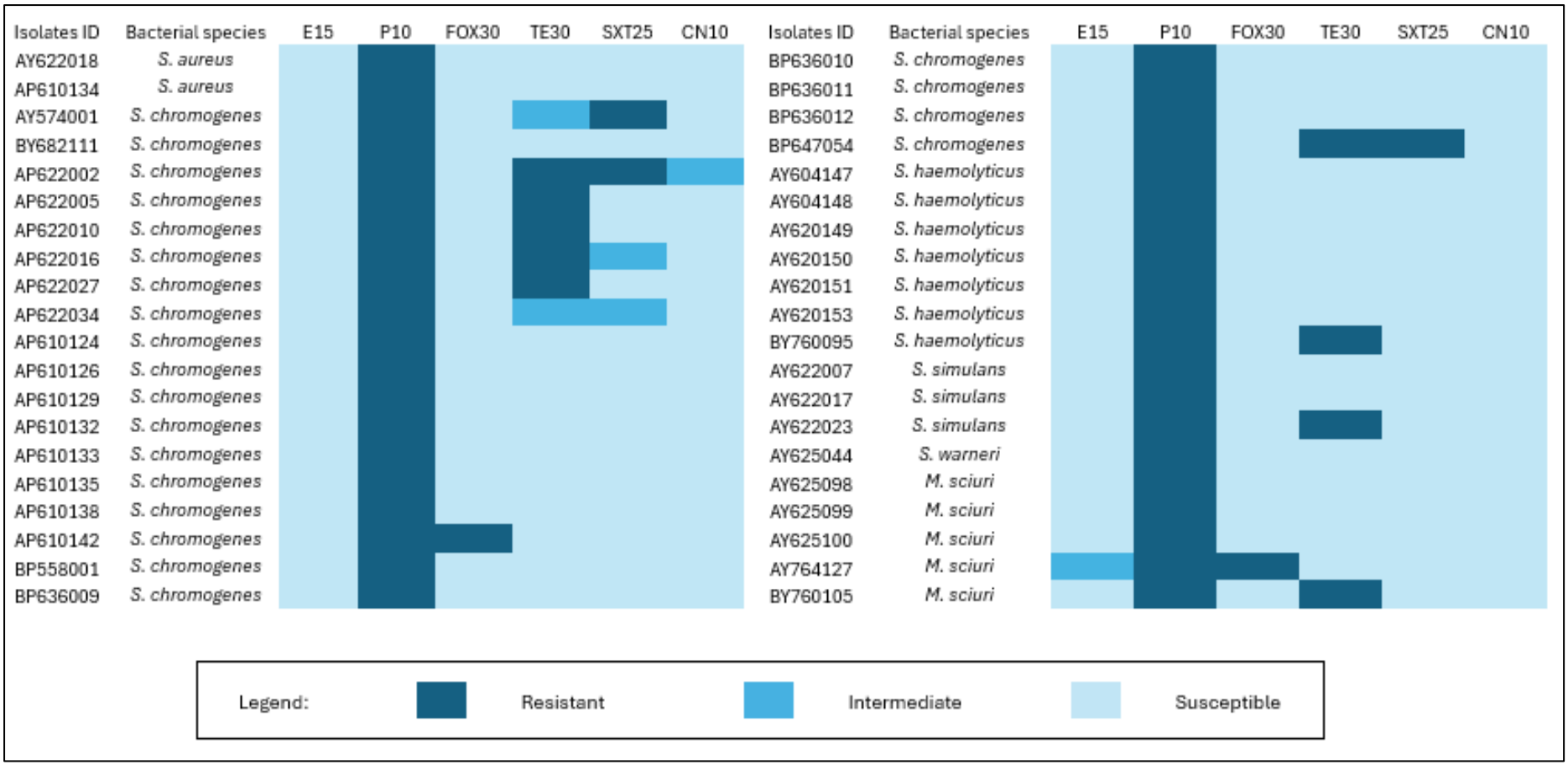


Figure 4.5 Heatmap Visualization of Antibiotic Susceptibility Profiles in *Staphylococcus* spp. (n = 35) and *M. sciuri* (n = 5) Isolates Against Six Antibiotics

Notes: E15: erythromycin (15 µg), P10: penicillin G (10 µg), FOX30: cefoxitin (30 µg), TE30: tetracycline (30 µg), SXT25: sulfamethoxazole-trimethoprim (25 µg), CN10: gentamicin (10 µg).

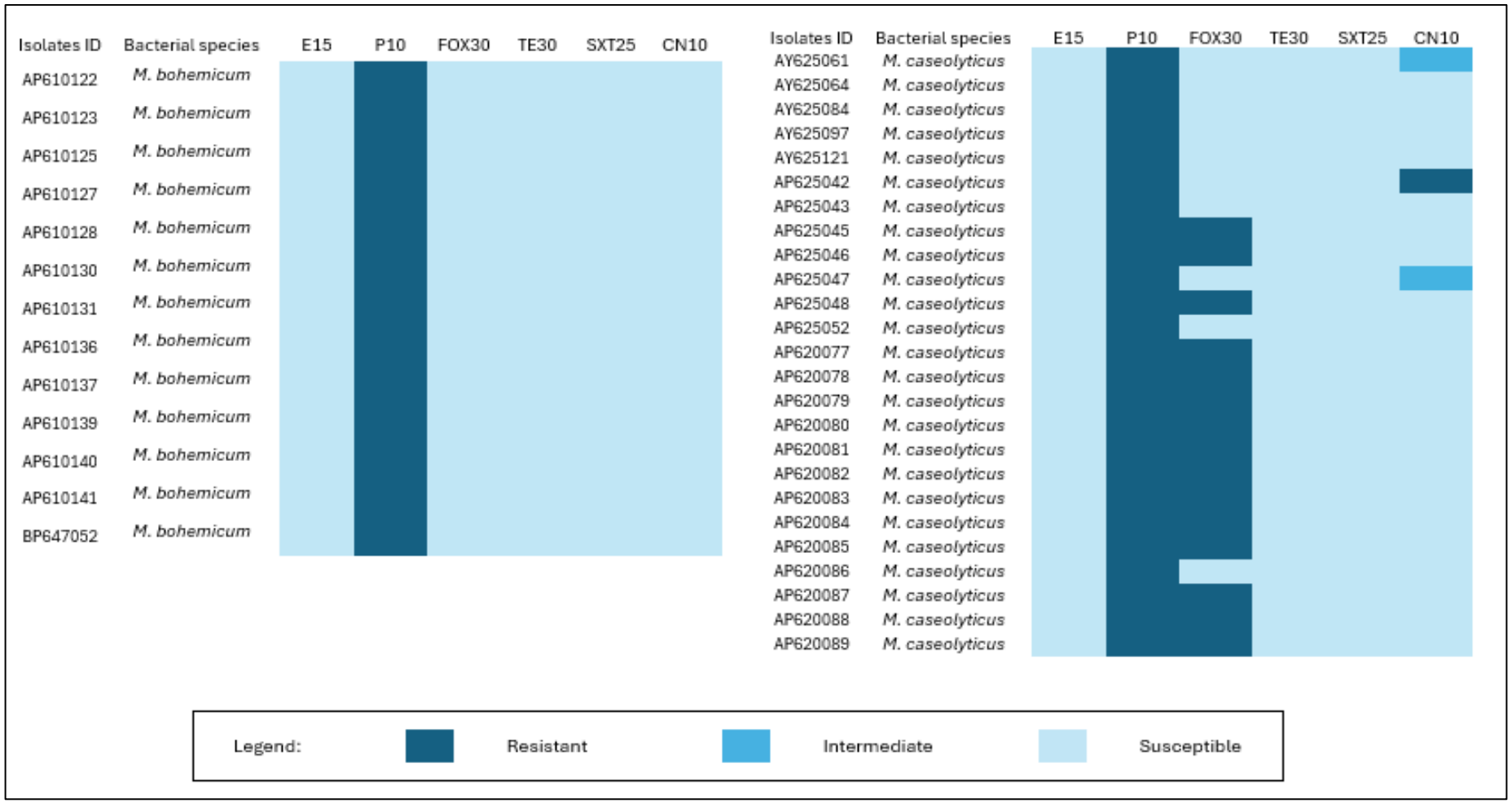


Figure 4.6 Heatmap Visualization of Antibiotic Susceptibility Profiles in *Macrococcus* spp. (n = 38) Isolates Against Six Antibiotics

Notes: E15: erythromycin (15 µg), P10: penicillin G (10 µg), FOX30: cefoxitin (30 µg), TE30: tetracycline (30 µg), SXT25: sulfamethoxazole-trimethoprim (25 µg), CN10: gentamicin (10 µg).

To ensure a standardized interpretation of the findings, antibiotic resistance prevalence were classified based on European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) guidelines (EFSA & ECDC, 2025). Therefore, the prevalence was categorized as very low (0.1% to 1%), low (> 1% to 10.0%), moderate (> 10% to 20%), high (> 20% to 50%), very high (> 50% to 70%) and extremely high (> 70%).

As shown in Table 4.4, penicillin resistance was found to be extremely high. The data revealed that 100% (n= 78) of the isolates exhibited resistance to at least one antibiotic, with resistance to penicillin G observed in all recovered isolates (as in Figure 4.5 and Figure 4.6). Specifically, this resistance phenotype encompassed 100% (n = 35) of *Staphylococcus* spp., 100% (n = 5) of *M. sciuri* and 100% (n = 38) of *Macrococcus* spp.. These elevated resistance patterns could be due to several factors, such as extensive use of penicillin, inappropriate therapeutic dosing practices or horizontal dissemination of mobile genetic elements in the organisms. As a narrow-spectrum β-lactam, penicillin is highly effective against the Gram-positive bacteria that cause the majority of bovine mastitis cases (Rodríguez *et al.*, 2023; Yang *et al.*, 2023; Saeed *et al.*, 2024). Importantly, the current findings demonstrate a marked increase in the prevalence of penicillin resistance compared to previous local studies. For instance, an earlier study by Othman and Bahaman (2005) revealed a penicillin resistance rate of 39% (n = 14) in *S. aureus*, which is lower than the rates detected in this study. Similarly, Ali *et al.* (2020) reported a low resistance level of 7.4% (n = 4). More recently, Saeed *et al.* (2022) found that 46% (n = 18) of *S. aureus* isolates exhibited penicillin resistance. It is suggested that this emerging resistance might be attributed to the capability of *S. aureus* to form biofilms (Saeed *et al.*, 2022). Biofilms act as a physical barrier that reduces the permeability of the bacterial cells, thereby preventing penicillin from effectively penetrating the cells (Ugwu *et al.*, 2025). In Thailand, Nitipan *et al.* (2025) also reported 100% (n = 14) penicillin resistance in bovine mastitis-associated *S. aureus*. These variations across studies likely reflect differences in herd management and antibiotic selective pressure specific to each farm (Moreno *et al.*, 2025; Nitipan *et al.*, 2025).

Regarding CoNS, the high resistance observed mirrors findings by Getahun *et al.* (2024), who demonstrated that 51.8% (n = 68) of CoNS possessed resistance to penicillin G. This resistance phenotype was consistent across species identified in both studies, including *S. chromogenes*, *S. haemolyticus*, *S. simulans* and *S. warneri*. Similar

substantial penicillin resistance rates were reported by Tarazona-Manrique *et al.* (2024) and Yang *et al.* (2023) with 55.1% (n = 27) and 71.3% (n = 114) penicillin resistance detected, respectively. The widespread presence of penicillin resistance across diverse species highlights the role of CoNS as a genetic reservoir for the *blaZ* gene (Mbindyo *et al.*, 2021). The *blaZ* gene has been reported to be the common gene that confers penicillin resistance in both *S. aureus* and CoNS, hence both species use the same resistance mechanism via the production of β-lactamase (Rodríguez *et al.*, 2023). This gene is frequently located on plasmid, facilitating horizontal gene transfer between species sharing the same environment (Hnini *et al.*, 2024; Dündar & Çakırlar, 2025). Meanwhile, penicillin resistance observed in *M. sciuri* isolates mirrors findings by Panigrahi *et al.* (2022), who similarly detected resistance in 100% (n = 6) of recovered isolates. This resistance pattern is likely related to *M. sciuri* capabilities to upregulate the expression of a chromosomal homologue of the *mecA* gene, which is frequently located on staphylococcal cassette chromosome elements (Crippa *et al.*, 2024). In contrast, Getahun *et al.* (2024) reported a lower resistance rate of 46.7% (n = 7), where the variation is likely attributable to differences in antibiotic usage patterns, disease epidemiology and farm management. For the *Macrococcus* isolates, the 100% (n = 38) resistance rate is supported by Machado *et al.* (2020), who reported that all *M. caseolyticus* (n = 14) isolated from pasteurized milk also exhibited penicillin resistance. Similarly, Foster and Paterson (2020) detected penicillin G resistance in *M. bohemicum* isolated from other clinical veterinary samples, canine otitis. These patterns suggest that commensal *Macrococcus* strains have emerged as opportunistic pathogens with a broad host range, capable of harbouring resistance determinants (Carroll *et al.*, 2023). Notably, mechanism of resistance in *Macrococcus* differs from that of *Staphylococcus* and *Mammaliicoccus*, as resistance in *Macrococcus* is often driven by the acquisition of the *mecD* gene (Schwendener *et al.*, 2019). Unlike the *blaZ* gene, which encodes an enzyme that hydrolyses penicillin, the *mecD* gene encodes modified penicillin-binding proteins (PBPs) (Schwendener *et al.*, 2017). This modification confers broad-spectrum resistance to all β-lactams by reducing the binding affinity of antibiotic to native PBPs (Schwendener & Perreten, 2022). Collectively, penicillin resistance observed in Staphylococcaceae isolates suggests that penicillin G is no longer a viable therapeutic option for mastitis treatment in this specific herd.

Overall, Staphylococcaceae isolates exhibited high phenotypic resistance pattern to cefoxitin as shown in Table 4.4, accounting for 22% (n = 17) of the total

isolates. At species level, however, the resistance phenotype varied. It was low at 5% (n =1) for *S. chromogenes*, moderate at 20% (n =1) for *M. sciuri* and high at 60% (n =15) for *M. caseolyticus*. According to CLSI, cefoxitin was used to indicate methicillin resistance pattern. Consistent with established practices in bovine mastitis research (Elemo *et al.*, 2017; Khan *et al.*, 2023), our study relied on cefoxitin resistance as a dependable phenotypic marker for detecting methicillin-resistant staphylococci. However, because phenotypic screening only provides a preliminary indication, definitive characterization of these isolates fundamentally relies on molecular confirmation of the *mec* genes. The low resistance rate of *S. chromogenes* against cefoxitin in this study was supported by Rodríguez *et al.* (2023) and Taponen *et al.* (2023), who also observed low or zero resistance rates at only 7.3% (n = 41) and 0% (n = 37) in *S. chromogenes*, respectively. Hence, the current finding aligns with previous literature stating that methicillin resistance remains rare among *S. chromogenes* isolates from bovine mastitis (Moser *et al.*, 2013; Duse *et al.*, 2021; Fergestad *et al.*, 2021). On another note, the current study demonstrated a significantly higher prevalence of cefoxitin-resistant *M. sciuri* compared to previous studies. According to Rodríguez *et al.* (2023) and Taponen *et al.* (2023), they observed a cefoxitin resistance pattern at 5.7% (n = 10) and 0% (n = 64) in *M. sciuri* (cited as *S. sciuri*) isolates associated with SCM. The cefoxitin resistance was conferred by acquisition of the *mecA* gene in *S. chromogenes and mecA1* gene in *M. sciuri* (Frey *et al.*, 2013; Regecová *et al.*, 2021; Zakaria *et al.*, 2026). The high cefoxitin resistance rate of *M. caseolyticus* in this study mirrors findings by MacFadyen *et al.* (2018), who reported 100% (n = 33) of *M. caseolyticus* isolated from bovine milk in England and Wales, indicating the high methicillin resistant-*M. caseolyticus* in dairy cattle isolates. This marked resistance profile is possibly caused by improper disposal of livestock manure containing residual antibiotics or poor sanitation and hygiene, which allows the resistant phenotype to spread to the communities. Molecularly, this high resistance pattern is likely mediated by distinct resistance genes such as *mecB* and *mecD* genes. As noted by Keller *et al.* (2022), these genes encode the modified PBPs (PBP2a) that confer broad spectrum β-lactam resistance including methicillin. Hence, presence of the resistant isolates poses an elevated zoonotic risk due to their capability for cross-species transmission and is facilitated by virulence factors that enable adaptation and colonization in different hosts, such as humans and bovines (Rodríguez *et al.*, 2023). Furthermore, the high

resistance rate in *M. caseolyticus* highlights its potential role as a reservoir for resistance genes in the dairy environment.

Regarding tetracycline susceptibility, the results indicated a moderate level of resistance, with 12% (n = 9) of the Staphylococcaceae isolates displaying the resistant phenotype (Table 4.4). However, resistance profiles showed variability across different species. Notably, high resistance rates were observed in CoNS isolates, including 27% (n = 6) *S. chromogenes* and 33% (n = 1) of *S. simulans*, whereas moderate resistance was identified in other CoNS species of 14% (n = 1) *S. haemolyticus* and 20% (n = 1) of *M. sciuri*. This species-specific variability aligns with previous global findings, which demonstrate a diverse antibiotic resistance profile among *Staphylococcus* and *Mammaliicoccus* species associated with bovine mastitis. For instance, in Ethiopia, an extremely high resistance pattern was reported by Getahun *et al.* (2025), who observed 90.9% (n = 9) of tetracycline resistance in *S. chromogenes* and 100% in both *S. haemolyticus* (n = 8) and *S. simulans* (n = 6) isolates recovered from bovine milk samples. In another study conducted by Mbindyo *et al.* (2021) in Kenya, a high tetracycline resistance of 22.8% (n = 21) was reported among CoNS isolates recovered from bovine mastitis. In contrast, a local study in Selangor by Haulisah *et al.* (2021) observed a low tetracycline resistance pattern, reporting a rate of 9.1% (n = 2) in CoNS isolated from ruminants. A low resistance rate was also reported by Tarazona-Manrique *et al.* (2024), who identified tetracycline resistance in only 4% (n = 5) of *S. chromogenes* isolates, with *M. sciuri* (cited as *S. sciuri*) and *S. haemolyticus* remaining fully susceptible. These differences can be attributed to geographical differences and herd-specific management practices (Getahun *et al.*, 2025). Specifically, the variation in resistance pattern is likely a consequence of farm-specific prophylactic and therapeutic usage of tetracycline. As noted by El-Razik *et al.* (2017), the routine employment of tetracycline as a prophylactic or therapeutic agent for mastitis management elevates antibiotic selective pressure, thereby driving the emergence and variation of resistance within individual dairy herds. This persistent selective pressure establishes CoNS and *M. sciuri* as genetic reservoirs for tetracycline resistance within the milking environment (Matheou *et al.*, 2025). Furthermore, this observation highlights the role of CoNS and *M. sciuri* as emerging opportunistic pathogens capable of harbouring resistance genes, particularly *tet* genes, which confer reduced susceptibility to tetracycline via efflux pumps or ribosomal protection (Phophi *et al.*, 2019; Zakaria *et al.*, 2026). Consequently, despite the moderate pattern of resistance,

tetracycline remains a viable therapeutic option for this herd if it is used prudently to sustain its efficacy.

In this study, sulfamethoxazole-trimethoprim susceptibility testing revealed a moderate level of resistance, with 14% (n = 3) of the Staphylococcaceae isolates (AY574001, AP622002 and BP647054) exhibiting the resistant phenotype. Interestingly, this resistance phenotype was species-specific, found exclusively in *S. chromogenes* isolates, while all other species remained fully susceptible. In contrast, Kenar *et al.* (2012) reported that *S. chromogenes* isolated from bovine subclinical milk in Turkey had high sulfamethoxazole-trimethoprim resistance rate. However, studies conducted by Rodríguez *et al.* (2023) in Colombia and Yang *et al.* (2023) China, reported low sulfamethoxazole-trimethoprim resistance rates of 10.3% (n = 4) and 0%, respectively, in *S. chromogenes* isolated from bovine mastitis. These differences in sulfamethoxazole-trimethoprim resistance are likely driven by geographical variations in the resistance profiles of *S. chromogenes*, which have a considerable impact on antibiotic prescribing practices. From an epidemiological perspective, the World Organization for Animal Health (WOAH) recorded that sulfonamides constitute only 6.14% of antibiotics used in veterinary practices (WOAH, 2025), which reflects limited usage of this antibiotic, including in Malaysia. This low percentage is not worrying, however, it does not deny the need for continuous surveillance of the AR profile to ensure this pattern of low resistance is maintained. The sulfamethoxazole-trimethoprim resistance trait may be attributed to the acquisition of *dfr* and *sul* genes, which encodes modified forms of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively (Brdová *et al.*, 2024). These modified enzymes possess a reduced binding affinity for the antibiotics, thereby rendering the treatment ineffective. Collectively, these findings suggest the regular monitoring and surveillance of AR, especially on sulfamethoxazole-trimethoprim to prevent the emergence of resistant isolates.

In this study, gentamicin resistance was categorized as very low, occurring in only 1% (n = 1) of the Staphylococcaceae isolates. This single resistant phenotype was identified exclusively in the *M. caseolyticus* strain AP625042. However, these findings are in contrast with a previous study. For instance, a study in England and Wales by MacFadyen *et al.* (2018) reported a 0% resistance to gentamicin among *M. caseolyticus* isolated from dairy cattle, based on antibiogram profiles generated using the Vitek 2 AST-P634 card. Nevertheless, *Macrococcus* spp. can potentially serve as reservoirs for

gentamicin resistance gene such as *aac(6')-Ie–aph(2'')-Ia* that are highly associated with conjugative plasmid and transposon, allowing for the spread and disseminations of this resistance genes. A study conducted by Keller *et al.* (2022) has detected the bifunctional gene *aac(6')-Ie–aph(2'')-Ia* in gentamicin-resistant *M. caseolyticus*, which encodes for aminoglycoside-modifying enzymes (AMEs), that prevents gentamicin from binding to the ribosome (Keller *et al*., 2022; Amini *et al*., 2024). Conversely, a complete absence of gentamicin-resistant *Staphylococcus* spp. was observed in current study. This pattern is corroborated by recent studies conducted in Uruguay by De los Santos *et al.* (2022) and Moreno *et al.* (2025), who also reported 0% gentamicin resistance in *Staphylococcus* spp. isolated from bovine mastitis. The low resistance level is probably due to the infrequent use of this antibiotic as a first-line treatment for staphylococcal bovine mastitis compared to β-lactams and tetracyclines (Getahun *et al.*, 2025; Moreno *et al.*, 2025). On another note, the lack of observation of gentamicin resistance among *M. sciuri* isolates in this study aligns with the findings of Panigrahi *et al.* (2022), who similarly reported a 0% resistance rate in *M. sciuri* associated with bovine mastitis. This finding likely reflects the absence of an acquired resistance gene in *M. sciuri* isolated from bovine mastitis. Collectively, these findings demonstrate that restricted usage has minimized selective pressure, preserving gentamicin's efficacy against mastitis-associated Staphylococcaceae, though continuous surveillance of potential resistance reservoirs like *M. caseolyticus* remains essential (Chaves & Tadi, 2024).

In the present study, none of the isolates exhibited resistance to erythromycin, demonstrating the high efficacy of this macrolide against the Staphylococcaceae isolates. However, a local study conducted by Saeed *et al.* (2022) previously observed a moderate resistance rate of 18% (n = 7) among *S. aureus* isolates, suggesting different patterns within the region. Global studies have reported high resistance patterns to erythromycin. For example, in China, a study conducted by Yang *et al.* (2023) demonstrated resistance rate of 22.1% (n = 38) and 28.8% (n = 46) in *S. aureus* and CoNS, respectively, isolated from bovine mastitis. Furthermore, a study conducted in Algeria reported resistance rates of 30% (n = 3) and 54.3% (n = 19) for CoPS and CoNS isolates, respectively (Saidi *et al.*, 2019). The absence of erythromycin resistance observed in this current study is likely a consequence of the prudent use of macrolides for mastitis cure and prevention in the studied herd, which has minimized the antibiotic selective pressure for resistance. Resistance to erythromycin in *Staphylococcus* spp.,

M. sciuri and *Macrococcus* spp. are typically mediated by ribosomal methylation encoded by *erm* genes, such as *ermA*, *ermB* and *ermC*, or by ribosomal protection proteins encoded by *msr* genes which are typically associated with mobile genetic elements such as conjugative transposons, plasmids and resistance islands (Schwendener *et al.*, 2020; Moawad *et al.*, 2023; Brdová *et al.*, 2024; Zakaria *et al.*, 2026). The absence of phenotypic resistance in this study likely suggests an absence of resistance genes among the isolates, reflecting minimal exposure to macrolide within this studied dairy herd. Despite this favourable susceptibility profile, continuous monitoring is essential, as *M. caseolyticus* has been increasingly identified elsewhere as a reservoir for transferable resistance islands that can be acquired by pathogenic staphylococci (Schwendener *et al.*, 2017).

4.8.1 Multidrug Resistance Profiles

Following phenotypic resistance, this study identified multidrug resistant (MDR) phenotypes among the recovered isolates. As illustrated in Figure 4.5, two specific strains (AP622002 and BP647054), representing 3% (n = 2) of the total isolates exhibited MDR profiles, of which both belong to the same species of *S. chromogenes*. Both isolates demonstrated the same resistance profile to three antibiotics: penicillin G, tetracycline and sulfamethoxazole-trimethoprim. Similarly, Schmidt *et al.* (2015) have also found multidrug resistant-*S. chromogenes* at the prevalence of 3% (n = 2) from bovine mastitis milk. These strains were observed to be resistant against similar classes of antibiotics, including penicillin and tetracycline (Schmidt *et al.*, 2015). The presence of MDR-*S. chromogenes* suggests that this species has emerged as a resistance reservoir in SCM (Waller *et al.*, 2023; Khasapane *et al.*, 2025). The MDR isolates found in this study were comparatively lower than the report of Getahun *et al.* (2024), who reported 100% (n = 3) of MDR *S. chromogenes* isolated from bovine milk in Ethiopia. These differences may be attributed to the variations in regional disease epidemiology (Getahun *et al.*, 2024). In another study, *S. chromogenes* with a high MDR rate of 58.1% (n = 68) was also reported, demonstrating resistance up to seven different antibiotic classes (Fazal *et al.*, 2023). The escalation of MDR rates is likely due to prolonged and repetitive administration of antibiotics or ability to form biofilm (Fazal *et al.*, 2023). Furthermore, emergence of this MDR phenotype possibly underscores co-selection pressures potentially via plasmids co-harbouring *tet* genes and *sul* or *dfr*

genes, which are common in *S. chromogenes* (Osada *et al.*, 2022; Naranjo-Lucena & Slowey, 2023). Interestingly, Khasapane *et al.* (2025) has reported a limited variety of plasmids carrying ARGs, including *dfr* and *tet* genes, in *S. chromogenes* derived from bovine mastitis. Among five isolates being screened, only two plasmid replicon types, which are rep13_9_rep (pSepCH) and rep22_1b_repB (pAMalpha1) were found in the mastitis associated *S. chromogenes* in bovines (Khasapane *et al.*, 2025). This relatively host-specific plasmids may attribute to the lower multidrug resistance prevalence observed in current findings compared to previous reports. Therefore, continuous surveillance of *S. chromogenes* MDR profiles, coupled with plasmid replicon typing and genomic surveillance, is imperative to mitigate its role as an emerging resistance reservoir threatening both bovine udder health and One Health antibiotic stewardship.

4.9 Genotypic Characterization of Antibiotic Resistance

The emergence of antibiotic resistance (AR) in veterinary pathogens poses a critical threat to both animal health and public health, particularly in the dairy sector, where zoonotic transfer is a concern. To characterize the genotypic characteristics of antibiotic resistance within the studied herd, all resistant Staphylococcaceae isolates were subjected to genotypic screening using polymerase chain reaction (PCR) for specific antibiotic resistance genes (ARGs) corresponding to their phenotypic resistant profiles, including *blaZ*, *mecA*, *mecD*, *tetK*, *tetM*, *dfrA* and *aacA-aphD*.

4.9.1 Relationship between Phenotypic and Genotypic Resistance in Staphylococcaceae

To statistically validate the relationship between phenotypic resistance and the detected resistance genes, Cohen's Kappa coefficient (κ) was used to measure concordance. Additionally, sensitivity and specificity were calculated to assess how accurately the presence of genes predicted phenotypic resistance (Table 4.5). The strength of agreement was interpreted based on established standards, where κ values ≤ 0 indicate poor agreement, 0.01 to 0.20 indicate slight agreement, 0.21 to 0.40 indicate fair agreement, 0.41 to 0.60 indicate moderate agreement, 0.61 to 0.80 indicate substantial agreement and 0.81 to 1.00 indicate almost perfect agreement (McHugh, 2012).

Table 4.5
Distribution of Phenotypic Resistance and Concordance with Resistance Genes Across Staphylococcaceae Species

Species (n)	Penicillin (P)	Cefoxitin (FOX)		Tetracycline (TET)		Sulfamethoxazole-trimethoprim (SXT)	Gentamicin (CN)
	Pheno / *blaZ* (%)	Pheno / *mecA* (%)	Pheno / *mecD* (%)	Pheno / *tetK* (%)	Pheno / *tetM* (%)	Pheno / *dfrA* (%)	Pheno / *aacA-aphD* (%)
***Staphylococcus* spp.**							
S. aureus (2)	2/2 (100)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)
S. chromogenes (22)	22/22 (100)	1/0 (0)	1/0 (0)	6/5 (83)	6/6 (100)	3/1 (33)	0/0 (0)
S. haemolyticus (7)	7/7 (100)	0/0 (0)	0/0 (0)	1/1 (100)	1/1 (100)	0 (0)	0/0 (0)
S. simulans (3)	3/3 (100)	0/0 (0)	0/0 (0)	1/1 (100)	1/1 (100)	0 (0)	0/0 (0)
S. warneri (1)	1/1 (100)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0 (0)	0/0 (0)
***Mammaliicoccus* spp.**							
M. sciuri (5)	5/5 (100)	1/1 (100)	1/0 (0)	1/1 (100)	1/1 (100)	0 (0)	0/0 (0)
***Macrococcus* spp.**							
M. bohemicum (13)	13/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0 (0)	0/0 (0)
M. caseolyticus (25)	25/10 (40)	15/0 (0)	15/15 (100)	0/0 (0)	0/0 (0)	0 (0)	1/1 (100)
Total (n = 78)	78/50	17/1	17/15	9/8	9/9	3/1	1/1
Statistical Analysis							
Concordance Rate (%)	64.1	5.9	88.2	88.9	100	33.3	100
Cohen's Kappa (κ)	0.000[a]	0.089	0.921	0.934	1.000	0.491	1.000
P-value (P)	1.000	0.057	0.000	0.000	0.000	0.000	0.000

Note: Pheno: number of isolates displaying phenotypic resistance in disc diffusion assay; PEN: penicillin; FOX: cefoxitin; TET: tetracycline; SXT: sulfamethoxazole-trimethoprim, GEN: gentamicin

[a] Specificity could not be calculated for penicillin because no susceptible isolates (true negatives) were observed in the sample population

Percentages (%) are calculated as the number of isolates harbouring the resistance gene divided by the total number of phenotypically resistant isolates.

4.9.2 Screening of *blaZ* Gene That Confers Resistance to Penicillin

All 78 penicillin-resistant isolates were subjected to genotypic detection of the *blaZ* gene. In this study, the successful amplification of the *blaZ* gene across representative resistant isolates was confirmed by the presence of a distinct DNA band at approximately 173 bp, as shown in Figure 4.7. A positive control strain was included during gel visualization to confirm primer efficiency and validate the PCR reaction conditions.

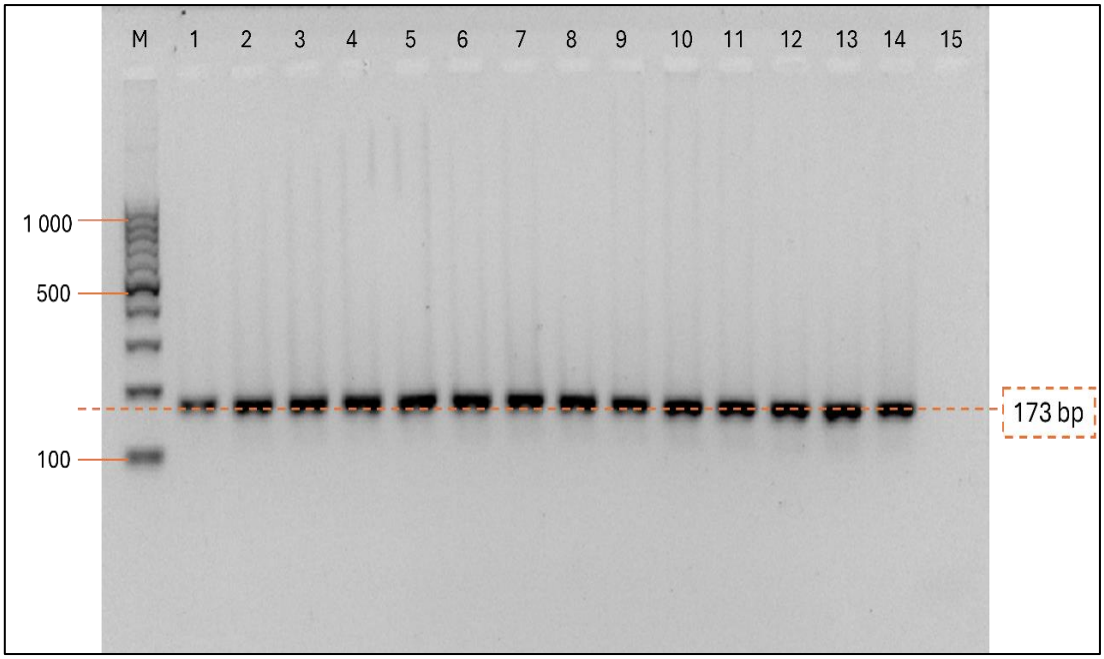


Figure 4.7 PCR Amplification of *blaZ* Gene (173 bp) in Representative Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (positive control); Lane 2 to 14: Tested Isolates (AP622005, AP622007, AP622010, AP622016, AP622023, AP622027, AP622017, AP622034, AP610124, AP610126, AP610129, AP610132 and AP610133, Respectively); Lane 15: Negative Control

Following this confirmation, the *blaZ* gene was detected in 64.1% (n = 50) of the total isolates, though the distribution patterns differed between genera. The *blaZ* gene was present in 100% (n = 35) of *Staphylococcus* spp. suggesting that this is a primary gene conferring penicillin resistance. Interestingly, current findings reveal a higher prevalence than what has been reported in previous studies. For example, a study conducted by Hnini *et al.* (2024) found that 67.3% (n = 35) of *Staphylococcus* spp. collected from bovine mastitis in Portugal harbours the *blaZ* gene. In another study conducted in China, it was found that 69.4% (n = 111) of *Staphylococcus* spp. isolates

were *blaZ*-positive isolates (Yang *et al.*, 2023). The association of *blaZ* gene with penicillin resistance is driven by its function to encode β-lactamase, an enzyme that cleaves the β-lactam ring of penicillin (Rajput *et al.*, 2024). This hydrolysis renders the antibiotic inactive, thereby preventing it from disrupting cell wall formation. Genetically, *blaZ* gene can be localized on either plasmid or chromosome and is frequently integrated into transposable elements such as the transposons Tn*552*, Tn*4002* and Tn*4201*, which facilitates its dissemination via horizontal gene transfer (Sansevere & Robinson, 2017; Mlynarczyk-Bonikowska *et al.*, 2022). The plasmid-borne localization is clinically significant because this plasmid has the capacity to co-harbour additional genes, including other ARGs, transposase genes and enterotoxin genes, allowing for the co-selection of multidrug resistance under the pressure of a single antibiotic (Schwarz *et al.*, 2014; Mlynarczyk-Bonikowska *et al.*, 2022). However, the low detection of MDR in this study suggests that the *blaZ*-carrying elements circulating in this herd may currently lack these additional resistance genes, particularly ARGs, thereby limiting the co-selection of broader resistance phenotypes despite the high prevalence of the *blaZ* gene.

Based on our findings, the reclassified *M. sciuri* isolates showing a 100% (n = 5) penicillin resistance phenotype were also found to be positive for *blaZ* screening. This is in contrast with a finding by Panigrahi *et al.* (2022), who found that 85.7% (n = 6) of penicillin resistant-*M. sciuri* did not carry the *blaZ* gene. Instead, it was suggested that the resistance traits of *M. sciuri* against β-lactams antibiotics was conferred by other ARGs such as *mecA*, *femA* or *femB* genes (Panigrahi *et al.*, 2022). On another note, *blaZ* was not detected or present at a lower rate among *Macrococcus* spp., where the gene was absent in 100% (n = 13) of *M. bohemicum* isolates and detected in only 40% (n = 10) of *M. caseolyticus* isolates. As a result, the overall Cohen's kappa analysis ($\kappa = 0.000$, $P = 1.000$) indicated no statistical agreement across the full dataset (as in Table 4.5). This discordance suggests that while *blaZ* is the main gene conferring penicillin resistance in the staphylococcal isolates, penicillin resistance in *Macrococcus* spp., particularly *M. bohemicum* is likely mediated by genus-specific variant, *blaZm*, which is not detected by the primers used in this study (Schwendener *et al.*, 2017). This highlights the potential ability of *Macrococcus* to persist as silent reservoir of resistance genes. Another possibility, highlighted by Schwendener *et al.* (2017), is the involvement of other resistance genes, such as plasmid-encoded *mecB* and *mecD* genes. Unlike the narrow-spectrum resistance conferred by *blaZ*, these *mecB* and *mecD* genes

encode alternative PBPs that mediate cross-resistance to widespread β-lactams, including penicillin, oxacillin and methicillin (Schwendener & Perreten, 2022).

4.9.3 Screening of *mec* Genes That Confer Resistance to Cefoxitin

To determine the genotypic characterization of cefoxitin resistance, screening of *mecA* and *mecD* genes was conducted. As shown in Figures 4.8 and 4.9, successful amplification of the *mecA* and *mecD* genes across representative cefoxitin-resistant isolates was confirmed by the presence of a distinct DNA band at approximately 147 bp and 867 bp, respectively. *S. epidermidis* ATCC 35984 was used as the positive control strain to validate primer binding specificity and optimize PCR conditions.

In the present study, *mecA* gene was selected for screening in *Staphylococcus* and *Mammaliicoccus* isolates as it is the primary, genus-specific gene of methicillin resistance (Schwendener & Perreten, 2022). Based on the phenotypic resistance profile, only two isolates (*S. chromogenes* (AP610142) and *M. sciuri* (AY764127)) were identified as cefoxitin-resistant, with the statistical analysis indicating poor agreement between the resistance phenotype and the presence of *mecA* gene ($\kappa = 0.089$, $P = 0.057$). This uncorrelation is likely attributable to the small sample size of resistant isolates ($n = 2$), where a single negative result significantly impacts the concordance rate.

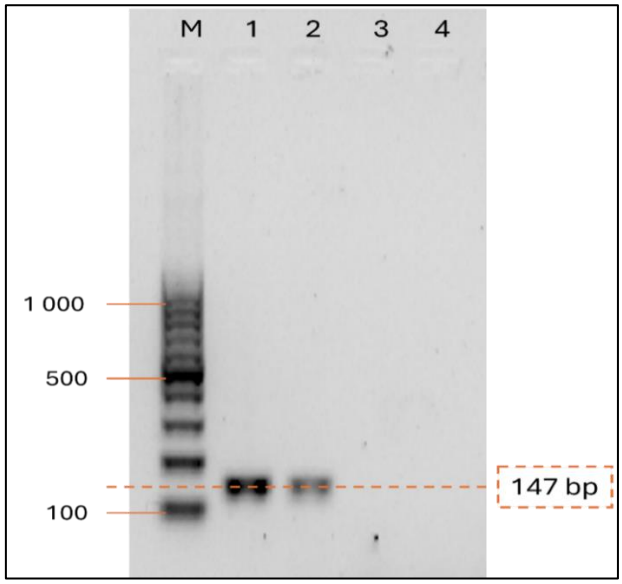


Figure 4.8 PCR Amplification of *mecA* Gene (147 bp) in *S. chromogenes* and *M. sciuri* Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2: *M. sciuri* Isolate AY764127; Lane 3: *S. chromogenes* Isolate AP610142; Lane 4: Negative Control

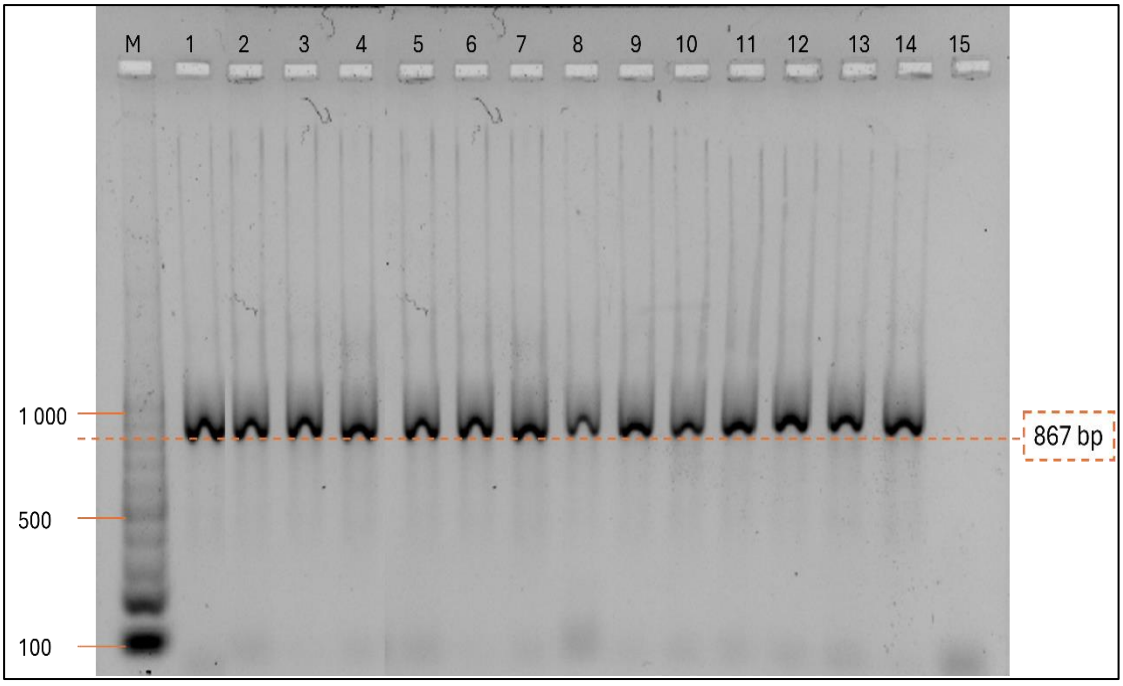


Figure 4.9 PCR Amplification of *mecD* Gene (867 bp) in Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2 to 14: Tested Isolates (AP625045, AP625046, AP625048, AP620077, AP620078, AP620079, AP620080, AP620081, AP620082, AP620083, AP620084, AP620085 and AP620087, Respectively); Lane 15: Negative Control

Based on the genotypic data, *mecA* gene was undetectable in AP610142 of cefoxitin resistant-*S. chromogenes* isolate. A study by Yang *et al.* (2023) similarly reported that *S. chromogenes* isolated from bovine mastitis did not harbour the *mecA* gene. In another study conducted by Dos Santos *et al.* (2016) also demonstrated a *mecA*-negative in two isolates of *S. chromogenes* isolated from bovine milk. The absence of *mecA* gene in *S. chromogenes* strains gives the possibility of methicillin resistance mediated by *mecC* gene, as its presence has been reported in a wide range of other host species, including livestock in European countries (Umami *et al.*, 2023). Moreover, the resistance phenotype could be attributed to alternative non-*mec* mechanisms, such as point mutations in native PBPs (resembling a borderline oxacillin-resistant *S. aureus*-like phenotype). This mechanism has been documented to elevate β-lactam minimum inhibitory concentrations (MICs) sufficiently to exceed the resistance breakpoint, resulting in phenotypic resistance even in the absence of the *mecA* gene (Argudín *et al.*, 2018).

In the present study, the *mecA* carriage was detected in AY764127 of *M. sciuri* isolate. The presence of *mecA* in *M. sciuri* aligns with a recent study from Colombia

that demonstrated 20% (n = 2) of *M. sciuri* isolates associated with bovine mastitis were positively detected carrying the *mecA* gene (Tarazona-Manrique *et al.*, 2024). Similarly, in a study conducted by Khazandi *et al.* (2018), a single of *M. sciuri* isolate was found to harbour the *mecA* gene. This validates the phenotypic resistance observed in *M. sciuri* is likely mediated by the PBP2a mechanism, which reduced the binding affinity for β-lactams, particularly cefoxitin (Pimenta *et al.*, 2023). In addition, this positive detection likely supports the hypothesis that the observed resistance is mediated by the mutation in species' native *mecA* homolog (*mecA1*), located within the SCC element (Antignac & Tomasz, 2009; Tsubakishita *et al*., 2010; Frey *et al.*, 2013; Zakaria *et al*., 2026). While native *mecA* homolog share 80% nucleotide sequence identity with *mecA gene*, it is typically does not confer resistance unless mutations occur that upregulates its expression (Wu *et al.*, 2001; Ito *et al*., 2012). Alternatively, *M. sciuri* can also acquire the classic *mecA* via horizontal gene transfer. This finding reinforces the role of *M. sciuri* as a potential evolutionary reservoir of *mecA* capable of disseminating resistance gene to other staphylococcal species.

The cefoxitin resistant-*Macrococcus* strains identified phenotypically in this study were screened for the *mecD* gene, as this gene is characteristically responsible for methicillin resistance in this genus. Notably, *mecD* gene shares ≤ 60% similarity with the nucleotide sequence of staphylococcal *mecA*, rendering common *mecA*-specific primers ineffective for detection in *Macrococcus* (Schwendener *et al.*, 2017). Based on the Table 4.5, the screening of *mecD* gene demonstrated a strong correlation with the cefoxitin resistance phenotype with concordance rate of 88.2%. Specifically, 100% (n = 15) of *Macrococcus* resistant isolates harboured the *mecD* gene. The presence of this gene was significantly aligned with phenotypic resistance ($\kappa = 0.921$, $P = < 0.001$), confirming that cefoxitin resistance in these *Macrococcus* species is primarily driven by the production of an alternative, *mecD*-encoded PBP2a. Data observed on this study aligns with Schwendener *et al.* (2019) who reported *mecD*-positive *M. caseolyticus* (n = 60) in bovine mastitis cases. The detection of *mecD* is concerning because the encoded PBP2a may confer resistance to the fifth-generation cephalosporins, such as ceftobiprole and ceftaroline. This suggests a potential for the emergence of highly resistant methicillin resistant-*Macrococcus* strains (Schwendener *et al.*, 2017), which poses a challenge to advanced therapeutic options typically reserved for multidrug resistant staphylococci associated with bovine mastitis (Brdová *et al.*, 2024). Furthermore, *mecD* gene is typically carried on a mobile *M. caseolyticus* resistance

island *mecD* (McRI$_{mecD}$) that are chromosomally integrated at the 3' end of the 30S ribosomal protein (*rpsI*) gene (Schwendener & Perreten, 2018). This mobility enhances horizontal gene transfer, contributing to the emergence of resistance profiles. On another note, methicillin resistance in *M. caseolyticus* can also be associated with *mecB* gene (Schwendener *et al.*, 2017; Foster & Paterson, 2020). This gene exhibits plasticity as it can be either localized on plasmid and integrated with transposon Tn*6045* or integrated into SCC*mec*-like element in *M. caseolyticus* (Schwendener *et al.*, 2017; Mašlaňová *et al.*, 2018). The location of both *mecD* and *mecB* genes in mobile genetic elements further highlights the genomic plasticity of *Macrococcus* species, which potentially serves as reservoir for methicillin resistance genes.

4.9.4 Screening of *aacA-aphD* Gene That Confers Resistance to Gentamicin

In the present study, a single gentamicin-resistant isolate, identified as *M. caseolyticus* (AP625042) was screened for the *aacA-aphD* gene (also known as *aac(6')-Ie – aph(2'')-Ia* gene). This gene was selected because it encodes bifunctional aminoglycoside acetyltransferases and phosphotransferases activities that confer resistance to a broad range of aminoglycoside antibiotics, including gentamicin (Schwendener *et al.*, 2019). Specifically, the acetyltransferases enzyme catalyze the action of an acetyl group to aminoglycosides, while phosphotransferases catalyze the transfer of a phosphate group to gentamicin molecule (Krause *et al.*, 2016). These modifications reduce the antibiotic's binding affinity to its target on 30S ribosomal subunit, thereby conferring resistance (Resende *et al.*, 2018). As shown in Figure 4.10, the isolate conferring phenotypic resistance to gentamicin (AP625042) yielded a positive result for *aacA-aphD* gene, with amplification occurring at the expected size of 227 bp. To confirm the PCR components were working well and PCR conditions were correct, the reference strain *Enterococcus faecium* strain S98b was incorporated as a positive control during gel electrophoresis. Consequently, statistical analysis revealed a complete agreement between the observed phenotypic resistance and genotypic profile ($\kappa = 1.000$, $P < 0.001$). This finding aligns with previous studies that identified the *aacA-aphD* gene as the primary determinant of gentamicin resistance. For example, Resende *et al.* (2018) reported that 47.6% (n = 100) of *M. caseolyticus* isolates derived from a dairy product, particularly cheese, harboured the *aacA-aphD* gene. In another study, *M. caseolyticus* isolates with gentamicin resistance phenotype were also

detected to carry the tandem *aacA-aphD* gene (Keller *et al.*, 2022). Furthermore, a study conducted in England and Wales by Schwendener *et al.* (2019) found that methicillin resistant-*M. caseolyticus* strains isolated from bovines exhibited the *aacA-aphD* gene with elevated gentamicin minimum inhibitory concentrations ranging from eight (8) to sixteen (16) µg/mL. The successful detection of *aacA-aphD* gene confirms that the observed phenotypic resistance in AP625042 is mediated by this resistance gene. Moreover, this gene is frequently localized on multidrug resistant plasmid, mirroring its classic location in staphylococci within transposon Tn*4001* (Mlynarczyk-Bonikowska *et al.*, 2022). This plasmid localization facilitates horizontal transfer among *Macrococcus* strains in the udder environment, as well as between *Macrococcus* and staphylococci, explaining the frequent detection of *aacA-aphD* in mastitis isolates. This finding reinforces the role of *M. caseolyticus* as a potential reservoir for aminoglycoside resistance determinants capable of disseminating to other pathogenic staphylococci in dairy environments, thereby highlighting the need for continuous molecular surveillance.

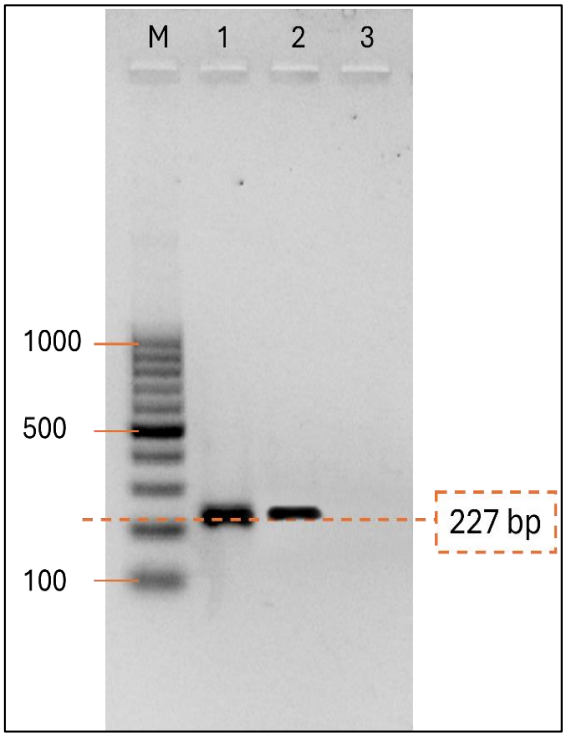


Figure 4.10 PCR Amplification of *aacA-aphD* Gene (227 bp) in a Resistant *M. caseolyticus* Isolate; Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *Enterococcus faecium* strain S98b (Positive Control); Lane 2: Tested Isolate (AP625042) and Lane 3: Negative Control

4.9.5 Screening of *tet* Genes That Confer Resistance to Tetracycline

Regarding tetracycline resistance, all nine resistant isolates were screened for *tet* genes (*tetK* and *tetM*). As shown in Figures 4.11 and 4.12, successful amplification of the *tetK* and *tetM* genes across all resistant isolates was confirmed by the presence of a distinct DNA band at approximately 360 bp and 158 bp, respectively. To assess assay performance and ensure optimal amplification conditions during gel visualization, *Enterococcus faecalis* strain E533 and *S. epidermidis* ATCC 35984 were employed as positive control strain for *tetK* and *tetM*, respectively.

This targeted approach addresses two resistance mechanisms in *Staphylococcus* spp. and *Mammaliicoccus* spp., which are active efflux pump and ribosomal protection (Schauer *et al.*, 2021; Shao *et al.*, 2025). In this study, 88.9% (n = 8) of the resistant isolates harbour the *tetK* gene. Among the *Staphylococcus* spp., this gene was detected in *S. chromogenes* (n = 5), *S. haemolyticus* (n = 1) and *S. simulans* (n = 1).

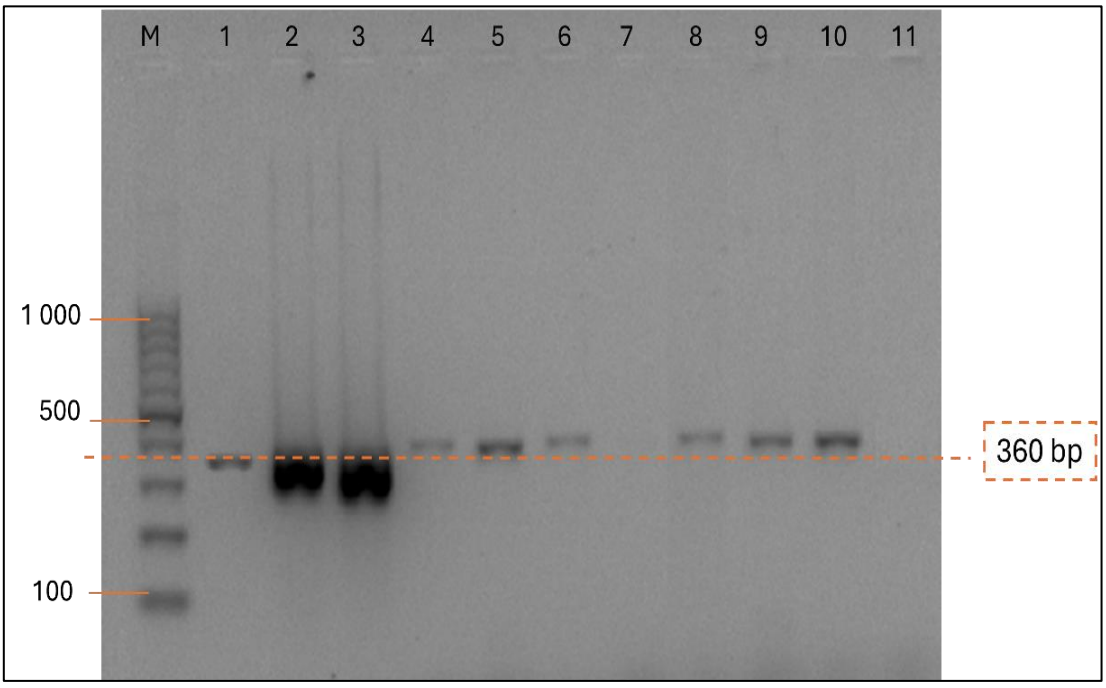


Figure 4.11 PCR Amplification of *tetK* Gene (360 bp) in Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *Enterococcus faecalis* strain E533 (Positive Control); Lane 2 to 10: Tested Isolates (AP622002, AP622010, AP622016, AP622027, BP647054, AP622005, BY760095, AP622023 and BY760105, Respectively); Lane 11: Negative Control

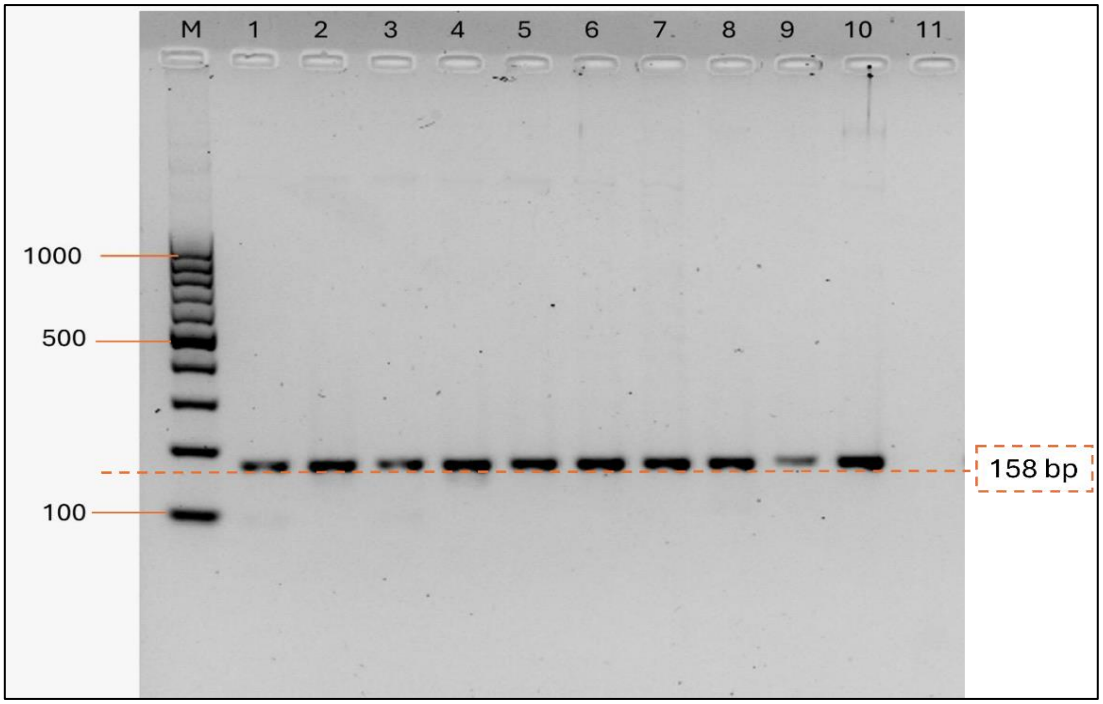


Figure 4.12 PCR Amplification of *tetM* Gene (158 bp) in Staphylococcaceae Isolates. Lane M: 100 bp DNA ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2 to 10: Tested Isolates (AP622002, AP622010, AP622016, AP622027, BP647054, AP622005, BY760095, AP622023 and BY760105, Respectively); Lane 11: Negative Control

This result was statistically analysed, showing a high correlation ($\kappa = 0.934$, $P < 0.001$) between genotypic and phenotypic tetracycline resistance, with only one isolate (AP622005) lacking this gene. This removal of tetracycline decreases the intracellular drug concentration, thereby conferring resistance (Ginn *et al.*, 2000; Jin *et al.*, 2002; Rajput *et al.*, 2024). In previous studies, a low prevalence of 6.9% (n = 3) and 17.5% (n = 28) of *tetK* genes were identified in tetracycline resistant-CoNS (Mbindyo *et al.*, 2021; Yang *et al.*, 2023). This is due to the fact that *tetM* gene has been screened to be the one that confers tetracycline resistance in most of the isolates. In contrast to the staphylococci, the detection of *tetK* in the single *M. sciuri* isolate is a notable finding. This aligns with observations by Schauer *et al.* (2021) and Zakaria *et al.* (2026), who reported a *tetK* prevalence of 2% (n = 2) and 25% (n = 1) in this species, respectively. The presence of a low-level silent reservoir of the *tetK* gene is likely influenced by limited sample size, which precludes definitive conclusions regarding the reliability of genotypic markers for antibiotic resistance in *M sciuri* (Zakaria *et al.*, 2026). The limited observation of *tetK* in both *Staphylococcus* spp. and *M. sciuri* may be attributed to the localization of the *tetK* gene on the plasmid pT181. This small, non-conjugative plasmid lacks the transfer and conjugation genes making it incapable of self-

mobilization (Leroy *et al.*, 2019; Gómez-Sanz *et al.*, 2021; Phillips *et al.*, 2025). Hence, dissemination of this gene is dependent on the integration of pT181 into a conjugative transposon or other conjugative plasmid that carry the *rep* and *pre* or *mob* genes, essential for replication and mobilization in a narrow host range (Ong *et al.*, 2017; Phillips *et al.*, 2025). Therefore, this reliance on helper elements restricts the horizontal spread of the *tetK* gene to a narrower range of staphylococcal lineages compared to the broadly distributed *tetM* gene.

The *tetM* gene was detected with a high occurrence of 100% (n = 9) in tetracycline resistant *Staphylococcus* and *Mammaliicoccus* isolates. Among *Staphylococcus* spp., the gene was detected in all *S. chromogenes* (n = 6), *S. haemolyticus* (n = 1) and *S. simulans* (n = 1). This finding was supported by statistical analysis that demonstrated a perfect agreement between resistance phenotype and the presence of *tetM* gene ($\kappa = 1.000$, $P < 0.001$), establishing *tetM* as the main tetracycline resistance determinant in the current study. Mechanistically, this gene confers resistance by encoding ribosomal protection proteins (RPPs) that induce conformational changes of the ribosome that actively dislodge tetracycline from its binding site (Tsiklauri *et al.*, 2025). This release allows protein synthesis to proceed, even in the presence of high tetracycline concentrations. The high prevalence of *tetM* gene observed in the current finding was in contrast with previous studies. For instance, low *tetM* prevalence rates among staphylococci isolated from bovine milk have been reported, including 16.3% (n = 8) by Jose and Vijayakumar (2023) and 16.2% (n = 7) by Mbindyo *et al.* (2021). These previous findings likely suggests that the phenotypic resistance in their herd was mediated by other unscreened tetracycline determinants (Jose & Vijayakumar, 2023). Notably, the presence of *tetM* gene was also detected in the single *M. sciuri* isolate. In a previous study, Schauer *et al.* (2021), has reported a *tetM* prevalence of 68% (n = 83) in this species. The high prevalence of *M. sciuri* harbouring *tetM* in previous study suggests the capabilities of the isolates to serve as a reservoir of *tetM* gene, which may facilitates its dissemination via mobile genetic elements within the herd (Kazemzadeh *et al.*, 2025).

The dominance of *tetM* gene observed in this study aligns with Mbindyo *et al.* (2021), who reported a higher prevalence of *tetM* than *tetK* in bovine mastitis CoNS isolates, as mentioned previously. This observation also aligns with current literature reporting non-*aureus* staphylococci (NAS) exhibited 22% (n = 35) *tetM* gene and 10% (n = 10) *tetK* gene (Cantekin *et al.*, 2024). The detection of *tetK* and *tetM* gene may be

driven by the unregulated accessibility of tetracycline or their indiscriminate use by farmers, including the frequent intramuscular injection of low concentration (30 mg/mL) commercial tetracycline without clinical justification (Cantekin *et al.*, 2024). Notably, the dominance of *tetM* is not limited to NAS found in bovine mastitis, it is also a prevailing determinant in bovine and human *S. aureus* (Ong *et al.*, 2017). For instance, a *tetM* prevalence of 45.5% (n = 15) and a *tetK* prevalence of 15.2% (n = 5) was reported in *S. aureus* isolated from bovine mastitis milk in Iran (Jamali *et al.*, 2014). Meanwhile, Ong *et al.* (2017) reported a higher detection of *tetM* compared to *tetK* in human methicillin-resistant *S. aureus* (MRSA) samples from Perak, with prevalence rates of 97.8% (n = 87) and 42.7% (n = 38), respectively. This parallel between bovine CoNS and human MRSA highlights the unrestricted host range of the *tetM* gene. This cross-host ubiquity is likely attributed to the common residence of *tetM* gene within the Tn*916*/Tn*1545* family of conjugative transposons (De Vries *et al.*, 2009; Ong *et al.*, 2017). These elements are highly mobilizable with a wide host range encompassing a diverse bacterial species (Ciric *et al.*, 2013; Wright & Grossman, 2016). It is capable of autonomous excision and subsequent chromosomal integration in the recipient cell. This integration ensures the genetic stability of the resistance determinant, allowing it to persist in the population even in the absence of antibiotic pressure (Wright & Grossman, 2016; Firth *et al.*, 2018). Therefore, the detection of this gene in *Staphylococcus* spp. and *Mammaliicoccus* spp. associated with bovine mastitis suggests that the veterinary environment may serve as a persistent reservoir for these mobile genetic elements. This persistence is likely driven by the selective pressure of constant tetracycline usage within the studied herd, necessitating immediate attention in veterinary therapeutic applications to mitigate further spread.

4.9.6 Screening of *dfrA* Gene That Confers Resistance to Sulfamethoxazole-Trimethoprim

To determine the genotypic association of sulfamethoxazole-trimethoprim resistance, molecular screening for the *dfrA* gene was performed on the three *S. chromogenes* isolates (AY574001, AP622002 and BP647054) exhibiting phenotypic resistance. The *dfrA* gene was chosen due to its reported mechanism for trimethoprim resistance in staphylococci associated with bovine mastitis (Shoaib *et al.*, 2023; Kazemzadeh *et al.*, 2025). Functionally, this gene is responsible for encoding the

modified dihydrofolate reductase (DHFR) enzyme, which bypasses the drug's inhibitory effect and ensures the synthesis of tetrahydrofolate (Mlynarczyk-Bonikowska *et al.*, 2022). As shown in Figure 4.13, PCR amplification revealed a distinct DNA band at 270 bp corresponding to the *dfrA* gene in only one of the three resistant isolates, specifically *S. chromogenes* (AY574001). The reference strain *S. epidermidis* ATCC 35984 was employed as a positive control to ensure that the chosen annealing temperature promoted sequence-specific primer binding and consistent target amplification. This observation was supported by statistical analysis, which demonstrated a substantial correlation between the resistance trait and the gene (κ = 0.387, $P < 0.001$). The detection of *dfrA* gene in a single isolate aligns with previous studies, which reported low prevalence of the gene in staphylococci. For instance, Nurjadi *et al.* (2021) and Shoaib *et al.* (2023) demonstrated that only 1% (n = 1) and 7.7% (n = 3) of staphylococcal isolates harboured this gene, respectively. The presence of *dfrA* gene in the *S. chromogenes* (AY574001) isolate likely indicates the acquisition of this gene via plasmids (Mlynarczyk-Bonikowska *et al.*, 2022). This gene can be mediated by the composite transposon Tn*4003*, which often co-harbour the *thyE* gene and is flanked by three copies of the insertion sequence IS257 (Schwarz *et al.*, 2014). The presence of the transposase enzyme within the IS257 element is of particular concern in bovine mastitis, as it facilitates the mobilization and rapid spread of this resistance gene among staphylococcal species within the dairy environment (Schwarz *et al.*, 2014). Consequently, the identification of *dfrA* gene in the *S. chromogenes* isolate AY574001 highlights its potential role as a genetic reservoir (Regecová *et al.*, 2021). This poses a risk of horizontally transferring sulfamethoxazole-trimethoprim resistance to other mastitis pathogens, potentially complicating therapeutic outcomes in dairy herds.

In contrast, the *dfrA* gene was not detected in the other resistant *S. chromogenes* isolates (AP622002 and BP647054). This suggests that resistance can be mediated by other ARGs. As noted by Nurjadi *et al.* (2021) and Khasapane *et al.* (2025), trimethoprim resistance is often conferred by alternative plasmid-borne genes, such as *dfrC*, *dfrD*, *dfrG,* or *dfrK*. These alternative genes function similarly to *dfrA* gene by modifying the DHFR to reduce its affinity for trimethoprim and are also host specific. Notably, the *dfrC* and *dfrG* genes have been specifically identified in *S. chromogenes* isolates associated with bovine mastitis (Klimiene *et al.*, 2016; Khasapane *et al.*, 2025).

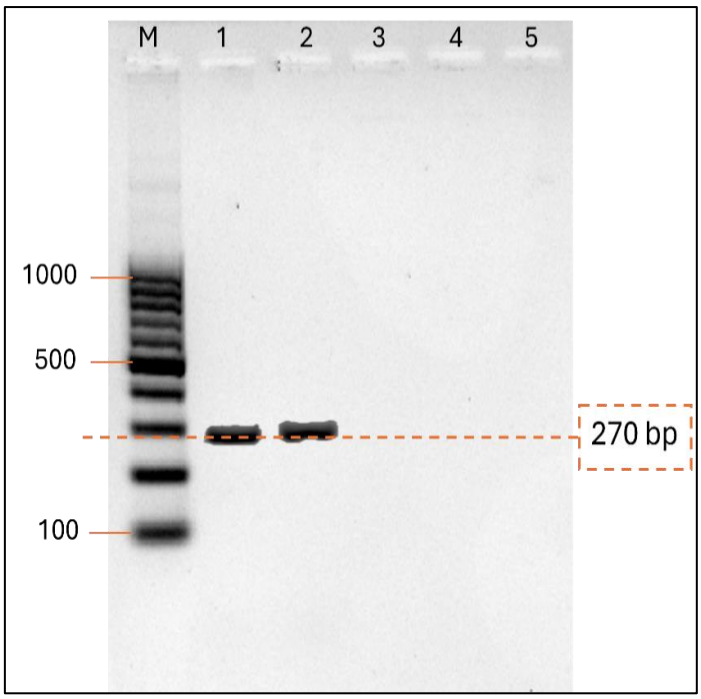


Figure 4.13 PCR Amplification of *dfrA* Gene (270 bp) in Resistant *S. chromogenes* Isolates. Lane M: 100 bp DNA Ladder (Vivantis); Lane 1: *S. epidermidis* ATCC 35984 (Positive Control); Lane 2 to 4: Tested Isolates (AY574001, AP622002 and BP647054, Respectively); Lane 5: Negative Control

Furthermore, it is important to recognize that sulfamethoxazole and trimethoprim act synergistically to inhibit folate synthesis. Consequently, the observed phenotypic resistance may not be solely driven by *dfr* genes but could also involve sulfonamides genes such as *sul* genes, which its screening was not included in this current study. According to Khan *et al.* (2022) and Shoaib *et al.* (2023), sulfonamides resistance in *Staphylococcus* can be mediated by genes such as *sul1*, *sul2*, or *sul3*, that target the sequential steps in folic acid pathway. These genes were identified as determinants of sulfonamides resistance in *Staphylococcus* species (Shoaib *et al.*, 2023). These *sul* genes encode for dihydropteroate synthase (DHPS) enzyme are typically located either on plasmid or integrons (Domínguez *et al.*, 2019; Venkatesan *et al.*, 2023). This genetic mobility facilitates the co-selection of multiple resistance determinants, thereby driving the emergence of multidrug-resistant *Staphylococcus*.

4.10 Biofilm Formation Activity of Staphylococcaceae Isolates Associated with Mastitis

To assess the biofilm formation activity, all 78 Staphylococcaceae isolates were subjected to microtiter plate-biofilm assay. Both positive and negative controls of *S.*

epidermidis were also included on every plate assay. Isolates were categorized using final optical density (OD) values measured relative to a cut-off threshold (OD_c), defined as three standard deviations above the mean OD of negative control (ODc = average OD of the negative control + (3 × ODc)) according to Stepanović *et al.* (2007). This cut-off threshold allowed standardized classification of isolates into four categories: non-biofilm, weak, moderate and strong biofilm formers. A summary of biofilm formation activity data was presented in Table 4.6, while the details regarding the ability of each isolates to form biofilm on microtiter plate are tabulated in Table 4.7.

Table 4.6
Distribution of Biofilm-Forming Strains of Staphylococcaceae (n = 78) Isolated from Bovine Milk Associated with SCM

Species (n)	Biofilm formation category			
	Non-biofilm (OD ≤ ODc) n (%)	**Weak (ODc < OD_{570} ≤ 2ODc) n (%)**	**Moderate (2ODc < OD_{570} ≤ 4ODc) n (%)**	**Strong (4ODc < OD_{570}) n (%)**
***Staphylococcus* spp.**				
S. aureus (2)	2 (100.0)	0 (0)	0 (0)	0 (0)
S. chromogenes (22)	16 (72.7)	3 (13.6)	2 (9.1)	1 (4.5)
S. haemolyticus (7)	5 (71.4)	1 (14.3)	1 (14.3)	0 (0)
S. simulans (3)	2 (66.7)	0 (0)	1 (33.3)	0 (0)
S. warneri (1)	1 (100.0)	0 (0)	0 (0)	0 (0)
***Mammaliicoccus* spp.**				
M. sciuri (5)	1 (20.0)	4 (80.0)	0 (0)	0 (0)
***Macrococcus* spp.**				
M. bohemicum (13)	13 (100.0)	0 (0)	0 (0)	0 (0)
M. caseolyticus (25)	8 (32.0)	10 (40.0)	7 (28.0)	0 (0)
Total strains (78) (%)	48 (61.5)	18 (23.1)	11 (14.1)	1 (1.3)

Note: n = total number of bacterial isolates; percentage (%) is calculated based on the total number of bacterial isolates.

Table 4.7
Biofilm-Forming Capabilities of Staphylococcaceae Isolates (n = 78)

Isolate ID	Species	Mean OD_{570}	Final OD	S.E.M	Fold Increase*	p (t-test)	Biofilm Phenotype
Negative	*Staphylococcus epidermidis* ATCC 12228	0.333	0.426	0.012	1.000	1.000	Non-biofilm
Positive	*Staphylococcus epidermidis* ATCC 35984	3.122	2.696	0.017	9.365	0.000	Strong
AY622018	*Staphylococcus aureus*	0.230	0.000	0.090	0.690	0.337	Non-biofilm
AP647134	*Staphylococcus aureus*	0.476	0.050	0.251	1.428	0.610	Non-biofilm
AP647135	*Staphylococcus chromogenes*	0.103	0.000	0.003	0.309	0.000	Non-biofilm
AP610138	*Staphylococcus chromogenes*	0.107	0.000	0.008	0.322	0.000	Non-biofilm
BY605111	*Staphylococcus chromogenes*	0.126	0.000	0.005	0.377	0.000	Non-biofilm
AP622002	*Staphylococcus chromogenes*	0.128	0.000	0.007	0.383	0.000	Non-biofilm
AP610142	*Staphylococcus chromogenes*	0.135	0.000	0.003	0.405	0.000	Non-biofilm
AP682126	*Staphylococcus chromogenes*	0.158	0.000	0.012	0.475	0.000	Non-biofilm
BP610011	*Staphylococcus chromogenes*	0.180	0.000	0.022	0.541	0.002	Non-biofilm
BP610010	*Staphylococcus chromogenes*	0.206	0.000	0.053	0.618	0.092	Non-biofilm
BP610012	*Staphylococcus chromogenes*	0.206	0.000	0.072	0.619	0.174	Non-biofilm
AP636010	*Staphylococcus chromogenes*	0.269	0.000	0.001	0.808	0.011	Non-biofilm
AP647129	*Staphylococcus chromogenes*	0.336	0.000	0.113	1.009	0.981	Non-biofilm
AP560034	*Staphylococcus chromogenes*	0.362	0.000	0.113	1.085	0.819	Non-biofilm
AP560027	*Staphylococcus chromogenes*	0.374	0.310	0.160	1.121	0.817	Non-biofilm
BP610009	*Staphylococcus chromogenes*	0.736	0.346	0.571	2.207	0.532	Non-biofilm
BP610001	*Staphylococcus chromogenes*	0.772	0.359	0.635	2.316	0.539	Non-biofilm
AP682124	*Staphylococcus chromogenes*	0.785	0.710	0.403	2.355	0.344	Weak
BP610054	*Staphylococcus chromogenes*	1.136	0.751	0.354	3.409	0.108	Weak
AY574001	*Staphylococcus chromogenes*	1.177	0.799	0.644	3.532	0.282	Weak
AP636016	*Staphylococcus chromogenes*	1.225	1.231	0.567	3.676	0.214	Moderate
AP647132	*Staphylococcus chromogenes*	1.657	1.498	0.424	4.972	0.052	Moderate

AP647133	***Staphylococcus chromogenes***	**1.924**	**1.792**	**0.635**	**5.773**	**0.087**	**Strong**
AP636005	***Staphylococcus chromogenes***	**2.218**	**0.000**	**0.615**	**6.653**	**0.055**	**Strong**
AY625153	*Staphylococcus haemolyticus*	0.140	0.000	0.003	0.419	0.000	Non-biofilm
AY625151	*Staphylococcus haemolyticus*	0.141	0.000	0.001	0.424	0.000	Non-biofilm
AY625149	*Staphylococcus haemolyticus*	0.151	0.000	0.014	0.454	0.000	Non-biofilm
BY620095	*Staphylococcus haemolyticus*	0.157	0.493	0.008	0.470	0.000	Non-biofilm
AY625148	*Staphylococcus haemolyticus*	0.564	0.138	0.011	1.692	0.000	Non-biofilm
AY625150	*Staphylococcus haemolyticus*	0.919	1.140	0.776	2.756	0.505	Weak
AY625147	*Staphylococcus haemolyticus*	1.565	0.000	0.234	4.696	0.013	Moderate
AP560023	*Staphylococcus simulans*	0.229	0.000	0.027	0.688	0.024	Non-biofilm
AP560017	*Staphylococcus simulans*	0.568	0.142	0.269	1.703	0.447	Non-biofilm
AP636007	*Staphylococcus simulans*	1.455	1.029	0.655	4.364	0.185	Moderate
AP625044	*Staphylococcus warneri*	0.796	0.370	0.026	2.387	0.000	Non-biofilm
BY620105	*Mammaliicoccus sciuri*	0.680	0.254	0.009	2.040	0.000	Non-biofilm
AY622098	*Mammaliicoccus sciuri*	0.887	0.461	0.023	2.662	0.000	Weak
AY622100	*Mammaliicoccus sciuri*	1.056	0.630	0.180	3.167	0.028	Weak
AY625127	*Mammaliicoccus sciuri*	1.138	0.712	0.065	3.413	0.001	Weak
AY622099	*Mammaliicoccus sciuri*	1.146	0.720	0.029	3.438	0.000	Weak
AP682122	*Macrococcoides bohemicum*	0.146	0.000	0.005	0.439	0.000	Non-biofilm
AP647128	*Macrococcoides bohemicum*	0.147	0.000	0.012	0.440	0.000	Non-biofilm
BP610052	*Macrococcoides bohemicum*	0.166	0.000	0.003	0.499	0.000	Non-biofilm
AP647136	*Macrococcoides bohemicum*	0.173	0.000	0.019	0.520	0.001	Non-biofilm
AP647130	*Macrococcoides bohemicum*	0.185	0.000	0.038	0.555	0.024	Non-biofilm
AP647131	*Macrococcoides bohemicum*	0.185	0.000	0.018	0.555	0.001	Non-biofilm
AP647137	*Macrococcoides bohemicum*	0.242	0.000	0.049	0.727	0.157	Non-biofilm
AP610141	*Macrococcoides bohemicum*	0.258	0.000	0.044	0.775	0.185	Non-biofilm
AP610140	*Macrococcoides bohemicum*	0.288	0.000	0.065	0.865	0.543	Non-biofilm
AP682123	*Macrococcoides bohemicum*	0.301	0.000	0.101	0.903	0.771	Non-biofilm
AP682125	*Macrococcoides bohemicum*	0.352	0.000	0.174	1.055	0.923	Non-biofilm
AP610139	*Macrococcoides bohemicum*	0.376	0.000	0.100	1.129	0.696	Non-biofilm
AP682127	*Macrococcoides bohemicum*	0.414	0.000	0.160	1.242	0.649	Non-biofilm

AY622064	*Macrococcus caseolyticus*	0.182	0.000	0.004	0.545	0.000	Non-biofilm
AP560042	*Macrococcus caseolyticus*	0.219	0.000	0.028	0.658	0.019	Non-biofilm
AP660052	*Macrococcus caseolyticus*	0.241	0.000	0.009	0.723	0.001	Non-biofilm
AP760084	*Macrococcus caseolyticus*	0.269	0.000	0.017	0.808	0.025	Non-biofilm
AY622061	*Macrococcus caseolyticus*	0.270	0.000	0.078	0.809	0.478	Non-biofilm
AY622097	*Macrococcus caseolyticus*	0.378	0.000	0.102	1.133	0.692	Non-biofilm
AP660047	*Macrococcus caseolyticus*	0.470	0.044	0.293	1.409	0.673	Non-biofilm
AY625121	*Macrococcus caseolyticus*	0.573	0.147	0.146	1.719	0.199	Non-biofilm
AP760087	*Macrococcus caseolyticus*	0.888	0.462	0.119	2.664	0.018	Weak
AP660048	*Macrococcus caseolyticus*	0.996	0.570	0.061	2.989	0.001	Weak
AP660046	*Macrococcus caseolyticus*	1.012	0.586	0.085	3.035	0.004	Weak
AP682089	*Macrococcus caseolyticus*	1.013	0.587	0.144	3.039	0.018	Weak
AP760086	*Macrococcus caseolyticus*	1.016	0.590	0.060	3.048	0.001	Weak
AP660045	*Macrococcus caseolyticus*	1.072	0.647	0.076	3.218	0.002	Weak
AP760083	*Macrococcus caseolyticus*	1.149	0.723	0.069	3.447	0.001	Weak
AP529082	*Macrococcus caseolyticus*	1.214	0.788	0.057	3.643	0.000	Weak
AP560043	*Macrococcus caseolyticus*	1.232	0.806	0.037	3.697	0.000	Weak
AP529080	*Macrococcus caseolyticus*	1.270	0.844	0.062	3.810	0.000	Weak
AP529079	*Macrococcus caseolyticus*	1.305	0.879	0.100	3.914	0.002	Moderate
AY622084	*Macrococcus caseolyticus*	1.374	0.948	0.066	4.121	0.000	Moderate
AP660077	*Macrococcus caseolyticus*	1.423	0.997	0.050	4.269	0.000	Moderate
AP529081	*Macrococcus caseolyticus*	1.444	1.018	0.040	4.333	0.000	Moderate
AP660078	*Macrococcus caseolyticus*	1.632	1.206	0.135	4.896	0.002	Moderate
AP760085	*Macrococcus caseolyticus*	1.641	1.215	0.129	4.922	0.002	Moderate
AP760088	*Macrococcus caseolyticus*	1.686	1.260	0.536	5.058	0.086	Moderate

Note: Mean OD = average of optical density reading; Final OD = mean OD of a stain – OD negative control (ODc); S.E.M = standard error of mean; *indicates the fold increase of biofilm formation as compared to the negative control strain *S. epidermidis* ATCC 12228.

Based on the Table 4.6, an overall 38.5% (n = 30) of the Staphylococcaceae isolates associated with bovine mastitis were determined as biofilm producers, exhibiting varying categories for biofilm formation. Specifically, most of the biofilm formers were classified as weak at 23.1% (n = 18), followed by moderate at 14.1% (n = 11) and strong biofilm at 1.3% (n = 1). According to bacterial species, *M. caseolyticus* exhibited the highest prevalence of biofilm formation activity at 68% (n = 17), followed by 27.2% (n = 6) of *S. chromogenes*, 28.6% (n = 2) of *S. haemolyticus*, 33.3% (n = 1) of *S. simulans* and 80% (n = 4) of *M. sciuri* (Table 4.6). The presence of this phenotype among all bacterial species highlights the capacity of these mastitis-associated causative pathogens to adhere to bovine epithelial tissue of mammary gland or milking equipment (Hordofa, 2022).

Interestingly, *M. caseolyticus* strains exhibited 40% (n = 10) as weak biofilm formers and 28% (n = 7) as moderate biofilm formers. The weak and moderate biofilm formers demonstrated the OD range value of 0.462 to 0.844 and 0.879 to 1.260, with a fold increase range of 2.664 to 3.810 and 3.914 to 5.058 (Table 4.7). Although this species has previously been considered a minor pathogen, the present findings highlight emerging challenges. The phenotypic data suggest that *Macrococcus* possesses virulence factors that allow it to persist in the udder environment comparable to those of established major pathogens such as *S. aureus*. Comparison with other study is limited as not many studies focused on *M. caseolyticus* isolates recovered from bovine mastitis. However, in one study, a similar finding has been documented by Mnif *et al.* (2020) who isolated *M. caseolyticus* from bulk milk storage tanks. In their study, the isolates were identified as strong biofilm formers that also produced extracellular proteases, mechanisms known to facilitate host cell invasion and colonization. In contrast, a study in Brazil by Machado *et al.* (2020) reported that *M. caseolyticus* isolates recovered from pasteurized milk did not exhibit biofilm-forming activity when evaluated using the Congo Red Agar assay. This variation in phenotypic expression is likely driven by distinct factors, which are sample recovery and methodological constraints employed in the study. The bacterial strains recovered in bovine milk associated with bovine mastitis may require a strong adherence mechanism compared to those surviving post-pasteurization. Additionally, differences in the biofilm formation assay between the qualitative and quantitative method may be attributed for this divergent observation. Collectively, these contrasting findings underscore the

phenotypic diversity within the genus *Macrococcus* and highlight the need for further genomic and phenotypic characterization of mastitis-associated isolates.

In *Macrococcus* strains biofilm formation can be driven by capsule- and protein-mediated mechanisms (Mašlaňová *et al.*, 2018; Zhang *et al.*, 2022). A study conducted by Mašlaňová *et al.* (2018) demonstrated that the capability of *M. caseolyticus* to form biofilms can be mediated by *veg* and *cap* genes. Specifically in clinical *M. caseolyticus* isolate, the *veg* gene encodes a regulatory protein that controls the production of extracellular matrix, while *cap* gene synthesizes a capsular polysaccharide to form the protective slime layer encasing the bacterial cells (Mašlaňová *et al.*, 2018). By forming this robust extracellular matrix, *M. caseolyticus* effectively reduces the penetration of antibiotics, shielding itself from treatment within udder environment. Given its genetic potential to establish biofilms in the udder environment, *M. caseolyticus* may complicate therapeutic interventions for mastitis, suggesting a need to reconsider its clinical relevance in dairy herd management.

In the biofilm-positive *Staphylococcus* spp. strains, 44.4% (n = 4) were identified as weak and 44.4% (n = 4) as moderate biofilm former with the OD range value and a fold increase range of 0.493 to 0.799 and 0.470 to 3.532 and 1.029 to 1.498 and 2.756 to 4.972, respectively (Table 4.7). Notably, a strong biofilm-forming strain was identified in only 11.1% (n = 1) of the *S. chromogenes* isolates, which was the isolate named AP622005. The biofilm formers in this study were categorized as CoNS isolates. The prevalence of biofilm production in current finding was in contrast to previous studies conducted in Brazil and Uruguay that have reported a high biofilm production rate of 65.8% (n = 25) and 94.5% (n = 34) in CoNS associated with bovine mastitis, respectively (Israel *et al.*, 2022; Moreno *et al.*, 2025). The high prevalence of biofilm production in CoNS suggests that the enhancement of invasive potential and the ability to cause bovine mastitis infection (Marek *et al.*, 2021). The determination of strong biofilm-formation activity in the *S. chromogenes* isolate aligns with a study conducted by Goetz *et al.* (2017), who also reported strong biofilm production in *S. chromogenes* obtained from mastitis pathogen culture collection in Canada. This is possibly due to the association of *icaA* gene by *S. chromogenes* at 61.5% (n = 40) higher than other CoNS isolates, enabling polysaccharide intercellular adhesin (PIA) production for a robust biofilm matrix (Lee & Lee, 2022). In other CoNS, the biofilm formation activity might be mediated by weaker PIA-independent mechanism that are protein-dependent, such as fibronectin-binding proteins (FnBPs), fibrinogen-binding

protein (Fib), clumping factors (Clfs) and serine-aspartate repeat family proteins (Sdrs) (O'Gara, 2007; Schilcher & Horswill, 2020). This allows CoNS to serve as a reservoir, potentially transferring antibiotic resistance genes or virulence factors to other pathogenic staphylococci thereby facilitating chronic infections that are increasingly difficult to eradicate.

Regarding *Mammaliicoccus* spp., which were represented exclusively by *M. sciuri* in this study, 80% (n = 4) were classified as weak biofilm formers. The weak biofilm formers were classified based on the OD range values of 0.461 to 0.720 with a fold increase range of 2.662 to 3.438. Current findings align with a study by Lira *et al.* (2016), who similarly observed that the single *M. sciuri* isolate recovered in their study was positive for biofilm production. Furthermore, the result of *M. sciuri* as a biofilm former was also reported by Gajewska and Chajęcka-Wierzchowska (2020), who found that 100% (n = 2) of *M. sciuri* isolated from raw bovine milk were capable of forming strong biofilms of the association of *aap* gene, which encode the accumulation associated protein (Gajewska & Chajęcka-Wierzchowska, 2020). These findings suggest that biofilm production facilitates the persistence of these opportunistic pathogens on milking equipment and milkers' hands (Ruiz-Romero & Vargas-Bello-Pérez, 2023). Consistent with other staphylococci, a local study in Terengganu and Kelantan by Zakaria *et al.* (2026) reported the presence of the *ica* gene cluster, specifically *icaA* and *icaC* as essential genes for biofilm production in *M. sciuri*. In addition to the *ica* cluster, Zakaria *et al.* (2026) also detected the presence of the *sspA* gene, which encodes an extracellular proteolytic enzyme in all four *M. sciuri* isolates obtained from farmed ruminants. This aligns with previous findings by Naushad *et al.* (2019) and Veken *et al.* (2022), which consistently report the presence of *sspA* in this species. Collectively, the presence of *ica* and *sspA*, suggests that these genes constitute the virulence profiles of *M. sciuri* strains.

4.10.1 Correlation between Antibiotic Resistance and Biofilm Formation

To evaluate the relationship between antibiotic resistance and biofilm formation activity among the bacterial isolates, a cross-tabulation analysis was conducted. The application of Pearson Chi-Square test was precluded, as 58.3% of the expected frequencies fell below the minimum threshold of five ($n < 5$). Consequently, Fisher's exact test was employed to accommodate this limited dataset, thereby ensuring the

reliability of the statistical analysis. The distribution of resistance counts across the biofilm formation categories is tabulated in Table 4.8. The detailed antibiotic resistance profile and biofilm formation capacity for each isolate are summarized in Appendix 5.

Based on Fisher's exact test, the results demonstrate a significant positive relationship between biofilm formation and antibiotic resistance profiles ($p = 0.001$). The analysis indicates that increased biofilm-forming activity is associated with increased antibiotic resistance profile. Descriptively, isolates exhibiting a mono-resistant phenotype (1R) were predominantly classified as biofilm non-producers, accounting for 77% ($n = 39$) of isolates. In contrast, isolates demonstrating resistance to two antibiotic classes (2R) exhibited a higher distribution of biofilm-producing phenotype, with 68% ($n = 17$) categorized as weak, moderate, or strong biofilm formers. This overall trend aligns with the studies by Adhikari *et al.* (2025) and Rychshanova *et al.* (2022), who similarly reported the positive correlation between biofilm formation and antibiotic resistance profiles. Furthermore, literature consistently demonstrates that biofilm-producing bacteria exhibit higher resistance rates in comparison to non-biofilm producers (Oliveira *et al.*, 2016; Rychshanova *et al.*, 2022). In the context of bovine mastitis, Adhikari *et al.* (2025) highlighted that isolates exhibiting a high level of multidrug-resistance phenotype are particularly adapted for persistence within the mammary gland. This environmental adaptation is further supported by Rychshanova *et al.* (2022), who found 43.8% of the MDR *S. aureus* isolates possessed the ability to form biofilms utilizing the matrix as a protective mechanism against antibiotics.

However, of the two multidrug-resistant (MDR) *S. chromogenes* isolates identified, one isolate (BP647054) exhibited a weak biofilm phenotype, whereas the other (AP622002) was a non-biofilm producer (Appendix 5). The observation of non-biofilm producing in MDR isolate likely reflects an evolutionary trade-off driven by biological fitness costs. The acquisition of multiple resistance genes impose a substantial metabolic burden on the bacterial cell, which reduces the energy available for exopolysaccharide synthesis (Arciola *et al.*, 2015; Marincola *et al.*, 2021; Ahmad *et al.*, 2025). Conversely, the weak biofilm-forming MDR isolate likely retains functional ica clusters or compensates with alternative surface-adhesion factors, such as *atl*, *ebh*, or *ebp* genes, that sustain minimal matrix production even in the presence of multidrug resistance traits (Asante *et al.*, 2022). Notably, the moderate biofilm formers exhibited resistance to only one or two antibiotics and the single strong biofilm-forming isolate (AP622005) demonstrated resistance to exactly two antibiotics (Appendix 5).

Table 4.8
Association Between Antibiotic Resistance Profiles and Biofilm Formation Activity in Staphylococcaceae Isolates (n = 78)

Resistance Profile/ Species (n)	Non-biofilm, n (%)	Weak, n (%)	Moderate, n (%)	Strong, n (%)
	1R			
S. aureus (2)	**2 (100)**	0 (0)	0 (0)	0 (0)
S. chromogenes (14)	**12 (86)**	0 (0)	**2 (14)**	0 (0)
S. haemolyticus (6)	**4 (67)**	**1 (17)**	**1 (17)**	0 (0)
S. simulans (2)	**1 (50)**	0 (0)	**1 (50)**	0 (0)
S. warneri (1)	**1 (100)**	0 (0)	0 (0)	0 (0)
M. sciuri (3)	0 (0)	**3 (100)**	0 (0)	0 (0)
M. bohemicum (13)	**13 (100)**	0 (0)	0 (0)	0 (0)
M. caseolyticus (10)	**6 (60)**	**2 (20)**	**2 (20)**	0 (0)
Total 1R (51)	**39 (77)**	**6 (12)**	**6 (12)**	0 (0)
	2R			
S. chromogenes (6)	**3 (50)**	**2 (33)**	0 (0)	**1 (17)**
S. haemolyticus (1)	**1 (100)**	0 (0)	0 (0)	0 (0)
S. simulans (1)	**1 (100)**	0 (0)	0 (0)	0 (0)
M. sciuri (2)	**1 (50)**	**1 (50)**	0 (0)	0 (0)
M. caseolyticus (15)	**2 (13)**	**8 (53)**	**5 (33)**	0 (0)
Total 2R (25)	**8 (32)**	**11 (44)**	**5 (20)**	**1 (4)**
	3R			
S. chromogenes (2)	**1 (50)**	**1 (50)**	0 (0)	0 (0)
Total 3R (2)	**1 (50)**	**1 (50)**	0 (0)	0 (0)

Note: Percentage (%) is calculated based on the total number of bacterial isolates (n). Abbreviations: 1R: resistance to one antibiotic; 2R: resistance to two antibiotics; 3R: resistance to three antibiotics. Statistical significance determined by Fisher's Exact Test ($p = 0.001$) due to limited number of isolates in specific subgroup ($n < 5$).

This observation highlights that while biofilm provides a robust phenotypic and mechanical defence, MDR can be driven by genotypic factor as mentioned in section 4.8.1 rather than dependent on biofilm formation capacity. Nevertheless, the significant positive correlation suggests that biofilm production remains a critical factor in treatment failure. The persistence of bacteria in the bovine udder encompasses complex survival strategies, including adaptive stress responses, physiological alteration and the development of dormant persister cells within the biofilm matrix (Omwenga & Awuor,

2024; Adhikari *et al.*, 2025). The ability of staphylococci to establish capsule-like exterior extracellular polymeric substance (EPS) layer contributes to the resilience of organisms by limiting the penetration of antibiotics into the mammary gland. Consequently, as Mirghani *et al.* (2022) noted, when the bacteria utilize this EPS shield, they can exhibit up to a thousand-fold increase in antibiotic resistance compared to bacteria in a planktonic state, making non-MDR strains incredibly difficult to eradicate.

CHAPTER 5
CONCLUSION

This research comprehensively characterizes the distribution, antibiotic resistance profiles, resistance genes and biofilm-forming potential, of mastitis-associated Staphylococcaceae recovered from a Malaysian dairy herd. While initial phenotypic screening suggested a predominance of *Staphylococcus* spp., subsequent molecular identification targeting the *sodA* gene in 78 isolates (selected based on preliminary antibiotic screening) revealed various members of Staphylococcaceae including *S. aureus* (n = 2), *S. chromogenes* (n = 22), *S. haemolyticus* (n = 7), *S. simulans* (n = 3), *S. warneri* (n = 1), *M. sciuri* (n = 5), *M. bohemicum* (n = 13) and *M. caseolyticus* (n = 25). Although *Staphylococcus* species are well-established causes of mastitis, these findings highlight the emergence of previously underreported species, specifically *M. caseolyticus* and *M. bohemicum*, as significant intramammary pathogens. The key novelty of this study is the definitive molecular identification of *Macrococcus* spp. as highly resistant, biofilm-forming mastitis pathogens within Malaysian dairy cattle. Consequently, this study demonstrates that reliance on phenotypic assays using selective and differential media and standard biochemical tests is fundamentally insufficient for the accurate surveillance of aetiological agents associated with bovine mastitis.

Furthermore, antibiotic susceptibility profiling revealed a concerning pattern of resistance directed particularly against β-lactams. Phenotypically, all isolates exhibited resistance to penicillin (100%), followed by cefoxitin (22%) and tetracycline (12%) while low resistance was observed for gentamicin (1%) and sulfamethoxazole-trimethoprim (4%). None of the isolates were found to be resistant against erythromycin. Notably, multidrug resistance (MDR) was restricted to 3% (n = 2) of the total isolates, represented by isolates AP622002 and BP647054, which belonged exclusively to *S. chromogenes* species. The relationship between phenotypic and genotypic characterization yielded variable levels of concordance across tested antibiotics. Molecular screening confirmed the presence of *blaZ* gene in 64.1% of penicillin-resistant isolates, demonstrating a poor statistical agreement with phenotypic resistance. This suggests that the presence of other resistance determinants

that may confer to this phenotype. Conversely, a fair statistical concordance was observed for other resistance genes, with *tetM* (100%) and *tetK* (88.9%) detected in tetracycline-resistant isolates, *aacA-aphD* (100%) in gentamicin-resistant isolate, and *dfrA* (33.3%) in sulfamethoxazole-trimethoprim-resistant isolates. The *mecA* gene was restricted to 5.9% found only in *M. sciuri*. A pivotal genotypic finding was the detection of the *mecD* gene in 88.2% of cefoxitin-resistant *M. caseolyticus*, which is clinically significant as it is residing in the *M. caseolyticus* resistance island *mecD* ($McRI_{mecD}$). This suggests that *Macrococcus* may serve as a hidden reservoir for the methicillin-resistance element, posing a potential risk of transferring the resistance gene to other bacterial species. Likewise, the molecular detection of tetracycline resistance revealed the presence of both *tetK* and *tetM* genes that were linked to a conjugative transposon. This finding highlights the need for routine molecular surveillance to monitor the horizontal gene transfer within the herd.

Regarding the extent of biofilm capability among resistant isolates, 38.5% of the recovered Staphylococcaceae isolates were biofilm producers, with *M. caseolyticus* exhibiting the highest prevalence of biofilm-formation activity. Stronger biofilm capacity was significantly associated with dual-antibiotic resistance pattern and Fisher's exact test ($p = 0.001$) confirmed a strong positive correlation between biofilm formation and antibiotic resistance phenotypes. This suggests that biofilm formation significantly contributes to bacterial persistence and reduces the effectiveness of standard antibiotic treatment, thereby, causing the mastitis infection more worsen.

Overall, this study highlights the necessity of integrating molecular detection with phenotypic observation to accurately characterize the aetiological agents of bovine mastitis. The substantial misidentification of *Macrococcus* species as *Staphylococcus* reveals the gap that compromises the accuracy of surveillance data for intramammary infections. Furthermore, the discovery of *mecD*-mediated methicillin resistance and tetracycline genes within *Macrococcus* isolates identifies them as a silent reservoir for antibiotic resistance, posing a significant risk of horizontal gene transfer within dairy herds. The significant association of biofilm formation capacity and antibiotic resistance profiles suggests that mastitis control strategies may require targeted re-evaluation to maintain their long-term efficacy. Consequently, adopting comprehensive molecular screening is essential to track these emerging pathogens, mitigate the silent dissemination of resistance genes and ensure that future veterinary interventions remain targeted, effective and sustainable within the One Health framework.

Based on these findings, several recommendations are proposed to enhance the management of bovine mastitis such as quantitative subclinical screening, enhancing farm hygiene practices and adhering strictly to therapeutic dosage. For instance, at farm level, immediate priority should be given to the implementation of rapid, reliable and quantitative tests, such as automated somatic cell counting via flow cytometry to overcome the subjective limitations of CMT assay, enabling early SCM detection to halt pathogen transmission. Concurrently, because 38.5% of isolates exhibited biofilm forming capacity, farm hygiene must be upgraded by integrating cleaning-in-place protocols with enzymatic detergents and post-milking disinfectant capable of disrupting bacterial extracellular polymeric substance matrices. Furthermore, the high prevalence of antibiotic resistance requiring farmers and veterinarians to adhere strictly to therapeutic dosages to mitigate selection pressure for multidrug-resistant phenotypes.

For future study, other specific primers targeting conserved housekeeping genes, such as the *rpoB* and *tuf* genes, alongside *sodA*, are recommended to guarantee high-resolution, species-level discrimination among staphylococci and emerging *Macrococcus* species in phylogenetic analysis. For a comprehensive genomic analysis, whole genome sequencing (WGS) is essential to elucidate the complete genetic profile of the identified pathogens. WGS is crucial for mapping the mobile genetic elements responsible for carrying the resistance determinants, thereby clarifying their potential for horizontal gene transfer. Simultaneously, molecular screening should be expanded to include specific biofilm-associated genes (such as *ica* and *bap* genes) to elucidate the specific genetic mechanisms driving the strong biofilm capacity in the Staphylococcaceae isolates. Finally, conducting longitudinal surveillance program is vital to continuously monitor the persistence and distribution of these pathogens. The resulting epidemiological data is necessary to implement rigorous antibiotic stewardship policies, guide prudent antibiotic use and establish effective, data-driven dairy management practices.

REFERENCES

Abdul-Aziz, A., Mohamad, S. A. S., & Abdullah, M. F. F. (2015). Identification of Coagulase-Negative Staphylococci by *sodA* Gene Sequence Analysis. *Current Research in Bacteriology*, *8*(3), 48.

Abebe, R., Hatiya, H., Abera, M., Megersa, B., & Asmare, K. (2016). Bovine mastitis: Prevalence, Risk Factors and Isolation of *Staphylococcus aureus* in Dairy Herds at Hawassa Milk Shed, South Ethiopia. *BMC Veterinary Research 2016 12:1*, *12*(1), 1–11.

Abebe, R., Markos, A., Abera, M., & Mekbib, B. (2023). Incidence Rate, Risk Factors and Bacterial Causes of Clinical Mastitis on Dairy Farms in Hawassa City, Southern Ethiopia. *Scientific Reports*, *13*, 10945.

Abed, A. H., Menshawy, A. M. S., Zeinhom, M. M. A., Hossain, D., Khalifa, E., Wareth, G., & Awad, M. F. (2021). Subclinical Mastitis in Selected Bovine Dairy Herds in North Upper Egypt: Assessment of Prevalence, Causative Bacterial Pathogens, Antimicrobial Resistance and Virulence-Associated Genes. *Microorganisms*, *9*(6), 1175.

Adhikari, S., Khanal, S., Marasini, A., Panthi, P., Adhikari, A., Luitel, H., & Pandeya, Y. R. (2025). Antimicrobial Resistance and Biofilm-Forming Ability in *Staphylococcus aureus* Causing Clinical Bovine Mastitis in Chitwan, Nepal. *Veterinary and Animal Science*, *30*, 100508.

Águila-Arcos, S., Álvarez-Rodríguez, I., Garaiyurrebaso, O., Garbisu, C., Grohmann, E., & Alkorta, I. (2017). Biofilm-Forming Clinical *Staphylococcus* Isolates Harbor Horizontal Transfer and Antibiotic Resistance Genes. *Frontiers in Microbiology*, *8*, 289347.

Ahmad, M., Aduru, S. V., Smith, R. P., Zhao, Z., & Lopatkin, A. J. (2025). The Role of Bacterial Metabolism in Antimicrobial Resistance. *Nature Reviews. Microbiology*, *23*(7), 439–454.

Ahmed, W., Neubauer, H., Tomaso, H., El Hofy, F. I., Monecke, S., Abdeltawab, A. A., & Hotzel, H. (2020). Characterization of Staphylococci and Streptococci Isolated from Milk of Bovides with Mastitis in Egypt. *Pathogens*, *9*(5), 381.

Ajose, D. J., Oluwarinde, B. O., Abolarinwa, T. O., Fri, J., Montso, K. P., Fayemi, O. E., Aremu, A. O., & Ateba, C. N. (2022). Combating Bovine Mastitis in the Dairy Sector in an Era of Antimicrobial Resistance: Ethno-veterinary Medicinal

Option as a Viable Alternative Approach. *Frontiers in Veterinary Science*, *9*, 287.

Aklilu, E., & Ying, C. H. (2020). First *mecC* and *mecA* Positive Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (*mecC* MRSA/LA-MRSA) from Dairy Cattle in Malaysia. *Microorganisms*, *8*(2).

Ali, B., Zakaria, Z., Firdaus, F., Ramanoon, S., & Mohd Lila, M. (2020). Prevalence and Antimicrobial Resistance Profiles of Bacterial Isolates Associated with Intramammary Infections in Malaysian Dairy Herds.

Ali, T., Kamran, Raziq, A., Wazir, I., Ullah, R., Shah, P., Ali, M. I., Han, B., & Liu, G. (2021). Prevalence of Mastitis Pathogens and Antimicrobial Susceptibility of Isolates From Cattle and Buffaloes in Northwest of Pakistan. *Frontiers in Veterinary Science*, *8*, 746755.

Alnakip, M. E., 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*, 659801.

Alzohairy, A. (2024). By Interpreting BLAST Result.

Amin, N. M., Nor, N. M., Bakar, M. Z. A., Sadiq, M. B., Omar, S., & Mansor, R. (2025). Prevalence and Risk Factors of Bubaline Subclinical Mastitis in Selected Peninsular Malaysian States. *Pertanika Journal of Tropical Agricultural Science*, *48*(4).

Amini, S., Omidi, A. H., Afkhami, H., Sabati, H., Mohsenzadeh, A., Soleymani, A., Zonobian, M. A., Ghanbarnejad, N., & Mohammadi, M. R. (2024). Frequency of *aac (6')-le-aph (2'')* Gene and Resistance to Aminoglycoside Antibiotics in *Staphylococcus aureus* Isolates. *Cellular, Molecular and Biomedical Reports*, *4*(2), 120–128. 6

Antignac, A., & Tomasz, A. (2009). Reconstruction of the Phenotypes of Methicillin-Resistant *Staphylococcus aureus* by Replacement of the Staphylococcal Cassette Chromosome *mec* with a Plasmid-Borne Copy of *Staphylococcus sciuri pbpD* Gene. *Antimicrobial Agents and Chemotherapy*, *53*(2), 435–441.

Arciola, C. R., Campoccia, D., Ravaioli, S., & Montanaro, L. (2015). Polysaccharide Intercellular Adhesin in Biofilm: Structural and Regulatory Aspects. *Frontiers in Cellular and Infection Microbiology*, *5*.

Argaw, A. (2016). Review on Epidemiology of Clinical and Subclinical Mastitis on Dairy Cows.

Argudín, M. A., Roisin, S., Nienhaus, L., Dodémont, M., de Mendonça, R., Nonhoff, C., Deplano, A., & Denis, O. (2018). Genetic Diversity among *Staphylococcus aureus* Isolates Showing Oxacillin and/or Cefoxitin Resistance Not Linked to the Presence of *mec* Genes. *Antimicrobial Agents and Chemotherapy*, *62*(7), e00091-18.

Asante, J., Abia, A. L. K., Anokwah, D., Hetsa, B. A., Fatoba, D. O., Bester, L. A., & Amoako, D. G. (2022). Phenotypic and Genomic Insights into Biofilm Formation in Antibiotic-Resistant Clinical Coagulase-Negative *Staphylococcus* Species from South Africa. *Genes*, *14*(1), 104.

Ashraf, A., & Imran, M. (2020). Causes, Types, Etiological Agents, Prevalence, Diagnosis, Treatment, Prevention, Effects on Human Health and Future Aspects of Bovine Mastitis. *Animal Health Research Reviews*, *21*(1), 36–49.

Aslam, B., Khurshid, M., Arshad, M. I., Muzammil, S., Rasool, M., Yasmeen, N., Shah, T., Chaudhry, T. H., Rasool, M. H., Shahid, A., Xueshan, X., & Baloch, Z. (2021). Antibiotic Resistance: One Health One World Outlook. *Frontiers in Cellular and Infection Microbiology*, *11*.

Ayeni, F. A., Andersen, C., & Nørskov-Lauritsen, N. (2017). Comparison of Growth on Mannitol Salt Agar, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry, VITEK® 2 with Partial Sequencing of 16S rRNA Gene for Identification of Coagulase-Negative Staphylococci. *Microbial Pathogenesis*, *105*, 255–259.

Azharollah, F. H., Faiz, M., Abdullah, F., Farah, S., Mohd Nawi, A., & Abdul-Aziz, A. (2021). High Prevalence of Methicillin-Resistant *Staphylococcus haemolyticus* Isolated from Commensals in Healthy Adults. *Malaysian Journal of Medicine and Health Sciences*, *17*(1), 2636–9346.

Bari, M. S., Rahman, M. M., Persson, Y., Derks, M., Sayeed, M. A., Hossain, D., Singha, S., Hoque, M. A., Sivaraman, S., Fernando, P., Ahmad, I., Samad, A., & Koop, G. (2022). Subclinical Mastitis in Dairy Cows in South-Asian Countries: A Review of Risk Factors and Etiology to Prioritize Control Measures. *Veterinary Research Communications*, *46*(3), 621–640.

Basuino, L., Jousselin, A., Alexander, J. A. N., Strynadka, N. C. J., Pinho, M. G., Chambers, H. F., & Chatterjee, S. S. (2018). PBP4 Activity and Its

Overexpression are Necessary for PBP4-Mediated High-Level β-lactam Resistance. *Journal of Antimicrobial Chemotherapy*, *73*(5), 1177–1180.

Bedruddin, M. (2023). Review on Mastitis and Public Health Importance of Mastitis Causing Pathogens in Raw Milk from Mastitis Infected Dairy Goat.

Bello, S., Mudassir, S. H., Rudra, B., & Gupta, R. S. (2023). Phylogenomic and Molecular Markers Based Studies on Staphylococcaceae and Gemella species. Proposals for an Emended Family Staphylococcaceae and Three New Families (Abyssicoccaceae fam. Nov., Salinicoccaceae fam. Nov. And Gemellaceae fam. Nov.) Harboring Four New Genera, *Lacicoccus* gen. nov., *Macrococcoides* gen. nov., *Gemelliphila* gen. nov., and *Phocicoccus* gen. nov. *Antonie van Leeuwenhoek*, *116*(10), 937–973.

Beuckelaere, L., De Visscher, A., Souza, F. N., Meyer, E., Haesebrouck, F., Piepers, S., & De Vliegher, S. (2021). Colonization and Local Host Response Following Intramammary *Staphylococcus chromogenes* Challenge in Dry Cows. *Veterinary Research*, *52*, 137.

Bhavana, R. N., & Chaitanya, R. K. (2022). Identification of Coagulase Negative Staphylococcal Species from Bovine Mastitis in India. *Iranian Journal of Veterinary Research*, *23*(4), 358–362.

Boukhalfa, N., Douifi, M., Berber, A., & Hakem, A. (2021). Prevalence and Antibiotic Susceptibility of *Staphylococcus aureus* Isolated from Cow Mastitis in Algeria. *Agricultura*, *120*(3–4).

Brdová, D., Ruml, T., & Viktorová, J. (2024). Mechanism of Staphylococcal Resistance to Clinically Relevant Antibiotics. *Drug Resistance Updates*, *77*, 101147.

Campos, B., Pickering, A. C., Rocha, L. S., Aguilar, A. P., Fabres-Klein, M. H., de Oliveira Mendes, T. A., Fitzgerald, J. R., & de Oliveira Barros Ribon, A. (2022). Diversity and Pathogenesis of *Staphylococcus aureus* from Bovine Mastitis: Current Understanding and Future Perspectives. *BMC Veterinary Research*, *18*(1), 115.

Camsing, A., Phetburom, N., Chopjitt, P., Pumhirunroj, B., Patikae, P., Watwiengkam, N., Yongkiettrakul, S., Kerdsin, A., & Boueroy, P. (2024). Occurrence of Antimicrobial-Resistant Bovine Mastitis Bacteria in Sakon Nakhon, Thailand. *Veterinary World*, *17*(6), 1202–1209.

Cantekin, Z., Ozmen, G., Demir, M., Er, Z., Gurturk, K., Solmaz, H., Ekin, I., Ozturk, D., Gözer, A., & Ergun, Y. (2024). Presence of Antibiotic Resistance Genes in

Staphylococci Isolated From Bovine Subclinical Mastitis. *Large Animal Review*, *30*, 99–104.

Carroll, L. M., Pierneef, R., Mafuna, T., Magwedere, K., & Matle, I. (2023). Genus-Wide Genomic Characterization of *Macrococcus*: Insights into Evolution, Population Structure and Functional Potential. *Frontiers in Microbiology*, *14*, 1181376.

Chadi, Z. D., Dib, L., Zeroual, F., & Benakhla, A. (2022). Usefulness of Molecular Typing Methods for Epidemiological and Evolutionary Studies of *Staphylococcus aureus* Isolated from Bovine Intramammary Infections. *Saudi Journal of Biological Sciences*, *29*(8), 103338.

Chaves, B. J., & Tadi, P. (2024). Gentamicin. In *StatPearls*. StatPearls Publishing.

Chen, X., Chen, Y., Zhang, W., Chen, S., Wen, X., Ran, X., Wang, H., Zhao, J., Qi, Y., & Xue, N. (2022). Prevalence of Subclinical Mastitis among Dairy Cattle and Associated Risks Factors in China during 2012–2021: A Systematic Review and Meta-Analysis. *Research in Veterinary Science*, *148*, 65–73.

Cheng, W. N., & Han, S. G. (2020). Bovine Mastitis: Risk Factors, Therapeutic Strategies and Alternative Treatments — A Review. *Asian-Australasian Journal of Animal Sciences*, *33*(11), 1699–1713.

Chowdhury, M. S. R., Hosen, M. M., Hossain, M. H., Islam, M. R., Uddin, M. B., Rahman, M. M., Hossain, M. M., & Rahman, M. M. (2025). Biofilm Production and Virulence Traits among Extensively Drug-Resistant and Methicillin-Resistant *Staphylococcus aureus* from Buffalo Subclinical Mastitis in Bangladesh. *Scientific Reports*, *15*(1), 34425.

Ciric, L., Jasni, A., Vries, L. E. de, Agersø, Y., Mullany, P., & Roberts, A. P. (2013). The Tn*916*/Tn*1545* Family of Conjugative Transposons. In *Madame Curie Bioscience Database [Internet]*. Landes Bioscience.

Cizmeci, S. U., Kose, A. M., Aydin, I., Sayin, Z., & Ali Dinc, D. (2019). Determination of the Effects of Different Dry Cow Therapy on Udder Health in Dairy Cows. *10*(1), 22–23.

Clinical and Laboratory Standard Institute (CLSI). (2023). *Performance Standards for Antimicrobial Susceptibility Testing* (33rd ed.).

Cloeckaert, A., Zygmunt, M. S., & Doublet, B. (2017). Editorial: Genetics of Acquired Antimicrobial Resistance in Animal and Zoonotic Pathogens. *Frontiers in Microbiology*, *8*, 2428.

Cobirka, M., Tancin, V., & Slama, P. (2020). Epidemiology and Classification of Mastitis. *Animals : An Open Access Journal from MDPI*, *10*(12), 2212.

Côté-Gravel, J., & Malouin, F. (2019). Symposium Review: Features of *Staphylococcus aureus* Mastitis Pathogenesis that Guide Vaccine Development Strategies. *Journal of Dairy Science*, *102*(5), 4727–4740.

Crippa, B. L., De Matos, L. G., Souza, F. N., & Silva, N. C. C. (2024). Non-*aureus* Staphylococci and Mammaliicocci (NASM): Their Role in Bovine Mastitis and One Health. *Journal of Dairy Research*, *91*(1), 44–56.

Dahl, G. E., Tao, S., & Laporta, J. (2020). Heat Stress Impacts Immune Status in Cows Across the Life Cycle. *Frontiers in Veterinary Science*, *7*, 116.

Daly, S. M., Sturge, C. R., & Greenberg, D. E. (2017). Inhibition of Bacterial Growth by Peptide-Conjugated Morpholino Oligomers. *Methods in Molecular Biology (Clifton, N.J.)*, *1565*, 115–122.

Danieli, B., Schogor, A. L. B., Zucchi, J., Neto, A. T., Danieli, B., Schogor, A. L. B., Zucchi, J., & Neto, A. T. (2025). Cows with High SCC Exhibit Poorer Performance and Milk Quality, Regardless of the Season. *Dairy*, *6*(4).

De Buck, J., Ha, V., Naushad, S., Nobrega, D. B., Luby, C., Middleton, J. R., De Vliegher, S., & Barkema, H. W. (2021). Non-*aureus* Staphylococci and Bovine Udder Health: Current Understanding and Knowledge Gaps. *Frontiers in Veterinary Science*, *8*.

De los Santos, R., González-Revello, Á., Majul, L., Umpiérrez, A., Aldrovandi, A., Gil, A., Hirigoyen, D., & Zunino, P. (2022). Subclinical Bovine Mastitis Associated with *Staphylococcus* spp. in eleven Uruguayan Dairy Farms. *Journal of Infection in Developing Countries*, *16*(4), 630–637.

de Vries, L. E., Christensen, H., Skov, R. L., Aarestrup, F. M., & Et., A. (2009). Diversity of the Tetracycline Resistance Gene *tetM* and Identification of Tn*916*- and Tn*5801*-like (Tn*6014*) Transposons in *Staphylococcus aureus* from Humans and Animals. *Journal of Antimicrobial Chemotherapy*.

Dego, O. K. (2020). Bovine Mastitis: Part I. In *Animal Reproduction in Veterinary Medicine*. IntechOpen.

Dego, O. K., & Vidlund, J. (2024). Staphylococcal Mastitis in Dairy Cows. *Frontiers in Veterinary Science*, *11*.

Dejyong, T., Chanachai, K., Immak, N., Prarakamawongsa, T., Rukkwamsuk, T., Tago Pacheco, D., & Phimpraphai, W. (2022). An Economic Analysis of High Milk

Somatic Cell Counts in Dairy Cattle in Chiang Mai, Thailand. *Frontiers in Veterinary Science*, *9*.

Dhaouadi, S., Soufi, L., Campanile, F., Dhaouadi, F., Sociale, M., Lazzaro, L., Cherif, A., Stefani, S., & Elandoulsi, R. B. (2020). Prevalence of Meticillin-Resistant and -Susceptible Coagulase-Negative Staphylococci with the First Detection of the *mecC* Gene among Cows, Humans and Manure in Tunisia. *International Journal of Antimicrobial Agents*, *55*(1), 105826.

Domínguez, M., Miranda, C. D., Fuentes, O., de la Fuente, M., Godoy, F. A., Bello-Toledo, H., & González-Rocha, G. (2019). Occurrence of Transferable Integrons and *sul* and *dfr* Genes Among Sulfonamide-and/or Trimethoprim-Resistant Bacteria Isolated from Chilean Salmonid Farms. *Frontiers in Microbiology*, *10*.

Dos Santos, F., Mendonça, L. C., Reis, D. R. D. L., Guimarães, A. D. S., Lange, C. C., Ribeiro, J. B., Machado, M. A., & Brito, M. A. V. P. (2016). Presence of *mecA*-Positive Multidrug-Resistant *Staphylococcus epidermidis* in Bovine Milk Samples in Brazil. *Journal of Dairy Science*, *99*(2), 1374–1382.

Dündar, T., & Köksal Çakırlar, F. (2025). Antimicrobial Resistance in Coagulase Negative Staphylococci: Genome Analysis and Role of Horizontal Gene Transfer. *Research in Microbiology*, *176*(5), 104298.

Duse, A., Persson-Waller, K., Pedersen, K., Duse, A., Persson-Waller, K., & Pedersen, K. (2021). Microbial Aetiology, Antibiotic Susceptibility and Pathogen-Specific Risk Factors for Udder Pathogens from Clinical Mastitis in Dairy Cows. *Animals*, *11*(7).

EFSA, E. F. S. A., & ECDC, E. C. for D. P. and C. (2025). The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2022–2023. *EFSA Journal*, *23*(3), 142.

Egyedy, A. F., & Ametaj, B. N. (2022). Mastitis: Impact of Dry Period, Pathogens, and Immune Responses on Etiopathogenesis of Disease and its Association with Periparturient Diseases. *Dairy*, *3*(4), Article 4.

Eid, H., Mahallawy, H., Mohammed, S., Mohammed, N., & Eidaroos, N. (2022). Multidrug-Resistant and Enterotoxigenic Methicillin-Resistant *Staphylococcus aureus* Isolated from Raw Milk of Cows at Small-Scale Production Units. *Journal of Advanced Veterinary and Animal Research*, *9*(1), 113.

Elemo, K. K., Sisay, T., Shiferaw, A., & Fato, M. A. (2017). Prevalence, Risk Factors and Multidrug Resistance Profile of *Staphylococcus aureus* Isolated from Bovine Mastitis in Selected Dairy Farms in and around Asella Town, Arsi Zone, South Eastern Ethiopia. *African Journal of Microbiology Research*, *11*(45), 1632–1642.

El-Razik, K. A. A., Arafa, A. A., Hedia, R. H., & Ibrahim, E. S. (2017). Tetracycline Resistance Phenotypes and Genotypes of Coagulase-Negative Staphylococcal Isolates from Bubaline Mastitis in Egypt. *Veterinary World*, *10*(6), 702–710.

El-Seedy, F. R., Radwan, I. A., Hassan, W. H., & Shehata, A. (2017). Coagulase Negative Staphylococci as an Emerging Cause of Bovine Mastitis: Prevalence, Antimicrobial Resistance and Biofilm Formation. *Journal of Veterinary Medical Research*, *24*(1), 1–11.

Emaneini, M., Bigverdi, R., Kalantar, D., Soroush, S., Jabalameli, F., Noorazar Khoshgnab, B., Asadollahi, P., & Taherikalani, M. (2013). Distribution of Genes Encoding Tetracycline Resistance and Aminoglycoside Modifying Enzymes in *Staphylococcus aureus* Strains Isolated from a Burn Center. *Annals of Burns and Fire Disasters*, *26*(2), 76.

Fazal, M. A., Rana, E. A., Akter, S., Alim, M. A., Barua, H., & Ahad, A. (2023). Molecular Identification, Antimicrobial Resistance and Virulence Gene Profiling of *Staphylococcus* spp. Associated with Bovine Subclinical Mastitis in Bangladesh. *Veterinary and Animal Science*, *21*, 100297.

Fergestad, M. E., De Visscher, A., L'Abee-Lund, T., Tchamba, C. N., Mainil, J. G., Thiry, D., De Vliegher, S., & Wasteson, Y. (2021). Antimicrobial Resistance and Virulence Characteristics in 3 Collections of Staphylococci from Bovine Milk Samples. *Journal of Dairy Science*, *104*(9), 10250–10267.

Fergestad, M. E., Stamsås, G. A., Morales Angeles, D., Salehian, Z., Wasteson, Y., & Kjos, M. (2020). Penicillin-Binding Protein PBP2a Provides Variable Levels of Protection toward Different β-lactams in *Staphylococcus aureus* RN4220. *MicrobiologyOpen*, *9*(8), e1057.

Fesseha, H., Mathewos, M., Aliye, S., & Wolde, A. (2021). Study on Prevalence of Bovine Mastitis and Associated Risk Factors in Dairy Farms of Modjo Town and Suburbs, Central Oromia, Ethiopia. *Veterinary Medicine: Research and Reports*, *12*, 271–283.

Fidelis, C. E., Orsi, A. M., Freu, G., Gonçalves, J. L., & dos Santos, M. V. (2024). Biofilm Formation and Antimicrobial Resistance of *Staphylococcus aureus* and *Streptococcus uberis* Isolates from Bovine Mastitis. *Veterinary Sciences*, *11*(4), 170.

Firth, N., Jensen, S. O., Kwong, S. M., Skurray, R. A., & Ramsay, J. P. (2018). Staphylococcal Plasmids, Transposable and Integrative Elements. *Microbiology Spectrum*, *6*(6), 10.1128/microbiolspec.gpp3-0030–2018.

Fischer, M., Thöny, B., & Leimkühler, S. (2010). 7.17—The Biosynthesis of Folate and Pterins and Their Enzymology. In H.-W. (Ben) Liu & L. Mander (Eds.), *Comprehensive Natural Products II* (pp. 599–648). Elsevier.

Fishovitz, J., Hermoso, J. A., Chang, M., & Mobashery, S. (2014). Penicillin-Binding Protein 2a of Methicillin-Resistant *Staphylococcus aureus*. *IUBMB Life*, *66*(8), 572.

Flügge, C., & Flügge, K. G. F. W. (1896). *Die Mikroorganismen: Mit besonderer berücksichtigung der ätiologie der infektionskrankheiten*. F. C. W. Vogel.

Foster, G., & Paterson, G. K. (2020). Methicillin-Resistant *Macrococcus bohemicus* Encoding a Divergent SCCmecB Element. *Antibiotics*, *9*(9), 590.

Foster, T. (1996). *Staphylococcus* (S. Baron, Ed.).

Foster, T. J., & Geoghegan, J. A. (2015). *Staphylococcus aureus*. In *Molecular Medical Microbiology* (pp. 655–674). Academic Press.

Fox, L. K., Bayles, K. W., & Bohach, G. A. (2001). *Staphylococcus aureus* Mastitis. In A. L. Honeyman, H. Friedman, & M. Bendinelli (Eds.), *Staphylococcus aureus Infection and Disease* (pp. 271–294). Springer US.

Freu, G., Garcia, B. L. N., Tomazi, T., Di Leo, G. S., Gheller, L. S., Bronzo, V., Moroni, P., & Dos Santos, M. V. (2023). Association between Mastitis Occurrence in Dairy Cows and Bedding Characteristics of Compost-Bedded Pack Barns. *Pathogens*, *12*(4), Article 4.

Frey, Y., Rodriguez, J. P., Thomann, A., Schwendener, S., & Perreten, V. (2013). Genetic Characterization of Antimicrobial Resistance in Coagulase-Negative Staphylococci from Bovine Mastitis Milk. *Journal of Dairy Science*, *96*(4), 2247–2257.

Gajewska, J., & Chajęcka-Wierzchowska, W. (2020). Biofilm Formation Ability and Presence of Adhesion Genes among Coagulase-Negative and Coagulase-Positive Staphylococci Isolates from Raw Cow's Milk. *Pathogens*, *9*(8), 1–12.

Garbacz, K., Wierzbowska, M., Kwapisz, E., Kosecka-Strojek, M., Bronk, M., Saki, M., & Międzobrodzki, J. (2021). Distribution and Antibiotic-Resistance of Different *Staphylococcus* Species Identified by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Isolated from the Oral Cavity. *Journal of Oral Microbiology*, *13*(1).

Gebremariam, N. M., Bitew, A., Tsige, E., Woldesenbet, D., & Tola, M. A. (2022). A High Level of Antimicrobial Resistance in Gram-Positive Cocci Isolates from Different Clinical Samples Among Patients Referred to Arsho Advanced Medical Laboratory, Addis Ababa, Ethiopia. *Infection and Drug Resistance*, *15*, 4203.

Gebremedhin, E. Z., Ararso, A. B., Borana, B. M., Kelbesa, K. A., Tadese, N. D., Marami, L. M., & Sarba, E. J. (2022). Isolation and Identification of *Staphylococcus aureus* from Milk and Milk Products, Associated Factors for Contamination, and Their Antibiogram in Holeta, Central Ethiopia. *Veterinary Medicine International*, *2022*.

Getahun, D. D., Tarekegn, H. T., Azene, B. T., Abebe, L. T., Belete, M. A., & Tessema, T. S. (2025). Virulence Genes and Antibiotic Resistance Profiling of *Staphylococcus S*pecies Isolated from Mastitic Dairy Cows in and around Bahir Dar, Ethiopia. *BMC Microbiology*, *25*, 210.

Getahun, Y. A., Abey, S. L., Beyene, A. M., Belete, M. A., & Tessema, T. S. (2024). Coagulase-Negative Staphylococci from Bovine Milk: Antibiogram Profiles and Virulent Gene Detection. *BMC Microbiology*, *24*(1), 263.

Ghebremedhin, B., Layer, F., König, W., & König, B. (2008). Genetic Classification and Distinguishing of *Staphylococcus* Species Based on Different Partial *gap*, 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* Gene Sequences. *Journal of Clinical Microbiology*, *46*(3), 1019–1025.

Gherardi, G., Di Bonaventura, G., & Savini, V. (2018). Staphylococcal Taxonomy. In *Pet-to-Man Travelling Staphylococci: A World in Progress* (pp. 1–10). Elsevier.

Ginn, S. L., Brown, M. H., & Skurray, R. A. (2000). The TetA(K) Tetracycline/H+ Antiporter from *Staphylococcus aureus*: Mutagenesis and Functional Analysis of Motif C. *Journal of Bacteriology*, *182*(6), 1492–1498.

Giri, D. (2018). Coagulase Test: Types, Principle, Procedure, Interpretation and Examples. LaboratoryTests.Org.

Girma, A., & Tamir, D. (2022). Prevalence of Bovine Mastitis and Its Associated Risk Factors among Dairy Cows in Ethiopia during 2005–2022: A Systematic Review and Meta-Analysis. *Veterinary Medicine International*, *2022*, 7775197.

Goetz, C., Tremblay, Y. D. N., Lamarche, D., Blondeau, A., Gaudreau, A. M., Labrie, J., Malouin, F., & Jacques, M. (2017). Coagulase-Negative Staphylococci Species Affect Biofilm Formation of Other Coagulase-Negative and Coagulase-Positive Staphylococci. *Journal of Dairy Science*, *100*(8), 6454–6464.

Gomes, F., Saavedra, M. J., & Henriques, M. (2016). Bovine Mastitis Disease/Pathogenicity: Evidence of the Potential Role of Microbial Biofilms. *Pathogens and Disease*, *74*(3), ftw006.

Gómez-Sanz, E., Haro-Moreno, J. M., Jensen, S. O., Roda-García, J. J., & López-Pérez, M. (2021). The Resistome and Mobilome of Multidrug-Resistant *Staphylococcus sciuri* C2865 Unveil a Transferable Trimethoprim Resistance Gene, Designated *dfrE* , Spread Unnoticed. *mSystems*, *6*(4), 10.1128/msystems.00511-21.

Gómez-Sanz, E., Schwendener, S., Thomann, A., Brawand, S. G., & Perreten, V. (2015). First Staphylococcal Cassette Chromosome *mec* Containing a *mecB*-Carrying Gene Complex Independent of Transposon Tn*6045* in a *Macrococcus caseolyticus* Isolate from a Canine Infection.

Gonal, B. N., Dalbanjan, N. P., Kadapure, A. J., Kumar, S. K. P., & Arakera, S. B. (2025). Epidemiology, Antimicrobial Resistance, Economic Burden and Management Approaches for *Staphylococcus aureus*-Associated Bovine Mastitis: A Systematic Review. *Animals and Zoonoses*.

González-Martín, M., Corbera, J. A., Suárez-Bonnet, A., María, &, Tejedor-Junco, T., Gonz Alez-Mart In, M., & Su Arez-Bonnet, A. (2020). Virulence Factors in Coagulase-Positive Staphylococci of Veterinary Interest Other than *Staphylococcus aureus*. *Veterinary Quarterly*, *40*(1), 118–131.

Götz, F., Bannerman, T., & Schleifer, K.-H. (2006). The Genera *Staphylococcus* and *Macrococcus*. *The Prokaryotes*, 5–75.

Grando, K., Nicastro, L. K., Tursi, S. A., De Anda, J., Lee, E. Y., Wong, G. C. L., & Tükel, Ç. (2022). Phenol-Soluble Modulins from *Staphylococcus aureus* Biofilms Form Complexes with DNA to Drive Autoimmunity. *Frontiers in Cellular and Infection Microbiology*, *12*, 884065.

Grima, L. Y. W., Leliso, S. A., Bulto, A. O., & Ashenafi, D. (2021). Isolation, Identification and Antimicrobial Susceptibility Profiles of *Staphylococcus aureus* from Clinical Mastitis in Sebeta Town Dairy Farms. *Veterinary Medicine International*, *2021*.

Halasa, T., & Kirkeby, C. (2020). Differential Somatic Cell Count: Value for Udder Health Management. *Frontiers in Veterinary Science*, *7*, 609055.

Hartman, P. G. (1993). Molecular Aspects and Mechanism of Action of Dihydrofolate Reductase Inhibitors. *Journal of Chemotherapy (Florence, Italy)*, *5*(6), 369–376.

Haulisah, N. A., Hassan, L., Bejo, S. K., Jajere, S. M., & Ahmad, N. I. (2021). High Levels of Antibiotic Resistance in Isolates from Diseased Livestock. *Frontiers in Veterinary Science*, *0*, 300.

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. *Dairy*, *3*(4), Article 4.

Heikens, E., Fleer, A., Paauw, A., Florijn, A., & Fluit, A. C. (2005). Comparison of Genotypic and Phenotypic Methods for Species-Level Identification of Clinical Isolates of Coagulase-Negative Staphylococci. *Journal of Clinical Microbiology*, *43*(5), 2286–2290.

Heilmann, C., Ziebuhr, W., & Becker, K. (2019). Are Coagulase-Negative Staphylococci Virulent? *Clinical Microbiology and Infection*, *25*(9), 1071–1080.

Hnini, R., Silva, E., Pinho, L., Najimi, M., & Thompson, G. (2024). Beta-Lactam Antimicrobials Activity and the Diversity of *blaZ* Gene in *Staphylococcus aureus* Isolates from Bovine Mastitis in the Northwest of Portugal. *Revista Brasileira de Zootecnia*, *53*, e20230024.

Hordofa, D. L. (2022). Review on Biofilm Forming Microbials in Cases of Bovine Mastitis and its Impact on Treatment. *13*.

Hosseinzadeh, S., & Saei, H. D. (2014). Staphylococcal Species Associated with Bovine Mastitis in the North West of Iran: Emerging of Coagulase-Negative Staphylococci. *International Journal of Veterinary Science and Medicine*, *2*(1), 27–34.

Howden, B. P., Giulieri, S. G., Wong Fok Lung, T., Baines, S. L., Sharkey, L. K., Lee, J. Y. H., Hachani, A., Monk, I. R., & Stinear, T. P. (2023). *Staphylococcus*

aureus Host Interactions and Adaptation. *Nature Reviews Microbiology 2023 21:6*, *21*(6), 380–395.

Huang, C.-H., & Kusaba, N. (2022). Association between Differential Somatic Cell Count and California Mastitis Test Results in Holstein Cattle. *JDS Communications*, *3*(6), 441–445.

Ibrahim, O. O. (2020). *Staphylococcus aureus* a Gram-positive Coccid Bacterium Causing Microbial Infections and Toxins Symptoms Including Food Poisoning.

Ibrahim, S., Wei Hoong, L., Lai Siong, Y., Mustapha, Z., C. W. Zalati, C. W. S., Aklilu, E., Mohamad, M., Kamaruzzaman, N. F., Hoong, L. W., Siong, Y. L., Mustapha, Z., Zalati, C. W. S. C. W., Aklilu, E., Mohamad, M., & Kamaruzzaman, N. F. (2021). Prevalence of Antimicrobial Resistance (AMR) *Salmonella* spp. and *Escherichia coli* Isolated from Broilers in the East Coast of Peninsular Malaysia. *Antibiotics 2021, Vol. 10, Page 579*, *10*(5), 579.

Idamokoro, E. M. (2022). Coagulase-Negative Staphylococci as an Evolving Mastitis Causing Organism in Cows: A Review. *F1000Research*, *11*, 824.

Israel, L. F. S., Rabello, R. F., Ferreira, L. B., Peruquetti, R. C., & Medeiros, L. S. (2022). Biofilm Production by *Staphylococcus* spp. Isolated from Bovine Mastitis in Dairy Herds in State of Acre, Brazil and Its Implications. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *74*, 563–575.

Ito, T., Hiramatsu, K., Tomasz, A., de Lencastre, H., Perreten, V., Holden, M. T. G., Coleman, D. C., Goering, R., Giffard, P. M., Skov, R. L., Zhang, K., Westh, H., O'Brien, F., Tenover, F. C., Oliveira, D. C., Boyle-Vavra, S., Laurent, F., Kearns, A. M., Kreiswirth, B., Ko, Kwan Soo, Grundmann, Hajo, Sollid, Johanna E., John Jr, Joseph F., Daum, Robert, Soderquist, Bo, Buist, G. (2012). Guidelines for Reporting Novel *mecA* Gene Homologues. *Antimicrobial Agents and Chemotherapy*, *56*(10), 4997–4999.

Jamali, H., Radmehr, B., & Ismail, S. (2014). Short Communication: Prevalence and Antibiotic Resistance of *Staphylococcus aureus* Isolated from Bovine Clinical Mastitis. *Journal of Dairy Science*, *97*(4), 2226–2230.

Jin, J., Guffanti, A. A., Bechhofer, D. H., & Krulwich, T. A. (2002). Tet(L) and Tet(K) Tetracycline-Divalent Metal/H+ Antiporters: Characterization of Multiple Catalytic Modes and a Mutagenesis Approach to Differences in Their Efflux Substrate and Coupling Ion Preferences. *Journal of Bacteriology*, *184*(17), 4722–4732.

Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA Gene Sequencing for Species and Strain-Level Microbiome Analysis. *Nature Communications*, *10*(1), 5029.

Johri, A., Arora, N., Maansi, & Mrigesh, M. (2023). California Mastitis Test (CMT): An Onsite Test for Screening of Subclinical Mastitis in Buffaloes. *The Pharma Innovation Journal*, *12*(7S), 947–950.

Jose, K. R., & Vijayakumar, K. (2023). Investigation of Tetracycline Resistance in Staphylococci Isolated from Bovine Mastitis.

Josse, J., Laurent, F., & Diot, A. (2017). Staphylococcal Adhesion and Host Cell Invasion: Fibronectin-Binding and Other Mechanisms. *Frontiers in Microbiology*, *8*, 2433.

Kaczorek-Łukowska, E., Małaczewska, J., Sowińska, P., Szymańska, M., Wójcik, E. A., & Siwicki, A. K. (2022). *Staphylococcus aureus* from Subclinical Cases of Mastitis in Dairy Cattle in Poland, What Are They Hiding? Antibiotic Resistance and Virulence Profile. *Pathogens*, *11*(12), 1404.

Kandeel, S. A., Morin, D. E., Calloway, C. D., & Constable, P. D. (2018). Association of California Mastitis Test Scores with Intramammary Infection Status in Lactating Dairy Cows Admitted to a Veterinary Teaching Hospital. *Journal of Veterinary Internal Medicine*, *32*(1), 497–505.

Kateete, D. P., Kimani, C. N., Katabazi, F. A., Okeng, A., Okee, M. S., Nanteza, A., Joloba, M. L., & Najjuka, F. C. (2010). Identification of *Staphylococcus aureus*: DNase and Mannitol Salt Agar Improve the Efficiency of the Tube Coagulase Test. *Annals of Clinical Microbiology and Antimicrobials*, *9*, 23.

Kazemzadeh, S., Korneeva, O., Shabunin, S., & Syromyatnikov, M. (2025). Antibiotic Resistance in Mastitis-Causing Bacteria: Exploring Antibiotic-Resistance Genes, Underlying Mechanisms and Their Implications for Dairy Animal and Public Health. *Open Veterinary Journal*, *15*(9), 3980–4006.

Keller, J. E., Schwendener, S., Neuenschwander, J., Overesch, G., & Perreten, Vincent. (2022). Prevalence and Characterization of Methicillin-Resistant *Macrococcus* spp. in Food Producing Animals and Meat in Switzerland in 2019. *Schweiz Arch Tierheilkd*, *2*(1), 153–164.

Kenar, B., Kuyucuoğlu, Y., & ŞEKER, E. (2012). Antibiotic Susceptibility of Coagulase-Negative Staphylococci Isolated from Bovine Subclinical Mastitis in Turkey. *Pakistan Veterinary Journal*, *32*, 390–393.

Khan, S. B., Khan, M. A., Ahmad, I., Khan, F. A., Khan, H., & Khan, S. A. (2022). Prevalence of Antibiotic Resistant Genes in *Staphylococcus aureus* Isolated from Bovine Mastitis. *Pakistan Journal of Zoology*, *54*(5).

Khan, S., Manjusha, K. M., Banu, A. S., Haritha, C. V., Kumar, R., Pawde, A., & Amarpal. (2020). Surgical Affections of Udder and Teat in Large Animals.

Khan, W., Khan, S., Khan, F., Khan, S., Ullah, I., Shah, A., Uddin, I., Khan, S., Khan, H., Uddin, M., Akbar, F., & Qayum, N. (2023). Therapeutic Potential of Natural Products and Antibiotics against Bovine Mastitis Pathogen of Cows and Buffaloes. *Veterinární Medicína*, *68*(7), 271–280.

Khasanah, H., Setyawan, H. B., Yulianto, R., & Widianingrum, D. C. (2021). Subclinical Mastitis: Prevalence and Risk Factors in Dairy Cows in East Java, Indonesia. *Veterinary World*, *14*(8), 2102–2108.

Khasapane, N. G., Byaruhanga, C., Thekisoe, O., Nkhebenyane, S. J., & Khumalo, Z. T. H. (2023). Prevalence of Subclinical Mastitis, Its Associated Bacterial Isolates and Risk Factors among Cattle in Africa: A Systematic Review and Meta-Analysis. *BMC Veterinary Research*, *19*, 123.

Khasapane, N. G., Nkhebenyane, S. J., Thekisoe, O., Ramatla, T., & Lekota, K. E. (2025). Population Structure, Resistome and Virulome of *Staphylococcus chromogenes* Strains from Milk of Subclinical Bovine Mastitis in South Africa. *Frontiers in Cellular and Infection Microbiology*, *15*.

Khatoon, H., Chavan, D., Anokhe, A., & Kalia, V. (2022). Catalase Test: A Biochemical Protocol for Bacterial Identification.

Khazandi, M., Al-Farha, A. A.-B., Coombs, G. W., O'Dea, M., Pang, S., Trott, D. J., Aviles, R. R., Hemmatzadeh, F., Venter, H., Ogunniyi, A. D., Hoare, A., Abraham, S., & Petrovski, K. R. (2018). Genomic Characterization of Coagulase-Negative Staphylococci Including Methicillin-Resistant *Staphylococcus sciuri* Causing Bovine Mastitis. *Veterinary Microbiology*, *219*, 17–22.

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

Kim, J., & Ahn, J. (2022). Emergence and Spread of Antibiotic-Resistant Foodborne Pathogens from Farm to Table. *Food Science and Biotechnology*, *31*(12), 1481.

Kim, S.-J., Moon, D. C., Park, S.-C., Kang, H. Y., Na, S. H., & Lim, S.-K. (2019). Antimicrobial Resistance and Genetic Characterization of Coagulase-Negative Staphylococci from Bovine Mastitis Milk Samples in Korea. *Journal of Dairy Science*, *102*(12), 11439–11448.

Kirkeby, C., Schwarz, D., Denwood, M., Farre, M., Nielsen, S. S., Gussmann, M., Toft, N., & Halasa, T. (2021). Dynamics of Somatic Cell Count (SCC) and Differential SCC During and Following Intramammary Infections. *Journal of Dairy Science*, *104*(3), 3427–3438.

Kirsanova, E., Heringstad, B., Lewandowska-Sabat, A., & Olsaker, I. (2019). Alternative Subclinical Mastitis Traits for Genetic Evaluation in Dairy Cattle. *Journal of Dairy Science*, *102*(6), 5323–5329.

Klempt, M., Franz, C. M. A. P., & Hammer, P. (2022). Characterization of Coagulase-Negative Staphylococci and Macrococci Isolated from Cheese in Germany. *Journal of Dairy Science*, *105*(10), 7951–7958.

Klimiene, I., Virgailis, M., Pavilonis, A., Siugzdiniene, R., Mockeliunas, R., & Ruzauskas, M. (2016). Phenotypical and Genotypical Antimicrobial Resistance of Coagulase-Negative staphylococci Isolated from Cow Mastitis. *Polish Journal of Veterinary Sciences*, *19*(3), 639–646.

Kloos, W. E., Ballard, D. N., George, C. G., Webster, J. A., Hubner, R. J., Ludwig, W., Schleifer, K. H., Fiedler, F., & Schubert, K. (1998). Delimiting the genus *Staphylococcus* through description of *Macrococcus caseolyticus* gen. Nov., comb. Nov. And *Macrococcus equipercicus* sp. Nov., *Macrococcus bovicus* sp. Nov. And *Macrococcus carouselicus* sp. Nov. *International Journal of Systematic and Evolutionary Microbiology*, *48*(3), 859–877.

Koch, F., Lamp, O., Eslamizad, M., Weitzel, J., & Kuhla, B. (2016). Metabolic Response to Heat Stress in Late-Pregnant and Early Lactation Dairy Cows: Implications to Liver-Muscle Crosstalk. *PLOS ONE*, *11*(8), e0160912.

Kosecka-Strojek, M., Sabat, A. J., Akkerboom, V., Becker, K., van Zanten, E., Wisselink, G., Miedzobrodzki, J., Kooistra-Smid, A. M. D. (Mirjam), & Friedrich, A. W. (2019). Development and Validation of a Reference Data Set for Assigning *Staphylococcus* Species Based on Next-Generation Sequencing

of the 16S-23S rRNA Region. *Frontiers in Cellular and Infection Microbiology*, *9*.

Kour, S., Sharma, N., Balaji, N., Kumar, P., Soodan, J. S., Santos, M. V. dos, & Son, Y. O. (2023). Advances in Diagnostic Approaches and Therapeutic Management in Bovine Mastitis. *Veterinary Sciences 2023, Vol. 10, Page 449*, *10*(7), 449.

Krause, K. M., Serio, A. W., Kane, T. R., & Connolly, L. E. (2016). Aminoglycosides: An Overview. *Cold Spring Harbor Perspectives in Medicine*, *6*(6), a027029.

Krebs, I., Zhang, Y., Wente, N., Leimbach, S., & Krömker, V. (2023). Severity of Clinical Mastitis and Bacterial Shedding. *Pathogens*, *12*(9), 1098.

Krishnamoorthy, P., Goudar, A. L., Suresh, K. P., & Roy, P. (2021). Global and Countrywide Prevalence of Subclinical and Clinical Mastitis in Dairy Cattle and Buffaloes by Systematic Review and Meta-Analysis. *Research in Veterinary Science*, *136*, 561–586.

Książczyk, M., Dębowska, K., & Bierowiec, K. (2026). Bacteriocins in Veterinary Medicine: From Antibiotic Limitations to Targeted Solutions. *International Journal of Molecular Sciences*, *27*(13), 5812.

Kuipers, A., Stapels, D. A. C., Weerwind, L. T., Ko, Y.-P., Ruyken, M., Lee, J. C., van Kessel, K. P. M., & Rooijakkers, S. H. M. (2016). The *Staphylococcus aureus* Polysaccharide Capsule and Efb-Dependent Fibrinogen Shield Act in Concert to Protect Against Phagocytosis. *Microbiology*, *162*(7), 1185–1194.

Langhorne, C., Gupta, S. D., Horsman, S., Wood, C., Wood, B. J., Barker, L., Deutscher, A., Price, R., McGowan, M. R., Humphris, M., Ranjbar, S., Henning, J., & Gibson, J. S. (2023). Bacterial Culture and Antimicrobial Susceptibility Results from Bovine Milk Samples Submitted to Four Veterinary Diagnostic Laboratories in Australia from 2015 to 2019. *Frontiers in Veterinary Science*, *10*, 1232048.

Lasa, I., & Penadés, J. R. (2006). Bap: A Family of Surface Proteins Involved in Biofilm Formation. *Research in Microbiology*, *157*(2), 99–107.

Lee, Y. J., & Lee, Y. J. (2022). Characterization of Biofilm Producing Coagulase-Negative Staphylococci Isolated from Bulk Tank Milk. *Veterinary Sciences*, *9*(8), 430.

Leroy, S., Christieans, S., & Talon, R. (2019). Tetracycline Gene Transfer in *Staphylococcus xylosus* In-Situ During Sausage Fermentation. *Frontiers in Microbiology*, *10*.

Lestari, D. S., Setiawan, B., Saputro, A. L., Mafruchati, M., Praja, R. N., & Prastiya, R. A. (2025). Environmental and Management Risk Factors for Goat Mastitis in Banyuwangi. *Journal of Basic Medical Veterinary*, *14*(2), 202–212.

Li, H., Wang, Z., Barkema, H. W., Li, X., Song, D., Ren, M., Tong, J., Liu, M., Gao, J., & Cheng, J. (2026). Virulence Factors of Bovine Mastitis Pathogens: Distribution, Pathogenesis and Emerging Vaccines Targeting Virulence Factors: A Literature Review. *Frontiers in Veterinary Science*, *12*, 1745390.

Lira, M. C., Givisiez, P. E. N., De Sousa, F. G. C., Magnani, M., De Souza, E. L., Spricigo, D. A., Gebreyes, W. A., & De Oliveira, C. J. B. (2016). Biofilm-Forming and Antimicrobial Resistance Traits of Staphylococci Isolated from Goat Dairy Plants. *Journal of Infection in Developing Countries*, *10*(9), 932–938.

Liu, Y. (2024). Investigating the Prevalence and Function of the *mecA* Gene and PBP2a Protein among *Staphylococcus* and *Mammaliicoccus* Species. *Theoretical and Natural Science*, *35*, 119–125.

MacFadyen, A. C., Fisher, E. A., Costa, B., Cullen, C., & Paterson, G. K. (2018). Genome Analysis of Methicillin Resistance in *Macrococcus caseolyticus* from Dairy Cattle in England and Wales. *Microbial Genomics*, *4*(8), e000191.

Machado, M. A. A., Ribeiro, W. A., Toledo, V. S., Ramos, G. L. P. A., Vigoder, H. C., & Nascimento, J. S. (2020). Antibiotic Resistance and Biofilm Production in Catalase-Positive Gram-Positive Cocci Isolated from Brazilian Pasteurized Milk. *Journal of Food Quality and Hazards Control*.

Madhaiyan, M., Wirth, J. S., & Saravanan, V. S. (2020). Phylogenomic Analyses of the Staphylococcaceae Family Suggest the Reclassification of Five Species within the genus *Staphylococcus* as Heterotypic Synonyms, the Promotion of Five Subspecies to Novel Species, the Taxonomic Reassignment of Five *Staphylococcus* species to *Mammaliicoccus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family Staphylococcaceae. *International Journal of Systematic and Evolutionary Microbiology*, *70*(11), 5926–5936.

Manyi-Loh, C., Mamphweli, S., Meyer, E., & Okoh, A. (2018). Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications. *Molecules*, *23*(4), Article 4.

Marek, A., Pyzik, E., Stępień-Pyśniak, D., Dec, M., Jarosz, Ł. S., Nowaczek, A., & Sulikowska, M. (2021). Biofilm-Formation Ability and the Presence of Adhesion Genes in Coagulase-Negative Staphylococci Isolates from Chicken Broilers. *Animals : An Open Access Journal from MDPI*, *11*(3), 728.

Marimuthu, M., Abdullah, F. F. J., Mohammed, K., Poshpum, S. S., Adamu, L., Osman, A. Y., Abba, Y., & Tijjani, A. (2014). Prevalence and Antimicrobial Resistance Assessment of Subclinical Mastitis In Milk Samples From Selected Dairy Farms. *American Journal of Animal and Veterinary Sciences*, *9*(1), 65–70.

Marincola, G., Liong, O., Schoen, C., Abouelfetouh, A., Hamdy, A., Wencker, F. D. R., Marciniak, T., Becker, K., Köck, R., & Ziebuhr, W. (2021). Antimicrobial Resistance Profiles of Coagulase-Negative Staphylococci in Community-Based Healthy Individuals in Germany. *Frontiers in Public Health*, *9*, 684456.

Markos, A., Begna, F., Afera, Y., & Tolosa, T. (2023). *Staphylococcus aureus* Isolates from Cow's Milk in Dairy Farms at Shinshicho Town, Kembata Tembaro Zone, Southern Ethiopia: Prevalence, Risk Factors and Antimicrobial Susceptibility Profile. *Ethiopian Veterinary Journal*, *27*(2), 23–44.

Mašlaňová, I., Wertheimer, Z., Sedláček, I., Švec, P., Indráková, A., Kovařovic, V., Schumann, P., Spröer, C., Králová, S., Šedo, O., Krištofová, L., Vrbovská, V., Füzik, T., Petráš, P., Zdráhal, Z., Ružičková, V., Doškař, J., & Pantuček, R. (2018). Description and Comparative Genomics of *Macrococcus caseolyticus* subsp. hominis subsp. nov., *Macrococcus goetzii* sp. nov., *Macrococcus epidermidis* sp. nov., and *Macrococcus bohemicus* sp. nov., Novel Macrococci from Human Clinical Material with Virulence Potential and Suspected Uptake of Foreign DNA by Natural Transformation. *Frontiers in Microbiology*, *9*, 1178.

Matheou, A., Abousetta, A., Pascoe, A. P., Papakostopoulos, D., Charalambous, L., Panagi, S., Panagiotou, S., Yiallouris, A., Filippou, C., Johnson, E. O., Matheou, A., Abousetta, A., Pascoe, A. P., Papakostopoulos, D., Charalambous, L., Panagi, S., Panagiotou, S., Yiallouris, A., Filippou, C., & Johnson, E. O. (2025). Antibiotic Use in Livestock Farming: A Driver of Multidrug Resistance? *Microorganisms*, *13*(4).

Mazhar, S., Kilcawley, K. N., Hill, C., & McAuliffe, O. (2020). A Systems-Wide Analysis of Proteolytic and Lipolytic Pathways Uncovers the Flavor-Forming Potential of the Gram-Positive Bacterium *Macrococcus caseolyticus* subsp. Caseolyticus. *Frontiers in Microbiology*, *11*.

Mazreku, N., Sylejmani, D., & Robaj, A. (2025). Prevalence of Subclinical Mastitis, Related Risk Factors and Antimicrobial Susceptibility of Bacteria Isolated from the Milk of Dairy Cows in Kosovo. *Veterinární Medicína*, *7*(4), 117–123.

Mbindyo, C. M., Gitao, G. C., Plummer, P. J., Kulohoma, B. W., Mulei, C. M., & Bett, R. (2021). Antimicrobial Resistance Profiles and Genes of Staphylococci Isolated from Mastitic Cow's Milk in Kenya. *Antibiotics*, *10*(7).

McHugh, M. L. (2012). Interrater Reliability: The Kappa Statistic. *Biochemia Medica*, *22*(3), 276–282.

Medina-Estrada, I., López-Meza, J. E., & Ochoa-Zarzosa, A. (2016). Anti-Inflammatory and Antimicrobial Effects of Estradiol in Bovine Mammary Epithelial Cells During *Staphylococcus aureus* Internalization. *Mediators of Inflammation*, *2016*, e6120509.

Mgonja, F. R., Charles, M. M., & Katakweba, A. S. (2023). Prevalence of Subclinical Mastitis and Associated Risk Factors in Dairy Cattle from Institution Farms in Morogoro Municipality. *World Journal of Veterinary Science*, *4*.

Michalik, M., Samet, A., Podbielska-Kubera, A., Savini, V., Międzobrodzki, J., & Kosecka-Strojek, M. (2020). Coagulase-Negative Staphylococci (CoNS) as a Significant Etiological Factor of Laryngological Infections: A review. *Annals of Clinical Microbiology and Antimicrobials*, *19*, 26.

Michira, L., Kagira, J., Maina, N., Waititu, K., Kiboi, D., Ongera, E., & Ngotho, M. (2023). Prevalence of Subclinical Mastitis, Associated Risk Factors and Antimicrobial Susceptibility Pattern of Bacteria Isolated from Milk of Dairy Cattle in Kajiado Central Sub-County, Kenya. *Veterinary Medicine and Science*, *9*(6), 2885–2892.

Miklasińska-Majdanik, M. (2021). Mechanisms of Resistance to Macrolide Antibiotics among *Staphylococcus aureus*. *Antibiotics*, *10*(11), 1406.

Miles, A. M., & Huson, H. J. (2021). Graduate Student Literature Review: Understanding the Genetic Mechanisms Underlying Mastitis. *Journal of Dairy Science*, *104*(1), 1183–1191.

Mirghani, R., Saba, T., Khaliq, H., Mitchell, J., Do, L., Chambi, L., Diaz, K., Kennedy, T., Alkassab, K., Huynh, T., Elmi, M., Martinez, J., Sawan, S., & Rijal, G. (2022). Biofilms: Formation, Drug Resistance and Alternatives to Conventional Approaches. *AIMS Microbiology*, *8*(3), 239–277.

Mlynarczyk-Bonikowska, B., Kowalewski, C., Krolak-Ulinska, A., & Marusza, W. (2022). Molecular Mechanisms of Drug Resistance in *Staphylococcus aureus*. *International Journal of Molecular Sciences 2022, Vol. 23, Page 8088*, *23*(15), 8088.

Mnif, S., Jardak, M., Yaich, A., & Aifa, S. (2020). Enzyme-Based Strategy to Eradicate Monospecies *Macrococcus caseolyticus* Biofilm Contamination in Dairy Industries. *International Dairy Journal*, *100*, 104560.

Moawad, A. A., El-Adawy, H., Linde, J., Jost, I., Tanja, G., Katja, H., Karsten, D., Neubauer, H., Monecke, S., & Tomaso, H. (2023). Whole Genome Sequence-Based Analysis of *Staphylococcus aureus* Isolated from Bovine Mastitis in Thuringia, Germany. *Frontiers in Microbiology*, *14*.

Modh, R., Islam, M., Nauriyal, D., Modi, R. J., & Wadhwani, K. (2018). Study on pH and Somatic Cell Count in Milk of Subclinical Mastitic Cows in Association with Udder and Teat Shape. 75–79.

Molineri, A. I., Camussone, C., Zbrun, M. V., Suárez Archilla, G., Cristiani, M., Neder, V., Calvinho, L., & Signorini, M. (2021). Antimicrobial Resistance of *Staphylococcus aureus* Isolated from Bovine Mastitis: Systematic Review and Meta-Analysis. *Preventive Veterinary Medicine*, *188*, 105261.

Monday, I. E., & Hosu, Y. S. (2022). Seasonal Distribution, Antimicrobial Activity and Risk Factors Associated with the Incidence of *Staphylococcus* Species in Bovine Milk from a Dairy Farm in Eastern Cape, South Africa. *Asian Journal of Agriculture and Rural Development*, *12*(1), 10–19.

Moormeier, D. E., & Bayles, K. W. (2017). *Staphylococcus aureus* Biofilm: A Complex Developmental Organism. *Molecular Microbiology*, *104*(3), 365–376.

Moreno, J., Diana, L., Martínez, M., Iribarnegaray, V., & Puentes, R. (2025). Comprehensive Analysis of Antimicrobial Resistance, Biofilm Formation and Virulence Factors of Staphylococci Isolated from Bovine Mastitis. *Heliyon*, *11*(4), e42749.

Moschovas, M., Pavlatos, G., Basdagianni, Z., Manessis, G., & Bossis, I. (2023). A Cross-Sectional Study of Risk Factors Affecting Milk Quality in Dairy Cows. *Animals*, *13*(22), Article 22.

Moser, A., Stephan, R., Ziegler, D., & Johler, S. (2013). Species Distribution and Resistance Profiles of Coagulase-Negative Staphylococci Isolated from Bovine Mastitis in Switzerland. *Schweizer Archiv Für Tierheilkunde*, *155*(6), 333–338.

Moubareck, C. A. (2020). Polymyxins and Bacterial Membranes: A Review of Antibacterial Activity and Mechanisms of Resistance. *Membranes (Basel)*, *10*(8), 1–30.

Moura de Sousa, J. A., Pfeifer, E., Touchon, M., & Rocha, E. P. C. (2021). Causes and Consequences of Bacteriophage Diversification via Genetic Exchanges across Lifestyles and Bacterial Taxa. *Molecular Biology and Evolution*, *38*(6), 2497–2512.

Mramba, R. P., & Mohamed, M. A. (2024). The Prevalence and Factors Associated with Mastitis in Dairy Cows Kept by Small-Scale Farmers in Dodoma, Tanzania. *Heliyon*, *10*(13), e34122.

Nainu, F., Masyita, A., Bahar, M. A., Raihan, M., Prova, S. R., Mitra, S., Emran, T. B., Simal-Gandara, J., Nainu, F., Masyita, A., Bahar, M. A., Raihan, M., Prova, S. R., Mitra, S., Emran, T. B., & Simal-Gandara, J. (2021). Pharmaceutical Prospects of Bee Products: Special Focus on Anticancer, Antibacterial, Antiviral, and Antiparasitic Properties. *Antibiotics*, *10*(7).

Nandi, S. (2021). Alteration of Milk pH, Somatic Cell Count (SCC), Lactate Dehydrogenase (LDH) and Alkaline Phosphatase (ALP) Activities in Buffalo Milk Related to Udder Health Status. *Buffalo Bulletin*, *40*(3).

Naranjo-Lucena, A., & Slowey, R. (2023). Invited Review: Antimicrobial Resistance in Bovine Mastitis Pathogens: A Review of Genetic Determinants and Prevalence of Resistance in European Countries. *Journal of Dairy Science*, *106*(1), 1–23.

National Mastitis Council (NMC). (2017). *Laboratory Handbook on Bovine Mastitis* (3rd ed.).

Naushad, S., Barkema, H. W., Luby, C., Condas, L. A. Z., Nobrega, D., Carson, D., & De Buck, J. (2016). Comprehensive Phylogenetic Analysis of Bovine Non-*aureus* Staphylococci Species Based on Whole-Genome Sequencing. *Frontiers in Microbiology*, *7*.

Naushad, S., Naqvi, S. A., Nobrega, D., Luby, C., Kastelic, J. P., Barkema, H. W., & De Buck, J. (2019). Comprehensive Virulence Gene Profiling of Bovine Non-*aureus* Staphylococci Based on Whole-Genome Sequencing Data. *mSystems*, *4*(2), 10.1128/msystems.00098-18.

Negash, S. (2023). Review on Bovine Mastitis and Assessments of Its Associated Risk Factors in Lactating Dairy Cows. *International Journal of Advanced Research in Biological Sciences*, *10*(3), 59–65.

Nguyen, H. T. T., Nguyen, T. H., & Otto, M. (2020). The Staphylococcal Exopolysaccharide PIA – Biosynthesis and Role in Biofilm Formation, Colonization and Infection. *Computational and Structural Biotechnology Journal*, *18*, 3324–3334.

Nitipan, S., Da-Oh, H., & Thongaupao, S. (2025). Characterization of *Staphylococcus aureus* from Bovine Mastitis: Genetic Markers and Antibiotic Resistance. *Trends in Sciences*, *22*(5), 9505–9505.

Nitz, J., Krömker, V., Klocke, D., Wente, N., Zhang, Y., & tho Seeth, M. (2020). Intramammary Infections in Heifers—Time of Onset and Associated Risk Factors. *Animals : An Open Access Journal from MDPI*, *10*(6), 1053.

Nomura, R., Nakaminami, H., Takasao, K., Muramatsu, S., Kato, Y., Wajima, T., & Noguchi, N. (2020). A Class A β-Lactamase Produced by Borderline Oxacillin-Resistant *Staphylococcus aureus* Hydrolyses Oxacillin. *Journal of Global Antimicrobial Resistance*, *22*, 244–247.

Notcovich, S., DeNicolo, G., Flint, S. H., Williamson, N. B., Gedye, K., Grinberg, A., & Lopez-Villalobos, N. (2018). Biofilm-Forming Potential of *Staphylococcus aureus* Isolated from Bovine Mastitis in New Zealand. *Veterinary Sciences*, *5*(1).

Nuñez, M. (2014). *Micrococcus*. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 627–633). Academic Press.

Nurjadi, D., Chanthalangsy, Q., Zizmann, E., Stuermer, V., Moll, M., Klein, S., Boutin, S., Heeg, K., & Zanger, P. (2021). Phenotypic Detection of Hemin-Inducible Trimethoprim-Sulfamethoxazole Heteroresistance in *Staphylococcus aureus*. *Microbiology Spectrum*, *9*(2).

Nurjadi, D., Zizmann, E., Chanthalangsy, Q., Heeg, K., & Boutin, S. (2021). Integrative Analysis of Whole Genome Sequencing and Phenotypic Resistance Toward

Prediction of Trimethoprim-Sulfamethoxazole Resistance in *Staphylococcus aureus*. *Frontiers in Microbiology*, *11*, 607842.

O'Gara, J. P. (2007). Ica and Beyond: Biofilm Mechanisms and Regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS Microbiology Letters*, *270*(2), 179–188.

Ogston, Alex. (1882). Micrococcus Poisoning. *Journal of Anatomy and Physiology*, *16*(Pt 4), 526–567.

Oknin, H., Kroupitski, Y., Shemesh, M., & Blum, S. (2023). Upregulation of ica Operon Governs Biofilm Formation by a Coagulase-Negative *Staphylococcus caprae*. *Microorganisms*, *11*(6), 1533.

Olatoye, O., Amosun, A., Ogbu, U., & Okunlade, Y. (2018). Bulk Tank Somatic Cell Count and Associated Microbial Quality of Milk from Selected Dairy Cattle Herds in Oyo State, Nigeria. *Italian Journal of Food Safety*, *7*(2), 95–100.

Oliveira, A. D., Pereira, V. C., Pinheiro, L., Riboli, D. F. M., Martins, K. B., & Cunha, M. D. L. R. de S. da. (2016). Antimicrobial Resistance Profile of Planktonic and Biofilm Cells of *Staphylococcus aureus* and Coagulase-Negative Staphylococci. *International Journal of Molecular Sciences*, *17*(9).

Omer, A. B., Eltom, K. H., Tawor, A. B., & Erganiş, O. (2025). Phylogenetic Footprints of Coagulase-Negative Staphylococci and *Mammaliicoccus* Isolated from Raw Caprine Milk. Preprints.

Omwenga, E. O., & Awuor, S. O. (2024). The Bacterial Biofilms: Formation, Impacts and Possible Management Targets in the Healthcare System. *The Canadian Journal of Infectious Diseases & Medical Microbiology*, *2024*, 1542576.

Ong, M. H.-L., Ho, W.-Y., Ng, W.-W., & Chew, C.-H. (2017). High Prevalence of *tetM* as Compared to *tetK* Amongst Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates from Hospitals in Perak, Malaysia. *Jundishapur Journal of Microbiology*, *10*(6), e13935.

Osada, M., Aung, M. S., Urushibara, N., Kawaguchiya, M., Ohashi, N., Hirose, M., & Kobayashi, N. (2022). Prevalence and Antimicrobial Resistance of *Staphylococcus aureus* and Coagulase-Negative *Staphylococcus*/*Mammaliicoccus* from Retail Ground Meat: Identification of Broad Genetic Diversity in Fosfomycin Resistance Gene fosB. *Pathogens*, *11*(4).

Othman, N., & Bahaman, A. R. (2005). Prevalence of Subclinical Mastitis and Antibiotic Resistant Bacteria in Three Selected Cattle Farms in Serdang, Selangor and Kluang, Johor. *Jabatan Veterinar Malaysia*, *17*(1), 27–31.

Ozbey, G., Otlu, B., Yakupogullari, Y., Celik, B., Tanriverdi, E. S., Kelestemur, N., Safak, T., Risvanli, A., Persad, A., & Sproston, E. (2022). Investigation of Bacterial Pathogens in Milk from Mastitic Dairy Cattle by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *The Thai Journal of Veterinary Medicine*, *52*(1), 63–73.

Page, M. G. P. (2012). Beta-Lactam Antibiotics. In T. J. Dougherty & M. J. Pucci (Eds.), *Antibiotic Discovery and Development* (pp. 79–117). Springer US.

Pangprasit, N., Srithanasuwan, A., Intanon, M., Suriyasathaporn, W., & Chaisri, W. (2023). Clinical Field Trial of Parenteral Amoxicillin for the Treatment of Clinical and Subclinical Mastitis in Smallholder Dairy Farms in the Upper Region of Northern Thailand. *Veterinary World*, 792–798.

Panigrahi, S., Biswal, S., Padhi, S. K., Pahari, A., Bhuyan, K., & Sahoo, N. (2022). Genotypic and Phenotypic Characterization of *Mammaliicoccus sciuri*—A MDR Strain Causing Clinical Mastitis in Cows of Odisha, India. *Indian Journal of Animal Research*, *56*(12).

Papich, M. G. (2016). Linezolid. In *Saunders handbook of veterinary drugs*.

Parnham, M. J., Haber, V. E., Giamarellos-Bourboulis, E. J., Perletti, G., Verleden, G. M., & Vos, R. (2014). Azithromycin: Mechanisms of Action and Their Relevance for Clinical Applications. *Pharmacology & Therapeutics*, *143*(2), 225–245.

Pascu, C., Herman, V., Iancu, I., & Costinar, L. (2022). Etiology of Mastitis and Antimicrobial Resistance in Dairy Cattle Farms in the Western Part of Romania. *Antibiotics*, *11*(1).

Peckler, G. L., & Adcock, S. J. J. (2025). Evaluating Somatic Cell Count, the California Mastitis Test and Infrared Thermography for Subclinical Mastitis Detection in Meat Ewes. *Research in Veterinary Science*, *193*, 105791.

Peng, Q., Tang, X., Dong, W., Sun, N., & Yuan, W. (2022). A Review of Biofilm Formation of *Staphylococcus aureus* and Its Regulation Mechanism. *Antibiotics*, *12*(1).

Petzer, I.-M., Labuschagne, C., Phophi, L., & Karzis, J. (2022). Species Identification and Cow Risks of Non-*aureus* Staphylococci from South African Dairy Herds. *The Onderstepoort Journal of Veterinary Research*, *89*(1), 2021.

Phillips, M. A., Petit, R. A., Weissman, D. B., & Read, T. D. (2025). Transition of *Staphylococcus aureus* Tetracycline Resistance Plasmid pT181 from Independent Multicopy Replicon to Predominantly Integrated Chromosomal Element over 65 years.

Phophi, L., Petzer, I.-M., & Qekwana, D. N. (2019). Antimicrobial Resistance Patterns and Biofilm Formation of Coagulase-Negative *Staphylococcus* Species Isolated from Subclinical Mastitis Cow Milk Samples Submitted to the Onderstepoort Milk Laboratory.

Pimenta, L. K. L., Rodrigues, C. A., Filho, A. R. G., Coelho, C. J., Goes, V., Estrela, M., de Souza, P., Avelino, M. A. G., Vieira, J. D. G., & Carneiro, L. (2023). *Staphylococcu*s spp. Causatives of Infections and Carrier of *blaZ*, *femA* and *mecA* Genes Associated with Resistance. *Antibiotics*, *12*(4), 671.

Plummer, P. J., & Plummer, C. (2012). Diseases of the Mammary Gland. In *Sheep and Goat Medicine* (pp. 442–465). W.B. Saunders.

Poyart, C., Quesne, G., Boumaila, C., & Trieu-Cuot, P. (2001). Rapid and Accurate Species-Level Identification of Coagulase-Negative Staphylococci by Using the *sodA* Gene as a Target. *Journal of Clinical Microbiology*, *39*(12), 4296–4301.

Pumipuntu, N., Kulpeanprasit, S., Santajit, S., Tunyong, W., Kong-ngoen, T., Hinthong, W., & Indrawattana, N. (2017). Screening Method for *Staphylococcus aureus* Identification in Subclinical Bovine Mastitis from Dairy Farms. *Veterinary World*, *10*(7), 721–726.

Pumipuntu, N., Tunyong, W., Chantratita, N., Diraphat, P., Pumirat, P., Sookrung, N., Chaicumpa, W., & Indrawattana, N. (2019). *Staphylococcus* spp. Associated with Subclinical Bovine Mastitis in Central and Northeast Provinces of Thailand. *PeerJ*, *2019*(3).

Qiu, Y., Zhou, Y., Chang, Y., Liang, X., Zhang, H., Lin, X., Qing, K., Zhou, X., & Luo, Z. (2022). The Effects of Ventilation, Humidity and Temperature on Bacterial Growth and Bacterial Genera Distribution. *International Journal of Environmental Research and Public Health*, *19*(22), 15345.

Qu, Y., Zhao, H., Nobrega, D. B., Cobo, E. R., Han, B., Zhao, Z., Li, S., Li, M., Barkema, H. W., & Gao, J. (2019). Molecular Epidemiology and Distribution

of Antimicrobial Resistance Genes of *Staphylococcus* Species Isolated from Chinese Dairy Cows with Clinical Mastitis. *Journal of Dairy Science*, *102*(2), 1571–1583.

Rahman, M. H., Shahadat, M. N., Siddique, A. B., & Ahmed, S. (2026). Antimicrobial Resistance Profiles of non-*aureus* Staphylococci Isolated from Farm Animals, Farm Environments and Companion Animals. *Scientific Reports*.

Rajamanickam, K., Yang, J., Chidambaram, S. B., & Sakharkar, M. K. (2020). Enhancing Drug Efficacy against Mastitis Pathogens - An In Vitro Pilot Study in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Animals : An Open Access Journal from MDPI*, *10*(11), 1–15.

Rajput, P., Nahar, K. S., & Rahman, K. M. (2024). Evaluation of Antibiotic Resistance Mechanisms in Gram-Positive Bacteria. *Antibiotics*, *13*(12), 1197.

Ramírez, N. F., Keefe, G., Dohoo, I., Sánchez, J., Arroyave, O., Cerón, J., Jaramillo, M., & Palacio, L. G. (2014). Herd- and Cow-Level Risk Factors Associated with Subclinical Mastitis in Dairy Farms from the High Plains of the Northern Antioquia, Colombia. *Journal of Dairy Science*, *97*(7), 4141–4150.

Ramuada, M., Tyasi, T. L., Gumede, L., & Chitura, T. (2024). A Practical Guide to Diagnosing Bovine Mastitis: A review. *Frontiers in Animal Science*, *5*.

Rather, M. A., Gupta, K., & Mandal, M. (2021). Microbial Biofilm: Formation, Architecture, Antibiotic Resistance and Control Strategies. *Brazilian Journal of Microbiology*, *52*(4), 1701–1718.

Ravaioli, S., Donno, A. D., Bottau, G., Campoccia, D., Maso, A., Dolzani, P., Balaji, P., Pegreffi, F., Daglia, M., & Arciola, C. R. (2024). The Opportunistic Pathogen *Staphylococcus warneri*: Virulence and Antibiotic Resistance, Clinical Features, Association with Orthopedic Implants and Other Medical Devices and a Glance at Industrial Applications. *Antibiotics*, *13*(10).

Regecová, I., Výrostková, J., Zigo, F., Gregová, G., & Kováčová, M. (2021). Detection of Antimicrobial Resistance of Bacteria *Staphylococcus chromogenes* Isolated from Sheep's Milk and Cheese. *Antibiotics*, *10*(5), 570.

Reiner, K. (2010). Catalase Test Protocol. *American Society for Microbiology*.

Resende, J. A., Fontes, C. O., Ferreira-Machado, A. B., Nascimento, T. C., Silva, V. L., & Diniz, C. G. (2018). Antimicrobial-Resistance Genetic Markers in Potentially Pathogenic Gram Positive Cocci Isolated from Brazilian Soft Cheese. *Journal of Food Science*, *83*(2), 377–385.

Reygaert, W. C. (2018). An Overview of the Antimicrobial Resistance Mechanisms of Bacteria. *AIMS Microbiology*, *4*(3), 482–501.

Reyher, K. K., & Dohoo, I. R. (2011). Diagnosing Intramammary Infections: Evaluation of Composite Milk Samples to Detect Intramammary Infections. *Journal of Dairy Science*, *94*(7), 3387–3396.

Rodríguez, M. F., Gomez, A. P., & Ceballos-Garzon, A. (2023). Antimicrobial Resistance Profiles of *Staphylococcus* Isolated from Cows with Subclinical Mastitis: Do Strains from the Environment and from Humans Contribute to the Dissemination of Resistance among Bacteria on Dairy Farms in Colombia? *Antibiotics*, *12*(11), Article 11.

Rosenbach, F. J. (1884). Mikro-organismen bei den Wund-Infections-Krankheiten des Menschen. J.F. Bergmann.

Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., & Thompson, F. (Eds.). (2014). The Family Staphylococcaceae. In *The Prokaryotes: Firmicutes and Tenericutes*. Springer Berlin Heidelberg.

Ruegg, P. L. (2017). *A 100-Year Review:* Mastitis Detection, Management and Prevention. *Journal of Dairy Science*, *100*(12), 10381–10397.

Ruiz-Romero, R. A., & Vargas-Bello-Pérez, E. (2023). Non-*aureus* Staphylococci and Mammaliicocci as a Cause of Mastitis in Domestic Ruminants: Current Knowledge, Advances, Biomedical Applications and Future Perspectives – A Systematic Review. *Veterinary Research Communications*, *47*(3), 1067–1084.

Rust, J. D., Christian, M. J., Vance, C. J., Bolajoko, M. B., Wong, J. T., Suarez-Martinez, J., Allan, F. K., & Peters, A. R. (2023). A Study of the Effectiveness of a Detergent-Based California Mastitis Test (CMT), using Ethiopian and Nigerian Domestic Detergents, for the Detection of High Somatic Cell Counts in Milk and Their Reliability Compared to the Commercial UK CMT. *Gates Open Research*, *5*, 146.

Rychshanova, R., Mendybayeva, A., Miciński, B., Mamiyev, N., Shevchenko, P., Bermukhametov, Z., Orzechowski, B., & Miciński, J. (2022). Antibiotic Resistance and Biofilm Formation in *Staphylococcus aureus* Isolated from Dairy Cows at the Stage of Subclinical Mastitis in Northern Kazakhstan. *Archives Animal Breeding*, *65*(4), 439–448.

Saeed, S. I., Kamaruzzaman, N. F., Gahamanyi, N., Nguyen, T. T. H., Hossain, D., & Kahwa, I. (2024). Confronting the Complexities of Antimicrobial Management

for *Staphylococcus aureus* causing Bovine Mastitis: An Innovative Paradigm. *Irish Veterinary Journal*, *77*(1), 4.

Saeed, S. I., Mat Yazid, K. A., Hashimy, H. A., Dzulkifli, S. K., Nordin, F., Nik Him, N. A., Bin Omar, M. F. F., Aklilu, E., Mohamad, M., Zalati, C. W. S., & Kamaruzzaman, N. F. (2022). Prevalence, Antimicrobial Resistance and Characterization of *Staphylococcus aureus* Isolated from Subclinical Bovine Mastitis in East Coast Malaysia. *Animals*, *12*(13).

Saglam, A. G., Sahin, M., Çelik, E., Çelebi, Ö., Akça, D., & Otlu, S. (2017). The Role of Staphylococci in Subclinical Mastitis of Cows and Lytic Phage Isolation against to *Staphylococcus aureus*. *Veterinary World*, *10*(12), 1481.

Sahar, G. A. E.-H., I.A. Attia, El-Dera, H. B., & El-Seedy, A. S. (2019). Prevalence and Antibiotic Resistance of *Staphylococcus aureus* Isolated From Raw Milk and Dairy Products Collected From Alexandria, Egypt. *16*(2).

Saidi, R., Mimoune, N., Baazizi, R., Benaissa, M. H., Khelef, D., & Kaidi, R. (2019). Antibiotic Susceptibility of Staphylococci Isolated from Bovine Mastitis in Algeria. *Journal of Advanced Veterinary and Animal Research*, *6*(2), 231–235.

Saito, K., Warrier, T., Somersan-Karakaya, S., Kaminski, L., Mi, J., Jiang, X., Park, S., Shigyo, K., Gold, B., Roberts, J., Weber, E., Jacobs, W. R., & Nathan, C. F. (2017). Rifamycin Action on RNA Polymerase in Antibiotic-Tolerant *Mycobacterium tuberculosis* Results in Differentially Detectable Populations. *Proceedings of the National Academy of Sciences*, *114*(24), E4832–E4840.

Sangwa, A. L., Kashoma, I. P., & Lupindu, A. M. (2025). Comparative Evaluation of Some Diagnostic Tests for on-farm Screening of Subclinical Mastitis in Crossbreed Dairy Cows. *Journal of Applied Veterinary Sciences*, *10*(2), 75–85.

Sansevere, E. A., & Robinson, D. A. (2017). Staphylococci on ICE: Overlooked Agents of Horizontal Gene Transfer. *Mobile Genetic Elements*, *7*(4), 1–10.

Satbige, A., & Mammani, V. S. (2021). Prevalence, Diagnosis and Therapeutic Management of Subclinical Mastitis in Deoni Cows. *Advances in Dairy Research*, *9*(6), 1–4.

Schauer, B., Szostak, M. P., Ehricht, R., Monecke, S., Feßler, A. T., Schwarz, S., Spergser, J., Krametter-Frötscher, R., & Loncaric, I. (2021). Diversity of Methicillin-Resistant Coagulase-Negative *Staphylococcus* spp. and Methicillin-Resistant *Mammaliicoccus* spp. Isolated from Ruminants and New World Camelids. *Veterinary Microbiology*, *254*, 109005.

Schiffer, C. J., & Ehrmann, M. A. (2025). *Macrococcus capreoli* sp. nov., A New Fosfomycin Resistant Species Isolated from Feces and Nasal Swabs of Deer. *Systematic and Applied Microbiology*, *48*(4), 126620.

Schilcher, K., & Horswill, A. R. (2020). Staphylococcal Biofilm Development: Structure, Regulation and Treatment Strategies. *Microbiology and Molecular Biology Reviews*, *84*(3), 10.1128/mmbr.00026-19.

Schleifer, K. H., & Kloos, W. E. (1975). Isolation and Characterization of Staphylococci from Human Skin I. Amended Descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and Descriptions of Three New Species: *Staphylococcus cohnii*, *Staphylococcus haemolyticus* and *Staphylococcus xylosus*. *International Journal of Systematic and Evolutionary Microbiology*, *25*(1), 50–61.

Schmidt, T., Kock, M. M., & Ehlers, M. M. (2015). Diversity and Antimicrobial Susceptibility Profiling of Staphylococci Isolated from Bovine Mastitis Cases and Close Human Contacts. *Journal of Dairy Science*, *98*(9), 6256–6269.

Schwarz, S., Shen, J., Wendlandt, S., Feßler, A. T., Wang, Y., Kadlec, K., & Wu, C.-M. (2014). Plasmid-Mediated Antimicrobial Resistance in Staphylococci and Other Firmicutes. *Microbiology Spectrum*, *2*(6), 10.1128/microbiolspec.plas-0020–2014.

Schwendener, S., Cotting, K., & Perreten, V. (2017). Novel Methicillin Resistance Gene *mecD* in Clinical *Macrococcus caseolyticus* Strains from Bovine and Canine Sources. *Scientific Reports*, *7*, 43797.

Schwendener, S., Donà, V., & Perreten, V. (2020). The Novel Macrolide Resistance Genes *mefD*, *msrF* and *msrH* are Present on Resistance Islands in *Macrococcus canis*, *Macrococcus caseolyticus* and *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *64*(5), e00160-20. https://doi.org/10.1128/AAC.00160-20

Schwendener, S., Nigg, A., Collaud, A., Overesch, G., Kittl, S., Phumthanakorn, N., & Perreten, V. (2019). Typing of *mecD* Islands in Genetically Diverse Methicillin-Resistant *Macrococcus caseolyticus* Strains from Cattle. *Applied and Environmental Microbiology*, *85*(19), e01496-19.

Schwendener, S., & Perreten, V. (2018). The Integrase of the *Macrococcus caseolyticus* Resistance Island *mecD* (McRI*mecD*) Inserts DNA Site-

Specifically into *Staphylococcus* and *Bacillus* Chromosomes. *Molecular Microbiology*, *110*(3), 455–468.

Schwendener, S., & Perreten, V. (2022). The *bla* and *mec* Families of β-Lactam Resistance Genes in the Genera *Macrococcus*, *Mammaliicoccus* and *Staphylococcus*: An In-Depth Analysis with Emphasis on *Macrococcus*. *Journal of Antimicrobial Chemotherapy*, *77*(7), 1796–1827.

Sehrawat, R., Rathee, P., Khatkar, S., Akkol, E., Khayatkashani, M., Nabavi, S., & Khatkar, A. (2023). Dihydrofolate Reductase (DHFR) Inhibitors: A Comprehensive Review. *Current Medicinal Chemistry*, *30*.

Severin, A., Wu, S. W., Tabei, K., & Tomasz, A. (2004). Penicillin-Binding Protein 2 is Essential for Expression of High-Level Vancomycin Resistance and Cell Wall Synthesis in Vancomycin- Resistant *Staphylococcus aureus* Carrying the Enterococcal *vanA* Gene Complex. *Antimicrobial Agents and Chemotherapy*, *48*(12), 4566–4573.

Shaheen, M., & Tantary, H. (2015). A Treatise on Bovine Mastitis: Disease and Disease Economics, Etiological Basis, Risk Factors, Impact on Human Health, Therapeutic Management, Prevention and Control Strategy. *Advances in Dairy Research*, *04*(01).

Shalaby, M.-A. W., Dokla, E. M. E., Serya, Rabah. A. T., & Abouzid, K. A. M. (2020). Penicillin Binding Protein 2a: An Overview and a Medicinal Chemistry Perspective. *European Journal of Medicinal Chemistry*, *199*, 112312.

Shao, K., Yang, Y., Gong, X., Chen, K., Liao, Z., & Ojha, S. C. (2025). Staphylococcal Drug Resistance: Mechanisms, Therapies and Nanoparticle Interventions. *Infection and Drug Resistance*, *18*, 1007–1033.

Sharma, T., Das, P. K., Ghosh, P. R., Banerjee, D., & Mukherjee, J. (2017). Association between Udder Morphology and In Vitro Activity of Milk Leukocytes in High Yielding Crossbred Cows. *Veterinary World*, *10*(3), 342–347.

Shi, H., Wang, L., Li, G., Li, D., Zhai, H., Ji, S., Hu, Y., Lv, T., & Yao, L. (2025). Characteristic Profiles of Molecular Types, Antibiotic Resistance, Antibiotic Resistance Genes and Virulence Genes of *Staphylococcus aureus* Isolates from Caprine Mastitis in China. *Frontiers in Cellular and Infection Microbiology*, *15*.

Shields, P., & Tsang, A. Y. (2016). Mannitol Salt Agar Plates Protocols.

Shoaib, M., Xu, J., Meng, X., Wu, Z., Hou, X., He, Z., Shang, R., Zhang, H., & Pu, W. (2023). Molecular Epidemiology and Characterization of Antimicrobial-Resistant *Staphylococcus haemolyticus* Strains Isolated from Dairy Cattle Milk in Northwest, China. *Frontiers in Cellular and Infection Microbiology*, *13*, 1183390.

Shrimali, M. D., Chauhan, H. C., Patel, S. S., Panchal, J. D., Patel, A. C., Sharma, K. K., Thakore, A. K., Mohapatra, S. K., & Prajapati, B. I. (2025). Comparative Analysis of *Staphylococcus aureus* and non-*aureus* Staphylococci and Mammaliicocci (NASM) in Bovine Milk: Species Distribution, Resistance Profiling and Genomic Characterisation. *Gene Reports*, *41*, 102372.

Silva, V., Capelo, J. L., Igrejas, G., & Poeta, P. (2020). Molecular Epidemiology of *Staphylococcus aureus* Lineages in Wild Animals in Europe: A Review. *Antibiotics*, *9*(3), Article 3.

Singh, A. K. (2022). A Comprehensive Review on Subclinical Mastitis in Dairy Animals: Pathogenesis, Factors Associated, Prevalence, Economic Losses and Management Strategies. *CABI Reviews*, cabireviews202217057.

Sivadon, V., Rottman, M., Quincampoix, J.-C., Avettand, V., Chaverot, S., De Mazancourt, P., Trieu-Cuot, P., & Gaillard, J.-L. (2004). Use of *sodA* Sequencing for the Identification of Clinical Isolates of Coagulase-Negative Staphylococci. *Clinical Microbiology and Infection*, *10*(10), 939–942.

Smith, K. L., & Hogan, J. S. (1993). Environmental Mastitis. *Veterinary Clinics of North America: Food Animal Practice*, *9*(3), 489–498.

Song, X., Huang, X., Xu, H., Zhang, C., Chen, S., Liu, F., Guan, S., Zhang, S., Zhu, K., & Wu, C. (2020). The Prevalence of Pathogens Causing Bovine Mastitis and Their Associated Risk Factors in 15 Large Dairy Farms in China: An Observational Study. *Veterinary Microbiology*, *247*, 108757.

Srednik, M. E., Tremblay, Y. D. N., Labrie, J., Archambault, M., Jacques, M., Cirelli, A. F., & Gentilini, E. R. (2017). Biofilm Formation and Antimicrobial Resistance Genes of Coagulase-Negative Staphylococci Isolated from Cows with Mastitis in Argentina. *FEMS Microbiology Letters*, *364*(8).

Stanek, P., Żółkiewski, P., & Januś, E. (2024). A Review on Mastitis in Dairy Cows Research: Current Status and Future Perspectives. *Agriculture*, *14*(8), Article 8.

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of Biofilm in Microtiter Plates: Overview of

Testing Conditions and Practical Recommendations for Assessment of Biofilm Production by Staphylococci. *APMIS*, *115*(8), 891–899.

Sumon, S. M. M. R., Parvin, S., Ehsan, A., & Islam, T. (2020). Relationship Between Somatic Cell Counts and Subclinical Mastitis in Lactating Dairy Cows. *Veterinary World*, *13*(8), 1709.

Tang, J., Kang, M., Chen, H., Shi, X., Zhou, R., Chen, J., & Du, Y. (2011). The Staphylococcal Nuclease Prevents Biofilm Formation in *Staphylococcus aureus* and Other Biofilm-Forming Bacteria. *Science China Life Sciences*, *54*(9), 863–869.

Tanni, N. S., Islam, Md. S., Kabir, M., Parvin, Mst. S., Ehsan, Md. A., & Islam, Md. T. (2021). Evaluation of Sodium Lauryl Sulfate for the Development of Cow-Side Mastitis Screening Test. *Veterinary World*, *14*(8), 2290–2295.

Tanvi, T. Z., Islam, M. S., Lovelu, M. A., Mir, D. A., & Bahar, F. (2024). Prevalence of Bovine Subclinical Mastitis and Associated Risk Factors at Sylhet District in Bangladesh. *Asian Journal of Research in Animal and Veterinary Sciences*, *7*(3), 208–215.

Taponen, S., Björkroth, J., & Pyörälä, S. (2008). Coagulase-Negative Staphylococci Isolated from Bovine Extramammary Sites and Intramammary Infections in a Single Dairy Herd. *Journal of Dairy Research*, *75*(4), 422–429.

Taponen, S., Tölli, H.-T., & Rajala-Schultz, P. J. (2023). Antimicrobial Susceptibility of Staphylococci from Bovine Milk Samples in Routine Microbiological Mastitis Analysis in Finland. *Frontiers in Veterinary Science*, *10*, 1235417.

Tarazona-Manrique, L. E., Vargas-Abella, J. C., & Andrade-Becerra, R. J. (2024). Antibiotic Resistance Profile of Coagulase-Negative Staphylococci Species Isolated from Subclinical Mastitis in Colombian Dairy Herds. *Revista de Investigaciones Veterinarias Del Perú*, *35*(5), e29246.

Taylor, T. A., & Unakal, C. G. (2025). *Staphylococcus aureus* Infection. In *StatPearls*. StatPearls Publishing.

Teotia, A., Bashir, S., Malik, T., Mohan, A., Tariq, M., kumar, A., & Singh, R. (2025). Sustainable Management of Bovine Mastitis Caused by Major Pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Streptococcus agalactiae* and *Streptococcus uberis*) using Medicinal Plants: A Review. *Dairy Science and Management*, *2*(1), 19.

Tezera, M., & Ali, E. (2021). Prevalence and Associated Risk Factors of Bovine Mastitis in Dairy Cows in and around Assosa Town, Benishangul-Gumuz Regional State, Western Ethiopia. *Veterinary Medicine and Science*, *7*(4), 1280–1286.

Thrusfield, M. (2005). *Veterinary Epidemiology*. Blackwell Science Ltd.

Todtong, P., Nilnont, T., & Pilachai, R. (2021). Prevalence and Antimicrobial Susceptibility on Bacterial Pathogens of Clinical and Subclinical Mastitis in Lactating Cows in Udon Thani Province. *KKU Veterinary Journal*, *31*(1), 29–37.

Tomanić, D., Samardžija, M., & Kovačević, Z. (2023). Alternatives to Antimicrobial Treatment in Bovine Mastitis Therapy: A Review. *Antibiotics 2023, Vol. 12, Page 683*, *12*(4), 683.

Torlak, E., Korkut, E., Uncu, A. T., & Şener, Y. (2017). Biofilm Formation by *Staphylococcus aureus* Isolates from a Dental Clinic in Konya, Turkey. *Journal of Infection and Public Health*, *10*(6), 809–813.

Touaitia, R., Ibrahim, N. A., Touati, A., Idres, T., Touaitia, R., Ibrahim, N. A., Touati, A., & Idres, T. (2025). *Staphylococcus aureus* in Bovine Mastitis: A Narrative Review of Prevalence, Antimicrobial Resistance and Advances in Detection Strategies. *Antibiotics*, *14*(8).

Traversari, J., van den Borne, B. H. P., Dolder, C., Thomann, A., Perreten, V., & Bodmer, M. (2019). Non-*aureus* Staphylococci Species in the Teat Canal and Milk in Four Commercial Swiss Dairy Herds. *Frontiers in Veterinary Science*, *6*.

Treangen, T. J., Maybank, R. A., Enke, S., Friss, M. B., Diviak, L. F., Karaolis, D. K. R., Koren, S., Ondov, B., Phillippy, A. M., Bergman, N. H., & Rosovitz, M. J. (2014). Complete Genome Sequence of the Quality Control Strain *Staphylococcus aureus* subsp. *aureus* ATCC 25923. *Genome Announcements*, *2*(6), e01110-14.

Trimble, M. J., Mlynárčik, P., Kolář, M., & Hancock, R. E. W. (2016). Polymyxin: Alternative Mechanisms of Action and Resistance. *Cold Spring Harbor Perspectives in Medicine*, *6*(10), a025288.

Tripathi, N., & Sapra, A. (2021). Gram staining. *StatPearls*.

Tsiklauri, R., Kobakhidze, S., & Kotetishvili, M. (2025). Interactive Networks of Donors and Recipients of the *tetM* Gene and Its Evolutionary Dynamics across the Bacterial Domain. *Scientific Reports*, *15*(1), 35312.

Tsubakishita, S., Kuwahara-Arai, K., Baba, T., & Hiramatsu, K. (2010). Staphylococcal Cassette Chromosome *mec*-Like Element in *Macrococcus caseolyticus*. *Antimicrobial Agents and Chemotherapy*, *54*(4), 1469–1475. https://doi.org/10.1128/AAC.00575-09

Uddin, T. M., Chakraborty, A. J., Khusro, A., Zidan, B. R. M., Mitra, S., Emran, T. B., Dhama, K., Ripon, Md. K. H., Gajdács, M., Sahibzada, M. U. K., Hossain, Md. J., & Koirala, N. (2021). Antibiotic Resistance in Microbes: History, Mechanisms, Therapeutic Strategies and Future Prospects. *Journal of Infection and Public Health*, *14*(12), 1750–1766.

Ugwu, C. N., Ezeibe, E. N., Emencheta, S. C., Nwagwu, C. S., Ogbonna, K. O., Ejiofor, C. V., Onugwu, A. L., Berebon, D. P., & Attama, A. A. (2025). Biofilms: Structure, Resistance Mechanism, Emerging Control Strategies and Applications. *RSC Pharmaceutics*, *2*(6), 1376–1407.

Umami, Z., Warni, A. I., & Wahyuni, D. (2023). *mec* Genes as Marker in Methicillin-Resistant *Staphylococcus aureus*. *Journal of Islamic Pharmacy*, *8*(2), 78–82.

Vargová, M., Zigo, F., Výrostková, J., Farkašová, Z., & Rehan, I. F. (2023). Biofilm-Producing Ability of *Staphylococcus aureus* Obtained from Surfaces and Milk of Mastitic Cows. *Veterinary Sciences*, *10*(6), 386.

Veken, D. V. der, Hollanders, C., Verce, M., Michiels, C., Ballet, S., Weckx, S., & Leroy, F. (2022). Genome-Based Characterization of a Plasmid-Associated Micrococcin P1 Biosynthetic Gene Cluster and Virulence Factors in *Mammaliicoccus sciuri* IMDO-S72. *Applied and Environmental Microbiology*, *88*(4), e02088-21.

Venkatesan, M., Fruci, M., Verellen, L. A., Skarina, T., Mesa, N., Flick, R., Pham, C., Mahadevan, R., Stogios, P. J., & Savchenko, A. (2023). Molecular mechanism of plasmid-borne resistance to sulfonamide antibiotics. *Nature Communications*, *14*, 4031.

Vestby, L. K., Grønseth, T., Simm, R., & Nesse, L. L. (2020). Bacterial Biofilm and its Role in the Pathogenesis of Disease. *Antibiotics (Basel, Switzerland)*, *9*(2), 59.

Veterinary Biologics & Medicine Sections & Division of Veterinary Public Health. (2021). *Malaysian veterinary antimicrobial guidelines*. Department of

Veterinary Services Malaysia, Ministry of Agriculture & Food Industry Malaysia.

Vidlund, J., Gelalcha, B. D., Swanson, S., Fahrenholz, I. costa, Deason, C., Downes, C., Dego, O. K., Vidlund, J., Gelalcha, B. D., Swanson, S., Fahrenholz, I. costa, Deason, C., Downes, C., & Dego, O. K. (2022). Pathogenesis, Diagnosis, Control and Prevention of Bovine Staphylococcal Mastitis. In *Mastitis in Dairy Cattle, Sheep and Goats*. IntechOpen.

Vimberg, V., Cavanagh, J. P., Novotna, M., Lenart, J., Nguyen Thi Ngoc, B., Vesela, J., Pain, M., Koberska, M., & Balikova Novotna, G. (2020). Ribosome-Mediated Attenuation of *vgaA* Expression Is Shaped by the Antibiotic Resistance Specificity of VgaA Protein Variants. *Antimicrobial Agents and Chemotherapy*, *64*(11), e00666-20.

Waller, K. P., Myrenås, M., Börjesson, S., Kim, H., Widerström, M., Monsen, T., Sigurðarson Sandholt, A. K., Östlund, E., & Cha, W. (2023). Genotypic Characterization of *Staphylococcus chromogenes* and *Staphylococcus simulans* from Swedish Cases of Bovine Subclinical Mastitis. *Journal of Dairy Science*, *106*(11), 7991–8004.

Wang, F., Zhou, H., Olademehin, O. P., Kim, S. J., & Tao, P. (2018). Insights into Key Interactions Between Vancomycin and Bacterial Cell Wall Structures. *ACS Omega*, *3*(1), 37–45.

Wang, L., Haq, S. U., Shoaib, M., He, J., Guo, W., Wei, X., Zheng, X., Wang, L., Haq, S. U., Shoaib, M., He, J., Guo, W., Wei, X., & Zheng, X. (2024). Subclinical Mastitis in Small-Holder Dairy Herds of Gansu Province, Northwest China: Prevalence, Bacterial Pathogens, Antimicrobial Susceptibility and Risk Factor Analysis. *Microorganisms*, *12*(12).

Weigel, L. M., Donlan, R. M., Shin, D. H., Jensen, B., Clark, N. C., McDougal, L. K., Zhu, W., Musser, K. A., Thompson, J., Kohlerschmidt, D., Dumas, N., Limberger, R. J., & Patel, J. B. (2007). High-Level Vancomycin-Resistant *Staphylococcus aureus* Isolates Associated with a Polymicrobial Biofilm. *Antimicrobial Agents and Chemotherapy*, *51*(1), 231–238.

Wilson, D. N., Hauryliuk, V., Atkinson, G. C., & O'Neill, A. J. (2020). Target Protection as a Key Antibiotic Resistance Mechanism. *Nature Reviews. Microbiology*, *18*(11), 637–648.

WOAH. (2025). *Annual Report on Antimicrobial Agents Intended for Use in Animals. 9th Report*. WOAH (World Organisation for Animal Health).

Wójcik-Bojek, U., Różalska, B., & Sadowska, B. (2022). *Staphylococcus aureus*—A Known Opponent against Host Defense Mechanisms and Vaccine Development—Do We Still Have a Chance to Win? *International Journal of Molecular Sciences*, *23*(2).

Wright, L. D., & Grossman, A. D. (2016). Autonomous Replication of the Conjugative Transposon Tn*916*. *Journal of Bacteriology*, *198*(24), 3355–3366.

Wu, S. W., de Lencastre, H., & Tomasz, A. (2001). Recruitment of the *mecA* Gene Homologue of *Staphylococcus sciuri* into a Resistance Determinant and Expression of the Resistant Phenotype in *Staphylococcus aureus*. *Journal of Bacteriology*, *183*(8), 2417–2424.

Xie, Z. (2021). The Methods and Tools for Mobile Genetic Element Detection and Their Application to Systems Medicine. *Systems Medicine: Integrative, Qualitative and Computational Approaches: Volume 1-3*, 203–207.

Yang, F., Shi, W., Meng, N., Zhao, Y., Ding, X., & Li, Q. (2023). Antimicrobial Resistance and Virulence Profiles of Staphylococci Isolated from Clinical Bovine Mastitis. *Frontiers in Microbiology*, *14*, 1190790.

Yang, F., Wang, Q., Wang, X. Rong, Wang, L., Li, X. Pu, Luo, J. Y., Zhang, S. Dong, & Li, H. Sheng. (2016). Genetic Characterization of Antimicrobial Resistance in *Staphylococcus aureus* Isolated from Bovine Mastitis Cases in Northwest China. *Journal of Integrative Agriculture*, *15*(12), 2842–2847.

Yazdankhah, S. P., Sørum, H., Larsen, H. J. S., & Gogstad, G. (2001). Rapid Method for Detection of Gram-Positive and -Negative Bacteria in Milk from Cows with Moderate or Severe Clinical Mastitis. *Journal of Clinical Microbiology*, *39*(9), 3228–3233.

Yu, W., Zhang, Z., Wang, Z., Lin, X., Dong, X., & Hou, Q. (2025). Comprehensive Prevention and Control of Mastitis in Dairy Cows: From Etiology to Prevention. *Veterinary Sciences*, *12*(9), 800.

Zaatout, N. (2022). An Overview on Mastitis-Associated *Escherichia coli*: Pathogenicity, Host Immunity and the Use of Alternative Therapies. *Microbiological Research*, *256*, 126960.

Zaatout, N., Ayachi, A., & Kecha, M. (2020). *Staphylococcus aureus* Persistence Properties Associated with Bovine Mastitis and Alternative Therapeutic Modalities. *Journal of Applied Microbiology*, *129*(5), 1102–1119.

Zakaria, P. N. M., Lean, S. S., Mohd Desa, M. N., Aklilu, E., Shamsir Omar, M. S., Yeo, C. C., & Suhaili, Z. (2026). Genomic Insights into Multidrug-resistant *Mammaliicoccus sciuri* Carrying Novel Staphylococcal Cassette Chromosome mec (SCCmec) Elements and Plasmids from Farm Ruminants on the East Coast of Peninsular Malaysia. *Current Microbiology*, *83*(1), 72.

Zhan, C., Zhang, M., Hao, G., Zhang, Y., & Wang, F. (2026). Research Progress on *Macrococcus*: From Basic Biology to Clinical Antimicrobial Resistance Challenges. *Pathogens*, *15*(6), 578.

Zhang, X., Zhou, Y., Fu, L., Zhou, L., Cheng, X., Zhang, W., & Tan, Z. (2024). WGS Analysis of *Staphylococcus warneri* Outbreak in a Neonatal Intensive Care Unit. *Infection and Drug Resistance*, *17*, 4279–4289.

Zhang, Y., Min, S., Sun, Y., Ye, J., Zhou, Z., & Li, H. (2022). Characteristics of Population Structure, Antimicrobial Resistance, Virulence Factors and Morphology of Methicillin-Resistant *Macrococcus caseolyticus* in Global Clades. *BMC Microbiology*, *22*(1), 266.

APPENDICES

APPENDIX 1

Table of Mean of Zone of Inhibition (ZOI) Diameters and Susceptibility Categorization of Staphylococcaceae Isolates (N = 153) and Two Control Strains

No.	Isolates ID	E15		P10		FOX30	
		Mean of ZOI (mm)	Interpretation	Mean of ZOI (mm)	Interpretation	Mean of ZOI (mm)	Interpretation
1	*S. aureus* ATCC 25923	25.3	S	32.0	S	34.7	S
2	S. *epidermidis* ATCC 35984	0	R	9.3	R	10.1	R
3	AY574001	30.7	S	22.9	R	36.5	S
4	AY684002	25.7	S	38.7	S	31.0	S
5	AY684003	28.8	S	40.5	S	33.5	S
6	AY684004	27.4	S	39.6	S	33.0	S
7	AY622014	26.1	S	38.5	S	30.4	S
8	AY624015	22.0	I	37.0	S	30.0	S
9	AY622016	28.8	S	42.3	S	32.7	S
10	AY622017	29.5	S	41.3	S	33.4	S
11	AY622018	31.8	S	27.1	R	36.6	S
12	AY622019	26.6	S	38.7	S	32.0	S
13	AY622020	27.5	S	39.9	S	31.6	S
14	AY622021	26.7	S	38.1	S	31.4	S
15	AY622023	25.9	S	38.0	S	31.3	S
16	AY625061	28.4	S	20.1	R	31.3	S
17	AY625064	27.9	S	16.7	R	29.6	S
18	AY625079	30.6	S	22.7	R	34.2	S

19	AY625084	26.3	S	16.3	R	28.3	S
20	AY625088	20.0	I	20.7	R	32.3	S
21	AY625097	26.0	S	16.6	R	28.8	S
22	AY625098	26.2	S	25.2	R	27.2	S
23	AY625099	24.6	S	25.5	R	25.3	S
24	AY625100	28.4	S	22.5	R	26.3	S
25	AY625101	29.1	S	36.9	S	28.6	S
26	AY625102	26.8	S	33.4	S	27.3	S
27	AY625103	28.6	S	33.5	S	28.3	S
28	AY625104	28.3	S	34.2	S	27.8	S
29	AY625105	26.6	S	33.9	S	27.4	S
30	AY625121	26.1	S	24.1	R	26.6	S
31	AY764123	28.3	S	31.5	S	29.3	S
32	AY764124	24.0	S	30.7	S	26.6	S
33	AY764125	25.4	S	30.9	S	26.0	S
34	AY764126	24.1	S	30.1	S	25.6	S
35	AY764127	21.7	I	24.0	R	19.0	R
36	AY764128	24.7	S	30.3	S	24.3	S
37	AY604146	28.7	S	42.0	S	30.7	S
38	AY604147	25.3	S	25.0	R	23.3	S
39	AY620148	23.0	S	14.7	R	27.7	S
40	AY620149	24.0	S	14.7	R	27.0	S
41	AY620150	25.6	S	16.9	R	30.6	S
42	AY620151	28.2	S	21.5	R	32.7	S
43	AY620153	28.7	S	20.0	R	31.7	S
44	AY605158	26.6	S	41.0	S	33.0	S

45	BY558001	22.9	S	33.5	S	26.1	S
46	BY558003	25.3	S	28.6	S	27.0	S
47	BY558007	25.1	S	31.0	S	26.7	S
48	BY558010	25.0	S	31.4	S	26.8	S
49	BY558016	23.6	S	28.7	S	27.0	S
50	BY636023	25.7	S	38.1	S	29.9	S
51	BY636024	25.4	S	38.1	S	30.1	S
52	BY636025	26.4	S	39.2	S	28.4	S
53	BY636026	26.7	S	38.5	S	29.9	S
54	BY560038	23.9	S	33.5	S	26.8	S
55	BY560039	23.7	S	32.8	S	26.4	S
56	BY560040	22.8	S	33.0	S	26.5	S
57	BY560041	20.4	I	29.8	S	24.6	S
58	BY560042	22.4	I	31.5	S	26.2	S
59	BY560043	21.0	I	32.7	S	25.2	S
60	BY660060	23.4	S	35.1	S	28.5	S
61	BY660061	24.2	S	36.4	S	28.7	S
62	BY660074	22.2	S	33.4	S	27.5	S
63	BY660075	23.5	S	33.1	S	27.7	S
64	BY660076	23.7	S	37.3	S	29.8	S
65	BY660077	22.3	I	33.4	S	27.4	S
66	BY660078	22.1	I	33.1	S	26.9	S
67	BY529090	22.9	S	34.8	S	26.8	S
68	BY529091	24.8	S	33.0	S	26.0	S
69	BY529093	37.7	S	50.0	S	44.9	S
70	BY760095	26.1	S	13.4	R	30.4	S

71	BY760096	24.1	S	37.6	S	29.4	S
72	BY760097	26.2	S	35.3	S	30.4	S
73	BY760100	26.1	S	37.0	S	29.9	S
74	BY760105	31.5	S	20.2	R	36.4	S
75	BY760106	26.9	S	36.1	S	29.7	S
76	BY682111	29.5	S	20.1	R	35.3	S
77	BY682112	28.7	S	39.8	S	31.6	S
78	BY682113	24.2	S	34.5	S	29.3	S
79	BY682114	24.9	S	33.7	S	29.2	S
80	BY682115	26.7	S	36.6	S	30.8	S
81	BY682116	20.3	I	32.2	S	28.3	S
82	BY647136	26.4	S	37.3	S	31.3	S
83	BY647137	26.2	S	37.0	S	31.2	S
84	BY647138	25.2	S	36.3	S	31.1	S
85	BY647139	25.5	S	35.9	S	30.8	S
86	BY647140	25.3	S	35.6	S	30.5	S
87	AP622002	27.3	S	20.7	R	31.7	S
88	AP622005	28.0	S	19.7	R	32.7	S
89	AP622007	25.0	S	10.7	R	27.0	S
90	AP622010	29.0	S	21.0	R	33.7	S
91	AP622016	26.3	S	18.3	R	30.7	S
92	AP622017	25.3	S	10.7	R	27.7	S
93	AP622023	30.0	S	22.3	R	36.6	S
94	AP622027	32.2	S	22.3	R	38.4	S
95	AP622034	33.3	S	23.5	R	40.6	S
96	AP625041	22.0	I	21.0	R	32.0	S

97	AP625042	22.7	S	18.3	R	26.0	S
98	AP625043	27.3	S	21.9	R	21.5	S
99	AP625044	27.0	S	37.5	S	31.0	S
100	AP625045	26.6	S	23.3	R	18.5	R
101	AP625046	30.2	S	15.6	R	12.4	R
102	AP625047	27.6	S	19.0	R	29.6	S
103	AP625048	27.6	S	24.0	R	14.6	R
104	AP625052	25.0	S	20.1	R	29.7	S
105	AP764066	28.7	S	33.7	S	35.2	S
106	AP620077	24.4	S	11.1	R	9.5	R
107	AP620078	24.8	S	13.6	R	11.4	R
108	AP620079	29.7	S	15.1	R	11.5	R
109	AP620080	29.4	S	14.6	R	11.5	R
110	AP620081	27.5	S	12.2	R	11.1	R
111	AP620082	26.7	S	11.5	R	9.5	R
112	AP620083	27.2	S	16.8	R	10.3	R
113	AP620084	26.8	S	17.2	R	10.2	R
114	AP620085	25.6	S	12.1	R	19.6	R
115	AP620086	27.7	S	17.2	R	21.5	S
116	AP620087	27.0	S	18.5	R	18.1	R
117	AP620088	25.5	S	17.8	R	18.3	R
118	AP620089	25.9	S	17.7	R	17.5	R
119	AP610122	30.3	S	17.2	R	32.6	S
120	AP610123	31.7	S	17.9	R	34.6	S
121	AP610124	38.8	S	24.2	R	45.8	S
122	AP610125	28.3	S	19.9	R	34.3	S

123	AP610126	28.3	S	18.2	R	34.3	S
124	AP610127	31.0	S	17.2	R	33.4	S
125	AP610128	29.5	S	16.0	R	30.3	S
126	AP610129	28.6	S	18.7	R	31.8	S
127	AP610130	30.5	S	16.3	R	31.7	S
128	AP610131	29.1	S	16.7	R	30.7	S
129	AP610132	29.4	S	20.2	R	34.9	S
130	AP610133	38.3	S	24.4	R	44.0	S
131	AP610134	30.7	S	16.5	R	30.0	S
132	AP610135	29.2	S	17.3	R	35.3	S
133	AP610136	29.4	S	16.4	R	26.6	S
134	AP610137	31.4	S	16.7	R	34.9	S
135	AP610138	31.1	S	17.4	R	34.1	S
136	AP610139	31.0	S	16.6	R	33.2	S
137	AP610140	29.2	S	16.0	R	30.7	S
138	AP610141	29.5	S	16.1	R	36.5	S
139	AP610142	38.8	S	23.2	R	20.1	R
140	BP558001	33.8	S	27.4	R	40.3	S
141	BP558002	28.4	S	38.4	S	30.8	S
142	BP636009	30.5	S	23.4	R	37.8	S
143	BP636010	30.1	S	22.1	R	36.3	S
144	BP636011	29.9	S	21.8	R	37.0	S
145	BP636012	30.4	S	22.7	R	37.7	S
146	BP560024	22.6	S	29.4	S	25.0	S
147	BP560025	24.6	S	37.0	S	29.7	S
148	BP660031	25.2	S	33.6	S	27.8	S

149	BP529032	32.0	S	36.5	S	28.1	S
150	BP682045	23.4	S	32.4	S	29.2	S
151	BP647051	22.5	S	30.0	S	27.4	S
152	BP647052	25.0	S	20.1	R	29.7	S
153	BP647054	31.3	S	41.8	S	35.8	S
154	BP647057	27.2	S	39.4	S	32.4	S
155	BP647058	26.9	S	30.6	S	31.0	S

Note: Abbreviation; S: susceptible, I: intermediate, R: resistance, E15: erythromycin (15 µg), P10: penicillin G (10 µg), FOX30: cefoxitin (30 µg), TE30: tetracycline (30 µg), SXT25: sulfamethoxazole-trimethoprim (25 µg), CN10: gentamicin (10 µg).

APPENDIX 2

Table of Identification of Staphylococcaceae Using Partial *sodA* Gene Sequence Genotyping

No.	Isolate ID	Identified Bacterial Strains	E-value	Max Identity (%)	Query cover	GenBank Accession No.
1	AY622018	*Staphylococcus aureus* strain Sa9 chromosome, complete genome	0	95.36	94	CP134620.1
2	AP610134	*Staphylococcus aureus* strain Sa9 chromosome, complete genome	0	95.36	94	CP134620.1
3	AY574001	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	98.81	92	AJ343944.1
4	BY6825111	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	98.54	100	AJ343944.1
5	AP622002	*Staphylococcus chromogenes* strain MA4 16S ribosomal RNA gene, partial sequence	0	98.96	98	MT072191.1
6	AP622005	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	98.81	92	AJ343944.1
7	AP622010	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	98.81	91	AJ343944.1
8	AP622016	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	98.81	91	AJ343944.1
9	AP622027	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	98.8	99	AJ343944.1
10	AP622034	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	98.57	95	AJ343944.1

11	AP610124	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	97	AJ343901.1
12	AP610126	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	97	AJ343901.1
13	AP610129	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	100	AJ343901.1
14	AP610132	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	99	AJ343901.1
15	AP610133	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	99	AJ343901.1
16	AP610135	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	97	AJ343901.1
17	AP610138	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	99	AJ343901.1
18	AP610142	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	96	AJ343901.1
19	BP558001	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	97	AJ343901.1
20	BP636009	*Staphylococcus chromogenes* strain IVB6199 chromosome, complete genome	0	99.76	97	AJ343901.1
21	BP636010	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	97	AJ343901.1
22	BP636011	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain NEM2006	0	99.76	97	AJ343944.1

23	BP636012	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	97	AJ343901.1
24	BP647054	*Staphylococcus chromogenes* partial *sodA* gene for superoxide dismutase, strain CIP 81.59 T	0	99.76	97	AJ343901.1
25	AY604147	*Staphylococcus haemolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 81.56 T	0	99.52	96	AJ343910.1
26	AY620148	*Staphylococcus haemolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 81.56 T	0	99.76	96	AJ343910.1
27	AY620149	*Staphylococcus haemolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 81.56 T	0	99.76	95	AJ343910.1
28	AY620150	*Staphylococcus haemolyticus* strain M62.3 chromosome, complete genome	0	99.76	96	AJ343910.1
29	AY620151	*Staphylococcus haemolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 81.56 T	0	99.76	99	EU652775.1
30	AY620153	*Staphylococcus haemolyticus* strain M62.3 chromosome, complete genome	0	99.76	97	AJ343910.1
31	BY760095	*Staphylococcus haemolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 81.56 T	0	99.07	93	EU652775.1
32	AY625098	*Staphylococcus sciuri* subsp. *rodentium* strain CIP 105829 superoxide dismutase (*sodA*) gene, partial cds	0	99.76	99	EU652776.1
33	AY625099	*Staphylococcus sciuri* subsp. *sciuri* partial *sodA* gene for superoxide dismutase, strain CIP 81.62 T	0	99.07	97	AJ343929.1
34	AY625100	*Staphylococcus sciuri* subsp. *rodentium* strain CIP 105829 superoxide dismutase (*sodA*) gene, partial cds	0	99.76	100	EU652776.1

35	AY764127	*Staphylococcus sciuri* subsp. *rodentium* strain CIP 105829 superoxide dismutase (*sodA*) gene, partial cds	0	99.76	91%	EU652776.1
36	BY760105	*Staphylococcus sciuri* subsp. *sciuri* partial *sodA* gene for superoxide dismutase, strain CIP 81.62 T	0	99.02	97	AJ343929.1
37	AP622007	*Staphylococcus simulans strain* ATCC 27848 superoxide dismutase (*sodA*) gene, partial cds	0	99.53	98	EU652777.1
38	AP622017	*Staphylococcus simulans* strain ATCC 27848 superoxide dismutase (*sodA) gene, partial cds*	0	99.76	97	EU652777.1
39	AP622023	*Staphylococcus simulans* strain ATCC 27848 superoxide dismutase (*sodA*) gene, partial cds	0	99.76	100	EU652777.1
40	AP625044	*Staphylococcus warneri* partial *sodA* gene for superoxide dismutase, strain CIP 81.65 T	0	99.76	96	AJ343932.1
41	AP610122	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	99.04	100	CP054482.1
42	AP610123	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	4.00E-155	97.85	99	CP054482.1
43	AP610125	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.6	100	CP054482.1
44	AP610127	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.37	99	CP054482.1
45	AP610128	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.61	99	CP054482.1
46	AP610130	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.78	100	CP054482.1

47	AP610131	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.6	100	CP054482.1
48	AP610136	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.81	100	CP054482.1
49	AP610137	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	99.05	100	CP054482.1
50	AP610139	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.4	100	CP054482.1
51	AP610140	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	99.04	99	CP054482.1
52	AP610141	*Macrococcoides bohemicum* strain 19Msa0383 chromosome, complete genome	0	98.82	98	CP079984.1
53	BP647052	*Macrococcoides bohemicum* strain 19Msa422 chromosome, complete genome	0	98.58	100	CP054482.1
54	AY625061	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	1.00E-166	92.4	92	AJ343934.1
55	AY625064	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	2.00E-168	92.64	92	AJ343934.1
56	AY625084	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	9.00E-168	92.45	93	AJ343934.1
57	AY625097	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	1.00E-166	92.42	92	AJ343934.1
58	AY625121	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	1.00E-165	92.91	90%	AJ343934.1

59	AP625042	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	6.00E-164	92.67	96	AJ343934.1
60	AP625043	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	98.81	99	AJ343934.1
61	AP625045	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	98.8	97	AJ343934.1
62	AP625046	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.02	99	AJ343934.1
63	AP625047	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP	5.00E-165	92.34	97	AJ343934.1
64	AP625048	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.02	99	AJ343934.1
65	AP625052	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	2.00E-164	92.33	99	AJ343934.1
66	AP620077	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	98.8	95	AJ343934.1
67	AP620078	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	98.81	91	AJ343934.1
68	AP620079	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	98.81	91	AJ343934.1
69	AP620080	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	2.00E-164	92.33	99	AJ343934.1
70	AP620081	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.04	97	AJ343934.1

71	AP620082	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.04	99	AJ343934.1
72	AP620083	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.04	99	AJ343934.1
73	AP620084	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.04	97	AJ343934.1
74	AP620085	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.04	97	AJ343934.1
75	AP620086	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	98.81	97	AJ343934.1
76	AP620087	*Macrococcus caseolyticus* partial sodA gene for superoxide dismutase, strain CIP 100755 T	0	99.27	97	AJ343934.1
77	AP620088	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.27	98	AJ343934.1
78	AP620089	*Macrococcus caseolyticus* partial *sodA* gene for superoxide dismutase, strain CIP 100755 T	0	99.03	98	AJ343934.1

APPENDIX 3

Table of Mean of Zone of Inhibition (ZOI) Diameters and Susceptibility Categorization of Staphylococcaceae Isolates (N = 78) and Two Control Strains

No.	Isolates ID	E15		P10		FOX30		TE30		SXT25		CN10		Resistant Phenotype
		Mean of ZOI (mm)	Interpretation	Mean of ZOI (mm)	Interpretation	Mean of ZOI (mm)	Interpretation	Mean of ZOI (mm)	Interpretation	Mean of ZOI (mm)	Interpretation	Mean of ZOI (mm)	Interpretation	
1	*S. aureus* ATCC 25923	25.3	S	32.0	S	34.7	S	28.0	S	30.0	S	24.3	S	-
2	*S. epidermidis* ATCC 35984	**0.0**	**R**	**9.3**	**R**	**10.1**	**R**	31.1	S	**0.0**	**R**	**10.0**	**R**	**E15-P10-FOX30-SXT25-CN10**
3	AY622018	31.8	S	27.1	R	36.6	S	31.1	S	35.4	S	28.9	S	P10
4	AP610134	30.7	S	16.5	R	30.0	S	33.6	S	30.9	S	26.7	S	P10
5	AY574001	30.7	S	22.9	R	36.5	S	14.8	I	9.5	R	27.1	S	P10-SXT25
6	BY682111	29.5	S	20.1	R	35.3	S	32.3	S	30.6	S	26.0	S	P10
7	**AP622002**	27.33	S	**20.7**	**R**	31.7	S	**13.7**	**R**	**13.3**	**R**	13.3	I	**P10-TE30-SXT25**
8	AP622005	28.0	S	19.7	R	32.7	S	13.0	R	16.3	S	25.0	S	P10-TE30
9	AP622010	29.0	S	21.0	R	33.7	S	13.0	R	19.7	S	26.0	S	P10-TE30
10	AP622016	26.3	S	18.3	R	30.7	S	12.0	R	13.7	I	23.3	S	P10-TE30
11	AP622027	32.2	S	22.3	R	38.4	S	13.6	R	15.6	S	27.6	S	P10-TE30
12	AP622034	33.3	S	23.5	R	40.6	S	16.9	I	12.8	I	34.0	S	P10
13	AP610124	38.8	S	24.2	R	45.8	S	48.8	S	41.3	S	35.7	S	P10
14	AP610126	28.3	S	18.2	R	34.3	S	31.3	S	30.1	S	24.9	S	P10
15	AP610129	28.6	S	18.7	R	31.8	S	32.2	S	31.0	S	26.4	S	P10
16	AP610132	29.4	S	20.2	R	34.9	S	31.4	S	31.7	S	26.4	S	P10

17	AP610133	38.3	S	24.4	R	44.0	S	46.4	S	39.2	S	33.2	S	P10
18	AP610135	29.2	S	17.3	R	35.3	S	32.2	S	29.7	S	24.9	S	P10
19	AP610138	31.1	S	17.4	R	34.1	S	34.3	S	31.3	S	26.6	S	P10
20	AP610142	38.8	S	23.2	R	20.1	R	20.4	S	27.7	S	23.4	S	P10-FOX30
21	BP558001	33.8	S	27.4	R	40.3	S	36.7	S	34.3	S	27.8	S	P10
22	BP636009	30.5	S	23.4	R	37.8	S	34.7	S	34.3	S	25.9	S	P10
23	BP636010	30.1	S	22.1	R	36.3	S	34.2	S	32.3	S	26.0	S	P10
24	BP636011	29.9	S	21.8	R	37.0	S	32.7	S	31.0	S	25.8	S	P10
25	BP636012	30.4	S	22.7	R	37.7	S	35.3	S	31.8	S	27.0	S	P10
26	**BP647054**	31.3	S	**26.7**	R	35.8	S	**12.2**	**R**	**0.0**	**R**	28.3	S	**P10-TE30-SXT25**
27	AY604147	25.3	S	25.0	R	23.3	S	21.3	S	25.0	S	21.0	S	P10
28	AY604148	23.0	S	14.7	R	27.7	S	30.0	S	24.7	S	22.3	S	P10
29	AY620149	24.0	S	14.7	R	27.0	S	29.0	S	24.3	S	22.0	S	P10
30	AY620150	25.6	S	16.9	R	30.6	S	28.6	S	25.4	S	23.4	S	P10
31	AY620151	28.2	S	21.5	R	32.7	S	35.3	S	27.7	S	26.4	S	P10
32	AY620153	28.7	S	20.0	R	31.7	S	33.0	S	28.7	S	25.3	S	P10
33	BY760095	26.1	S	13.4	R	30.4	S	10.5	R	26.0	S	24.6	S	P10-TE30
34	AY625098	26.2	S	25.2	R	27.2	S	26.4	S	26.7	S	22.8	S	P10
35	AY625099	24.6	S	25.5	R	25.3	S	24.3	S	28.2	S	20.9	S	P10
36	AY625100	28.4	S	22.5	R	26.3	S	23.2	S	28.6	S	21.6	S	P10
37	AY764127	21.7	I	24.0	R	19.0	R	24.3	S	24.7	S	19.0	S	P10-FOX30
38	BY760105	31.5	S	20.2	R	36.4	S	12.7	R	32.8	S	29.3	S	P10-TE30
39	AP622007	25.0	S	10.7	R	27.0	S	26.3	S	20.3	S	21.0	S	P10
40	AP622017	25.3	S	10.7	R	27.7	S	26.7	S	21.3	S	22.7	S	P10
41	AP622023	30.0	S	22.3	R	36.6	S	13.7	R	19.9	S	27.0	S	P10-TE30
42	AP625044	27.0	S	27.0	R	31.0	S	28.9	S	33.5	S	33.5	S	P10
43	AP610122	30.3	S	17.2	R	32.6	S	33.9	S	30.9	S	26.3	S	P10

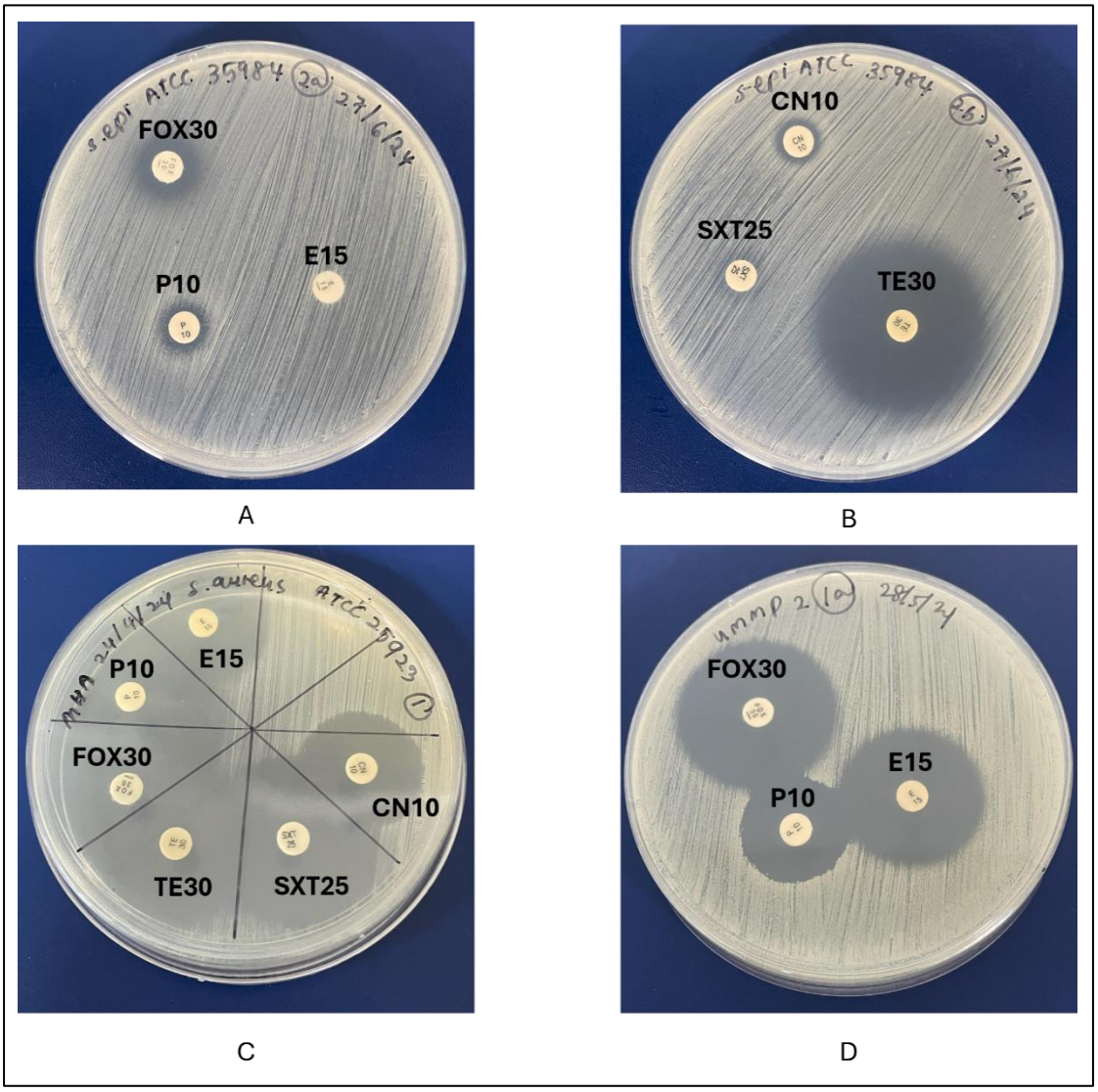
44	AP610123	31.7	S	17.9	R	34.6	S	35.2	S	31.8	S	26.6	S	P10
45	AP610125	28.3	S	19.9	R	34.3	S	32.8	S	30.5	S	25.3	S	P10
46	AP610127	31.0	S	17.2	R	33.4	S	31.2	S	29.9	S	24.3	S	P10
47	AP610128	29.5	S	16.0	R	30.3	S	33.2	S	28.7	S	25.2	S	P10
48	AP610130	30.5	S	16.3	R	31.7	S	33.6	S	29.6	S	24.7	S	P10
49	AP610131	29.1	S	16.7	R	30.7	S	33.2	S	27.7	S	25.1	S	P10
50	AP610136	29.4	S	16.4	R	26.6	S	34.1	S	30.2	S	25.8	S	P10
51	AP610137	31.4	S	16.7	R	34.9	S	35.8	S	33.2	S	27.3	S	P10
52	AP610139	31.0	S	16.6	R	33.2	S	33.5	S	30.0	S	25.3	S	P10
53	AP610140	29.2	S	16.0	R	30.7	S	32.1	S	29.4	S	24.9	S	P10
54	AP610141	29.5	S	16.1	R	36.5	S	33.5	S	31.3	S	25.6	S	P10
55	BP647052	25.0	S	20.1	R	29.7	S	28.0	S	25.9	S	16.6	S	P10
56	AY625061	28.4	S	20.1	R	31.3	S	31.7	S	29.6	S	12.8	I	P10
57	AY625064	27.9	S	16.7	R	29.6	S	29.7	S	27.5	S	25.3	S	P10
58	AY625084	26.3	S	16.3	R	28.3	S	26.0	S	25.0	S	23.7	S	P10-CN10
59	AY625097	26.0	S	16.6	R	28.8	S	28.5	S	26.2	S	23.2	S	P10
60	AY625121	26.1	S	24.1	R	26.6	S	19.2	S	25.0	S	21.5	S	P10-FOX30
61	AP625042	22.7	S	18.3	R	26.0	S	25.7	S	24.0	S	11.0	R	P10-FOX30
62	AP625043	27.3	S	21.9	R	21.5	S	32.6	S	29.5	S	21.9	S	P10
63	AP625045	26.6	S	23.3	R	18.5	R	33.3	S	30.1	S	23.0	S	P10-FOX30
64	AP625046	30.2	S	15.6	R	12.4	R	27.4	S	30.5	S	24.1	S	P10
65	AP625047	27.6	S	19.0	R	29.6	S	18.5	S	31.3	S	14.2	I	P10-FOX30
66	AP625048	27.6	S	24.0	R	14.6	R	29.7	S	29.0	S	21.9	S	P10-FOX30
67	AP625052	25.0	S	20.1	R	29.7	S	28.0	S	25.9	S	16.6	S	P10-FOX30
68	AP620077	24.4	S	11.1	R	9.5	R	25.6	S	25.2	S	21.8	S	P10-FOX30
69	AP620078	24.8	S	13.6	R	11.4	R	26.6	S	22.7	S	21.5	S	P10-FOX30
70	AP620079	29.7	S	15.1	R	11.5	R	31.9	S	31.2	S	24.7	S	P10-FOX30

71	AP620080	29.4	S	14.6	R	11.5	R	33.7	S	31.0	S	24.7	S	P10-FOX30
72	AP620081	27.5	S	12.2	R	11.1	R	33.4	S	29.6	S	24.5	S	P10-FOX30
73	AP620082	26.7	S	11.5	R	9.5	R	34.3	S	31.0	S	23.8	S	P10-FOX30
74	AP620083	27.2	S	16.8	R	10.3	R	29.9	S	29.1	S	22.2	S	P10
75	AP620084	26.8	S	17.2	R	10.2	R	32.8	S	33.3	S	23.7	S	P10-FOX30
76	AP620085	25.6	S	12.1	R	19.6	R	30.3	S	30.0	S	21.7	S	P10-FOX30
77	AP620086	27.7	S	17.2	R	21.5	S	30.3	S	30.4	S	21.7	S	P10-FOX30
78	AP620087	27.0	S	18.5	R	18.1	R	30.5	S	31.8	S	22.4	S	P10
79	AP620088	25.5	S	17.8	R	18.3	R	30.6	S	32.3	S	22.2	S	P10
80	AP620089	25.9	S	17.7	R	17.5	R	29.6	S	31.2	S	21.8	S	P10-CN10

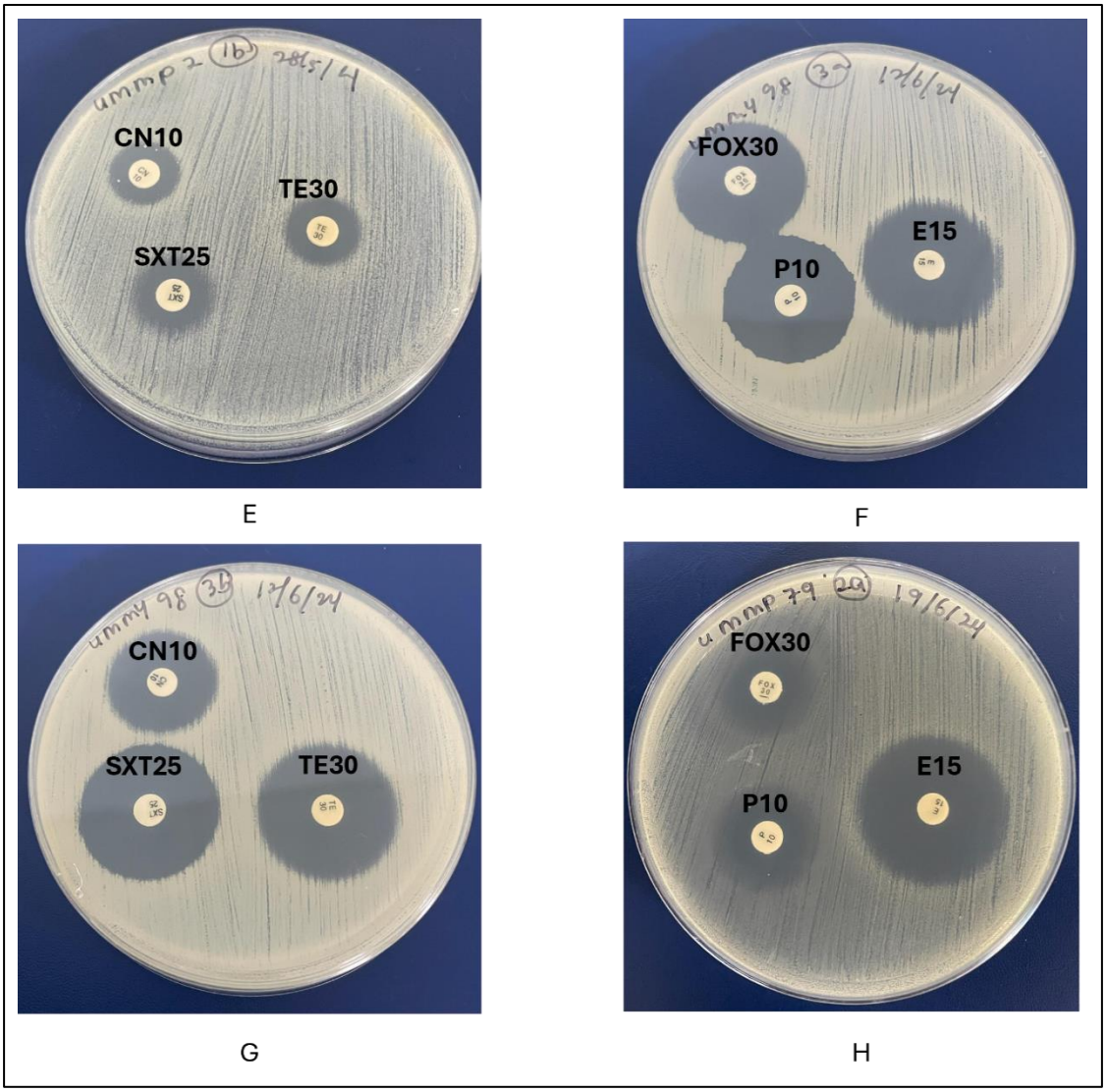
Note: Abbreviation; S: susceptible, I: intermediate, R: resistance, E15: erythromycin (15 µg), P10: penicillin G (10 µg), FOX30: cefoxitin (30 µg), TE30: tetracycline (30 µg), SXT25: sulfamethoxazole-trimethoprim (25 µg), CN10: gentamicin (10 µg).

APPENDIX 4

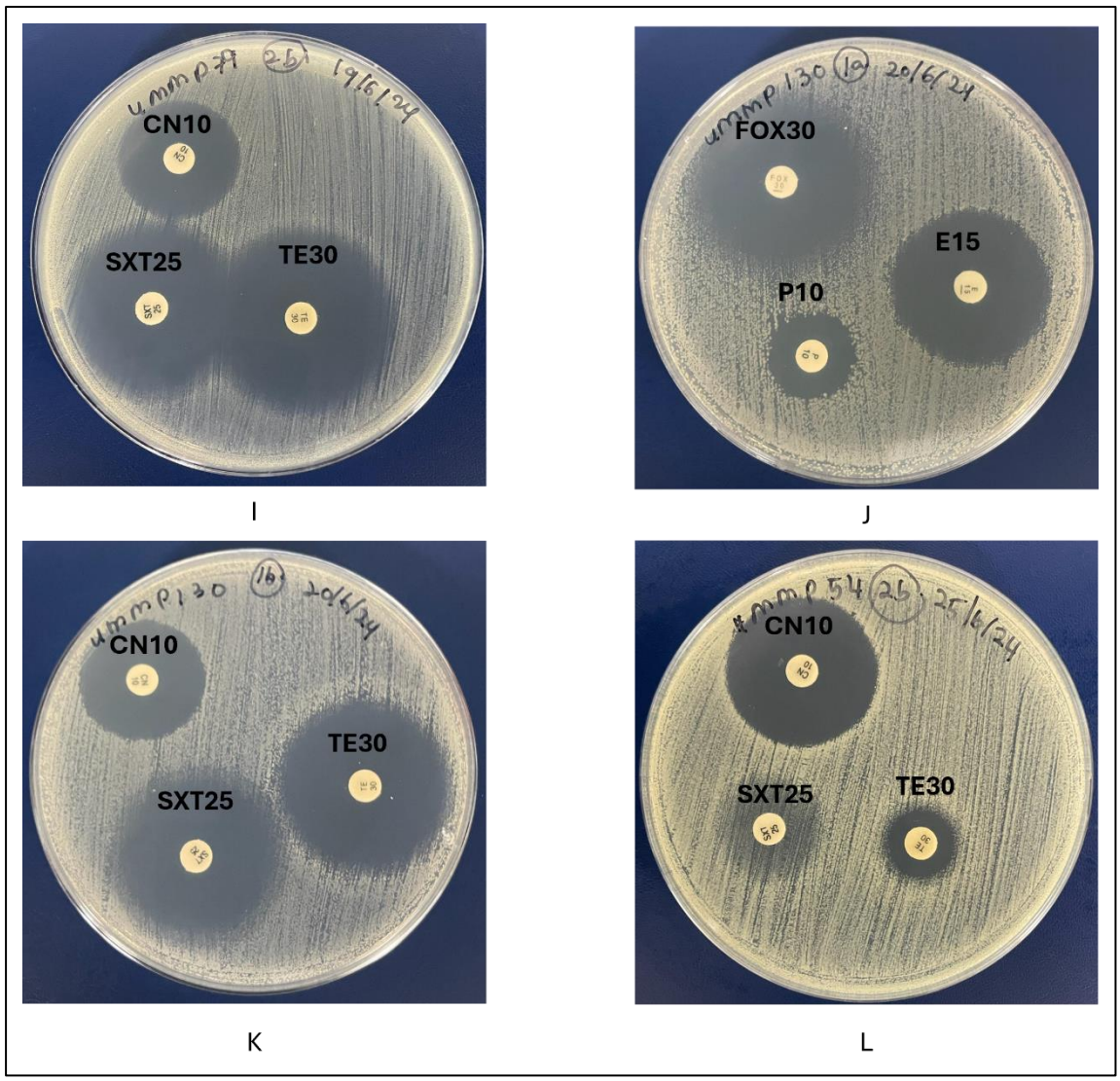
Representative Disk Diffusion Assay Showing Zone of Inhibition Patterns of Staphylococcaceae Isolates (A) *S. epidermidis* ATCC 35984 with Tested Antibiotics FOX30, P10 and E15 (Positive Control) (B) *S. epidermidis* ATCC 35984 with Tested Antibiotics CN10, SXT25 and TE30 (Positive Control) (C) *S. aureus* ATCC 25923 (Negative Control) (D) *S. chromogenes* with Tested Antibiotics FOX30, P10 and E15 (AP622002, MDR Isolate)



Representative Disk Diffusion Assay Showing Zone of Inhibition Patterns of Staphylococcaceae Isolates (E) *S. chromogenes* with Tested Antibiotics CN10, SXT25 and TE30 (AP622002, MDR Isolate) (F) *M. sciuri* with Tested Antibiotics FOX30, P10 and E15 (AY625098) (G) *M. sciuri* with Tested Antibiotics CN10, SXT25 and TE30 (AY625098) (H) *M. caseolyticus* with Tested Antibiotics FOX30, P10 and E15 (AY625079)



Representative Disk Diffusion Assay Showing Zone of Inhibition Patterns of Staphylococcaceae Isolates (I) *M. caseolyticus* with Tested Antibiotics CN10, SXT25 and TE30 (AY625079) (J) *M. bohemicum* with Tested Antibiotics FOX30, P10 and E15 (AP610130) (K) *M. bohemicum* with Tested Antibiotics CN10, SXT25 and TE30 (AP610130) (L) *S. chromogenes* with Tested Antibiotics CN10, SXT25 and TE30 (AP647054, MDR Isolate)



APPENDIX 5

Table of Antibiotic Resistance Profiles and Biofilm Formation Capacity of Staphylococcaceae Isolates (n : 78) and Two Control Strains

No.	Isolate ID	Bacterial Species	Biofilm formation category	Antibiotic Resistance Phenotype	Antibiotic Resistance Count
1	*S. epidermidis* 12228	Negative control	Non-biofilm	NT	NT
2	*S. aureus* 35984	Positive control	Strong	E15-P10-FOX30-SXT25-CN10	5
3	AY622018	*Staphylococcus aureus*	Non-biofilm	P10	1
4	AP610134	*Staphylococcus aureus*	Non-biofilm	P10	1
5	AP610135	*Staphylococcus chromogenes*	Non-biofilm	P10	1
6	AP610138	*Staphylococcus chromogenes*	Non-biofilm	P10	1
7	BY682111	*Staphylococcus chromogenes*	Non-biofilm	P10	1
8	**AP622002**	***Staphylococcus chromogenes***	**Non-biofilm**	**P10-TE30-SXT25**	**3**
9	AP610142	*Staphylococcus chromogenes*	Non-biofilm	P10-FOX30	2
10	AP610126	*Staphylococcus chromogenes*	Non-biofilm	P10	1
11	BP636011	*Staphylococcus chromogenes*	Non-biofilm	P10	1
12	BP636010	*Staphylococcus chromogenes*	Non-biofilm	P10	1
13	BP636012	*Staphylococcus chromogenes*	Non-biofilm	P10	1
14	AP636010	*Staphylococcus chromogenes*	Non-biofilm	P10-TE30	2
15	AP610129	*Staphylococcus chromogenes*	Non-biofilm	P10	1

16	AP622034	*Staphylococcus chromogenes*	Non-biofilm	P10	1
17	AP622027	*Staphylococcus chromogenes*	Non-biofilm	P10-TE30	2
18	BP636009	*Staphylococcus chromogenes*	Non-biofilm	P10	1
19	BP558001	*Staphylococcus chromogenes*	Non-biofilm	P10	1
20	AP610124	*Staphylococcus chromogenes*	Non-biofilm	P10	1
21	**BP647054**	***Staphylococcus chromogenes***	**Weak**	**P10-TE30-SXT25**	**3**
22	AY574001	*Staphylococcus chromogenes*	Weak	P10-SXT25	2
23	AP622016	*Staphylococcus chromogenes*	Weak	P10-TE30	2
24	AP610132	*Staphylococcus chromogenes*	Moderate	P10	1
25	AP610133	*Staphylococcus chromogenes*	Moderate	P10	1
26	AP622005	*Staphylococcus chromogenes*	Strong	P10-TE30	2
27	AY620153	*Staphylococcus haemolyticus*	Non-biofilm	P10	1
28	AY620151	*Staphylococcus haemolyticus*	Non-biofilm	P10	1
29	AY620149	*Staphylococcus haemolyticus*	Non-biofilm	P10	1
30	BY760095	*Staphylococcus haemolyticus*	Non-biofilm	P10-TE30	2
31	AY620150	*Staphylococcus haemolyticus*	Weak	P10	1
32	AY620148	*Staphylococcus haemolyticus*	Non-biofilm	P10	1
33	AY604147	*Staphylococcus haemolyticus*	Moderate	P10	1
34	AP622023	*Staphylococcus simulans*	Non-biofilm	P10-TE30	2

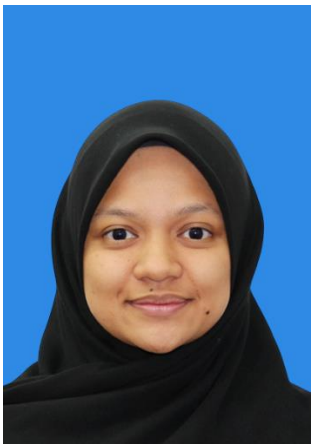
35	AP622017	*Staphylococcus simulans*	Non-biofilm	P10	1
36	AP622007	*Staphylococcus simulans*	Moderate	P10	1
37	AP625044	*Staphylococcus warneri*	Non-biofilm	P10	1
38	BY760105	*Mammaliicoccus sciuri*	Non-biofilm	P10-TE30	2
39	AY625098	*Mammaliicoccus sciuri*	Weak	P10	1
40	AY625100	*Mammaliicoccus sciuri*	Weak	P10	1
41	AY764127	*Mammaliicoccus sciuri*	Weak	P10-FOX30	2
42	AY625099	*Mammaliicoccus sciuri*	Weak	P10	1
43	AP610122	*Macrococcoides bohemicum*	Non-biofilm	P10	1
44	AP610128	*Macrococcoides bohemicum*	Non-biofilm	P10	1
45	BP647052	*Macrococcoides bohemicum*	Non-biofilm	P10	1
46	AP610136	*Macrococcoides bohemicum*	Non-biofilm	P10	1
47	AP610130	*Macrococcoides bohemicum*	Non-biofilm	P10	1
48	AP610131	*Macrococcoides bohemicum*	Non-biofilm	P10	1
49	AP610137	*Macrococcoides bohemicum*	Non-biofilm	P10	1
50	AP610141	*Macrococcoides bohemicum*	Non-biofilm	P10	1
51	AP610140	*Macrococcoides bohemicum*	Non-biofilm	P10	1
52	AP610123	*Macrococcoides bohemicum*	Non-biofilm	P10	1
53	AP610125	*Macrococcoides bohemicum*	Non-biofilm	P10	1

54	AP610139	*Macrococcoides bohemicum*	Non-biofilm	P10	1
55	AP610127	*Macrococcoides bohemicum*	Non-biofilm	P10	1
56	AY625064	*Macrococcus caseolyticus*	Non-biofilm	P10	1
57	AP625042	*Macrococcus caseolyticus*	Non-biofilm	P10-CN10	2
58	AP625052	*Macrococcus caseolyticus*	Non-biofilm	P10	1
59	AP620084	*Macrococcus caseolyticus*	Non-biofilm	P10-FOX30	2
60	AY625061	*Macrococcus caseolyticus*	Non-biofilm	P10	1
61	AY625097	*Macrococcus caseolyticus*	Non-biofilm	P10	1
62	AP625047	*Macrococcus caseolyticus*	Non-biofilm	P10	1
63	AY625121	*Macrococcus caseolyticus*	Non-biofilm	P10	1
64	AP620087	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
65	AP625048	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
66	AP625046	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
67	AP620089	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
68	AP620086	*Macrococcus caseolyticus*	Weak	P10	1
69	AP625045	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
70	AP620083	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
71	AP620082	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
72	AP625043	*Macrococcus caseolyticus*	Weak	P10	1

73	AP620080	*Macrococcus caseolyticus*	Weak	P10-FOX30	2
74	AP620079	*Macrococcus caseolyticus*	Moderate	P10	1
75	AY625084	*Macrococcus caseolyticus*	Moderate	P10	1
76	AP620077	*Macrococcus caseolyticus*	Moderate	P10-FOX30	2
77	AP620081	*Macrococcus caseolyticus*	Moderate	P10-FOX30	2
78	AP620078	*Macrococcus caseolyticus*	Moderate	P10-FOX30	2
79	AP620085	*Macrococcus caseolyticus*	Moderate	P10-FOX30	2
80	AP620088	*Macrococcus caseolyticus*	Moderate	P10-FOX30	2

Note: Abbreviation; E15: erythromycin (15 μg), P10: penicillin G (10 μg), FOX30: cefoxitin (30 μg), TE30: tetracycline (30 μg), SXT25: sulfamethoxazole-trimethoprim (25 μg), CN10: gentamicin (10 μg); NT: not tested.

AUTHOR'S PROFILE



Intan Nurfarzana binti Mohd Safini obtained Bachelor of Science (Hons.) in Molecular Biology in 2023 from Universiti Teknologi MARA, Shah Alam and Diploma in Microbiology (2020) from Universiti Teknologi MARA, Kuala Pilah. She is currently pursuing a Master of Science (Molecular Biology) at the same university. Her current research focusses on Staphylococcaceae isolated from bovine milk associated with subclinical mastitis. The study aims to investigate the occurrence of these species in bovine milk, their AR profiles, associated resistance genes and biofilm formation ability, emphasizing the importance of understanding AR dissemination in environmental reservoirs and its implications for public health.

LIST OF PUBLICATIONS

Safini, I. N. M.; Saad, Muhammad Iqbal Hafiz; Mohamad, Zuraifah Asrah; Hatta, Siti Khairiyah Mohd; Aziz, Aziyah Abd.; Bakon, Sophia Karen; and Jamil, Norashirene Mohamad (2026) "Patterns of Antibiotic Resistance in Staphylococci Associated with Bovine Subclinical Mastitis,"*Baghdad Science Journal*: Vol. 23: Iss. 1, Article 15.

Safini, I. N. M., Zakaria, N. F. S., Saad, M. I. H., Yahya, M. F. Z., & Jamil, N. M. (2024). Understanding bacterial persistence under antibiotic pressure: a review. *Science Letters*, *18*(2), 56-69.

Saad, M. I. H., **Safini, I. N. M**., Mohamad, Z. A., Bakon, S. K., Yahya, M. F. Z. R., Hatta, S. K. M., Abdul-Aziz, A., & Jamil, N. M. (2025). Prevalence and antibiotic resistance of *Enterococcus faecium* and *Enterococcus faecalis* in municipal wastewater integrated with hospital effluent in northern Malaysia. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, 50–61.

Saad, M. I. H., **Safini, I. N. M**., Mohamad, Z. A., Bakon, S. K., Yahya, M. F. Z. R., Hatta, S. K. M., Abdul-Aziz, A., & Jamil, N. M. (2025). Characterization of bacterial isolates and antibiotic resistance profiles from faecal samples of healthy and unhealthy sheep at a farm in Gajah Mati, Kedah. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, 167-175.