

**Molecular characterization, antimicrobial resistance and virulence
genotyping of *Staphylococcus* isolates from subclinical mastitis dairy cows
in the Thabo Mofutsanyane District Municipality, South Africa**

Thesis submitted in fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY

ENVIRONMENTAL HEALTH

in the

Department of Life Sciences

Faculty of Health and Environmental Sciences

at the

Central University of Technology, Free State

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DECLARATION

I, **Khasapane Ntelekwane George**, declare that the thesis entitled “**Molecular characterization, antimicrobial resistance and virulence genotyping of *Staphylococcus* isolates from subclinical mastitis dairy cows in Thabo Mofutsanyane District Municipality, South Africa**”, submitted for the degree of *Doctor of Philosophy in Environmental Health*, has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

Signature:

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GENERAL ABSTRACT

Mastitis is one of the economically important diseases in the dairy industry. *Staphylococcus* species are amongst the bacteria that cause bovine mastitis (BM) worldwide. These bacteria produce and contain a spectrum of extracellular protein toxins, virulence factors, and antimicrobial resistant properties that are thought to contribute to the pathogenicity of the organism. The aims of this study were to:

1. To explore the microbiota of milk from dairy cows with subclinical mastitis (SCM) from small-scale dairy farms in the Thabo Mofutsanyane District Municipality of the Free state Province, South Africa.
2. To determine the virulence and antibiotic resistance profiles as well as genetic characterization of *Staphylococcus* spp., from milk of SCM dairy cows in the Thabo Mofutsanyane District Municipality of the Free State Province, South Africa.

Prior to the experimental analyses, a systematic review and meta-analysis of literature concerning SCM published from January 2010 to December 2020 was conducted with the aim of understanding the prevalence of SCM, its associated risk factors, and major causative agents in Africa. The results showed that the prevalence of SCM was 48.2% (95% CI: 43.6-52.8%) in 11 countries of five African regions. Subgroup and meta-regression analyses further showed that East Africa had a significant ($p < 0.0092$) highest pooled prevalence estimate (PPE) of SCM at 67.7% (95% CI: 55.7-78.7), followed by West Africa at 50.5 (95% CI: 31.4-69.5) and North Africa at 40.3% (95%: 32.2-48.6). Other significant moderators for SCM were age ($p < 0.0001$), breed ($p < 0.0002$), lactation stage ($p < 0.019$), and parity ($p < 0.0008$) of cattle. The *Staphylococcus* species (43.7%) were the most predominant pathogens detected from SCM cow milk, followed by *Streptococcus* spp. (18.2%) and *Escherichia* spp. (9.5%).

In the current study, a total of 166 dairy cows were randomly screened for SCM from seven (7) small-scale farms using the California mastitis test and somatic cell count assay. The results showed that 33.13% of all cows had SCM at cow level, while the quarter-level prevalence was 54%. Thereafter, representative samples of 10 each from SCM and non-SCM dairy cow milk were sequenced to determine abundance and compare core microbiota through 16S rRNA metabarconding. Alpha-diversity metrics showed significant differences between the SCM cows and their non-SCM counterparts. The beta-diversity metrics on Principal Coordinate

Analysis significantly clustered samples by type (PERMANOVA test, $P < 0,05$), while Non-Metric Dimensional Scaling did not cluster the samples by type (PERMANOVA test, $p > 0,07$).

Four phyla, namely Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria collectively accounted for more than 97% of all sequencing reads from both the SCM and non-SCM cow milk samples. The most detected bacterial classes were Actinobacteria, Bacilli, Bacteroidia, Clostridia, and Gammaproteobacteria in healthy cow samples, whilst SCM cow milk samples mainly had Actinobacteria, Alphaproteobacteria, Bacilli, Clostridia, and Gammaproteobacteria. The dominant bacterial species in healthy cow samples were *Anthropic* spp., *Pseudomonas azotoformans*, *Pseudomonas fragi*, *Acinetobacter guillouiae*, *Enterococcus italicus*, and *Lactococcus lactis*, whilst *P. azotoformans*, *Micrococcus bovis*, *Pseudomonas fragi*, *Acinetobacter guillouiae*, and *Pseudomonas koreensis* were dominant in the SCM cow milk samples.

In addition to the above-mentioned findings, the study sought to detect *Staphylococcus* spp. from samples of SCM cow milk using MALDI-TOF MS, PCR assays (*nuc*, *tuf* and 16S rRNA), and gene sequencing to further determine their virulence and antimicrobial resistance (AMR) profiles. In 50 of 70 samples bacterial isolates were detected and characterised as *Staphylococcus* spp., and it was found that *S. aureus* (76%) was the most prevalent species, followed by *S. chromogenes* (12%), *S. epidermidis* (4%), and *S. haemolyticus* (4%) using MALDI-TOF MS. The 16S rRNA and species-specific gene sequences (*nuc* and *tuf*) indicated that *S. aureus* was the dominant species at 76%, followed by *S. chromogenes*, *S. agnetis*, *S. argenteus*, and *S. devriesei* at 10%, 4%, 4% and 1% respectively. The AMR test showed that 86% of the isolates were resistant to penicillin, followed by resistance to ciprofloxacin (80%) and ceftiofur (52%). Moreover, the detection of antimicrobial resistant and virulence genes showed that the *mecA* gene was present in 16% of all the isolates, while 52% of all the isolates carried the *LgG* gene, followed by the *coa* and *spa* (x-region) genes at 42% and 40% respectively. Moreover, the *hla* and *hly* genes were detected in 38% of the isolates, whereas the *Sea* and *bap* genes were detected in 10% and 2% of the isolates respectively.

In the second phase of the study, whole-genome sequencing technique was conducted to genetically characterize 38 mastitis-associated *S. aureus*. The results revealed that 38 *S. aureus* isolates appeared in seven sequence types (STs), namely, ST97, ST352, ST152, ST243,

ST8495, ST8500 and ST8501, whereby the latter three STs are novel. The 38 strains were divided into seven (7) primary clusters by core genes. The *Spa*-type t2883 accounted for most isolates at 12 (31.57%), followed by t416 at 11 (28.94%) and t2844 at 5 (13.15%) among the nine (9) distinct *spa*-types that were detected. The data also revealed the presence of four (4) plasmid replicONS, with *Rep_N* (rep20) accounting for the majority (17) of the isolates at 44.73%, followed by 2 Inc18 (repUS5). These isolates included 11 distinct antimicrobial resistance (ARGs) genes and 23 genes that were linked to virulence. All the isolates used in this investigation were methicillin susceptible.

In conclusion, it was found that diverse microbial populations were detected in the sampled milk from SCM-infected cows. Furthermore, the study also showed pathogenic potential and antimicrobial resistant status of *S. aureus* and coagulase negative staphylococci (CNS) through detection of virulence and AMR genes. These *S. aureus* isolates had varying levels of antibiotic responsiveness with some showing multiple drug resistance traits. The findings of this study will contribute significantly improvement of strategies for control and prevention of *Staphylococcus*-related mastitis on dairy farms.

Keywords: *Staphylococcus aureus*; subclinical mastitis; 16S metagenomics; antimicrobial resistance; virulence genes; MALDI-TOF MS; whole-genome sequencing; sequencing types; *spa*-types.

RESEARCH OUTPUTS

Published full-length articles

Khasapane, N.G., Byaruhanga, C., Thekiso, O., Nkhebenyane, S.J. and Khumalo, Z.T., 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(1)**: 1-16.

Khasapane, N.G., Khumalo, Z.T.H., Kwenda, S., Nkhebenyane, S.J. and Thekiso, O., 2023. Characterisation of Milk Microbiota from Subclinical Mastitis and Apparently Healthy Dairy Cattle in Free State Province, South Africa. *Veterinary Sciences*, **10(10)**, p.616.

Khasapane, N.G., Koos, M., Nkhebenyane, S.J., Khumalo, Z.T., Ramatla, T. and Thekiso, O., 2024. Detection of Staphylococcus Isolates and Their Antimicrobial Resistance Profiles and Virulence Genes from Subclinical Mastitis Cattle Milk Using MALDI-TOF MS, PCR and Sequencing in Free State Province, South Africa. *Animals*, **14(1)**, p.154.

Khasapane, N.G., Kwenda, S., Khumalo, Z.T.H., Nkhebenyane, S.J. and Thekiso, O., 2023. Three novel sequencing types from seventeen Staphylococcus aureus genomes isolated from dairy cows milk in the Free State Province of South Africa. *Microbiology Resource Announcements*, **12(12)**, pp.e00739-23.

Conference papers

Ntelekwane G Khasapane, Khumalo Z.T.H., Nkhebenyane S.J., Kwenda S., Thekiso M.M.O. Comprehensive analysis of Staphylococcus aureus isolates from subclinical mastitis dairy cows in Free state Province, South Africa using whole genome sequencing. The 20th Annual SASVEPM Congress. 23 – 25 September 2023. Gaborone, Botswana.

Khasapane N.G., Myburgh K, Nkhebenyane S.J., Khumalo Z.T.H., Ramatla T., Thekiso O. Detection of Staphylococcus species from subclinical mastitis cattle milk using MALDI-TOF MS, PCR and sequencing. South African Association for Food Science and Technology. 28-30 August 2023. Cape Town, South Africa.

Ntelekwane George Khasapane, Zamantungwa Khumalo, Stanford Kwenda, Sebolelo Jane Nkhebenyane, Oriel Thekiso. Characterization of milk microbiota from subclinical mastitis and healthy dairy cattle in Free State Province, South Africa. South African Society for Microbiology. 17-20 September 2023: Stellenbosch, South Africa.

DEDICATION

This study is dedicated to my dearly departed son, and my family.
“So do not fear, for I am with you; do not be dismayed, for I am your God. I
will strengthen you and help you; I will uphold you with my righteous right
hand.”

(Isaiah 41:10)

ACKNOWLEDGEMENTS

This thesis would not have been completed without the assistance of various people over the past three years. First and foremost, I acknowledge God for my perfect health and wisdom. Secondly, I thank my promoter, Prof. Oriel M.M. Thekiso, for believing in me and for his mentorship and desire to make me the scientist I am today. I thank him for the discussions we had about data interpretation, statistics, reviewing manuscripts, grant writing, and availing resources for me to go to scientific conferences.

I also thank my co-promoters, Dr Sebolelo J. Nkhebenyane and Dr Zamantungwa T.H. Khumalo, for their support and advice throughout this study.

I extend my appreciation and gratitude to Dr Stanford Kwenda (National Institute for Communicable Diseases) for exposing me to bioinformatics training that was so valuable for this work, as well as Dr Tsepo Ramatla (North-West University) for the continuous support and encouragement.

I also extend my appreciation to my family, partner, the Centre for Applied Food Sustainability and Biotechnology (CAFSAB) staff, and my friends for their immeasurable academic, emotional, social, and spiritual support during my tenure in graduate school.

I thank the National Research Foundation (nGAP scholar fund), Central University of Technology, through the Department of Higher Education and Training, University Capacity Development Programme (DHET-UCDP) and Fleming Fund Regional Grant SEQAFRICA for their financial support.

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LIST OF ABBREVIATIONS

3'	: (Reverse Primer) an oligonucleotide that flanks the 3'-end of the Amplicon
5'	: (Forward Primer) or an oligonucleotide that flanks the 5'-end of the Amplicon
AMR	: Antimicrobial resistance
ASV	: Amplicon sequence variant
<i>aur</i>	: Aureolysin
<i>bap</i>	: Biofilm-associated protein
BHI	: Brain Heart Infusion
BTS	: Bacterial test standard
<i>cadD</i>	: Cadmium resistance gene
CCS	: Consensus circular sequence
CI	: Confidence interval
CMT	: California mastitis test
CNS	: Coagulase negative staphylococci
<i>coa</i>	: Coagulase gene
<i>dfrG</i>	: Dihydrofolate reductase
DNA	: Deoxyribonucleic acid
gDNA	: Genomic Deoxyribonucleic acid
<i>hla</i>	: Alpha-hemolysin gene
<i>hly</i>	: Beta-hemolysin gene
<i>HlgAB</i>	: Gamma hemolysin A & B subunit
<i>hlyC</i>	: Gamma hemolysin C subunit
HPLC	: High-performance liquid chromatography
I	: Intermediate
I^2	: Proportion of observed variation after elimination of sampling error
IMI	: Intramammary infection
<i>Inc18</i>	: Incompatibility (Inc) 18 group
LF	: Left Front
LH	: Left Hind
<i>lmrS</i>	: Lincomycin resistance protein

<i>lukD</i>	: Leucocidin_D
<i>lukE</i>	: Leucocidin_E
<i>lukF.PV</i>	: Panton-Valentine leucocidin_F
<i>lukS.PV</i>	: Panton-Valentine leucocidin_S
MALDI-TOF MS	: Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MDR	: Multi-drug resistance
<i>mepA</i>	: Multidrug export protein
MH	: Muller Hinton
MLEE	: Multilocus enzyme electrophoresis
MLST	: Multiple-locus sequence typing
MLVA	: Multiple-locus VNTR (variable number of tandem repeats) analysis
MRSA	: Methicillin-resistant <i>Staphylococcus aureus</i>
MSA	: Mannitol salt agar
NAP	: Nutrient agar plate
NGS	: Next generation sequencing
NMC	: National mastitis council
<i>nuc</i>	: Thermocuclease
PacBio	: Pacific biosciences
PCR	: Polymerase chain reaction
PFGE	: Pulsed-field gel electrophoresis
PRISMA	: Preferred Reporting Items for Systematic Reviews and Meta-Analysis
R	: Resistant
RAPD	: Random amplification of polymorphic DNA
<i>Rep_N</i>	: Replication protein
RF	: Right Front
RH	: Right Hind
rRNA	: Ribosomal ribonucleic acid
RT	: Room temperature
S	: Susceptible
<i>sak</i>	: Staphylokinase
SCC	: Somatic cell count
SCM	: Subclinical mastitis
<i>scn</i>	: Staphylococcal complement inhibitor

<i>sea</i>	: Staphylococcal enterotoxin type A
<i>seg</i>	: Staphylococcal enterotoxin type G
<i>sei</i>	: Staphylococcal enterotoxin type I
<i>sem</i>	: Staphylococcal enterotoxin type M
<i>sen</i>	: Staphylococcal enterotoxin type N
<i>seo</i>	: Staphylococcal enterotoxin type O
<i>seu</i>	: Staphylococcal enterotoxin type U
SMRTbell	: Single molecule real time sequencing bell
<i>spa</i>	: Staphylococcal protein A
<i>SplA</i>	: Serine protease-like protein A
<i>SplB</i>	: Serine protease-like protein B
<i>SplE</i>	: Serine protease-like protein E
STs	: Sequence types
tet (38)	: Tetracycline
tetK	: Tetracycline efflux protein K
tetM	: Tetracycline efflux protein M
tuf	: Elongation factor Tu
WGS	: Whole-genome sequencing
wgSNP	: Whole genome single nucleotide polymorphism

LIST OF UNITS

±	: plus minus
:	: is to
>	: greater than
<	: less than
%	: percentage
/	: per
°C	: Degree Celsius
g	: Gram
L	: Litre
Mg	: Milligram
µ/g	: Microgram/gram
µ/mL	: Microgram/millilitre
mL	: Millilitre
mm	: Millimetre
nm	: Nanometer
µg	: Micro gram
µL	: Micro litre
µm	: Micro metre
Kb	: Kilo base pairs

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Mastitis is inflammation of the mammary gland parenchyma seen by physical, chemical, and bacteriological alterations in milk, as well as pathological abnormalities in animal glandular tissues (Nordin *et al.*, 2019). The existence of infectious organisms, host resistance, and environmental variables all contribute to the prevalence of this illness in bovines (Gera and Guha, 2011). This infection can be recognized clinically when the udder of a cow exhibits an inflammatory reaction that causes clotting and colour changes in the milk, or it can occur sub-clinically, in which case the farmer may not notice any signs of the disease (Mpatswenumugabo *et al.*, 2017). Subclinical mastitis (SCM) is often characterized by changes in milk quality, necessitating the use of specialized testing for diagnosis (Libera *et al.*, 2021). The fact that SCM has a detrimental impact on the freshness and quantity of raw milk remains a major source of concern among raw milk producers due to the associated financial losses (Khasapane *et al.*, 2021).

Several studies have been conducted over the world to evaluate and analyse the prevalence of SCM in cattle (Bangar *et al.*, 2015; Ndahetuye *et al.*, 2020; Mbinyo *et al.*, 2020; Selim *et al.*, 2022). Katsande *et al.* (2013) engaged in cross-sectional research to investigate the prevalence of both clinical and SCM on smallholder farms in Zimbabwe. They reported that the most common species were coagulase-negative staphylococci (CNS), *Escherichia coli* and *Staphylococcus aureus* at 27.6%, 25.2% and 16.3% respectively. According to Abrahmsén *et al.* (2014), CNS was found in 54.7% of small-scale farms in Kampala among bovines with SCM. More recently, Damian *et al.* (2020) undertook cross-sectional research to investigate the prevalence of SCM on smallholder farms in Northeast Tanzania and found that the major bacterial species that were isolated included *Staphylococcus aureus* (55.4%) and *S. epidermidis* (10.8%). Recent studies have reported not only the harmful effects of CNS on udder health but have also discussed the possibility that these organisms may have a favourable influence on them (Reyher *et al.*, 2012; Martins *et al.*, 2016). However, the latter authors highlight the need for more research into the primary mastitis organisms and their protective benefits against pre-

existing infectious mastitis pathogens. According to a study conducted by dos Santos Nascimento (2005), some CNS species such as *S. epidermidis*, *S. simulans*, *S. hominis*, *S. saprophylicus*, and *S. arlettae* have antimicrobial substances that prevent the multiplication of major mastitis-causing agents.

The main source of worry in the management of mastitis infection is the mastitis-causing agents' resistance to antimicrobials. Pathogens that cause mastitis have been shown to develop multi-drug resistance strains against veterinary drugs, while CNS have been characterized as being more resistant to antimicrobials than *S. aureus* (Zigo *et al.*, 2022). Furthermore, *Staphylococcus* species may include a variety of virulence genes, and the capacity of these organisms to generate biofilm is the most concerning aspect of their pathogenicity (Franca *et al.*, 2021). Biofilms can shield bacteria from immune system components that cause phagocytosis (Ghosh *et al.*, 2021; Ciofu *et al.*, 2022). Apart from creating virulence factors such as biofilm, staphylococci are also responsible for manufacturing enterotoxins, which are transmitted to food and cause food poisoning due to their thermostable nature (Singh *et al.*, 2023). Greater attention has been paid to the nature of these toxins in *S. aureus*, but very little data for other staphylococci species are known, which prompted this study.

Biochemical tests and morphological features of isolates are often used to identify and differentiate *Staphylococcus* species. However, due to misidentification of several phenotypic features, many tests have proven ineffective (Rasool *et al.*, 2022). As a result, molecular biology approaches, such as PCR for identifying the 16S rRNA gene and the internal transcribed spacer (ITS) gene as well as sequencing for the *tuf*, *soda*, *gap*, and *HSP60* genes, have been used (Martins *et al.*, 2017; Hussein, 2021). Furthermore, typing approaches such as random amplified polymorphism DNA-PCR, amplified fragment length polymorphism, and pulsed field gel electrophoresis have expanded our understanding of the distribution and distinctions in clinical mastitis caused by these species (Valckenier *et al.*, 2021; Meroni, 2022). Therefore, the current study utilised the culture method as well as biochemical and molecular techniques to investigate the prevalence of the *Staphylococcus* species and its related virulence and antimicrobial resistant statuses in SCM dairy cows in the Thabo Mofutsanyana District Municipality of the Free State Province, South Africa.

1.2 Problem Statement

The staphylococci species are dangerous pathogens that cause infections in both humans and animals. These infections may include, amongst others, toxic shock syndrome, food poisoning, staphylococcal scalded-skin syndrome in humans, and mastitis infection in animals, primarily in dairy cows (Sur and Turkyilmaz, 2020). *Staphylococcus aureus* is believed to be the pathogen of significance in all mastitis cases, as opposed to other species such as coagulase negative staphylococcus (CNS), owing to its opportunistic character. However, in recent years, this perspective has shifted due to the notion that CNS have been more commonly isolated in intramammary infections in South Africa and other African countries (Karzis *et al.*, 2021). This study investigated both coagulase negative and positive *Staphylococcus* species to gain a better understanding of their prevalence in subclinical mastitis cases. Furthermore, studies have been conducted to better understand the virulence factors in *S. aureus* from bovine mastitis cases, such as haemolysins (*hla/b*), leucocidin (*luk*), exfoliative toxins (*eta-etd*), toxic-shock syndrome toxin-1 (*tsst-1*), and biofilm formation (Zecconi *et al.*, 2006; Haveri *et al.*, 2008; Xu *et al.*, 2015). These virulence factors pose a significant risk to both people and animals (Pan *et al.*, 2020; Algammal *et al.*, 2020). Subsequently, the *Staphylococcus* species have also developed the risk of resisting antimicrobial agents due to the extensive use of antibiotics resulting in low cure rate (Chang *et al.*, 2022). Amongst all antimicrobials used to treat mastitis, the emergence of methicillin-resistant *S. aureus* is of importance due to the public health concerns it poses (Khairullah *et al.*, 2020). Xu *et al.* (2015) found that CNS developed antimicrobial resistance faster than *S. aureus*. Thus, the current study attempted to understand the virulence and resistance statuses of the *Staphylococcus* species to further assist farmers and veterinarians to prevent and monitor this infection.

1.3 Research aims and objectives.

Aims

1. To explore the microbiota of milk from dairy cows with SCM on small-scale dairy farms in the Thabo Mofutsanyane District Municipality of Free state Province, South Africa.
2. To determine the species, virulence, and antibiotic susceptibility characteristics of *Staphylococcus* spp., from milk of SCM dairy cows in the Thabo Mofutsanyane District Municipality of the Free State Province, South Africa.

Specific objectives

1. To conduct a systematic review and meta-analysis on the prevalence of SCM, its associated bacterial isolates, and risk factors among dairy cattle in Africa.
2. To screen dairy cows from selected farms for SCM using the California mastitis test and somatic cell count assay.
3. To determine the core microbiota associated with SCM by 16S rRNA metabarcoding.
4. To isolate *Staphylococcus* spp. from milk samples collected from selected small-scale farms using the culture method and characterize them using MALDI-TOF MS, PCR, and whole genome sequencing.
5. To determine the antimicrobial resistance and putative virulence factors of *Staphylococcus* spp. from bovine milk samples using documented gene markers and whole genome sequencing.

1.4 Hypotheses

Hypothesis 1: Different microbial populations exist in raw milk from SCM dairy cows.

Hypothesis 2: Various *Staphylococcus* species are involved in the development of subclinical mastitis infection, and they express various antimicrobial resistance and virulence genes.

1.5 Thesis chapter layout

Chapter one: General introduction

- Includes background, problem statement, aims, specific objectives, hypotheses, and description of potential impact of the study.

Chapter two: Literature review

- Provides and outlines an overview of the literature relating to bovine mastitis with reference to staphylococcal mastitis.

Chapter three: Prevalence of subclinical mastitis, its associated bacterial isolates and risk factors among cattle in Africa: A systematic review and meta-analysis

- A systematic review and meta-analysis of published literature were undertaken to better understand the prevalence of, and risk factors associated with subclinical mastitis in dairy cows across the African continent.

Chapter four: Characterization of core microbiota from subclinical mastitis dairy cows on small-scale farms in the Thabo Mofutsanyana District Municipality, Free State Province

- Focuses on the characterization of core microbiota associated with SCM by utilizing single molecule real time sequencing (SMRT) targeting the 16S rRNA gene.

Chapter five: Characterisation of the *Staphylococcus* species from subclinical mastitis dairy cows on small-scale farms in the Thabo Mofutsanyana District Municipality of Free State Province

- Focused on the microbial identification of various *Staphylococcus* species by means of biochemical tests. Furthermore, PCR was used to screen and validate antimicrobial genes and virulence factors of interest.

Chapter six: Distribution of antimicrobial resistance and virulence genes of *Staphylococcus* species isolated from dairy cows with subclinical mastitis in Thabo Mofutsanyana District Municipality of Free State Province

- Findings based on the antimicrobial resistance patterns of *Staphylococcus* spp., are presented. The investigation was conducted by means of phenotypic and genetic methods. The virulence statuses of these isolates using PCR assay were also determined.

Chapter seven: Comprehensive analysis of *Staphylococcus aureus* isolates genomes from subclinical mastitis dairy cows in Thabo Mofutsanyana District Municipality of Free State Province

- This chapter provides an intense genetic characterization of *S. aureus* isolates and their antimicrobial resistance and virulence statuses using the novel whole-genome sequencing method.

Chapter eight: General conclusion, and recommendations

A general discussion, key conclusions, and recommendations based on the findings are presented in this chapter.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Small-scale farmers are defined as marginalized individuals who find it difficult to access resources, finance, knowledge, and technology in developing nations (IFAD, 2013). In African countries, these farmers face enormous challenges that stifle their growth and capacity to contribute to overall food security (Mbanjwa, 2016). Some of the issues they experience are related to a lack of access to land as well as a lack of physical and institutional infrastructure (Dlamini and Tabit, 2014). In South Africa, small-scale farmers are typically located in rural regions, mostly in former homelands. One of the key constraints encountered by small-scale farmers is also a lack of stable markets. Many of these farmers get poor prices for their products because they sell them at the farm gate or at local markets (van den Berg, 2013).

The International Fund for Agricultural Development (IFAD) reported in 2012 that there were almost 500 million small-scale farmers worldwide at the time of the survey (Lowder *et al.*, 2021), whilst Lues *et al.* (2012) stated in their study that 750-900 million people lived on dairy farms globally. Recently, Mbanjwa (2016) predicted that 1 million litres of milk produced per year might provide employment for about 200 people. On the African continent, milk is produced by both small-and large-scale dairy farmers who differ in terms of the kind of cattle they raise and the manner in which they manage their farms. Small-scale dairy farming is characterised by a variety of operations such as cropping, using agricultural by-products such as cattle fodder, and applying manure to crops (Doughrate *et al.*, 2013).

In 2012, the Food and Agriculture Organization (FAO) characterized small-scale farmers' strengths as follows: (i) they expend minimal costs on production, (ii) have large profit margins, (iii) are burdened by few liabilities or obligations, (iv) have low liquidity risk, and (v) experience relative resistance to rising feed prices. Their potential is vested in the need for dairy products in third-world nations, the likelihood of milk price increases, and the creation of jobs in the dairy value chain. Small-scale farmers have only a few animals, which are mostly crossbreeds, and have a modest input on the production system (Nicholson *et al.*, 2004). These farmers all face similar issues in terms of animal health problems. Controlling animal illnesses

does not begin and end with treating sick animals or waiting for the condition to be reported. According to Miller (2012), small-scale farmers are suffering from hunger and poor animal health husbandry, which suppresses milk output and fertility. Chaminuka *et al.* (2014) argue that, in the Limpopo Province in South Africa, dairy cattle are only dipped each time they are milked, which is usually twice a day, and this is usually done young children at about 5 am in the morning.

Various studies have been conducted on the challenges that small-scale farmers face which, amongst others, include limited utilization of new technologies, lack of training and finances, low milk prices, feed shortage, poor farm management, low productive and reproductive performance, and high disease prevalence (Chinogaramombe *et al.*, 2008; Banda *et al.*, 2021). Marandure *et al.* (2020) stated that low resource grants, low- and poor-quality milk production, and limited access to land and water are also challenges that these famers face. Additional challenges that small-scale farmers face includes policy and institutional trials such as poor roads for access to markets, poor water supply, and a poor regulatory connection between input and output markets for farmers' organisations (Espinoza-Ortega *et al.*, 2007; Salami *et al.*, 2010).

2.2 Bovine mastitis

Bovine mastitis is regarded as the most prevalent mammary gland irritation affliction that is widely recognized as harmful to dairy businesses worldwide (Paramasivam *et al.*, 2023). This infection occurs mostly when microbial organisms, chemicals, or traumatic events affect the mammary gland, resulting in infection. Several bacteria have been implicated in bovine intramammary infection, with staphylococci being the most prevalent in all instances and their infection rates being the highest (Pal and Gizaw, 2019; Mengistie, 2022). Environmental and contagious mastitis are two types of bovine mastitis, with *Staphylococcus aureus* being the most detected bacterium in contagious mastitis (Cheng and Han, 2020). According to Zadoks *et al.* (2002) and Skarbye *et al.* (2022), milking machine teat liners that are used to milk cows are key in the spread of staphylococcal mastitis. As a result, it is critical that diseased cattle be detected quickly and that appropriate management measures be taken to break the cycle of bacterial transmission (Sharun *et al.*, 2021). Other *Staphylococcus* spp., referred to as coagulase negative staphylococci (CNS), have been identified as opportunistic pathogens in mastitis infection, while pathogens such as *Escherichia coli* are the primary cause of environmental mastitis on dairy farms (Sharifi *et al.*, 2022; Cobirka *et al.*, 2020).

2.2.1 Classification of bovine mastitis

Bovine mastitis can be classified into three classes based on the degree of inflammation, namely clinical, sub-clinical, and chronic mastitis (Khasanah and Widianingrum, 2021). Clinical bovine mastitis is easily detected in dairy cows by visible abnormalities, such as a red and swollen udder and fever (Paramasivam *et al.*, 2023). Clinical mastitis can be further subdivided into pre-acute, acute, and sub-acute infection depending on the degree of the inflammation (Kibebew, 2017). Severe cases of clinical mastitis can also be fatal (Le Page *et al.*, 2023). Contrary to clinical mastitis, sub-clinical mastitis shows no visible abnormality in the udder or milk, but milk production decreases with an increase in the somatic cell count (SCC) (Abebe *et al.*, 2016; Abdullah *et al.*, 2023). The financial losses caused by subclinical mastitis are difficult to quantify, but experts agree that it accounts for a higher financial burden than clinical cases (Abdullah *et al.*, 2023).

2.2.2 Diagnosis of bovine mastitis

Generally, the kind and category of the infection can be used to diagnose bovine mastitis. Clinical mastitis manifests as apparent indications of inflammation, such as udder swelling, reddening, soreness, heat, and irregular milk consistency (Egyedy and Ametaj, 2022). Clinical mastitis can range from moderate to severe and can progress to systemic disease, resulting in fever, depression, lack of appetite, and, in severe cases, death of an animal (Ibrahim and Kirmani, 2021). There are no apparent indications of inflammation, and the milk seems normal in subclinical mastitis (Ariffin *et al.*, 2019; Saleh *et al.*, 2022). The diagnosis of clinical mastitis is rather simple; it is usually based on a visual examination to detect inflammation (Contreras and Rodriguez 2011).

Subclinical mastitis is diagnosed using one of two approaches. The first is a milk bacteriologic culture to identify the presence of an infectious pathogen. This is the standard approach for determining intramammary infection (IMI) and mastitis (Koskinen *et al.*, 2010; Adkins *et al.*, 2018). In the second approach, the somatic cell count (SCC) of a cow or quarter is counted to determine the measure of inflammation (Halasa and Kirkeby, 2020). The rise in SCC is caused by leukocyte infiltration into the mammary gland and is widely thought to be the result of an immunological response to IMI (Zigo *et al.*, 2021).

According to recommendations by the National Mastitis Council (NMC), a SCC of more than 200,000 cells/mL usually characterizes a subclinical mastitis case in dairy cows, while cut point values ranging from 100,000 to 300,000 cells/mL are typical in the literature (Addis *et al.*, 2020). The intensity of the inflammatory reaction is associated with the increase in SCC (Halasa and Kirkeby, 2020). When compared to CNS, *S. aureus* causes a more severe inflammatory response. Meta-analysis conducted by Djabri *et al.* (2002) found a geometric mean SCC of 138,000 and 357,000 cells/mL in CNS- and *S. aureus*-infected cow quarters respectively. While uninfected quarters had a mean of 68,000 cells/mL and infected quarters had a mean of 68,000 cells/mL (Djabri *et al.*, 2002; Mugabi, 2018).

The N-acetyl-beta-D-glucosaminidase activity, pH change, lactose content, electrical conductivity change, milk flow measurement, quantification of acute phase proteins (milk amyloid A, serum amyloid A, and haptoglobin), and the level of pro-inflammatory cytokines (interleukin or TNF) are some other diagnostic techniques that can be used (Pyorala, 2003; Simojoki *et al.*, 2011; Kalmus *et al.*, 2013). Some PCR-based diagnostic methods that use extracted DNA from whole milk to diagnose IMI caused by major mastitis pathogens have also been developed (Koskinen *et al.*, 2010). Recently, a commercial real-time PCR kit (Thermo Fisher Scientific's PathoProof Mastitis PCR Assay) is now utilized directly on milk samples without microbial culturing. The test is more sensitive and quicker than bacterial culture methods (Koskinen *et al.*, 2010). Given the group's heterogeneity, applying a PCR-based test to the CNS might be difficult. Nonetheless, focusing on a few frequent CNS species that cause IMI might be viable.

The detection of persistent subclinical mastitis or IMI is economically important because total economic losses per cow per year for subclinical mastitis are estimated to be higher for chronic subclinical cases compared to clinical mastitis. This is due to increased production losses associated with subclinical mastitis (Motaung *et al.*, 2017). Previous studies (Barlow *et al.*, 2013; Niedziela *et al.*, 2020) have highlighted the significance of strain type in defining chronic IMI from serial samples. The lack of a repeatable and transportable strain typing system for CNS species is currently a restriction.

2.2.3 Economic impact of mastitis

Mastitis is the primary cause of production losses for dairy industry in the United States, and it is a serious problem that impacts the economic value of this industry globally (Paramasivam

et al., 2023). Losses are mostly the consequence of decreased or lost milk production, high veterinary consultation and treatment expenses, the cost of additional labour, inferior product quality resulting in low premiums, and herd replacement costs because of culling (Overton and Dhuyvetter, 2020). Even after recuperation, an affected cow's udder may not be fully functional (Akers and Nickerson, 2011), resulting in protracted output losses. Despite the dearth of recent national statistics, estimates from the 1990s indicated that mastitis losses in the United States ranged between US\$1.5-2.0 billion per year (Middleton *et al.*, 2014). These cost estimates did not include the expenditures of preventative treatments such as teat disinfection, dry cow therapy, and others. The real cost varies based on the pathogenic organism that causes the IMI, the kind of infection (clinical or subclinical), and the severity or length of the illness (Paramasivam *et al.*, 2023).

This illness has been found to occur in 30% of African countries, but a cohesive presentation of data from multiple studies on this illness in Africa is required, which will be addressed in this section. According to publications from 2011 to 2014 (Basdew and Laing, 2011; Saidi *et al.*, 2013; Kassa *et al.*, 2014), the detrimental impact of bovine mastitis on the African economy could be massive and require evidence-based intervention. Reports published between 2000 and 2014 based on surveys from small and big dairy farms in sub-Saharan Africa, plus a few from the Middle East and north Africa, showed that attempts to create efficient and cost-effective disease treatment approaches were accelerated in some regions (Motaung *et al.*, 2017). However, the large-scale economic effect of bovine mastitis in Africa is unknown due to a dearth of published evidence. As a result, mastitis-related production losses and expenditure in African and other developing nations are frequently overestimated and potentially misjudged (FAO, 2014). This means that a uniform technique for estimating the economic consequences of bovine mastitis is required. In Africa, the research environment for analysing the economic impacts of bovine mastitis is wide open (Motaung *et al.*, 2017).

2.3 Strategies to control bovine mastitis

2.3.1 Hygiene control measures

Contagious mastitis pathogens, including *Streptococcus agalactiae* and *Staphylococcus aureus*, have been successfully controlled by current mastitis control programs that were developed in the 1960s. These measures are based on teat disinfection, antibiotic therapy, and culling of chronically infected cows. Unfortunately, environmental infections like *S. uberis* and *E. coli*, which cause a considerable amount of subclinical and clinical mastitis in lactating and

nonlactating cows and heifers, make these methods far less successful (Dego, 2020). In an effort to reduce the prevalence of mastitis, the National Mastitis Council created a 5-point mastitis control program in 1969. This program entails (i) dipping teats in an antiseptic solution before and after milking, (ii) proper maintenance of milking equipment, (iii) identifying and treating animals as soon as they become infected, (iv) treating dry cows with long-acting antibiotics to shorten the duration of an existing infection and prevent new intramammary infection, and (v) culling animals with chronic infections (Ndahetuye *et al.*, 2020). Later, a 10-point plan was developed to include additional steps like setting udder health goals, maintaining a clean, dry, and comfortable environment, using proper milking techniques, maintaining milking equipment properly, keeping meticulous records, managing clinical mastitis during lactation, managing dry cows effectively (including blanket dry cow therapy), maintaining good biosecurity for contagious pathogens, and marketing chronically infected cows (Gooder, 2014). While these sanitary milking procedures and infection prevention methods reduce bacterial dissemination, transmission, and eventual illness, they do not completely shield dairy cows against developing infections (Kahinda, 2021).

2.3.2 Antibiotic therapy

For the treatment of mastitis and other ailments that affect dairy cows such as metritis, endometritis, retained placenta, lameness, and pneumonia, antibiotics are also widely utilized on dairy farms (Pinedo *et al.*, 2020). This method boosts the usage of antimicrobials in dairy farms and exposes a huge number of animals to them. Intramammary infusions and parenteral administration of antimicrobials are both used to treat clinical (acute or pre-acute) mastitis in dairy herds (Tempini *et al.*, 2018). Some farms additionally feed waste milk to heifer calves, which puts antibiotic pressure on their gastrointestinal tract (GIT). This milk usually contains microbiota as it is milk that is not used during antibiotic treatment or milk not permitted into the bulk tank after parturition (Dego, 2020). These techniques have increased the use of antimicrobials on dairy farms and exposed a lot of healthy cows to them, which unfortunately puts a lot of strain on the microorganisms that live in farm surroundings and on the bodies of animals.

Mastitis may develop from an intramammary infection either clinically or sub-clinically. Antimicrobials are typically used to treat clinically infected udders; however, sub-clinically infected udders may not be detected right away and treated, but they may instead continue to be infected and shed germs through milk throughout lactation (Chakraborty, 2019). The

percentage of cure after mastitis treatment varies, and the variation in cure rate is multifactorial. These factors are cow-based (a cow's age or parity number, the lactation stage, and the duration of infection), management factors (the time of detection and diagnosis to treatment, the availability of balanced nutrition, and sanitation), antimicrobial use patterns (type, dose, route, frequency, and duration), and pathogen factors (tumor type, titer, and duration) (Sharun *et al.*, 2021).

Lincosamide (19.4%), cephalosporins (53.2%), and non-cephalosporin-lactam (19.1%) are the antibiotics that are most often used to treat mastitis (Cáceres *et al.*, 2023). The issue associated with administering non-selective blanket antimicrobials to dairy cows as a preventative measure to control mastitis is that they exert selection pressure on both commensal bacteria and mastitis-causing bacteria in the animals' bodies. The use of antimicrobials for the prevention and treatment of mastitis and other diseases in dairy cattle may not change the final outcome, but the bacteria in the gut, in the mammary glands, and dairy farm environments are exposed to different levels of antibiotics and their bio-transformed products (Rainard, 2017; Merrill, 2018). This selective pressure may lead to the development of germs that are resistant to antibiotics, persistent on farms, and contagious to animals. Antimicrobials are used by dairy farmers either as a prophylactic measure to prevent mastitis or as therapies to treat mastitis cases. Due to the poor effectiveness of medications in treating *S. aureus* mastitis, the animal may need to be put to death (Weber *et al.*, 2021).

Several antibiotics have been used internationally to treat intramammary infections, and various investigations have shown resistance mechanisms such as β -lactamase synthesis, encoded by *blaZ*, and the formation of low-affinity penicillin-binding proteins (PBP2a), encoded by *mecA* or *mecC* (Zhang *et al.*, 2003; Cuny *et al.*, 2011). The *MecA* gene, which is present in the mobile genetic element known as staphylococcal cassette chromosome (SCCmec), allows *S. aureus* to be resistant to methicillin. The resistance of staphylococcal species to methicillin implies that they are resistant to the majority of currently available β -lactam antimicrobials (Brakstad and Maeland 1997; Srednik *et al.*, 2017). The CNS linked with IMI may serve as a reservoir for antibiotic resistance genes, particularly methicillin resistance genes, which can then be transmitted to significant veterinary or zoonotic diseases (Srednik *et al.*, 2017). As a result, given the emergence of different antibiotic resistance, several researchers have identified this reservoir as a possible public health risk (Hanssen, Kjeldsen

and Sollid 2004; Sawant, Gillespie and Oliver 2009; Febler *et al.*, 2010; Sampimon *et al.*, 2011).

2.4 Genus *Staphylococcus*

Staphylococcus species are salt-tolerant, Gram-positive, and catalase-positive cocci with low a guanine-cytosine (G+C) concentration measuring 0.5–1 μm in diameter. They typically develop in groups, pairs, and occasionally chains (Foster, 1996). The *Staphylococcus* genus includes some species that are typically commensals on both human and animal skin. Some of these species are also pathogenic and can cause disease, while other species are more prevalent in the environment and can be isolated from fermented foods and only rarely cause disease (Kastman *et al.*, 2016). They are the main source of IMI in cattle, which results in mastitis (Taponen and Pyorala, 2009). As of 2014 (Becker *et al.*, 2014), the genus had at least 47 species and 23 subspecies, and two new species (*S. schweitzeri* and *S. argenteus*), which are closely related to *S. aureus*, have recently been identified (Tong *et al.*, 2015). Coagulase-positive staphylococci (CPS) and coagulase-negative staphylococci are the two main subgroups of staphylococci that can coagulate blood plasma (CNS). The rest are categorized as CNS. The CPS consists of *S. argenteus*, *S. argenteus*, *S. aureus*, *S. intermedius*, *S. pseudintermedius*, *S. delphini*, *S. hyicus*, *S. lutrae*, *S. agnetis*, and *S. hyicus*. There are coagulase-negative and -positive subspecies of *S. schleiferi* that are referred to as *S. schleiferi* subsp. *schleiferi* and *S. schleiferi* subsp. *coagulans* respectively (van Duijkeren *et al.*, 2011; Becker *et al.*, 2014; Tong *et al.*, 2015). To prevent ambiguity, some researchers divide staphylococci into *S. aureus* and non-aureus staphylococci (NAS) (Condas *et al.*, 2017). Staphylococci were recently divided into 15 groups and six species groups based on DNA sequences from four genes (*16S rRNA*, *dnaj*, *rpoB*, and *tuf*). These groups include Auricularis, Hyicus-Intermedius, Epidermidis-Aureus, Saprophyticus, Simulans, and Sciuri (Lamers *et al.*, 2012).

2.4.1 Identification and characterization

Mastitis identification and diagnosis depend on parameters such as specificity, sensitivity, and the cost of the procedures used. To avoid the challenges associated with traditional methods of diagnosis and identification, DNA-based approaches are now being used to focus on the DNA content of microbes rather than on their phenotypic expression (Singh Saharan *et al.*, 2023). Recent advances in proteomics and genomics, such as polymerase chain reaction (PCR) and mass spectroscopy, have been developed to detect novel biomarkers and discover proteins and genes associated with mastitis (Perera *et al.*, 2022). Proteins such as prostaglandin D

synthetase, serotransferrin, and bovine serum are utilized in tests such as ELISA to diagnose this infection. These tests result in a reliable and effective diagnosis, but they can be costly at times (Yazdankhah *et al.*, 1998; Cremonesi *et al.*, 2005). Furthermore, Viguiet *et al.* (2009) discovered that methods such as multiplex PCR and real time quantitative PCR may both identify mastitis-causing bacteria in bovine milk at the same time. Shome *et al.* (2011) demonstrated that a two-tube PCR can identify almost ten pathogens at once, including *S. aureus*, *S. chromogenes*, *S. epidermidis*, *S. cuiri*, *S. haemolyticus*, *S. simulans*, streptococci species, and *E. coli*. Several investigations have also employed alternative molecular approaches to characterize staphylococcal species detected in raw bovine milk, such as PCR of the *16S rRNA* gene, *internal transcribed spacer (ITS)* PCR, and sequencing-based identification of the *16S rRNA*, *HSP60*, *turf*, *soda*, and *rpoB* genes (Mellmann *et al.*, 2006; Irlinger, 2008; Martins *et al.*, 2017).

In addition to the approaches listed above, whole genome sequencing (WGS) is now widely utilized to study disease epidemiology (Salipante *et al.*, 2015; Park *et al.*, 2017). Whole genome sequencing has become by far the most often utilized technology due to its selective capacity (Harris *et al.*, 2013). This approach has the strength and capability of distinguishing among closely related bacterial strains (Lowder *et al.*, 2009). In this approach, sequence readings are matched to a reference genome to find single nucleotide polymorphisms (SNPs) in the core genome (Price *et al.*, 2012; Spoor *et al.*, 2013; Tong *et al.*, 2015). To read and analyse bacterial genetic relatedness, a phylogenetic tree must be formed utilizing core genome SNP analysis (Price *et al.*, 2012). WGS is expected to replace most of the current strain-typing approaches.

2.4.2 Pathogenicity of *Staphylococcus* species in mastitis

Bacterial infections and their consequences are caused by three major abilities: to infiltrate the host, to colonize the host, and to proliferate inside the bacterial host (Sender *et al.*, 2017). These three abilities are akin to staphylococcal pathogenicity and virulence factors (González-Martín *et al.*, 2020). Furthermore, several studies have identified secreted proteins, cell surface-bound proteins, and cell wall components as virulence factors implicated in staphylococcal infection pathogenesis (Kumar *et al.*, 2020; Zheng *et al.*, 2021; Cheung *et al.*, 2021). Superantigens such as enterotoxins, exfoliative toxins, and toxic shock syndrome toxin-1 have also been identified as key pathogenic factors in staphylococcal infections by Abril *et al.* (2020).

The genetic diagnosis of staphylococcal pathogenicity and virulence is challenging and frequently convoluted. This is due to the continual variations in genetic expression of staphylococcal accessory regulator (*sar*) and accessory gene regulator (*agr*) (Sender *et al.*, 2017). Several investigations have been carried out to investigate and define the virulence factors such as leucocidins, exfoliative toxins, slime, and biofilm in *S. aureus* isolates from mastitis cases (Castro *et al.*, 2020; Little *et al.*, 2021; Aslantas *et al.*, 2022). However, Krishnamoorthy *et al.* (2016) states that only a few research studies have focused on the virulence factors on CNS species isolated from mastitis patients. In their investigation, Kuroishi *et al.* (2003) observed that both *S. aureus* and CNS isolates from mastitis patients generated enterotoxins and toxic shock syndrome toxin-1. The same study reported that these enterotoxins and toxic shock syndrome toxin-1 were more highly expressed in the CNS such as *S. epidermidis*, *S. chromogenes*, *S. hyicus*, and *S. xylosu* than in *S. aureus* in instances of subclinical mastitis. Several genes, such as *loci ica*, *bap*, *agr*, and *sar*, have been utilized to study biofilm development in staphylococcal species from animals with bovine mastitis and humans (Acheek *et al.*, 2020).

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CHAPTER THREE

PREVALENCE OF SUBCLINICAL MASTITIS, ITS ASSOCIATED BACTERIAL ISOLATES, AND RISK FACTORS AMONG CATTLE IN AFRICA: A SYSTEMATIC REVIEW AND META-ANALYSIS¹

ABSTRACT

Subclinical mastitis (SCM) is one of the most economically important diseases as it affects the dairy industry adversely. Compared to clinical mastitis, SCM does not cause visible changes in the udder of female bovines or results in physical changes in their milk. However, to understand this disease better, an in-depth overview of its prevalence and the risk factors associated with it in different regions on the African continent are still lacking. The objective of this review was to investigate the prevalence of SCM, assess the risk factors associated with it, and determine which dominant bacterial pathogens cause this disease in dairy bovines in Africa.

Literature published in English from January 2010 to December 2020 concerning SCM was identified based on certain inclusion criteria and systematically reviewed using two databases (PubMed and Web of Science). Meta-analysis was conducted using the ‘meta’ and ‘metafor’ packages in the R statistical software. A total of 258 studies were retrieved and, at the end of the screening process, 82 full texts were eligible for inclusion in the meta-analysis. These revealed that the prevalence of SCM was reported in 11 African countries located in five African regions. The random-effects model showed that the weighted pooled prevalence estimate (PPE) was 48.2% (95% CI: 43.6-52.8%). Heterogeneity was high and statistically significant as I^2 (proportion of observed variation) was 98.1% (95% CI: 98.0-98.3%), the τ^2 (true between-study variance) was 0.0433 (95% CI: 0.0322-0.0611), and the Cochran Q statistic was 4362.8 ($p < 0.0001$). Subgroup and meta-regression analyses showed that East Africa had significantly ($p = 0.0092$) the highest PPE of SCM (67.7%, 95% CI: 55.7-78.7) followed by West Africa (50.5%, 95% CI: 31.4-69.5), while the lowest was in North Africa (40.3%, 95% CI: 32.2-48.6). Other significant moderators for SCM were age ($p < 0.0001$), breed ($p = 0.0002$), lactation stage ($p = 0.019$), and parity ($p = 0.0008$). The *Staphylococcus* species (prevalence 43.7%) were the

¹ Published in *BMC Veterinary Research*

most predominant pathogens, followed by *Streptococcus* spp. (18.2%) and *Escherichia* spp. (9.5%).

The study showed a high variation of SCM prevalence in various parts of Africa, although the need for more data from some regions was identified. The reported high prevalence of SCM is a clear sign of inappropriate management practices of cattle herds and an indicator of the threat that SCM poses to the dairy industry. The information about the predisposing factors may guide effective management and control strategies to reduce transmission of the disease.

Keywords: Dairy, subclinical mastitis, Africa, meta-analysis, prevalence

3.1 Introduction

Mastitis is one of the most virulent diseases in animal husbandry and affects the dairy industry worldwide. The disease has an economic impact on farmers, either directly or indirectly, through reduced milk production and quality, high culling rate of affected cows, decreased reproductive performance, as well as treatment and control costs (Radostits *et al.*, 2007; Hogeveen *et al.*, 2011; Cheng and Han, 2020). Mastitis is estimated to cost the dairy industry in the region of US\$38 million per annum, while its costs can reach as high as US\$17.5 to US\$18.8 million per annum in developing countries such as Ethiopia, Madagascar, and India. However, due to inadequate research in Africa and other developing nations, the economic costs and production losses related to mastitis are likely to be underestimated or calculated incorrectly (Motaung *et al.*, 2017).

This disease is typically caused by a wide range of microorganisms such as *Escherichia coli*, *Streptococcus agalactiae*, *S. uberis*, *S. dysgalactiae*, *Staphylococcus aureus*, and *Mycoplasma* spp. However, in some cases, it may be caused by trauma to the mammary gland (Cobirka *et al.*, 2020). Mastitis is classified as either clinical or subclinical. The cost of sub-clinical mastitis (SCM) is often higher (70-80% of total losses) than that of clinical mastitis (Motaung *et al.*, 2017). Whereas clinical mastitis is distinguished by visible abnormal milk appearance as well as a swollen, reddened, hot and painful udder, there are no visible changes when SCM occurs (Cheng and Han, 2020).

The California mastitis test (CMT) is used to qualitatively estimate the concentration of white blood cells in milk. This test is most helpful in detecting SCM but serves little purpose in the detection of acute clinical mastitis (Moroni *et al.*, 2020). It is noted by Sumon *et al.* (2020) that the prevalence of this disease varies from one study report to another, which could be due to differences in locations and seasons, the total number of studied animals, their breed, the lactation stage of cows, parity number, and on-farm management practices. Apart from clinical classification, mastitis can be categorised according to transmission as either contagious or environmental (Alhussein *et al.*, 2018). To control mastitis effectively, it is necessary to systematically determine its prevalence under different systems and to identify the causal agents of SCM (de Vlieghe *et al.*, 2012). Although there have been reports of the widespread occurrence of SCM in dairy herds in countries in Africa, an extensive overview of the prevalence and epidemiological dynamics among cattle on this continent is still lacking.

In light of the above, the aim of this review was to determine the prevalence of SCM among cattle on the African continent and to assess the risk factors associated with this disease by identifying causative pathogens. The systematic review and meta-analysis approaches were used.

3.2 Materials and methods

3.2.1 Search strategy

The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines were followed in conducting the meta-analysis (Page *et al.*, 2020). A literature search related to bovine SCM in Africa was conducted using a group of search topics and search terms that were separated by the Boolean operators AND and OR respectively, as follows: (Mastitis) AND (Bovine OR Cattle OR Cow) AND (Sub-clinical OR Subclinical OR Sub clinical) AND (Prevalence OR Incidence OR Occurrence) AND (Africa OR Algeria OR Angola OR Benin OR Botswana OR Burkina Faso OR Burundi OR Cameroon OR Cabo Verde OR Central African Republic OR Chad OR Comoros OR DR Congo OR Democratic Republic of Congo OR Zaire OR Côte d'Ivoire OR Ivory Coast OR Djibouti OR Equatorial Guinea OR Egypt OR Eritrea OR Ethiopia OR Gabon OR Gambia OR Ghana OR Guinea OR Guinea-Bissau OR Kenya OR Lesotho OR Liberia OR Libya OR Madagascar OR Malawi OR Mali OR Mauritania OR Mauritius OR Morocco OR Mozambique OR Namibia OR Niger OR Nigeria OR Rwanda OR Sao Tome and Principe OR São Tomé and Príncipe OR Senegal OR Seychelles OR Sierra Leone OR Somalia OR Somaliland OR Puntland OR South Africa OR South Sudan OR Sudan OR Swaziland OR Eswatini OR Tanzania OR Zanzibar OR Togo OR Tunisia OR Uganda OR Zambia OR Zimbabwe)). Somali's autonomous regions of Puntland and Somaliland and Tanzania's semi-autonomous region of Zanzibar were included in the search strategy.

The bibliographic databases PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) and Web of Science-All Databases option (<https://www-webofscience-com.uplib.idm.oclc.org/wos/alldb/basic-search>) were searched from January to June 2021 using the above combination of terms. Non-peer reviewed or grey literature was not included in this review. Literature searches were limited to articles published in the English language dated from January 2010 to December 2020.

3.2.2 Selection of studies and data extraction

The studies that were identified and are discussed in this chapter were retrieved, screened, and reviewed using four stages: (i) identification of titles, (ii) screening of titles and abstracts, (iii) full-text retrieval and screening for eligibility, and (iv) review of eligible full texts and extraction of data (**Figure 3.1**).

The retrieved studies were managed using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) and EndNote version 20 (1500 Spring Garden Street, Fourth Floor, Philadelphia, PA 19130, USA). Upon compilation of the search titles and abstracts from the two databases, duplicate records were removed. This was followed by screening of the titles and abstracts for eligibility, using the following inclusion criteria: (i) peer-reviewed articles in English, (ii) cross-sectional studies that investigated the prevalence of SCM in cattle, (iii) studies conducted in African countries and published from January 2010 to December 2020, (iv) studies that reported SCM results based on the California mastitis test (CMT), and (v) studies that reported on the total sample size, positive samples and/or the prevalence rates, and with a sample size greater than 35. After screening the titles and abstracts, the full texts of eligible studies were evaluated using the same criteria as those listed above.

Data extracted from eligible studies included the total number of cattle examined and the number of positive mastitis cases (at individual and udder-quarter levels). Other retrieved data were authors' name(s) and year of publication as well as the age categories, breed, parity, lactation stage, production system, and milk yield associated with the sampled cows.

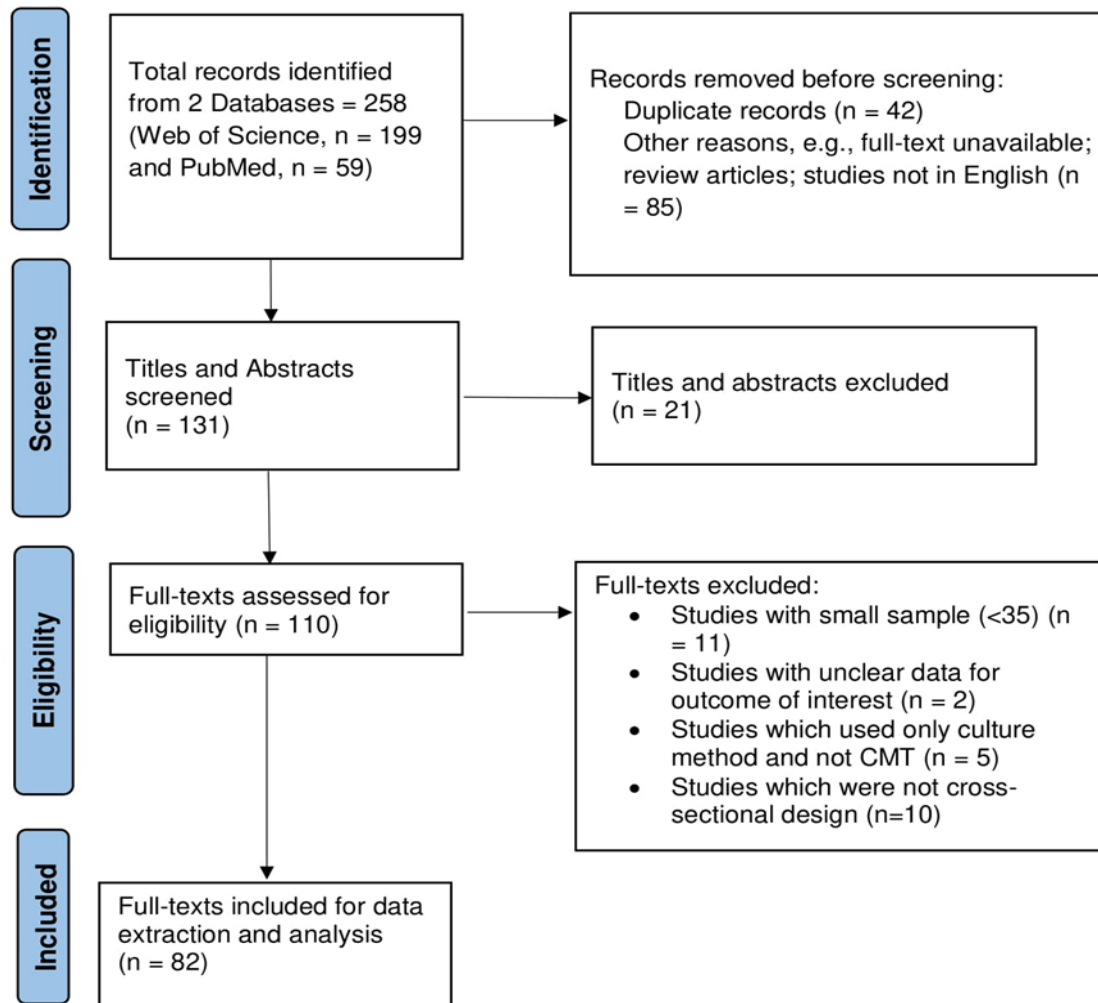


Figure 3.1: PRISMA flow diagram demonstrating the search and selection process for studies on the prevalence of sub-clinical mastitis among cattle in Africa (2010-2020). CMT = California mastitis test.

3.2.3 Quality assessment of the studies

The quality of each study was evaluated using the Joanna Briggs Institute (JBI) Critical Appraisal Tool for prevalence studies (Munn *et al.*, 2014). The JBI tool has ‘Yes’, ‘No’, ‘Unclear’ or ‘Not applicable’ question types and scores were assigned as 1 for ‘Yes’ and 0 for ‘No’. The final checklist contained five questions concerning: (i) appropriateness of sample frame, (ii) appropriateness of sampling procedure, (iii) adequacy of sample size, (iv) description of study subjects and setting, and (v) appropriateness of statistical analyses. The ‘Yes’ scores for each study were added and the percentage score computed by dividing by the total number of questions. The studies were classified as: low quality (less than 50%), moderate quality (50 to 74%), and high quality ($\geq 75\%$). All studies were included irrespective of the score, provided that they met the inclusion criteria listed above.

3.2.4 Data analysis

The statistical packages ‘meta’ (Schwarzer *et al.*, 2015) and ‘metafor’ (Viechtbauer, 2010) were used to estimate the models for meta-analysis and visualize the results. In the primary analysis, the overall prevalence of SCM and detected bacterial isolates were estimated using both fixed and random-effects models, which take into account within-study variances only or both within- and between-study variances respectively. The prevalences were presented along with the 95% confidence intervals (Wang *et al.*, 2010). Estimation of the models was performed using the restricted maximum likelihood method (REML) estimator (Raudenbush and Bryk, 1985), and data were transformed to conform to normal distribution using the double-arcsine transformation (PFT) method (Miller, 1978). The transformed proportions were then converted back to proportions for reporting purposes. Heterogeneity across the studies was tested and quantified using the Cochran’s Q statistic (Cochran, 1954) and the I^2 statistic (Hunter and Schmidt, 2000) respectively, in order to assess the proportion of total variation that is attributable to between-study variation rather than to within-study variation (chance). Heterogeneity was considered significant if the p -value was less than 0.05 in the Cochran Q test and I^2 was greater than 50%, given the commonly used benchmarks for I^2 heterogeneity levels as 25%, 50%, and 75% for small, moderate and high respectively (Higgins *et al.*, 2003). The true between-study variance (τ^2) and standard deviation (τ) were also determined using the tau statistic to estimate the amount of heterogeneity (Raudenbush and Bryk, 1985).

The study further looked for potential sources of heterogeneity in mastitis prevalence using subgroup analysis and meta-regression analysis (Cuijpers *et al.*, 2021). This analysis employed mixed-effects models in which the random-effect models were used to combine study effects within each subgroup, and fixed-effect models were used to test whether the effects across the subgroups varied significantly from one another. Common between-study variance was assumed across subgroups, and the within-group estimates of τ^2 were pooled. The considered moderators were geographical region (East Africa, Horn of Africa, North Africa, West Africa, and southern Africa), year of publication (before 2015 vs. after 2015), age of cattle in years (2 to 5, 6 to 9, >9), breed (local, crossbreed, exotic), lactation stage (early, mid, late), milk yield in litres (<7, 7 to 15, >15), parity (1 to 3, 4 to 6, ≥ 7), and production system (extensive, semi-intensive, intensive).

Forest plots were created to visualize the prevalence of heterogeneity and the 95% confidence intervals across the included studies. Although funnel plots for analysis for publication bias

can be problematic for meta-analysis of proportions (Hunter and Schmidt, 2000), the review visualized the asymmetry and additionally analyzed this using the unweighted Egger's regression test. The latter assesses small-study bias by evaluating if the association between estimated effects and study size is larger than might be expected by chance (Egger *et al.*, 1997). The number of included studies was greater than 10 (i.e., $n=82$), and therefore Egger's regression test was appropriate for supporting the presence of symmetry. The significance of udder-quarter – left forward (LF), right forward (RF), left hind (LH), and right hind (RH) – with regards to the prevalence of SCM was assessed using the Analysis of Variance (ANOVA). The udder-level prevalence data were first log transformed before analysis. All statistical analyses were performed at 5% significance level using R software version 4.2.1 (R Core Team, 2022).

3.3 Results

3.3.1 Search results

A total of 258 articles were retrieved from the database search. After removing the duplicates ($n=42$) and excluding studies for other reasons ($n=85$), 131 studies were screened based on title and abstract. Subsequently, from the full-text evaluation of the 110 studies, 38 were excluded for reasons such as absence of clear data on mastitis prevalence, study design (only cross-sectional studies were selected), unclear information on the outcome of interest, a small sample size of fewer than 35 cattle, and the use of only the culture method without an initial CMT diagnosis. Finally, only 82 studies were included for data extraction and analysis. The included studies were categorised based on region (Horn of Africa = 43, East Africa = 10, West Africa = 4, North Africa = 23, and Southern Africa = 2) and publication period (before 2015 = 36 and after 2015 = 46).

3.3.2 Meta-analysis for the prevalence of SMC among cattle in Africa

The random-effects model showed that the weighted pooled prevalence estimate (PPE) of SCM among cattle was 48.2% (95% CI: 43.6, 52.8) (**Figure 3.2**). Heterogeneity was high and significant as I^2 (proportion of observed variation after elimination of sampling error) was 98.1 (95% CI: 97.5, 98.7), and this was statistically significant ($Q=4362.83$, $p<0.0001$). Moreover, the prediction interval, which represents the expected range of highly probable prevalence values in future studies, covered a wider range (11.9 to 85.5%) than the 95% CI (**Figure 3.2**). The true between-study variance, τ^2 , was 0.0433 (0.0322, 0.0611), implying a similar amount of within-group heterogeneity and further confirming heterogeneity across studies. Subgroup

analyses and meta-regressions were therefore conducted to identify factors that could explain differences in effect sizes across studies.

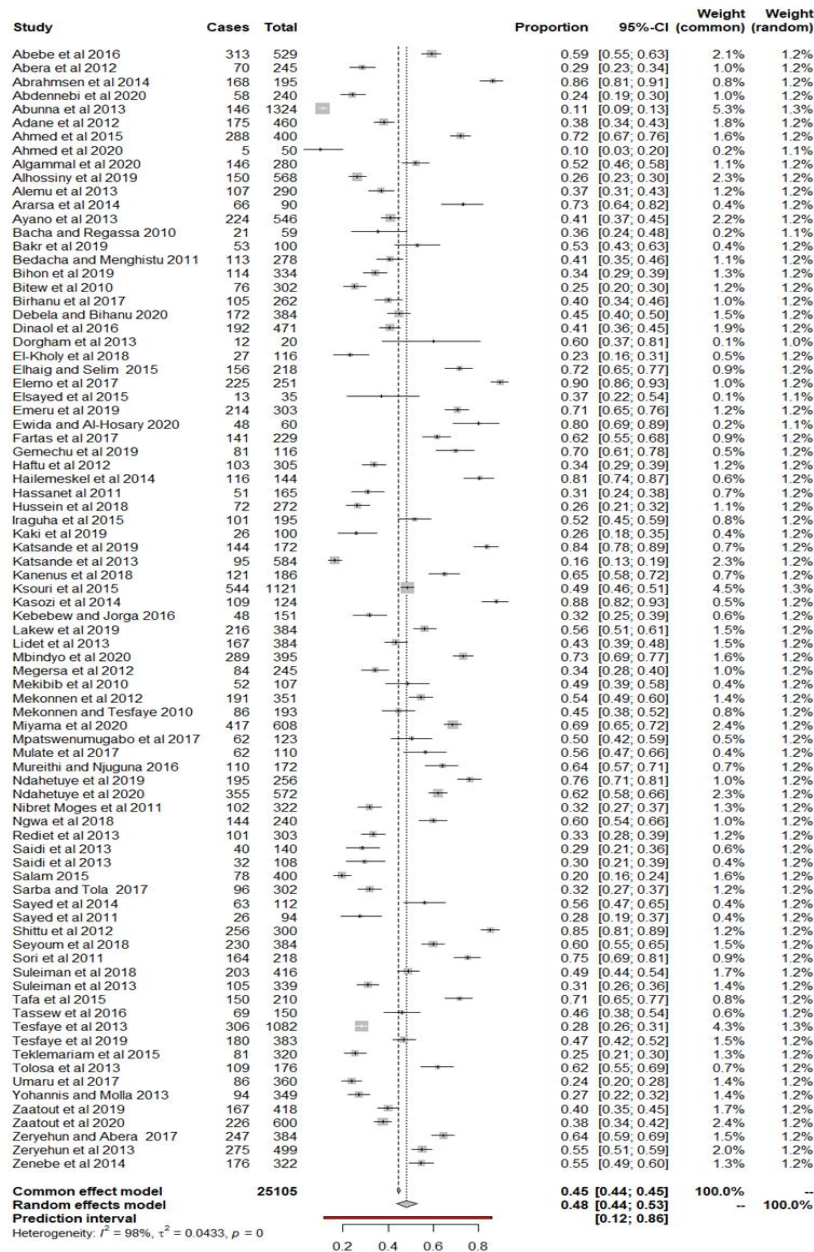


Figure 3.2: Forest plot of 82 studies included in a mixed-effects meta-analysis for prevalence of sub-clinical mastitis among cattle in Africa from January 2010 to December 2020. The diamond at the bottom represents the summarized prevalence. The grey boxes and horizontal lines through the boxes represent the weighed prevalence and the 95% confidence interval respectively for each included study. A shorter horizontal line suggests better precision of the study result. ‘Cases’ is the number of cattle that tested positive using the California mastitis test, ‘Total’ is the number tested, while ‘Proportion’ is the prevalence divided by 100. The weights that each study contributed to the summarized effect size (both fixed and random effects models) are shown as percentages in the last two columns. CI = confidence interval as

an index of precision for estimation of prevalence. Meta-analysis was conducted using ‘meta’ and ‘metafor’ packages in R version 4.2.1 (R Core Team, 2022).

3.3.3 Publication bias

The funnel plot was symmetrical (**Figure 3.3**) and the unweighted Egger’s regression test was not significant ($z=0.792$, $p=0.429$), suggesting that there were no small-study effects and probably no publication bias in the studies included in this meta-analysis.

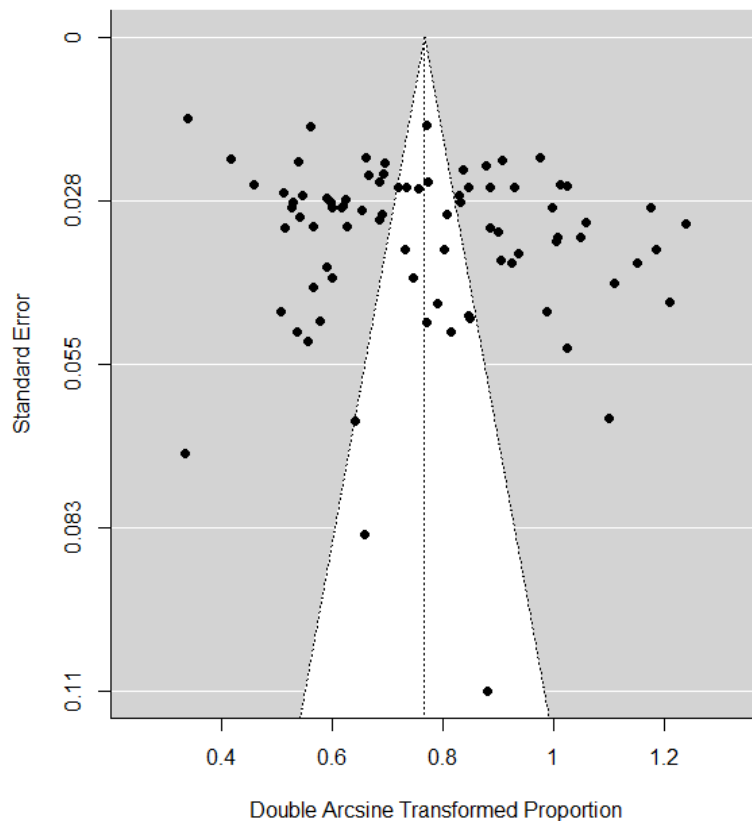


Figure 3.3: A funnel plot assessing publication bias for studies regarding the prevalence of sub-clinical mastitis in cattle in Africa (from 2010 to 2020). The number of studies included was 82. The x-axis is a measure of prevalence estimates (double arcsine transformed). The y-axis is the precision of the study size (standard error) of corresponding study. The vertical line is situated at the transformed value of the summarised prevalence on the funnel plot. Circles that represent smaller studies are broadly spread towards the bottom and further from the centre of the funnel plot (less similar to summarised prevalence), whereas circles from larger studies are narrowly distributed towards the upper part of the graph and symmetrically clustered around the vertical line. The two limit lines symbolise the 95% CI around the summary prevalence value. More circles lie beyond the two limit lines, indicating high heterogeneity.

Note: funnel plot as a measure of publication bias needs to be interpreted with caution because sometimes studies with undesirable results are not published due to various factors.

3.3.4 Quality assessment of individual studies

The quality of 82 included studies was scored and, of these, 51 were classified as high quality ($\geq 75\%$ score), 19 as low quality (< 50 score), and 9 as moderate quality (50 to 74% score). Other studies ($n=3$) were categorized as “Not applicable” to at least 3 of the 5 scoring items.

3.3.5 Subgroup analysis for publication period and study region

Subgroup analysis and meta-regression showed a significant effect of study region on the prevalence estimates of SCM ($Q [df=4] = 13.47, p=0.0092$), accounting for 10.8% (R^2) of the true heterogeneity (**Table 3.1**). The highest prevalence occurred in the East African region (67.7%; 95% CI 55.7, 78.6) and the lowest in North Africa (40.3; 95% CI 32.3, 48.6) (**Table 3.1 and Figure 3.4**). The study period, on the other hand, showed no significant moderating effect ($Q [df=1] = 0.66, p=0.417$) on SCM; i.e., the summarized prevalence was 46.0 (95% CI 39.1-52.9) for studies ‘before 2015’ and 49.9 (95% CI 43.7-55.9) for studies ‘after 2015’ (**Table 3.1**). This is supported by the absolute value of true heterogeneity, τ^2 , (amount of within-group heterogeneity across the two subgroups), which was almost the same (0.04) between the two subgroups ‘before 2015’ and ‘after 2015’.

Table 3.1: Heterogeneity statistics for prevalence of sub-clinical mastitis among cattle in Africa based on location and publication period. Data sets for the period 2010 to 2020.

Variable	Subgroup	No. of studies	Cattle tested by CMT			Heterogeneity			Univariate meta-regression			
			No. tested	No. positive	% (95% CI)	χ^2	p-value	I ² (%)	p-value	τ^2 , H ²	Cochran Q statistic	R ² (%)
Period									0.417	0.04, 53.6	0.66 (df=1)	0.00
Region	Before 2015	36	10947	4225	46.0 (39.1-52.9)	1545.06	<0.01	98 (97 to 99)				
	After 2015	46	14158	7076	49.9 (43.7-55.9)	1778.64	<0.0001	98 (97 to 99)				
	Total	82	25105	11301								
									0.0092	0.04, 47.0	13.47 df=(4)	10.84
	Horn of Africa ^a	43	14208	6040	47.4 (41.4-53.3)	1318.46	<0.01	97 (96 to 98)				
	East Africa	10	3056	2009	67.7 (55.7-78.7)	186.91	<0.01	95 (94 to 96)				
	West Africa	4	1239	591	50.5 (31.4-69.5)	361.02	<0.01	99 (98 to 100)				
	North Africa	23	5846	2422	40.3 (32.3-48.6)	671.61	<0.01	97 (96 to 98)				
	Southern Africa ^a	2	756	239	49.4 (23.2-75.8)	289.34	<0.01	100				
	Total	82	25105	11301								

CI: Confidence intervals. 95% CIs were calculated as described by Higgins and Thompson (2003).

I² (%): Residual heterogeneity (unaccounted heterogeneity)

R²: Proportion of between-study variance explained

QM: Coefficient of test for heterogeneity between subgroups; df=degrees of freedom

τ^2 (tau squared) = between-study variance - estimated variance of the distribution of true prevalence in the population of sub-clinical mastitis studies

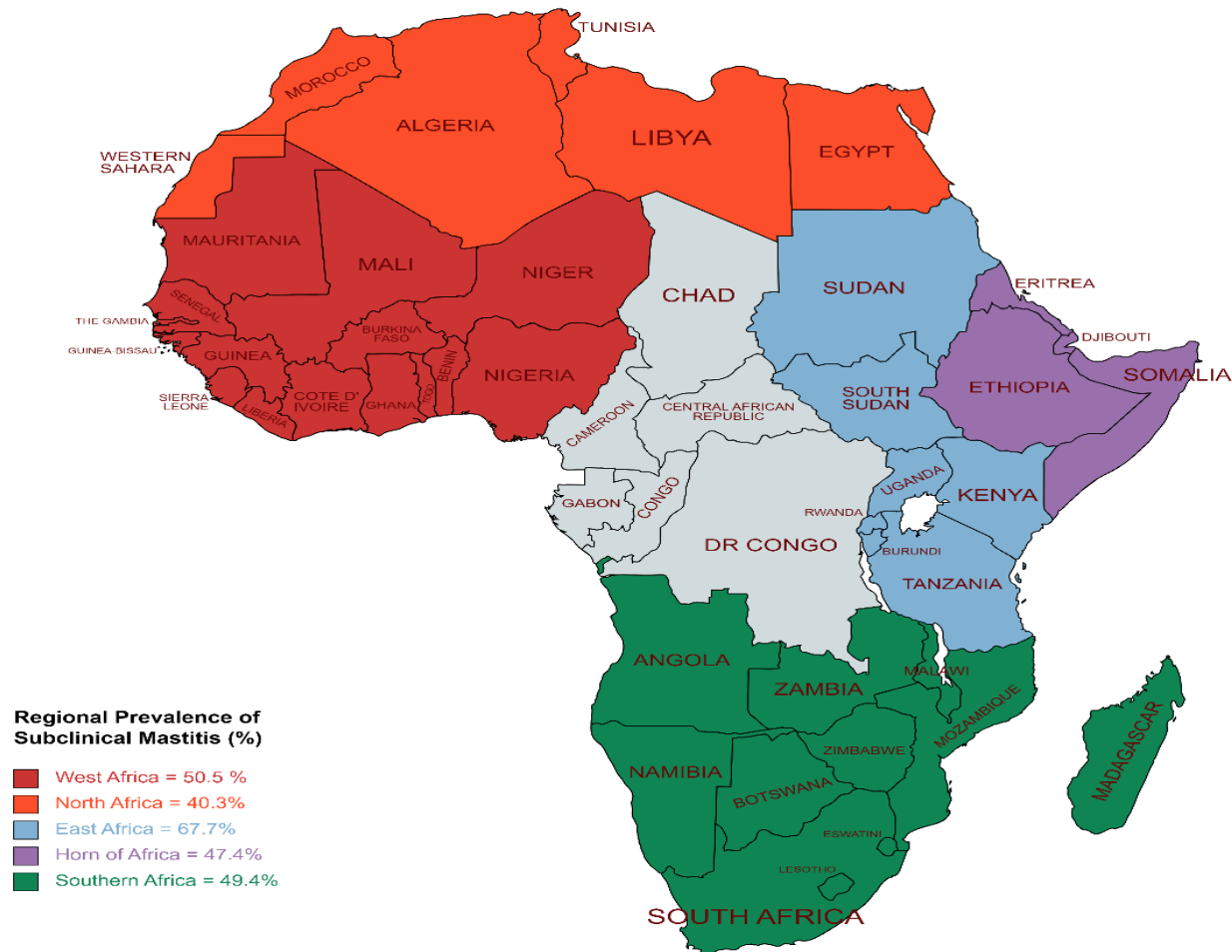


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H^2 = unaccounted variability (sampling variability) – the ratio of the standard deviation of the estimated overall prevalence from the random-effects model compared to the standard deviation from the fixed-effects model.

a Studies were from: Horn of Africa (Ethiopia); Southern Africa (Zimbabwe)

CMT = California mastitis test; df = degrees of freedom



Created with mapchart.net

Figure 3. 4: Map of Africa showing the prevalence estimates for subclinical mastitis among dairy cattle based on a summary of studies from 2010 to 2020.

3.3.6 Univariate meta-regression analysis for association between SCM and animal- or herd-level factors

Meta-regression analyses showed that the significant moderators for SCM were age (QM=26.2, $p<0.0001$), breed (QM=17.3, $p=0.0002$), lactation stage (QM=7.8, $p=0.019$) and parity (QM=14.2, $p=0.0008$) of cattle, while milk yield (QM=1.93, $p=0.38$) and production system (QM=0.47, $p=0.79$) had no statistically significant effect on the prevalence of SCM (**Table 3.2**). The prevalence of mastitis increased with age from 33.9% (95% CI 26.8, 41.6) among animals 2 to 5 years of age to 51.4% (43.8, 58.9) and 67.6% (56.9, 77.5) for animals 6 to 9 years or >9 years of age respectively. The prevalence was highest among exotic breeds (59.3%; 95% CI 49.1, 69.1%) followed by crossbreeds (50.2%; 95% CI 42.9, 57.5%), while it was the lowest for local breeds (33.5%, 95% CI 26.1, 41.3%) (**Table 3.2**). Exotic breeds (e.g., Jersey and Holstein-Friesian) were considered as cattle breeds that originated from continents such as Europe and North America. The prevalence of SCM increased with an increase in milk yield as follows: from 38.8% (95% CI 20.7, 58.4) among animals that yielded less than 7 litres to 48.5% (95% CI 31.0, 66.2) among animals that yielded 7 to 15 litres, and it was the highest among cattle that yielded more than 15 litres (59.6%, 95% CI 37.7, 79.6). Cattle of parity ≥ 7 (63.9%; 95% CI 50.2, 76.6%) had a higher prevalence of SCM than those of parity 4 to 6 (58.3.8%; 95% CI 50.0, 66.3%) and 1 to 3 (39.5%; 95% CI 31.8, 47.5%) (**Table 3.2**).

Table 3.2: Analysis of herd- and animal-level exposure factors for prevalence of sub-clinical mastitis among cattle in Africa from 2010 to 2020

Variable	Level	No. of datasets	Cattle tested by CMT			Heterogeneity			Univariate meta-regression (test for moderator association with sub-clinical mastitis)			
			No. tested	No. positive	% (95% CI)	χ^2	p-value	I^2 (%)	p-value	τ^2 , H^2	Coefficient QM	R^2 (%)
Age									<0.0001	0.045, 27.2	26.2 (df=2)	24.3
	2 to 5 years	31	4524	1587	33.9 (26.8, 41.6)	557.1	<0.01	96.5				
	6 to 9 years	32	5962	2779	51.4 (43.8, 58.9)	991.7	<0.01	97.2				
	>9 years	17	1161	754	67.6 (56.9, 77.5)	208.8	<0.01	96.8				
	Total					2191.3	<0.0001	96.3				
Breed									0.0002	0.033, 20.5	17.3 (df=2)	21.5
	Crossbreed	26	4824	2349	50.2 (42.9, 57.5)	519.4	<0.01	96.1				
	Exotic	13	2268	1223	59.3 (49.1, 69.1)	280.8	<0.01	95.8				
	Local	24	2447	823	33.5 (26.1, 41.3)	361.7	<0.01	95.2				
	Total					1407.8	<0.01	95.1				
Lactation stage									0.019	0.04, 19.6	7.8 (df=2)	5.9
	Early	36	4236	1978	48.4 (41.5, 55.4)	663.6	<0.01	95.2				
	Mid	33	4096	1813	46.8 (39.6, 53.9)	643.1	<0.01	95.1				
	Late	35	3380	1988	59.9 (52.8, 66.7)	324.1	<0.01	94.9				

CI: Confidence intervals. 95% CIs were calculated as described by Higgins and Thompson (2003).
 I^2 (%): Residual heterogeneity (unaccounted heterogeneity)
 R^2 : Proportion of between-study variance explained
 QM : Coefficient of test for heterogeneity between subgroups; df = degrees of freedom
 τ^2 (tau squared) = between-study variance - estimated variance of the distribution of true prevalence in the population of sub-clinical mastitis studies
 H^2 = unaccounted variability (sampling variability) – the ratio of the standard deviation of the estimated overall prevalence from the random-effects model compared to the standard deviation from the fixed-effects model.
CMT = California mastitis test

3.3.7 Comparison of prevalence of bovine SCM among udder quarters

Udder level data were available for only 20 studies, and the analyses showed no significant difference in the prevalence of SCM among the four quarters (RF, LF, RH and LH [$F=0.054$, $p=0.983$]). The mean prevalence and standard error were 41.1 ± 4.4 for the LF, 43.9 ± 4.6 for RF, 42.0 ± 4.6 for LH, and 47.4 ± 5.4 for RH.

3.3.8 Bacterial isolates

The proportion of cattle that had tested positive for bacterial isolates *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp., *Escherichia* spp., and *Pseudomonas* spp. were assessed separately for each pathogen genera across the various studies (16 to 61 studies). There was moderate to high heterogeneity across studies for each of the isolates ($I^2=67.3$ to 98.4%), and there was variation in PPE across these isolates (**Table 3.3**). The highest summarized prevalence was recorded for *Staphylococcus* spp. (43.7%) followed by *Streptococcus* spp. (18.2%), while the lowest was for *Pseudomonas* spp. (4.3%) (**Table 3.3**).

Table 3.3: Pooled prevalence estimate analysis of bacterial isolates from bovine sub-clinical mastitis cases among cattle in Africa from 2010 to 2020.

Pathogen genus	No. of studies	Total cultures	No. of isolate cases	Prevalence ^a % (95% CI)	95% prediction interval	Heterogeneity			
						Cochran Q statistic	p-value	I ² (%)	τ ² , H ²
<i>Staphylococcus</i>	61	15496	5364	43.7 (37.7, 49.9)	5.9, 86.7	3678.1	<0.0001	98.4 (98.2 to 98.5)	0.056, 61.3
<i>Streptococcus</i>	42	9270	1504	18.2 (13.8, 23.1)	0.08, 53.7	1549.9	<0.0001	97.4 (96.9 to 97.7)	0.036, 37.8
<i>Klebsiella</i>	18	4614	183	4.3 (2.7, 6.3)	0.03, 13.4	127.4	<0.0001	86.7 (80.4, 90.9)	0.0062, 7.5
<i>Escherichia</i>	36	7294	826	9.5 (6.7, 12.8)	0.0, 33.3	686.3	<0.0001	94.9 (93.8 to 95.8)	0.022, 19.6
<i>Pseudomonas</i>	16	3688	202	4.2 (2.8, 5.8)	0.6, 10.1	45.9	<0.0001	67.3 (44.9, 80.6)	0.003, 3.1

CI: Confidence interval

Meta-analysis performed using mixed effects (both random and fixed) models.

3.4 Discussion

Bovine mastitis is one of the most significant threats to dairy animals and is an expensive disease to control. This review was undertaken to investigate the prevalence of and risk factors for SCM at individual cow and quarter level. Prior to this study, assessment of the overall prevalence of mastitis, especially SCM, in dairy cattle on the African continent had been scant, and it was argued that this gap in research could compromise the implementation of specific strategies to prevent and control this disease.

The review indicated that heterogeneity was high, and this affirms the notion that various factors are responsible for the occurrence of sub-clinical mastitis. The I^2 (ratio of true to total variance) values were high for SCM prevalence, or moderate to high for the different bacterial isolates, suggesting a moderate to high standard deviation of observed prevalence across studies compared to the mean standard error from individual studies, and therefore a high level of uniqueness of each study prevalence (little overlap across confidence intervals) was evident. The minimal overlap in confidence intervals is evidence that prevalence varied from one cattle population (or herd) to another, and that the underlying differences were genuine and not due to chance. The high dispersion in prevalence across studies can be attributed to a variety of factors, such as the genetic make-up of the cows, parity, sanitation practices, dry cow therapy, nutrition, hygiene, and proportion of cows in early or late lactation in between-study phases.

Based on the review data, the weighted pooled prevalence estimate (PPE) of SCM (48.2%) was similar to that of the 45% global prevalence reported by Krishnamoorthy *et al.* (2021) as well as the rate reported for North America (46%) and Asia (42%); however, it was higher than the rate reported for Bangladesh (29.5%) (Islam *et al.*, 2011), Europe (37%), Oceania (36%), and Latin America (34%) (Krishnamoorthy *et al.*, 2021). On the other hand, the prevalence value was lower than the value reported previously for Malaysia (82%) (Krishnamoorthy *et al.*, 2021).

Subgroup analysis showed that the prevalence of SCM differed significantly across geographical regions, which may be attributed to differences in management practices and emphasis by farmers and veterinarians regarding disease control. However, only a few published studies were conducted in West Africa, North Africa, and southern Africa, which complicated comparative information. Among the African countries that were studied to report

on the prevalence of SCM in dairy cows, about half were conducted in Ethiopia which is a notoriously poverty-stricken country, and this may indicate great investment in livestock disease research as well as a significant problem in dairy animals in this country due to the poor economic status of dairy farmers.

Across all the studies, the prevalence of SCM was significantly linked to age, breed, lactation stage, and parity. Prevalence was higher in cows of old age, which can be attributed to poor teat canal integrity due to ageing as this may allow easy access of bacterial infection to the mammary gland after milking. Moreover, pendulous udders, which are more prone to injury and entry by pathogens, are more common in older cows than younger cows, and this may result in increased susceptibility of the former to mastitis (Ndahetuye *et al.*, 2019). The review revealed an increase in SCM with an increase in the number of parities. The higher prevalence of SCM in cows with more than three parities could be attributed to diminished immunity, or resistance of mastitis-causing bacteria to treatment caused by the indiscriminate use of antimicrobials for the treatment of mastitis in previous parities/lactations (Girma and Tamir, 2022). Numerous studies that investigated the risk factors for mastitis (Sarker *et al.*, 2013; Oliveira *et al.*, 2015), including those that focused on smallholder farmers (Kivaria *et al.*, 2007), consistently showed that multiparous cows had a higher risk of mastitis than primiparous cows.

The review also revealed a higher likelihood of SCM in later lactation stages than in earlier ones. This is in agreement with findings by studies that focused on dairy farms in other parts of the world, for example Brazil (Silva *et al.*, 2021; Cardozo *et al.*, 2015) and Nepal (Khanal and Pandit, 2013). The reason for the higher prevalence of mastitis at the end of lactation may be related to accumulated exposure to infectious microorganisms (i.e., cumulative infection) during the various lactation stages (Almaw *et al.*, 2008). Moreover, the effect of lactation stage on SCM can be related to the accumulation of chronic infections that may not have been identified during early lactation stages (Silva *et al.*, 2021).

The review also indicated that the prevalence of SCM increased with the age of the sampled cattle, which is a finding that is corroborated by Kayesh *et al.* (2014) whose study was conducted in Bangladesh. In the latter study, the highest SCM prevalence was recorded for the age group 9 to 12 years. Increase in prevalence in conjunction with age can be attributed to the

weakening or deterioration of the sphincter muscles following aging of the udder tissue and vaginal canal walls (Kayesh *et al.*, 2014; Tiwari *et al.*, 2022).

SCM was most prevalent in exotic breeds in Africa, such as Jersey and Holstein-Friesian, followed by exotic X indigenous zebu with the lowest rates occurring in indigenous zebu breeds. Breed variability as a factor in susceptibility to mastitis in dairy cattle was studied by Argaw (2016), Bangar *et al.* (2015), Hoque *et al.* (2018), and Bi *et al.* (2016). It has been argued that exotic breeds, such as Jersey and Holstein-Friesian, have larger udder sizes and that the genetic make-up of their teat canal muscles and keratin prevalence therefore increase their vulnerability to infection (Souto *et al.*, 2008; Bi *et al.*, 2016). However, the significance of genetic variability is often diluted by environmental variations. In Africa, exotic dairy animals are often reared under a zero grazing system but with intensive management practices, which predisposes these cows to mastitis compared to indigenous animals that are reared under an extensive grazing system. Given the superior milk production of exotic animals to local breeds, the higher susceptibility of the former to mastitis is likely to pose a challenge in the improvement of host resistance to mastitis through breeding (Biffa *et al.*, 2005).

Dispersion was observed among the studies for the different bacterial isolates assessed, and moderate to high heterogeneity values were revealed. *Staphylococcus* spp., followed by *Streptococcus* spp. and *Escherichia* spp., were the most prevalent pathogens associated with mastitis in Africa, which is consistent with a report by Rana *et al.* (2022) who conducted their study in Uruguay. The detection of a high prevalence of *S. aureus* suggests that transmission may have occurred during milking. The common practice of hand milking and the lack of dry cow therapy by farmers who rear dairy herds may contribute to the long-term transmission of contagious pathogens. *S. aureus* and other contagious microorganisms, such as *S. agalactiae*, are commonly found in teat canals, on teat or udder skin, and in infected udders (Schnitt and Tenhagen, 2020), and these organisms are the most common source of infection between infected and uninfected udder quarters as well as between infected and uninfected cows. This cross-infection usually occurs during milking. Even though farmers may apply hygienic practices such as udder washing, drying, and post-milking teat dip, these practices alone may not reduce the challenge that contagious mastitis pathogens pose because these pathogens, particularly *S. aureus*, are widely prevalent among cows in dairy herds. Furthermore, antibiotic therapy for *S. aureus* infections during lactation has a low cure rate, and therefore dry cow therapy and culling of chronically infected cows should be used. Pre- and post-milking teat

disinfection should also be improved to slow the spread of both contagious and environmental pathogens.

The review indicated that *Escherichia coli* was the most commonly isolated coliform species in the studies that were reviewed. This pathogen is a significant public health threat because it causes diarrhoea in humans (Jang *et al.*, 2017). Coliforms, that cause environmental mastitis, are primarily found in moisture, mud, faeces, and other organic matter around dairy animals. Poor hygiene, husbandry practices, and milking techniques may also increase the risk of environmental mastitis and milk contamination (Jang *et al.*, 2017). These Gram-negative bacteria can enter the mammary gland through the teat canal. One study that was reported in in South Africa showed that regular teat dipping for mastitis control was unfortunately not a common practice among small-scale farmers (Petzer *et al.*, 2018).

The high prevalence of SCM that was evident in the review, in addition to its serious economic impact, long duration, and limited obvious clinical manifestation, are points that need to be emphasised when control strategies are considered. Sub-clinically affected cows are a continuous source of infection for herd mates, and therefore there is a need for high sensitisation among farmers about the substantial losses that will be incurred due to SCM, and also the possible control measures they could apply.

Many regions on the African continent could not be evenly represented in the reviewed studies, and this was one of the limitations of this review. This uneven representation may be attributed to limited research and/or limited online publication of data from most African countries. This lapse made it difficult to assess the true status of bovine mastitis in Africa. There was also incomplete or a lack of data on other potentially important predictors, such as housing and hygiene practices.

3.5 Conclusion

There was relatively high PPE for SCM mastitis among cattle in Africa. Predisposing factors for SCM were age, breed, parity, and lactation stage of cattle. There was also a significant effect of geographical area on the prevalence of SCM. These findings may facilitate decision-makers in their efforts to develop effective prevention and control strategies against mastitis. Clearly, more research on bovine mastitis in most African nations is still required. There is a need for timely and effective diagnosis and therapeutic measures by field veterinarians as well

as the scientific management of dairy farms if the prevalence of mastitis in Africa is to be curbed and ultimately prevented.

3.6 References

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CHAPTER FOUR

CHARACTERIZATION OF MILK MICROBIOTA FROM SUBCLINICAL MASTITIS AND NON-SUBCLINICAL MASTITIS DAIRY CATTLE²

ABSTRACT

Inflammation and infection of bovine mammary glands, commonly known as mastitis, results in significant losses each year in the dairy industry worldwide. This infection is characterized by physical, chemical, and biological changes in the udder and milk. While several different bacterial species have been identified as causative agents of mastitis, many subclinical mastitis (SCM) cases remain culture negative. The aim of this phase of the larger study was to characterize milk microbiota from subclinical mastitis and apparently healthy dairy cows by 16S rRNA sequencing. All downstream analyses were performed using applications in R Studio (v3.6.1). Alpha-diversity metrics showed significant differences between SCM-infected cows and their healthy counterparts. The beta-diversity metrics on Principal Coordinate Analysis significantly clustered samples by type (PERMANOVA test, $P < 0,05$), while Non-Metric Dimensional Scaling did not (PERMANOVA test, $p=0.07$). Overall analysis revealed a total of 95 phyla, 33 classes, 82 orders, 124 families, 202 genera, and 119 bacterial species in the analysed samples. Four phyla, namely Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria collectively accounted for more than 97% of all sequencing reads from SCM and healthy cow milk samples. The most abundant bacterial classes were Actinobacteria, Bacilli, Bacteroidia, Clostridia, and Gammaproteobacteria in healthy cow samples, whilst SCM cow samples mainly contained Actinobacteria, Alphaproteobacteria, Bacilli, Clostridia, and Gammaproteobacteria. The dominant bacterial species in healthy cow samples were *Anthropi* spp., *P.s azotoformans*, *P. fragi*, *A. guillouiae*, *E. italicus*, and *Lactococcus lactis*, while *P. azotoformans*, *M. bovis*, *P. fragi*, *A. guillouiae*, and *P. koreensis* were dominant in SCM cow samples. To conclude, the current study observed differences in bacterial species between SCM and healthy cow's milk which proved to be quite diverse, hence the need for more epidemiological studies is recommended.

² Published in *Veterinary Sciences Journal*

Key words: Subclinical mastitis, microbiota, metabarcoding, cattle milk.

4.1. Introduction

Mastitis is a mammary gland irritation in female bovines and it is recognized as a significant illness that has a global impact on the dairy sector (Hagnestam-Nielsen and Ostergaard, 2009). The sudden onset of this infection is mainly caused by the entrance of pathogenic bacteria into the mammary gland, leading to the rupture of the gland's physical barriers. This necessitates immediate and effective host defenses to prevent colonization and further disease pathology (Aitken, Corl and Sordillo, 2011). The latter authors also note that when the illness advances, dysbiosis of the milk microbiome occurs, and this milk is characterized by an increasing number of pathogenic bacteria and fewer commensal bacteria. Until recently, the description of microbiota associated with mastitis relied on the discovery and isolation of a single bacterium (Hoque *et al.*, 2019).

If the condition is caused by epidemiologically distinct groups of microbes, it might be classed as infectious or environmental mastitis depending on the causal agents (Kateete *et al.*, 2013). *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Mycoplasma* spp., and *Corynebacterium bovis* are among the organisms that cause contagious mastitis (Falentin *et al.*, 2016), but *Escherichia coli* is the most common cause of environmental mastitis (Levison *et al.*, 2016). Despite the extensive understanding of bacteria that cause mastitis in cows, Lin *et al.* (2016) emphasizes that additional microbes such as archaea, viruses, and fungi may be involved in the disease process, arguing that these should be explored as well (Rinaldi, Li and Capuco, 2010). Furthermore, because this illness is produced by a range of microbial species and there is a possibility of antibiotic resistance, it is preferable to accurately identify the responsible species in order to treat the sickness (Pyorala, 2009).

The identification of these causative agents was accomplished using microbiological culture technique, which is a common technology in veterinary medicine (National Mastitis Council, 2001; Lago *et al.*, 2011). Despite, the fact that the microbiological culture of causative agents does not always result in bacterial growth, molecular approaches can enhance the detection of mastitis with high sensitivity and specificity (Phuektes *et al.*, 2003; Oultram *et al.*, 2017). Significant advancements in high throughput next generation sequencing (NGS) and bioinformatics techniques have facilitated the transition from culture-based methods to genomic sequence-based characterization of the microbiome associated with mastitis infection

in both humans and animals (D'Argenio *et al.*, 2014; Cremonesi *et al.*, 2018). Amongst these NGS approaches, 16S rRNA gene sequencing has remained the most extensively utilized technique in characterizing mastitis microbiota in recent years (Catozzi *et al.*, 2017).

4.2 Materials and methods

4.2.1 Study design and setting

This was a cross-sectional study which was conducted on samples of milk obtained from seven (7) small-scale farms in three local municipalities, namely Mantsopa (Latitude: 29.1283°S, Longitude: 27.2676°E), Setsoto (Latitude: 28.5302°S, Longitude 27.6435°E), and Maluti-a-Phofung (Latitude: 28°16'21.94"S, Longitude: 29°07'46.06"E). These are managed by the Thabo Mufutsanyana District Municipality in the Free State Province of South Africa (**Figure 4.1**).

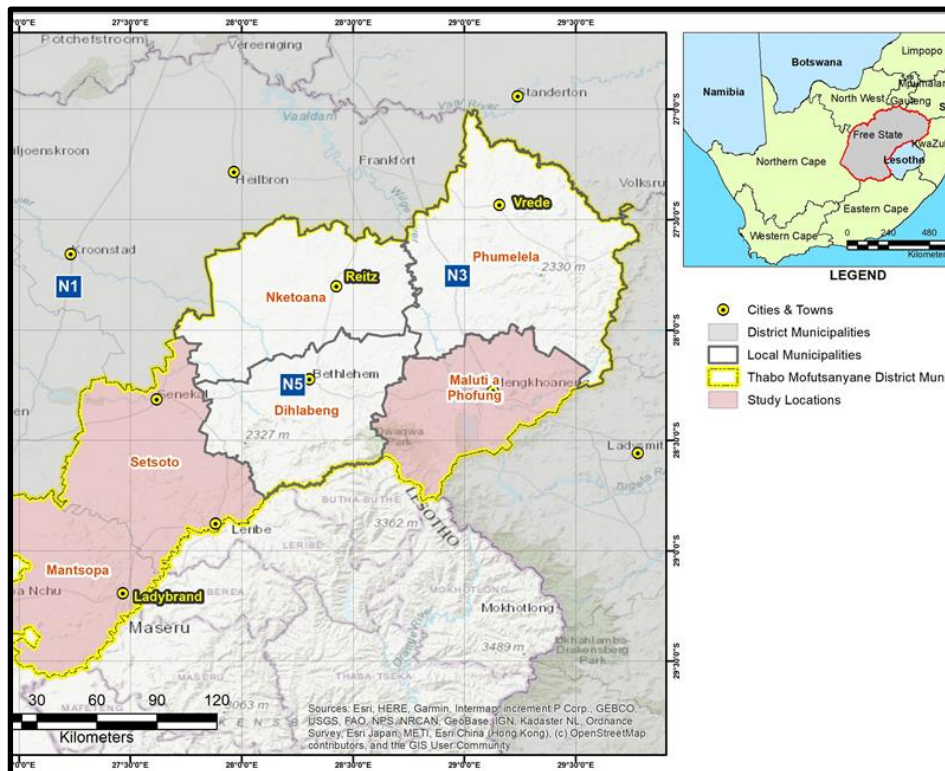


Figure 4. 1: Map of the Free State Province. The study region is indicated in pink.

4.2.2 Study population and sampling strategy

Samples were collected using normal techniques on selected farms. Milk samples were collected at the same time of day (early in the morning) following the customary pre-milking udder preparation by the farmer or farm workers and before connection of the milking machine. Before milk sample collection, the udders of the cows were washed with distilled water and dried with disposable paper towel to prevent any cross contamination. Thereafter, to ensure that milk samples were collected aseptically, 70% ethanol was applied to each udder before pure milk samples were collected using 50 mL sterile Falcon tubes. The initial milk streaks were discarded. A total of 166 composite milk samples from individual cows were randomly screened for intramammary infection by means of somatic cell count (SCC) assay using flow cytometry from an outsourced company (Mérieux NutriSciences, South Africa).

Thereafter, based on the SCC results, only 220 individual quarters from 55 of 166 cows were subjected to the California mastitis test (CMT) according to the manufacturer's instructions (DeLaval, South Africa). One or two squirts of milk per quarter were collected in each paddle compartment after foremilk had been removed. The paddle was tilted to allow most of the milk to run out leaving about 1 to 2 teaspoons (5 to 10 mL) in each compartment. CMT reagent was added to each compartment in a volume equal to the retained milk. The milk reagent mixture was swirled in a circular motion and the visually detected presence of gel or slime was recorded for each quarter. It was the CMT reagent reacting with the DNA of the leukocytes that produced the measurable response in the paddle and subsequently only 160 quarter milk samples were collected in duplicate using sterile 50 mL Falcon tubes; i.e., one batch for another round of somatic cell count (SCC) assay was collected for the outsourced company (Mérieux NutriSciences, South Africa) while another batch was used for microbiological analysis. The CMT results were scored and interpreted as 0 (negative [healthy quarter], somatic cell count [SCC] $\leq 100,000$ cells/ml milk), 1+ (weak positive, $100,000 < \text{SCC} < 500,000$ cells/ml milk), 2+ (distinct positive, $500,000 < \text{SCC} < 1,000,000$ cells/ml milk), and 3+ (strong positive, $\text{SCC} \geq 1,000,000$ cells/ml milk) as recommended by Karzis *et al.*, (2017). Hence, samples that had SCC of $100,000 < \text{SCC} < 500,000$ cells/ml and CMT 1+ were considered to be subclinically infected while those that had SCC of $\leq 100,000$ cells/ml milk and CMT 0 were considered to be healthy.

4.3 DNA extraction

The Zymbiomics DNA Miniprep Kit (Zymo Research, California, USA) was used to isolate gDNA from samples for investigation following the manufacturer's instructions. In a nutshell,

the sample was placed in a ZR BashingBead™ Lysis tube and augmented with 750 µl ZymoBIOMICS™ Lysis solution. The solution was then placed in a bead beater equipped with a 2 ml tube holder assembly and centrifuged at 10,000 x g for 1 min. The supernatant (400 µl) was then transferred to the Zymo-Spin™ III-F filter in a collection tube and centrifuged for 1 min at 8,000 x g. The filtrate in the collecting tube was then mixed with 1,200 µl of ZymoBIOMICS™ DNA Binding Buffer. Afterwards, 800 µl of the mixture was transferred to a Zymo-Spin™ IICR Column in a collection tube and centrifuged for 1 min at 10,000 x g. The flow-through from the collection tube was discarded, and step 6 was repeated. A 400 µl ZymoBIOMICS™ DNA Wash Buffer 1 solution was poured into the Zymo-Spin™ IICR Column in a fresh collection tube and spun at 10,000 x g for 1 min before discarding the flow-through. The flow-through was removed after 700 µl ZymoBIOMICS™ DNA Wash Buffer 2 had been poured into the Zymo-Spin™ IICR Column in a collection tube and spun at 10,000 x g for 1 min. In a collection tube, 200 µl ZymoBIOMICS™ DNA Sash Buffer 2 was applied to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube, and 100 µl (50 l minimum) ZymoBIOMICS™ DNase/RNase free water was added directly to the column matrix and incubated for 1 min before centrifugation at 10,000 x g for 1 min to elute the DNA. The Zymo-Spin™ III-HRC filter was inserted into a new collection tube, and 600 µl of ZymoBIOMICS™ HRC prep solution was added. The sample was centrifuged for 3 minutes at 8,000 x g. Finally, the eluted DNA (Step 11) was transferred to a clean 1.5 ml microcentrifuge tube containing a prepared Zymo-Spin™ III-HRC filter and centrifuged at exactly 16,000 x g for 3 min before being kept at -70 C° until analyzed by PCR.

4.3.1 Evaluation of the quantity and quality of isolated DNA

The gDNA extracted from samples was quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and the 1X dsDNA HS assay (ThermoFisher, Waltham, Massachusetts, USA). The purity of the DNA was evaluated using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer's absorbance ratio at 260 and 280 nm (A260/A280) (Thermo Fisher Scientific).

4.3.2 16S rRNA gene amplification and sample barcoding

The diversity of bacterial communities in the milk samples collected at the selected farms were analysed using single molecule real-time PacBio sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). Full-length 16S rRNA gene was amplified from genomic DNA using bacterial-specific primer 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3' as advised by Pootakham *et al.* (2017). To allow

multiplexing of amplicons, the 5'-ends of the 16S rRNA forward and reverse primers were tagged with the universal M13F (TGTAACGACGGCCAGT) and M13R (GGAAACAGCTATGACCATG) sequences respectively. Furthermore, 5'-block (5'-NH₄-C6) was added to 16S-specific primers to ensure that carry-over amplicons from the first round PCR were not ligated to the SMRTbell adapters in subsequent steps. A set of five barcoded M13F and five barcoded M13R primers were designed to generate PacBio sequencing-ready amplicons from 16S rRNA target sequence flanked by M13 universal overhangs. All primers were synthesised and HPLC-purified (according to PacBio's SMRT sequencing recommendation) by Integrated DNA Technology (San Jose, CA, USA). First rounds of PCR were performed using M13-tagged 16S-specific forward and reverse primers in a final volume of 25 µl (NEB Q5 HotStart MasterMix) consisting of 12.5 µl Q5 High-Fidelity 2X Master Mix, 1.25 µl of each 10 µM forward and reverse primers, 3 µl of DNA template, and 7 µl nuclease-free water.

The PCR conditions were as follows: pre-incubation at 98°C for 2 min, followed by 10 cycles of denaturation at 98°C for 30 s, annealing at 66°C for 15 s, elongation at 72°C for 45 s, and 10 cycles of denaturation at 98°C for 30 s, annealing at 68°C for 15 s, elongation at 72°C for 45 s, and a final extension step at 72°C for 5 min. Prior to the second PCR amplification, PCR products from the first round were loaded on an agarose gel for visual inspection using the Agilent 2100 Bioanalyzer System. Thereafter, the second PCR amplification was run using the same conditions. Barcoded 16S rRNA amplicons obtained from the secondary PCR were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using a Qubit® 2.0 Fluorometer and a Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Purified amplicons were then pooled in equimolar concentrations, and 500 ng of DNA was used for library preparation. Two PacBio libraries were constructed, each containing a pool of barcoded amplicons from nine samples.

4.4 PacBio sequencing

SMRTbell libraries were created using the SMRTbell™ Template Prep Kit 1.0 (Pacific Biosciences, CA, USA) following instructions in the protocol "Procedure & Checklist - Amplicon Template Preparation and Sequencing" (part number 100-801-600-04). Briefly, the quantity and purity of each barcoded amplicon were assessed using Qubit® Fluorometer (Invitrogen, Carlsbad, USA) and NanoPhotometer® NP80 (Implen GmbH, Munich, Germany) respectively. Amplicon samples were pooled at equimolar concentrations and purified with

AMPure® PB beads (Pacific Biosciences, CA, USA). Quality assessment (concentration, purity, and size confirmation) was performed for each purified amplicon pool using the Qubit® Fluorometer (Invitrogen, Carlsbad, USA), NanoPhotometer® NP80 (Implen GmbH, Munich, Germany) and Agilent 4200 TapeStation (Agilent Technologies, CA, USA) respectively. DNA damage repair, end-repair, ligation of blunt adapter, and purification were performed according to the manufacturer's instructions (Pacific Biosciences, CA, USA). The resulting SMRTbell templates were bound to the sequencing primer and Sequel polymerase using PacBio's Sequel® Binding Kit 2.1 according to the manufacturer's instructions. Each SMRTbell complex was loaded on Sequel® SMRT Cell 1M v2 with 600 min on the PacBio Sequel System. Sequencing was done using the Sequel® Sequencing Kit 2.1 (Pacific Biosciences, CA, USA) with an on-plate loading concentration of 4 pM.

4.5 Sequencing analysis

The Divisive Amplicon Denoising Algorithm 2 (DADA2) analysis workflow (Callahan *et al.*, 2016), implemented in R (v3.6.1) within R studio, was used to analyse raw amplicon sequencing data generated using the PacBio Sequel System (Pacific Biosciences, CA, USA). Briefly, raw amplicon data were initially processed using the PacBio SMRT Link software (v 7.0) to generate consensus circular sequence (CCS) reads in FASTQ format. As part of the DADA2 workflow, primer sequences were filtered, and the PacBio CCS reads were subjected to quality control following the standard DADA2 pre-processing steps for PacBio CCS reads (<https://www.biorxiv.org/content/10.1101/392332v2.full>). Inference of the amplicon sequence variants (ASVs) was performed using the DADA2 method. Taxonomy and ASV abundances were determined and assigned using DADA2-formatted training sequence sets based on the SILVA reference database (v138; <https://zenodo.org/record/4587955#.Y2F0p3ZBw2w>).

All downstream analyses, including abundance bar plots, alpha and beta diversity ordinations, richness and differential abundance estimates, statistical analyses, and visualizations, were done using Phyloseq (v1.28.0) (McMurdie and Holmes, 2013), ggplot2 (v3.2.1; Wickham, 2011), AmpVis2 (v2.6.4; Andersen *et al.*, 2018), and DESeq2 (v1.24.0; Love *et al.*, 2014) packages in R (v3.6.1). Group level significant differences in NMDS ordination plots were assessed using PERMANOVA (permutation test with pseudo-F ratios) as implemented in the adonis function in the Vegan package (<https://github.com/vegan-devs/vegan>). To compare alpha diversity among groups, Kruskal-Wallis rank-sum tests were performed. UpsetR (v1.4.0) and the Venn method (v1.6.20) was used to generate Venn diagrams (Conway *et al.*, 2017).

4.6 Results

4.6.1 Somatic cell counts (SCC)

The findings of this study revealed that, out of 166 cows evaluated for subclinical mastitis, only 55 (33.13%) had SCC $>100,000 < 500,000$ cells/ml milk and were thought to have intramammary infection/subclinical mastitis at a cow level, based on the criterion established by the National Mastitis Council's recommendations and Karzis *et al.*, (2017).

4.6.2 Sequence analyses

A total of 499544 (minimum = 9152; mean = 24977.2; maximum = 48172) CCSs of 10 raw milk samples from cows that seemed to be in good condition and 10 samples from cows that had subclinical infection were used to produce a total of 209357 (minimum = 3869; mean = 10467.85; maximum = 25205) non-chimeric reads. Through DADA2, the readings from the analysed sample groups were combined into 883 unique ASVs at the Kingdom (bacteria) level. A recognized phylum could be ascribed to at least 98,8% of the sequences (**Appendix T1**).

4.6.3 Taxonomic profile

The SMRT sequencing of the full-length 16S rRNA gene was performed to obtain accurate bacterial profiles of raw milk associated with subclinical mastitis in comparison to bacterial profiles of healthy raw milk. The ASVs were observed from 17 bacterial phyla with an average relative abundance of 5.8%. It was found that four of these phyla (Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria) collectively accounted for more than 97% of all sequencing reads of infected and healthy samples (**Figure 4.2**).

Further analysis of the core microbiome showed that the most abundant bacterial classes in non-subclinical mastitis samples were Actinobacteria, Bacilli, Bacteroidia, Clostridia, and Gammaproteobacteria, while in SCM samples they were Actinobacteria, Alphaproteobacteria, Bacilli, Clostridia, and Gammaproteobacteria (**Figure 4.3**). It is noteworthy that the reference sample showed quite a different taxonomic profiling compared to the healthy and subclinically-infected samples. The results showed that Kingdom classification was indeed bacteria, namely phylum Bacteroidota, class Bacteroidia, bacterial order Flavobacteriales, family *Weeksellaceae*, genus *Chryseobacterium*, and one specie was identified as *Chryseobacterium hominis* (**Table 4.1**).

Table 4.1: The taxonomic classification of reference sample UHT milk

otu	Kingdom	Phylum	Class	Order	Family	Genus	Species
ASV1	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	<i>Chryseobacterium</i>	<i>hominis</i>
ASV2	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	<i>Chryseobacterium</i>	<i>hominis</i>
ASV3	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	<i>Chryseobacterium</i>	<i>hominis</i>
ASV4	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	<i>Chryseobacterium</i>	<i>hominis</i>
ASV5	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	<i>Chryseobacterium</i>	<i>hominis</i>

The study further observed that the orders of bacteria were diverse in non-subclinical mastitis samples as they contained Enterobacterales, Flavobacteriales, Lactobacillales, Pseudomonadales, and Xanthomonadales, while the infected samples contained Corynebacteriales, Erysipelotrichales, Lactobacillales, Peptostreptococcales-Tissierellales, and Pseudomonadales (**Figure 4.4**). Subsequently, it was observed that both groups of samples comprised 15 bacterial genera respectively which, amongst others, included *Acinetobacter*, *Pseudomonas*, *Clostridium sensu stricto* 1 *Corynebacterium*, *Dietzia*, *Enterococcus*, *Kocuria*, *Lactococcus*, *Leuconostoc*, *Methylobacterium-Methylorubrum*, *Paeniclostridium*, *Romboutsia*, *Sphingomonas*, *Streptococcus*, and *Turicibacter* (**Figure 4.5**).

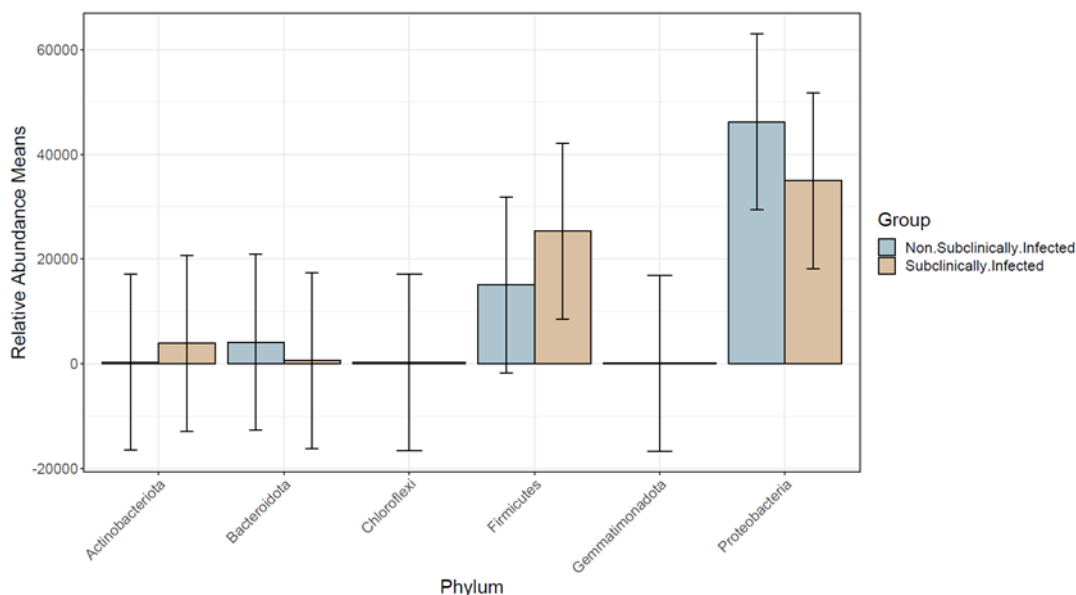


Figure 4.2: Distribution of four most abundant phylum in SCM and non-SCM milk samples.

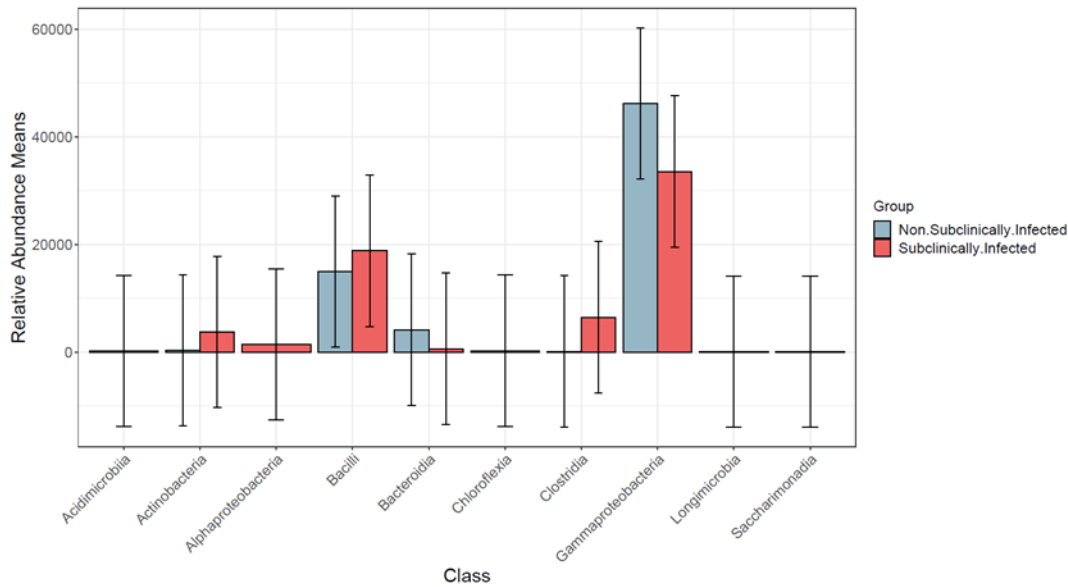


Figure 4.3: Distribution of five most abundant class in SCM and non-SCM cow milk samples.

Lastly, the sequences were analysed to species level, and it was found that both groups contained 15 dominant bacterial species that were diverse. Those found in healthy samples were: *Anthropi*, *P. azotoformans*, *P. fragi*, *A. guillouiae*, *E. italicus*, *L. lactis*, *P. lundensis*, *L. mesenteroides*, *C. otitidis*, *S. parauberis*, *psychrophile*, *P. putida*, *rhizophila*, *C. shigense*, *P. synxantha*, while *P. azotoformans*, *M. bovis*, *P. fragi*, *A. guillouiae*, *P. koreensis*, *L. lactis*, *P. lundensis*, *M. marinum*, *L. mesenteroides*, *S. parauberis*, *P. putida*, *L. raffinolactis*, and *P. synxantha* were found in subclinical mastitis samples (**Figure 4.6**).

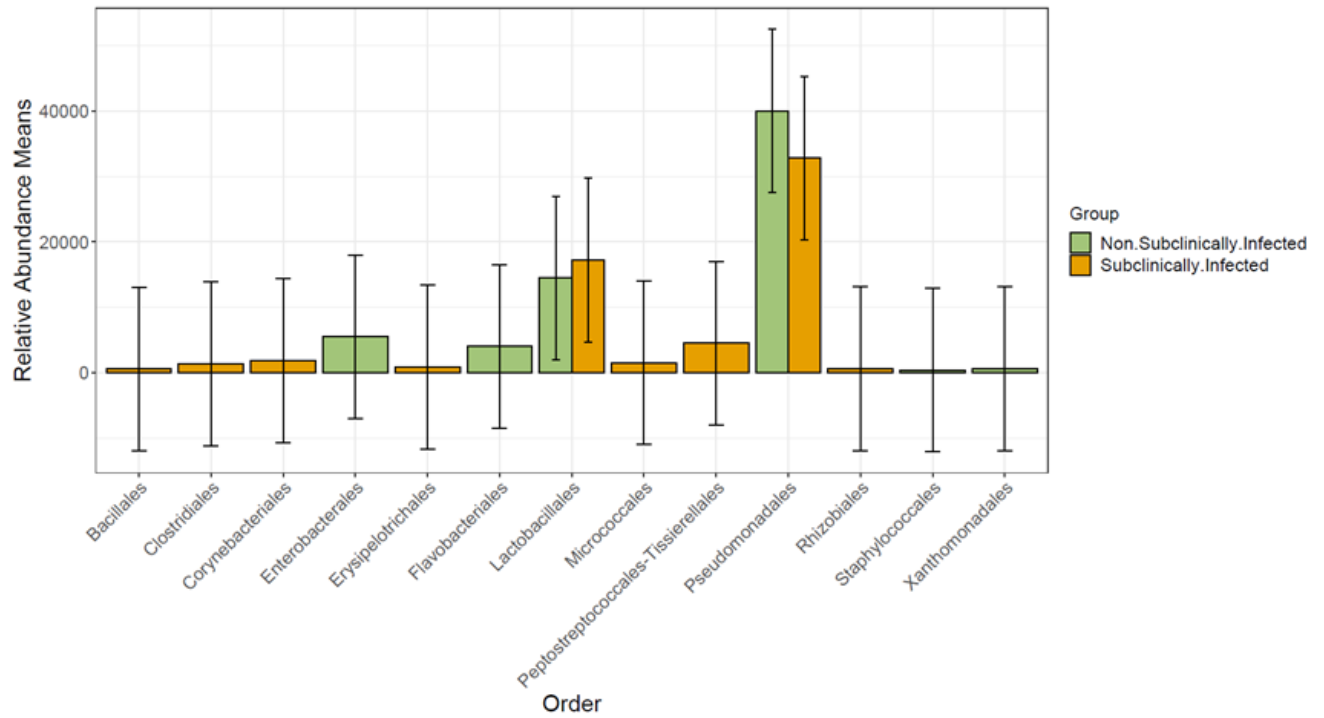


Figure 4.4: Distribution of five most abundant bacterial order in SCM and non-SCM cow milk samples.

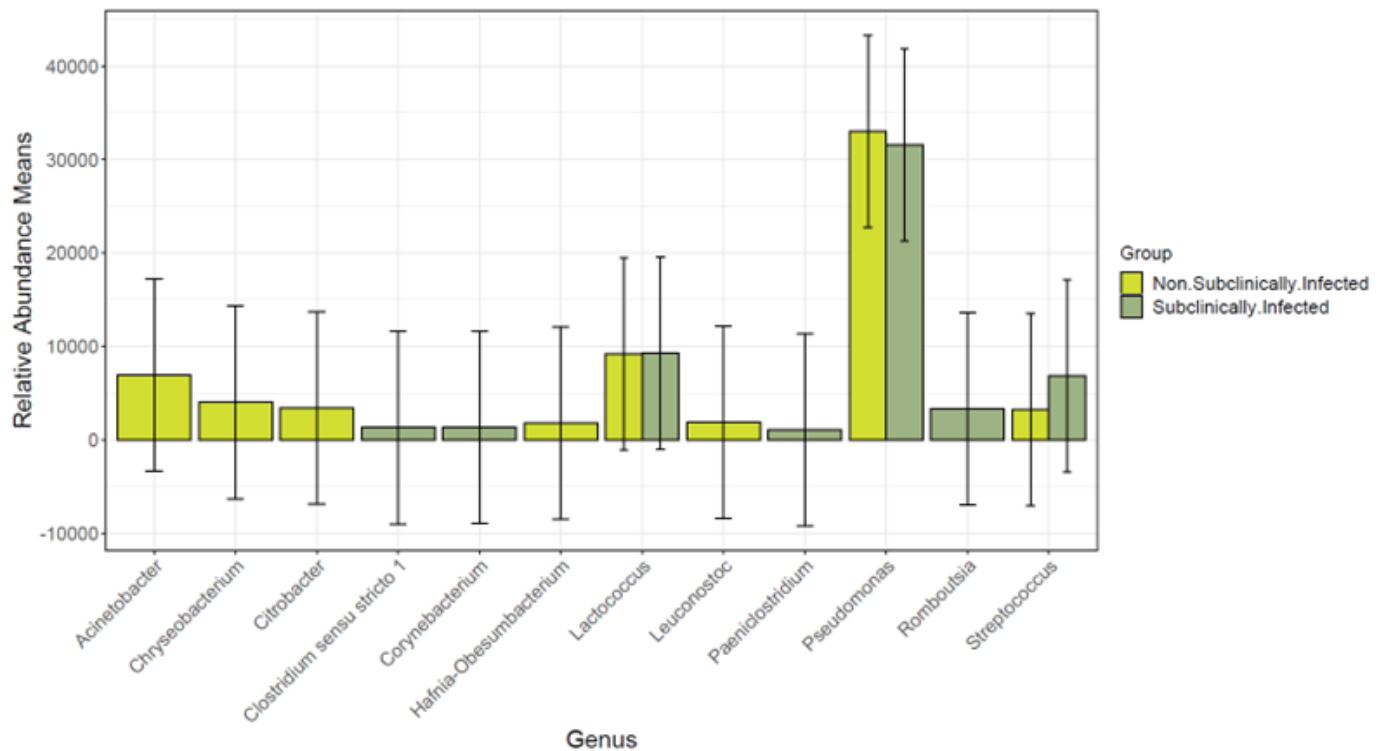


Figure 4.5: Distribution of fifteen most abundant genera in SCM and non-SCM cow milk samples.

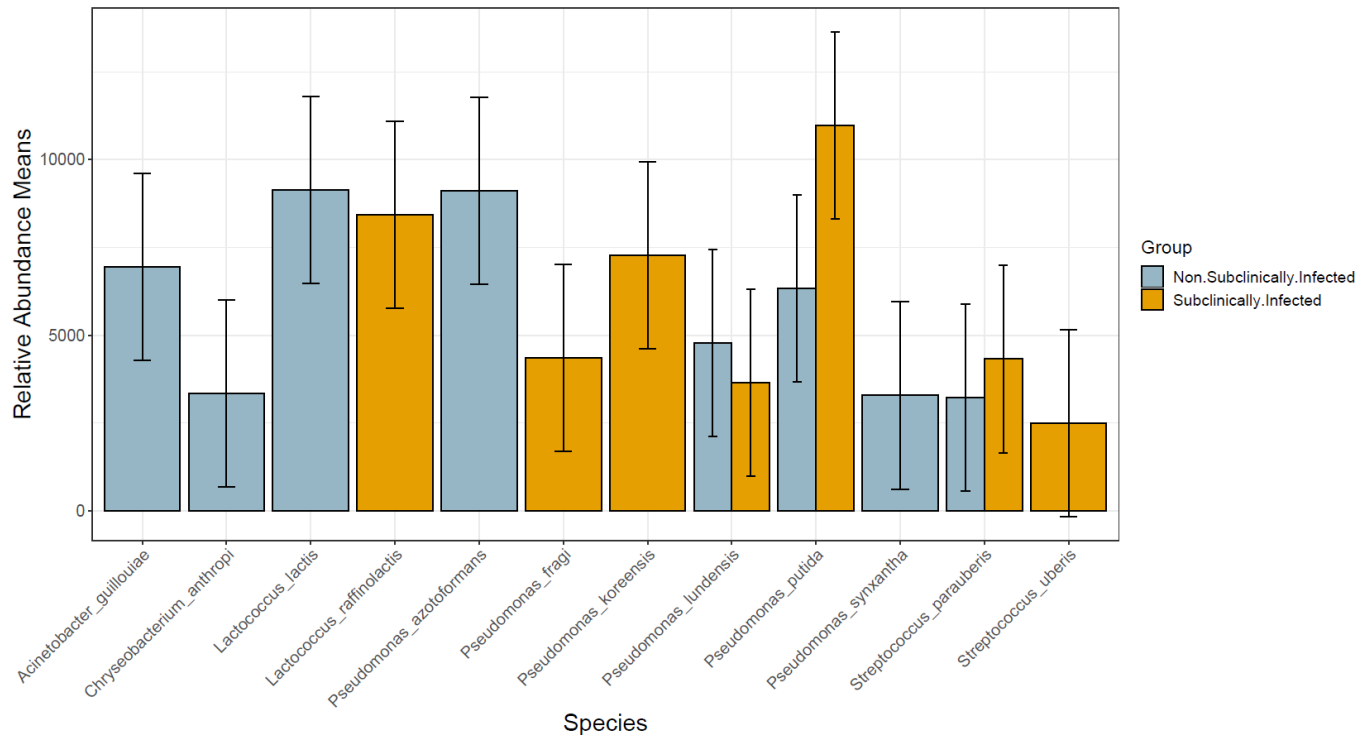


Figure 4. 6: Distribution of fifteen most abundant species in SCM and non-SCM cow milk samples.

4.6.4 Comparison of bacterial species and differential abundance in subclinical and non-subclinical mastitis samples

When examining the overlap in the bacterial species that had been identified, both groups of samples had 22 families in common. However, there were other families which were different in each group; the Non-SCM group shared three (3) families, while the SCM group shared 22 families (**Figure 4.7**). When examining the overlapping genera, the two groups had 28 genera in common. Separately, the subclinical mastitis samples shared 165 genera, while the Non-SCM cows shared 9 (**Figure 4.8**). When examining species composition, the SCM samples had 82 unique species, while the Non-SCM samples had 22. The two groups shared 31 species (**Figure 4.9**). When examining differential abundance between the healthy and SCM groups, all the samples had Firmicutes, Actinobacteriota, and Proteobacteria as dominant phyla. However, *Romboutsia*, *Corynebacterium*, *Pseudomonas*, and *Clostridium sensu stricto 1* were the most abundant genera while *Acinetobacter* was the least abundant.

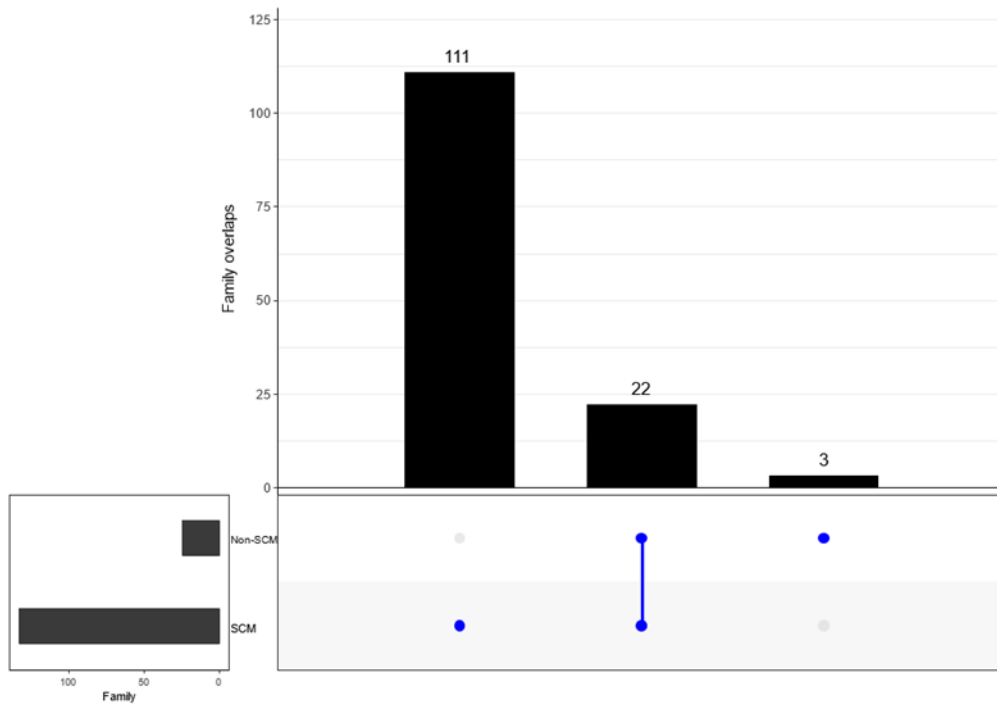


Figure 4. 7: UpSetR intersection plot showing number of unique and shared taxa at family level between the healthy and SCM groups.

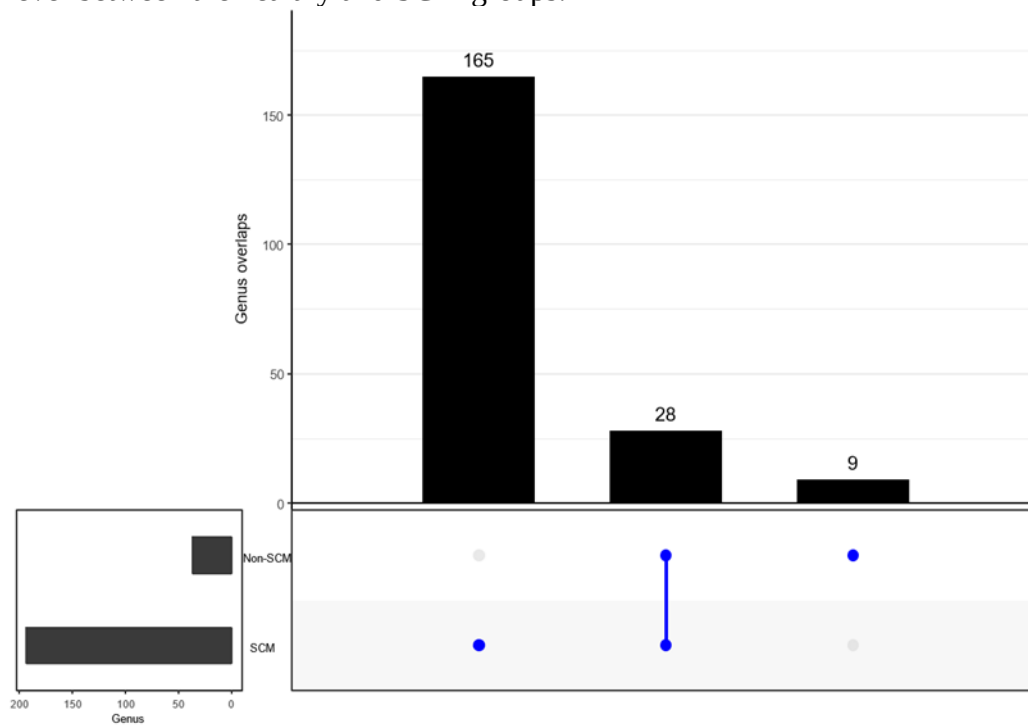


Figure 4. 8: UpSetR intersection plot showing the number of unique and shared taxa at genus level between the healthy and SCM groups.

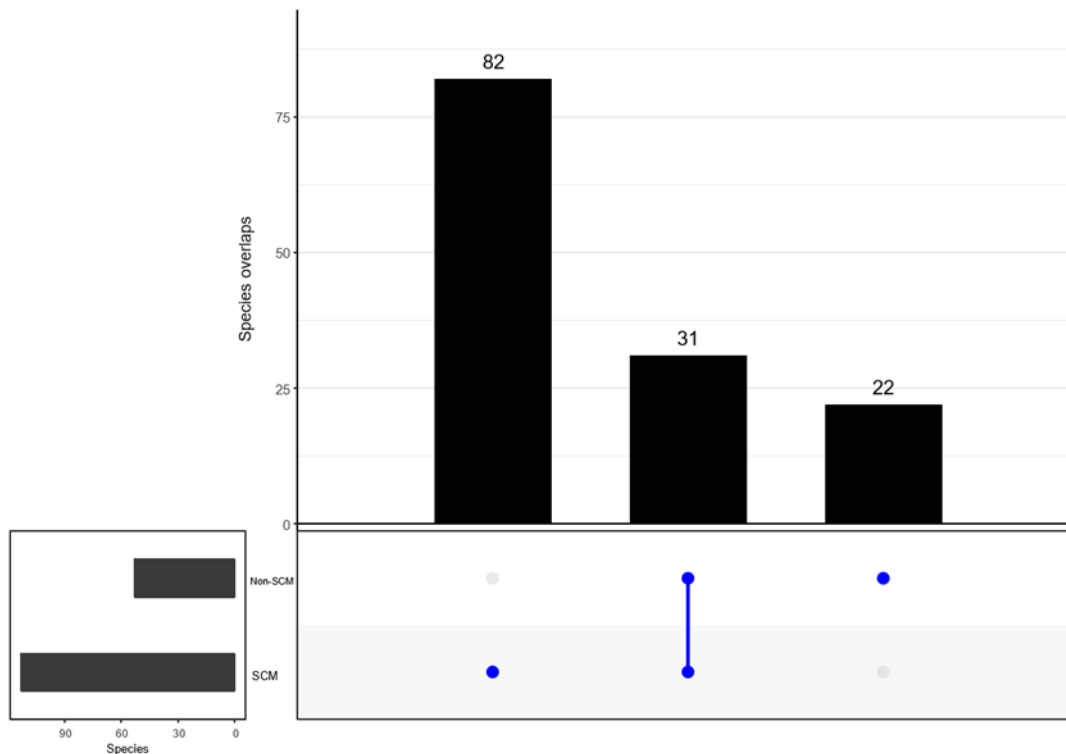


Figure 4.9: UpSetR intersection plot showing the number of unique and shared taxa at species level between the Non-SCM and SCM groups.

4.6.5 Alpha and beta diversity analyses

4.6.5.1 Alpha diversity analysis

When comparing the alpha diversity of the complete groups of samples, the species richness (Chao1 and ACE) and species evenness (Shannon and Simpson) indices indicated that SCM and Non-SCM cow milk samples were not significantly different. The Chao1 indices also did not indicate a significant difference between the non-SCM and infected samples (Kruskal-Wallis one-way ANOVA: $p > 0.47$) (**Appendix F1**). Similarly, the ACE indicators showed that there was no significant difference between the two groups (Kruskal-Wallis one-way ANOVA: $p > 0.28$) (**Appendix F2**). On the other hand, the overall Shannon diversity indices of the groups did not differ (Kruskal-Wallis one-way ANOVA: $p = 0.58$) (**Appendix F3**), while the Simpson's diversity was also not significantly different (Kruskal-Wallis one-way ANOVA: $p = 0.8$) (**Appendix F4**). The variation in the findings was confirmed by rarefaction analysis, and it was found that some of the non-SCM sample's diversity was the greatest in all samples.

4.6.5.2 Beta diversity analysis

For the purpose of this study, two β -diversity analyses were performed, and both had the s patterns. NMDS analysis showed that non-SCM and infected samples were not clustered together and were not significantly different from each other (PERMANOVA $p = 0.07$; $F =$

1.53) (**Appendix F5**). Principal coordinates analysis (PCA) confirmed the NMDS analysis indicating the (PERMANOVA) as $p = 0.05$ and $F = 1.53$ (**Appendix F6**).

4.7 Discussion

In general, mastitis research and the quest to discover and mitigate its causes remain important, yet the application of omics technology to find answers to this problem has been very limited (Sokolov *et al.*, 2021). SCM is an IMI that is caused by either underlying infections that do not heal in a timely manner or new infections that emerge during dry climatic conditions (Birhanu *et al.*, 2017). The presence of SCM in nursing cows has also been linked to the onset and progression of clinical mastitis. Furthermore, knowing the causes of mastitis and the microorganisms that cause it will aid in the development of preventative strategies to lower the incidence of SCM. In this study, the SMRT sequencing approach was used to compare milk microbiota associated with bovine subclinical mastitis and non-subclinical mastitis.

Various research studies were undertaken that utilized diverse sequencing technologies to further comprehend the prevalence of natural bacterial communities; nevertheless, these studies were limited to the identification of microorganisms to the genus level (Wiegel *et al.*, 2006; O'Connell *et al.*, 2016; Kamimura *et al.*, 2020). As a result, third generation sequencing, such as PacBio SMRT sequencing, was developed. The latter was used in this study because of its advantages over other sequencing platforms, such as its ability to generate longer or full-length sequence reads. Furthermore, the resolution of the SMRT sequencing approach allowed for the identification of bacteria at species level due to better taxonomic resolution.

Since 1960, somatic cell counts (SCCs) from quarter samples have been widely accepted and used as the operational measure of inflammation in the bovine lactating gland (Barkema *et al.*, 1999; Schukken *et al.*, 2003; Heeschen, 2010). The SCCs have also been used as an indicator of the severity of IMI (Harmon 1994) and of potential economic losses (Barkema *et al.*, 1999; Schukken *et al.*, 2003; Heeschen, 2010). However, the SCC threshold level used to characterize normal milk, IMI, and subclinical mastitis has been, and continues to be a contentious issue (Heeschen, 2010). As a result, from a South African perspective this study considered the Petzer *et al.* (2017) criterion, and therefore the sampled bovine milk DNA was subjected to 16S rRNA metabarcoding to better understand the microbiome of dairy cows suspected of having subclinical mastitis in comparison to that of non-SCM cows. It was found that Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes dominated the bovine milk

microbiome across both groups, and their relative abundance was diverse among samples of non-subclinical and sub-clinically infected cows. These findings were consistent with those of previous bovine (Hoque *et al.*, 2019; Derakhshani *et al.*, 2018; Oikonomou *et al.*, 2014) and human studies (Patel *et al.*, 2017) that focused on mastitis microbiomes. Proteobacteria and Firmicutes were previously identified as the predominant phyla in bovine SCM (Bhatt *et al.*, 2012), which supports the findings of the current study.

The SCM samples were dominated by several strains of *Pseudomonas*, *Lactococcus*, and *Streptococcus*, thus supporting the findings of previous studies such as the one by Oikonomou *et al.* (2020). Many of these microbiomes may operate as potential opportunists, thus interfering with metabolism, host defence and immunological development (Lee *et al.*, 2017; Klaas and Zadoks, 2018; Ma *et al.*, 2017) which results in other infections of varying severity (Lee *et al.*, 2017). The current research also revealed the existence of *Methylobacterium*, which is a genus of strictly aerobic, Gram-negative bacteria found in soil, freshwater, and lake sediments (Bracke *et al.*, 2014). These bacteria have been linked to opportunistic infections in immunocompromised individuals (Sanders *et al.*, 2000). *Methylobacterium* was also found in milk samples by Bracke *et al.* (2014), but no findings have linked it to bovine mastitis. In the current study, *Streptococcus* and *Corynebacterium* were found to have higher relative abundances among the dominating genera in both groups. *Streptococcus* species, such as *S. agalactiae*, *S. uberis*, and *S. dysgalactiae*, are bacteria that are well-known for causing mastitis (Klaas and Zadoks, 2017; Pang *et al.*, 2017).

Streptococcus parauberis, previously *S. uberis* type II was found in some of the samples. It is well known for causing mastitis in cows, which is an inflammatory illness that affects bovine mammary glands, as it was isolated in up to 20% of cases (Zhang *et al.*, 2020). *Corynebacterium* spp., which has been frequently described as infectious, has also been identified as the bacteria responsible for mastitis in dairy cows (Oultram *et al.*, 2017). Previous research also found *Corynebacterium* spp. in bulk tank milk samples obtained from 894 dairy herds in China at a 17.0% frequency level (Bi *et al.*, 2016) and from 1,242 dairy cows in Brazil at a 22.9% frequency level (Bi *et al.*, 2016; Goncalves *et al.*, 2016). In the current study, microbiome diversity (alpha and beta diversity) measurements revealed that microbial dysbiosis differed between the milk samples of non-SCM and diseased cows. Moreover, microbial diversity and species richness were discovered in both SCM and healthy controls in

this study. Previously, beta diversity also demonstrated a significant microbial difference between healthy controls and subclinical mastitis milk samples (Patel *et al.*, 2017).

Most of the bacterial species found in contaminated milk samples in the current study were *Pseudomonas*. This genus contains psychoactive Gram-negative bacteria that are abundant and widespread in nature and are considered the most frequent dairy product spoiler (Zhang, 2020). Although *Pseudomonas* spp. are not regarded as important human or animal pathogens, numerous species of this genus have been linked to human and animal diseases. Due to a lack of suitable identification methods for these organisms, non-pathogenic *Pseudomonas* spp. (*P. psychrophila*, *P. putida*, *P. koreensis*, among others) have been misidentified as pathogenic (Irorere *et al.*, 2017).

Several *Lactococcus* spp. (*L. raffinolactis* and *L. lactis*) were also commonly found in this investigation. *Lactococcus* bacteria survive transit through the gastrointestinal system, which is thought to be the mechanism of infection in humans (Hadjisymeou *et al.*, 2013). The pathogenicity of *L. lactis* subsp. *cremoris* remains unclear, as is the mode of infection (Inoue *et al.*, 2014). Other research employing culture-independent techniques revealed that *Lactococcus* was broadly dispersed in raw milk, pasteurized milk, and raw milk from bovine mastitis (Kuang *et al.*, 2009; Quigley *et al.*, 2013). As a result, the prevalence of the *Lactococcus* genus in mastitic and healthy milk samples was investigated. The findings revealed a greater relative abundance of *Lactococcus* in mastitic milk, indicating that species in this genus might be disease etiologic agents.

It was also discovered that *L. mesenteroides* sp. *Mesenteroides* was present in the milk samples. This bacteriocin inhibits additional *Leuconostoc* strains as well as various *Enterococcus* and *Listeria* spp. strains. Most Lactobacillales (LAB) are on ‘qualified presumption of safety’ (QPS) and ‘generally recognized as safe’ (GRAS) lists, meaning that their safety for use in food is ensured (Alnakip *et al.*, 2016). However, some LAB’s are excluded from these benefits, such as *Enterococcus* sp., because of their roles in causing certain human infections and contributing to the spread of antibiotic resistance (Ouwehand *et al.*, 2016). Their presence in milk could also indicate unsanitary production and faecal pollution by either human or animal routes, or both, because they are ubiquitously found in the intestinal microflora of humans and animals (Alnakip *et al.*, 2016).

The study also detected *Acinetobacter* spp. (*A. guillouiae*), which is of importance because little is known about the role of foods in the transmission of *Acinetobacter* spp., most likely because there are no standard protocols for recovering them from foods (Carvalho *et al.*, 2016; Amorim and Nascimento, 2017). Many articles, however, have documented the presence of *Acinetobacter* spp. in raw and pasteurized bovine milk, dairy products, and powdered milk (Neubeck *et al.*, 2015; Cho *et al.*, 2018). *Acinetobacter* spp. are common microbes found throughout nature. As a result, the presence of *Acinetobacter* in raw milk might simply be attributable to contamination. *Acinetobacter* can contaminate bulk tank milk from teats, udder surfaces, diseased mammary glands, milking machines, transportation systems, and contaminated water used to clean the milking equipment, as well as from the dairy farm environment (Straley *et al.*, 2006).

Finally, diverse strains of ambient, gastrointestinal, and animal skin-originating microorganisms dominated the healthy milk microbiome. Although the pathogenic mechanisms of these commensal microbes are largely unknown, they can cause opportunistic infections in the mammary glands and/or quarters of bovines with or without varying degrees of clinical episodes by producing various virulence factors (Maga *et al.*, 2017), especially in immunocompromised hosts (Gomes *et al.*, 2016; Klaas and Zadoks, 2018).

4.8 Conclusion

The study used 16S metagenomics to characterize the milk microbiome of subclinical mastitis dairy cows in comparison to milk from healthy control animals. The metagenomic analyses clearly indicated that the microbiome composition in SCM and HC differed considerably. Based on the findings, it is obvious that the 16S rRNA gene sequencing technique may be highly useful in characterizing raw milk microbiota. However, while 16S rRNA gene sequencing is a strong tool for describing bacterial communities and facilitates the identification of many unexpected evolutionary lineages, there are some constraints to consider (Salter *et al.*, 2014; Oultram *et al.*, 2017).

It is noteworthy that the study found significant differences in specific dominating CCS in the milk samples that had not been reported by previous investigations. Surprisingly, alpha-diversity analysis found that the richness and diversity of microbiota in subclinical mastitis samples were greater than in healthy samples. Beta-diversity analysis revealed a significant difference between subclinical and non-subclinical mastitis milk. Overall, the study revealed

complex diversity among the microbial communities of the milk sampled from dairy farms that operate under varying conditions. The study thus encourages that milk microbiota be examined on a regular basis for bovine mammary gland health management, which can provide complementary information such as raw milk microbial ecology and the detection of fastidious bacteria and polymicrobial infections.

4.9 References

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CHAPTER FIVE

DETECTION OF STAPHYLOCOCCUS ISOLATES AND THEIR ANTIMICROBIAL RESISTANCE PROFILES AND VIRULENCE GENES FROM SUBCLINICAL MASTITIS CATTLE MILK USING MALDI-TOF MS, PCR AND SEQUENCING IN FREE STATE PROVINCE, SOUTH AFRICA³

ABSTRACT

Staphylococcus species are amongst the bacteria that cause bovine mastitis worldwide, whereby they produce a wide range of protein toxins, virulence factors, and antimicrobial-resistant properties which are enhancing the pathogenicity of these organisms. This study aimed to detect *Staphylococcus* spp. from the milk of cattle with subclinical mastitis using MALDI-TOF MS and 16S rRNA PCR as well as screening for antimicrobial resistance (AMR) and virulence genes. Our results uncovered that from 166 sampled cows, only 33.13% had subclinical mastitis after initial screening, while the quarter-level prevalence was 54%. Of the 50 cultured bacterial isolates, MALDI-TOF MS and 16S rRNA PCR assay and sequencing identified *S. aureus* as the dominant bacteria by 76%. Furthermore, an AMR susceptibility test showed that 86% of the isolates were resistant to penicillin, followed by ciprofloxacin (80%) and ceftiofur (52%). Antimicrobial resistance and virulence genes showed that 16% of the isolates carried the *mecA* gene, while 52% of the isolates carried the *Lg G-binding* region gene, followed by *coa* (42%), *spa* (40%), *hla* (38%), and *hly* (38%), whereas *sea* and *bap* genes were detected in 10% and 2% of the isolates, respectively. The occurrence of virulence factors and antimicrobial resistance profiles highlights the need for appropriate strategies to control the spread of these pathogens.

Keywords: *Staphylococcus*, antimicrobial resistance, virulence, subclinical mastitis, cattle

³ Published in journal: *Animals*.

5.1 Introduction

The Staphylococci that cause mastitis in dairy cattle are divided into two major groups. These are (1) coagulase-positive *Staphylococcus aureus* and (2) Non-aureus staphylococci (NAS). The coagulase-positive *S. aureus* is considered a major pathogenic species whereas NAS are considered minor pathogens (Markey, 2013; Schleifer, 2015; Becker and von Eiff, 2011). The majority (About 95%) of coagulase-positive *Staphylococcus* species from bovine mastitis are *S. aureus* (Fox and Hancock, 1989) but about 15-20% of cases of mastitis are caused by NAS which comprises mainly coagulase-negative (more than 50 species), some coagulase-positive (*S. intermedius*, *S. pseudointermedius*, *S. coagulans* they are major pathogens of dogs and cats but also infect humans and occasionally mastitis) and variable [*S. hyicus* (major pathogen of swine (De Buck *et al.*, 2021) but also cause mastitis in dairy cattle), *S. agnetis*- cause mastitis in dairy cattle)] staphylococci (Markey, 2013; Devriese *et al.*, 2005; Bannoehr *et al.*, 2007; Sasaki *et al.*, 2007; Roberson *et al.*, 1996; Gotz *et al.*, 2006). *Staphylococcus* species, particularly *S. aureus*, are among the most prominent and prevalent etiological agents of bovine mastitis (Devriese *et al.*, 2005; Bannoehr *et al.*, 2007). Despite being known as a cause for illnesses with low clinical frequency, non-aureus *Staphylococcus* (NAS) and Coagulase-negative *Staphylococcus* have been discovered as frequent pathogens causing mastitis in a number of countries (Sasaki *et al.*, 2007; Roberson *et al.*, 1996). According to Xu *et al.*, (2015), it is essential to monitor the epidemiology, prevalence, and incidence of bacteria that cause bovine mastitis, particularly *Staphylococcus* species, to create programs and strategies for protecting human health in accordance with the “One Health” policy and preventing financial loss for dairy producers. Invasiveness, biofilm formation, toxin-mediated virulence factors, and antibiotic resistance are factors which influence the pathogenicity, cure rate, and ability of these organisms to survive in the host environment (Xu *et al.*, 2015; Argudín *et al.*, 2012). Hemolysins, leukocidins, enterotoxins, and superantigens are virulence factors produced by *S. aureus* that promote intramammary infection (IMI) and enable mastitis-causing bacteria to evade the host immune system (Oliveira *et al.*, 2018). Significantly, there are four distinct haemolysin types that *S. aureus* bacteria produce to enhance their pathogenicity, namely, beta, delta, gamma, and toxic shock syndrome toxin-1 (TSST-1), as well as staphylococcal enterotoxins and exfoliative toxins (Grazul *et al.*, 2023; Santos *et al.*, 2023). The family Staphylococcaceae, which includes the genera *Abyssicoccus*, *Aliicoccus*, *Auricoccus*, *Corticoccus*, *Jeotgalicoccus*, *Macrooccus*, *Nosocomiicoccus*, *Salinicoccus*, and *Staphylococcus*, has 98 validly documented species as of the writing of this paper, according

to Madhaiyan, Wirth, and Saravanan, (2020). Gram-positive, non-spore-forming, spherical or coccoid cells with sizes ranging from 0.5 to 2.5 μm are members of this family. They are also non-motile, occurring singly, in pairs, or in tetrads. They are strictly aerobic to facultatively anaerobic, catalase-positive (usually), variable for oxidase, and chemo-organotrophs capable of both fermentative and aerobic metabolism (Fox and Hancock, 1989). *Staphylococcus*, which has 23 subspecies and 55 validly recognised species, is the most common genus in this family (Magro, 2018; Madhaiyan, Wirth, and Saravanan, 2020; Wald *et al.*, 2018; Guliev *et al.*, 2020). A high frequency of antibiotic resistance and a natural reservoir of genes linked to virulence, which particularly favours traits for strains that prove to be more contagious and resistant to antibiotic treatments, are further features shared by all 23 species of coagulase-negative staphylococci (CNS) (Michels *et al.*, 2020). Furthermore, CNS are also capable of secreting numerous exotoxins (alpha, beta, gamma, and delta) (Dinges *et al.*, 2000).

Additionally, the existence of antibiotic-resistant bacteria in bovine mastitis cases, as well as the possibility of transmission to humans through consumption of unpasteurized dairy products, are two significant public health concerns. Antibiotics are frequently used to treat mastitis by farmers (Jain Neelam *et al.*, 2022). However, the overuse of antibiotics in livestock therapy can result in the emergence of antimicrobial-resistant strains and financial losses and further reduce the benefits of mastitis prevention and management (Poizat *et al.*, 2017). Moreover, *S. aureus*, a bacterium that causes mastitis and is related to food-borne diseases, has also been found to be resistant to several antibiotics (Aslam *et al.*, 2018). Additionally, the majority of methicillin-resistant *S. aureus* (MRSA) isolates have been detected from humans (Pereira *et al.*, 2021), livestock (Windria *et al.*, 2016), and the environment (Kayode and Okoh, 2022). Notably, some bacteria that cause mastitis contain genes that make them resistant to antibiotics, including the *mecA* gene for methicillin resistance (Hoekstra *et al.*, 2020).

The morphological and biochemical features of the isolates, as well as molecular biology techniques such as PCR combined with Sanger sequencing, which targets the 16S rRNA gene, and comparison of the isolates gene sequences with classified references in commonly used databases are generally used to identify bacteria (Hoekstra *et al.*, 2020). Due to its advantages over molecular identification methods and biochemical-based tests in terms of speed, cost, and labour savings, MALDI-TOF MS has therefore gained popularity as a substitute method for microbiological identification (Hoekstra *et al.*, 2020; Algammal *et al.*, 2020; Shoaib *et al.*, 2020).

5.2 Materials and methods

5.2.1 Study site, sampling, and screening of cows

A detailed description of the study area and the sampling and screening methods that were used was presented in Chapter four.

5.2.2 Bacterial isolation and identification

All isolates were identified according to a method proposed by Hoque *et al.* (2018) and endorsed by Liu *et al.* (2022). Briefly, 10 μ L aliquots were streaked out on mannitol salt agar (Oxoid, ThermoFisher, South Africa) (MSA) plates and incubated for 24 to 48 hours at 37°C. Colony morphology on MSA was used to identify the colonies (CNS: colourless or red colonies with red zones; *S. aureus*: yellow colonies with yellow zones). Thereafter, the gram staining and catalase test was performed. Two to three suspected staphylococcal colonies were sub-cultured on nutrient agar plates (NAP) and incubated at 37°C for 24 to 48 hours, which allowed for the establishment of pure cultures. Additionally, all pure *Staphylococcus* species colonies were thereafter kept at -80°C in BHI broth with 15% glycerol pending further research.

5.2.3 Gram staining

The *Staphylococcus* colonies were characterized morphologically by means of Gram's stain according to the method explained by Quirk (2010). A slide smear was prepared by picking a drop of the suspended culture from nutrient agar (NA) (Merck, Wadeville, South Africa) and transferring it onto a slide. It was then smeared, air dried, and heat fixed. After performing the Gram stain, the smear was examined under a light microscope. All Gram-negative and Gram-positive cocci bacteria were further subjected to the Catalase test.

5.2.4 Catalase test

A solution containing a drop of 3% aqueous hydrogen peroxide was placed on a clean microscope slide. A colony of test culture from nutrient agar (Merck, Wadeville, South Africa) was then placed on the hydrogen peroxide drop. *Staphylococcus* was considered positive when bubbles appeared on the surface of the culture material.

5.2.5 Matrix-assisted laser desorption ionisation time-of-flight mass spectrometric (MALDI-TOF MS) analysis of the isolates

5.2.5.1 Culture procedure of the isolates

The mass spectrometry system used in this study was outsourced from the Faculty of Natural and Agricultural Sciences, University of Pretoria (Private Bag X20 Hatfield, 0028, South Africa). Before shipping the bacterial isolates, the prescribed protocol by the University of Pretoria (SOP/GT066) was followed. Briefly, the isolates were streaked out and grown on

nutrient agar (Calle Forja 9, 28850, Torrejón de Ardoz, Madrid, distributed by Separation Scientific SA [Pty] Ltd, 1480 Cranberry St, Honeydew, Roodepoort, 2040).

Thereafter, the visible quantity of the fresh growing cells (between one single colony and 5-10 mg) from the agar plates were harvested and used to create a homogenous suspension in 300 µl sterile distilled water in an Eppendorf 1.5 ml microtube by pipetting up and down in the tube or by vortexing. Then, 900 µl of 100% HPLC grade ethanol was added to the above prepared suspension and mixed by pipetting up and down in the tube or by vortexing. Subsequently, the tubes were labelled and stored in a freezer at -20°C until shipped.

5.2.5.2 Bacterial identification using MALDI-TOF spectroscopy.

Staphylococcal species or genera were identified using the Biotyper 3.1 program (Bruker, Johannesburg 2191, South Africa). The *Escherichia coli* DH5α Bacterial Test Standard (BTS) was used to calibrate the Autoflex Speed equipment (Bruker Daltonics, Billerica, MA). Additionally, all bacterial isolates were identified as proposed by Nonnemann *et al.* (2013) and endorsed by Cameron *et al.* (2017). Briefly, pure colony was transferred to individual 1.5 mL microcentrifuge Safe-Lock Tubes (Sigma-Aldrich, Brøndbyvester, Denmark) containing 300 µL of ultra-HPLC grade water (Merck, Hellerup, Denmark) using sterile 1.0 µL disposable plastic inoculating loops. Tubes were vortexed briefly to create a homogeneous suspension followed by the addition of 900 µL of 100% ethanol (Merck), which was vortexed for an additional 15 seconds. Tubes were centrifuged for 3 min at $12,200 \times g$ at room temperature (RT), supernatants were poured and discarded, and tubes were centrifuged again for 3 min at $12,200 \times g$ at RT. The remaining ethanol/water was carefully aspirated using a micropipette. Cell pellets were air-dried for 3 min before the addition of an appropriate volume (15 to 50 µL) of 70% formic acid. The optimal volume of formic acid was determined by visually sizing the pellet. After up to 3 min, an equal volume of 100% acetonitrile was added to each sample and mixed carefully. Samples were centrifuged for 3 min at $12,200 \times g$ at RT. Eight 1 µL volumes of supernatant were carefully placed on a ground steel target plate and allowed to air dry before each spot was overlaid with 1.0 µL of cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics) dissolved in 50% acetonitrile with 2.5% trifluoroacetic acid (Sigma-Aldrich). Analysis of each isolate was run in duplicate. Isolates were declared unidentified if they were not resolved after two rounds of MALDI-TOF MS analysis. For reliability of the analysis, a cut-off score of ≥ 1.7 was used as a threshold for bacterial identification.

5.2.6 Bacterial DNA extraction

A detailed description DNA extraction method was presented in Chapter four.

5.2.7 Determination of the successful extraction of genomic DNA

The purity of DNA fragment for *Staphylococcus* spp. was measured using the Nano-Drop ND-1000 UV Spectrophotometer (Thermo-Fisher Scientific Inc., USA). This process was required before the amplification of the 16S rDNA gene. A 260/280 ratio between 1.8 and 2.0 was indicative of pure DNA, while a ratio above 2.0 indicated phenol and contamination and a ratio below 1.8 indicated contamination by proteins, as proposed by Lara *et al.* (2019).

5.2.8 Detection of Staphylococci

For identification of *Staphylococcus* species using 16S rRNA, the following primers were used: 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-TACCTTGTTACGACTT-3' (Li *et al.*, 2012). Species-specific *nuc* (F- 5'GCGATTGATGGTGATACGGTT3'and R-5'-AGCCAAGCCTTGACGAACTAAAGC-3') and *tuf* (F-5'CCAATGCCACAACTCGT-3' and R-5'-CCTGAACCAACAGTACGT-3') genes encoding for *S. aureus* (267bp) and CNS (412bp) were respectively used from published primers. The PCR reaction contained NEB OneTaq 2X Master Mix with standard buffer (New England Biolabs, USA), genomic DNA (10-30 ng/μl), forward primer (10 μM), reverse primer (10 μM), and nuclease-free water (Catalogue No. E476). The thermocycler conditions used were 94°C for 5 min, 94°C for 30 s, then 50°C for 30 s, 68°C for 1 min for 35 cycles, and 68°C for 10 min, then lastly held at 4°C. The PCR amplicons were visualized on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) and stained with EZ-vision® Bluelight DNA Dye. Furthermore, fragments were enzymatically purified using the ExoSAP procedure (NEB M0293L; NEB M0371). The 16S rRNA PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

5.2.9 DNA sequencing

PCR products were sequenced using an ABI PRISM® 3500XL DNA sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd (Pretoria, South Africa). The acquired sequences were aligned against a GenBank database using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) from the National Centre for Biotechnology Information (NCBI) to identify sequences with high similarity (Hendrickson *et al.*, 2002).

5.2.10 Accession numbers

The 16S rDNA sequences obtained from the current study were recorded in the GenBank database and were given accession numbers from OP341380-OP341389. All assigned accession numbers are indicated in **Appendix T2**.

5.2.11 Detection of *mecA* Gene from *Staphylococcus* species

All isolates were further screened for the *mecA* gene encoding for methicillin antibiotic resistance traits. Primers were as follows: *mecA*-1: 5'-AAAATCGATGGTAAAGGTTGGC-3'; *mecA*-2: 5'-AGTTCT GCAGTACCGGATTTGC3'. The amplicon sizes were 533 bp (Seki *et al.*, 1998). PCR was performed in a 12.5 μ L reaction volume containing OneTaq 2 $\times$ master mix with standard buffer (New England Biolabs, Ipswich, MA, USA) (4.25 μ L), genomic DNA (1–2 ng) (2 μ L), 1 μ L each of 10 μ M forward primer and 10 μ M reverse primer, and nucleasefree water (4.25 μ L) with the following thermocycling conditions: 94 $^{\circ}$ C for 2 min, followed by 25 cycles of 94 $^{\circ}$ C for 15 s, 55 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 10 min [40] using ProFlex PCR System (Applied Biosystems, Waltham, MA, USA).

5.2.12 Detection of Virulence Genes

For the purpose of this study, all isolates were further screened for virulence genes, namely, *lg*, *spa*, *coa*, *bap*, *hla*, *hly*, and *sea*, using published primers (Table 5.1). The binding of immunoglobulin G (*Lg G*) is known for its involvement in the neutralization and elimination of microbes. The *Lg G-binding* polyptide gene is associated with the *Lg G-binding* protein, while the *spa* gene encodes for staphylococcal protein-A. *Lg G-binding* ability is common among clinical strains of *S. aureus*. Lastly, *bap* and *coa* genes encode for biofilm-associated protein and coagulase, respectively, while *hla*, *hly*, and *sea* encode for haemolysins and enterotoxins. Individual PCR assays for each molecular identification of virulent and antimicrobial genes were set up in a 12.5 μ L volume. The PCR reaction and conditions are described above.

Table 5.1: Primer sequences specific to different putative virulence and antimicrobial resistance genes in *Staphylococcus* spp.

Target Gene	Primer set sequences (5' - 3')	Product (bp)	Reference
<i>LgG</i>	F-CACCTGCTGCAAATGCTGCG R-GGCTTGTTGTTGTCTTCCTC	920	Seki <i>et al.</i> , 1998
<i>Spa</i>	F-CAAGCACCAAAAGAGGAA R-CACCAGGTTTAACGACAT	Variable (130, 253, 315)	Kalorey <i>et al.</i> , 2007
<i>Coa</i>	F-ATAGAGATGCTGGTACAGG R-GCTTCCGATTGTTCGATGC	Variable (627, 710, 910)	Kalorey <i>et al.</i> , 2007
<i>Bap</i>	F-CCCTATATCGAAGGTGTAGAATTG	971	Simojoki <i>et al.</i> , 2012

	R-		
	GCTGTTGAAGTTAATACTGTACCTG		
	C		
<i>hla</i>	F-GGTTTAGCCTGGCCTTC	550	Booth <i>et al.</i> , 2001
	R-CATCACGAACTCGTTTCG		
<i>hlb</i>	F-GCCAAAGCCGAATCTAAG	840	Booth <i>et al.</i> , 2001
	R-GCGATATACATCCCATGGC		
<i>Sea</i>	F-GCAGGGAACAGCTTTAGGC	521	Lovseth <i>et al.</i> , 2004
	R-GTTCTGTAGAAGTATGAAACACG		

5.2.13 Antimicrobial Susceptibility Testing

Disk diffusion was performed for isolate phenotypic susceptibility testing using only four antibiotics on a 90 mm plate (Faza *et al.*, 2023). After being subcultured on nutritional agar (Merck, Wadeville, South Africa), pure *Staphylococcus* species were then incubated for 24 h at 37 °C. Thereafter, tests for antibiotic sensitivity were conducted using newly propagated overnight cultures. Using a sterile cotton swab, 100 µL aliquots from the suspensions were spreadplated on Mueller Hinton agar (MH), and the plates were incubated at 37 °C for 24 h. To evaluate the susceptibility of *Staphylococcus* species to widely used antimicrobial drugs, the single disk diffusion technique was utilized. Antibiotic discs (ThermoFisher, South Africa) comprising gentamicin (CA, 10 µg), ampicillin (AMP, 10 µg), tetracycline (TE, 30 µg), penicillin (P, 10 µg), erythromycin (E, 15 µg), ciprofloxacin (CIP, 5 µg), and ceftiofur (FOX, 15 µg) were utilized in this investigation. The Clinical Laboratory Standards Institute (CLSI, 2023) guidelines, which are interpreted as intermediate (I), sensitive (S), and resistant (R) (Table 5.2), were used to assess the antimicrobial profile of isolated staphylococci to different antibiotics using the quality control strain *Staphylococcus aureus* ATCC 25923 (Dinges *et al.*, 2000). Multidrug-resistant (MDR) isolates were defined as those that exhibited resistance to at least three different classes of antibiotics.

Table 5.2: Zone diameter breakpoints for *Staphylococcus* isolates.

Antibiotic Disks	Concentration (µg)	Abbreviations	Resistant (mm)	Intermediate (mm)	Susceptibility (mm)
Ampicillin	10	AMP	≤28		≥29
Ciprofloxacin	5	CIP	≤15	16-20	≥21
			≤15	16-18	≥19
			≤20	21-23	≥24
Erythromycin	15	E	≤13	14-22	≥23
			≤13	14-17	≥ 18
			≤13	14-17	≥ 18
Gentamicin	10	CN	≤12	13-14	≥15
Penicillin	10	P	≤28		≥29
Tetracycline	30	TET	≤14	15-18	≥19
Cefoxitin	30	FOX	≤24		≥25

5.2.14 Data Analysis

The antimicrobial resistance of an isolate was calculated as the percentage of isolates among the group that were resistant to a single antibiotic or a number of antibiotics (Kayode *et al.*, 2022). We further used tables and graphs to display the relationships of different variables and also for comparative analysis of data on antimicrobial resistance pattern of *Staphylococcus* species. The heatmap plots of the antibiotic resistance profile were generated using ChipPlot (<https://www.chipplot.online/#>, accessed on 30 October 2023).

5.3 Results

5.3.1 California mastitis test and somatic cell counts.

The results pertaining to screening techniques were discussed in Chapter four.

5.3.2 Morphological and bacteriological analysis of isolates

After screening all milk samples for subclinical mastitis, an even number of 10 positive samples from each farm was subjected to microbiological analysis. The results showed that a total of

70 presumptive isolates were detected, i.e., preliminary tests such as gram staining and catalase tests pre-confirmed them to be *Staphylococcus* species (**Appendix T3**).

5.3.3 MALDI-TOF MS identification

For identification of species using MALDI-TOF MS, a log score between 0-3 was calculated using the Biotyper algorithm (Bruker Daltonics, Bremen, Germany). The analysis for each isolate was run in duplicate. After two rounds of MALDI-TOF, the unidentified isolates were considered as not possible for identification. A cut-off score of ≥ 1.7 was regarded as a reliable threshold for the bacterial identification of *Staphylococci* at species level, as proposed by Cameron *et al.* (2017a, 2018). Of the 50 bacterial culture isolates, 48 (96%) were identified at species level by MALDI-TOF as *Staphylococcus* species: i.e., *S. aureus* (36/72%), *S. epidermidis* (2/4%), *S. chromogenes* (6/12%), and *S. haemolyticus* (2/4%), while two samples were mixed cultures between *S. aureus* and *S. hyicus* (4%). The remaining 2 (4%) were identified as *Enterococcus faecalis* (1/2%) and *Cytobacillus oceanisediminis* (1/2%) after two submissions to MALDI TOF (**Table 5.3**).



Table 5.3: Bacterial isolates from subclinical bovine mastitis identified by MALDI-TOF MS

Bacteria	Log score	Bacteria	Log score	Bacteria	Log score	Bacteria	Log store	Bacteria	Log Score
<i>S. aureus</i>	≥ 2	<i>S. chromogenes</i>	≤ 2	<i>S. chromogenes</i>	≥ 2	<i>S. aureus</i>	$\geq 2 \leq 2$	<i>S. aureus</i>	≤ 2
<i>S. aureus</i>	≥ 2	<i>S. chromogenes</i>	≤ 2	<i>S. aureus</i>	≤ 2	<i>S. aureus</i>	$\leq 2 \geq 2$	<i>S. aureus</i>	≤ 2
<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≥ 2	<i>E. faecalis</i>	$\geq 2 \leq 2$	<i>S. aureus</i>	$\leq 2 \geq 2$	<i>S. aureus</i> / <i>S. hyicus</i>	≤ 2
<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≤ 2	<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≥ 2
<i>S. aureus</i>	≤ 2	<i>S. aureus</i>	≤ 2	<i>S. aureus</i>	≥ 2	<i>S. haemolyticus</i>	≥ 2	<i>S. aureus</i> / <i>S. hyicus</i>	≤ 2
<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≤ 2	<i>S. aureus</i>	≥ 2	<i>S. haemolyticus</i>	≤ 2	<i>S. aureus</i>	≥ 2
<i>S. aureus</i>	≥ 2	<i>S. chromogenes</i>	≤ 2	<i>S. aureus</i>	≥ 2	<i>S. chromogenes</i>	≤ 2	<i>S. aureus</i>	≤ 2
<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≤ 2	<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≥ 2
<i>S. aureus</i>	≤ 2	<i>S. epidermidis</i>	≤ 2	<i>S. aureus</i>	≤ 2	<i>S. chromogenes</i>	≤ 2	<i>C. oceanisediminis</i>	≤ 2
<i>S. aureus</i>	≥ 2	<i>S. epidermidis</i>	≤ 2	<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≥ 2	<i>S. aureus</i>	≥ 2

A log score between 0-3 was calculated using the Biotyper algorithm (Bruker Daltonics, Bremen, Germany). Log scores of <1.7 provided no identification, while log scores of $1.7 \geq x < 2.0$ indicated genus identification, and log scores of ≥ 2 indicated species identification. All identifications ≥ 2.0 expressed specific species identification. The next closest score always specified the same species if the score was ≥ 2.0 .

5.3.4 Detecting *Staphylococcus* species using *Tuf*, *Nuc* and 16S rDNA Sanger sequencing.

Staphylococcus species were further confirmed using conventional PCR targeting the 16S rDNA (**Figure 5.1**) and the species-specific *tuf* gene for coagulase-negative staphylococci and the *nuc* gene for *S. aureus*. A total of 50 isolates were confirmed to be *Staphylococcus* species by PCR, and they were identified as *S. aureus*, *S. chromogenes*, *S. agnetis*, *S. argenteus*, and *S. devriesei* at 38/50 (76%), 5/50 (10%), 4/50 (4%), 2/50 (4%) and 1/50 (1%) (Accession number: OP341380- OP341389) respectively.

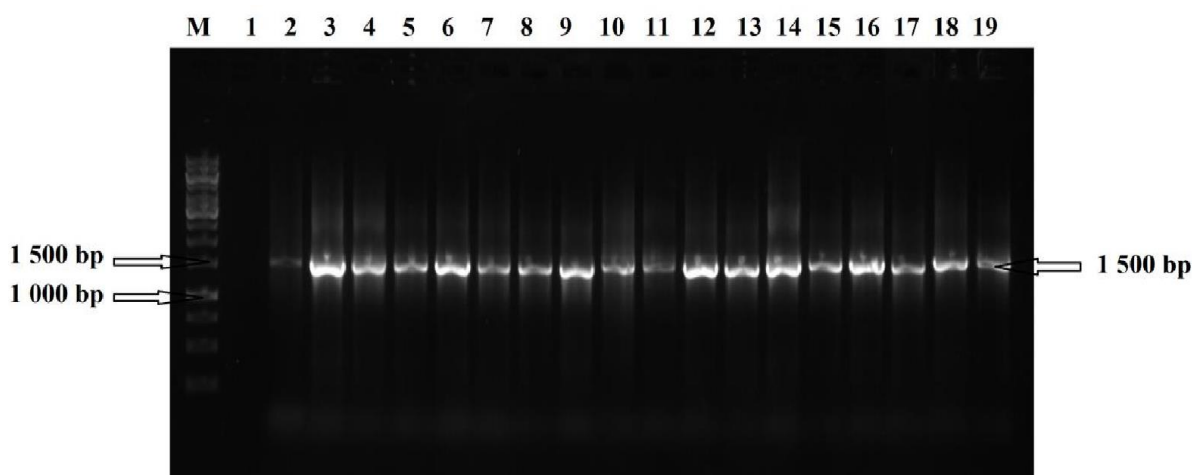


Figure 5. 1: Amplified 16S rDNA of *Staphylococcus* strains; molecular weight marker (1kb DNA ladder Lane M); (Lane 1) distilled water, Lane 2-19 (*Staphylococcus* species)

5.3.5 Detection of Methicillin Resistance and Virulence Genes

Out of 50 genomic DNA samples that were randomly selected, the *mecA* gene encoding for methicillin resistance was amplified in 8/50 (16%) isolates. Thereafter, we screened 50 selected isolates for virulence genes using PCR. The results of this investigation revealed that 52% (26/50) of all the isolates carried the *Lg G-binding* region gene, followed by 42% (21/50) and 40% (20/50) of the isolates carrying the *coa* and *spa* genes, respectively. The *hla* and *hly* genes were both detected in 38% (19/50) of the isolates, whereas the *sea* and *bap* genes were detected in 10% (5/50) and 2% (1/50) of the isolates, respectively.

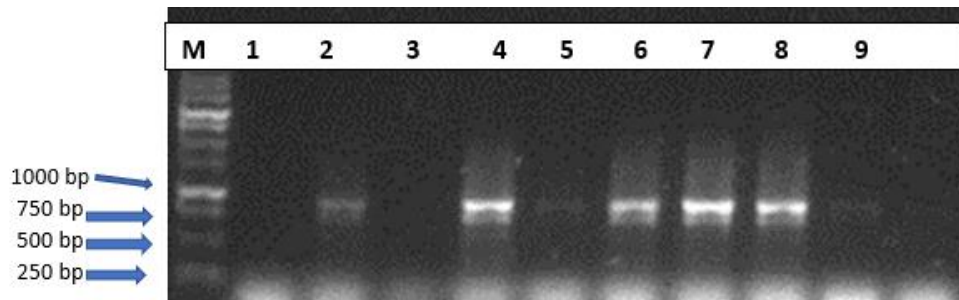


Figure 5.2: Detection of the 590 bp *LgG* gene fragments from nine representative isolates by agarose gel electrophoresis: Lane M 1kb bp marker; Lane 1 negative control; Lane 2–8 test samples.

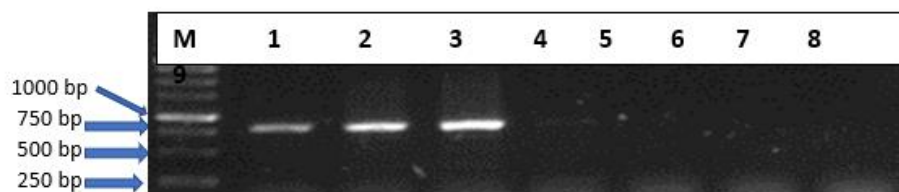


Figure 5. 3: Detection of the 627 bp *coa* gene fragments from six representative isolates by agarose gel electrophoresis: Lane M 1kb marker; Lane 1–8 test samples.

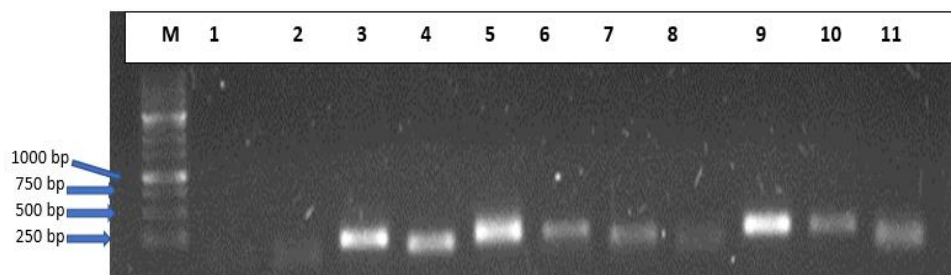


Figure 5.4: Detection of the 220 bp *Spa* gene fragments from ten representative isolates by agarose gel electrophoresis: Lane M 1kb marker; Lane 1 negative control; Lane 2–8 test samples.

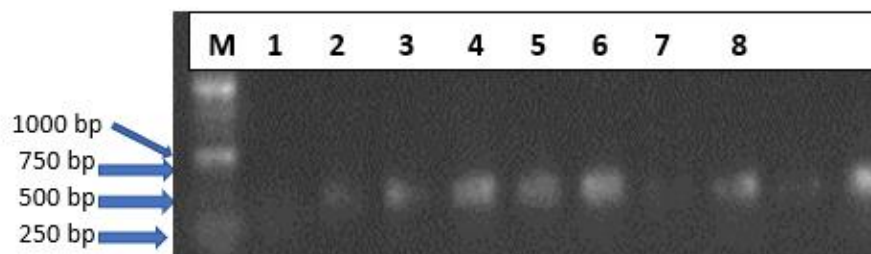


Figure 5. 5: Detection of the 550 bp *hla* gene fragments from seventeen representative isolates by agarose gel electrophoresis: Lane M 1kb marker; Lane 1 negative control; Lane 2–8 test samples.

5.3.6 Phenotypic Antimicrobial Resistance Test

The 43/resistance to penicillin, 40/(80%) to ciprofloxacin, 39/50 (76%) to vancomycin, and 26/50 (52%) to ceftiofur. Resistance against gentamycin, ampicillin, tetracycline, and erythromycin was observed in 18/50 (36%), 14/50 (28%), 9/50 (18%), and 9/50 (18%), respectively. Among all staphylococcal species, *S. aureus* had the highest percentage of resistance to all antibiotics followed by *S. chromogenes*, *S. agnetis*, *S. argenteus*, and *S. devriesei* (Table 5.4).

Table 5 1: Percentage of antimicrobial resistance among *Staphylococcus* species

Antibiotics	Isolates				
	<i>S. aureus</i> (n=38)	<i>S. chromogenes</i> (n=5)	<i>S. agnetis</i> (n=4)	<i>S. argenteus</i> (n=2)	<i>S. devriesei</i> (n=1)
Penicillin	32 (84.2%)	5 (100%)	4 (100%)	1 (50%)	1 (100%)
Erythromycin	6 (15.7%)	-	1 (25%)	1 (50%)	-
Tetracycline	9 (23.68%)	-	-	1 (50%)	-
Ampicillin	10 (26.31%)	2 (40%)	-	1 (50%)	1 (100%)
Gentamycin	15 (39.47%)	2 (40%)	1 (25%)	-	-
Ciprofloxacin	30 (78.94%)	5 (100%)	4 (100%)	2 (100%)	1 (100%)
Ceftiofur	19 (50%)	2 (40%)	3 (75%)	1 (50%)	1 (100%)

Multidrug resistance analysis showed that *S. aureus* had the highest number of resistant isolates at 34, followed by *S. chromogenes* with 5 isolates. Lastly, *S. agnetis* had four isolates with MDR, followed *S. argenteus* and *S. devriesei* with one isolate each as depicted in Table 5.5.



Table 5.2: *Staphylococcus* antimicrobial resistance and the prevalence of resistant strains

AMR phenotypes	Isolates				
	<i>S. aureus</i>	<i>S. chromogenes</i>	<i>S. agnetis</i>	<i>S. argenteus</i>	<i>S. devriesei</i>
P, CIP	3	—	1	—	—
P, FOX	1	—	1	—	—
P, CN,	1	—	—	—	—
P, CIP, , CN	—	1	—	—	—
P, FOX, AMP	1	—	—	—	—
P, CIP, FOX	—	1	—	—	—
P, CIP, AMP	1	1	—	—	—
P, CIP, CN	1	1	—	—	—
P, CIP, AMP	1	—	—	—	—
P, CIP, TET	1	—	—	—	—
P, FOX, E	—	—	1	—	—
P, CIP, CN	1	—	—	—	—
P, , FOX, TE,	1	—	—	—	—
P, CIP, CN	1	—	—	—	—
P, CIP, FOX	1	—	—	—	—
CN, CIP, E	1	—	—	—	—
P, CIP, CN, AMP	1	—	—	—	—
P, CIP, AMP, TET	1	—	—	—	—



TET, CIP, FOX	2	—	—	—	—
P, CIP, AMP, E	1	—	—	—	—
P, CIP, GEN, TET	1	—	—	—	—
P, CIP, FOX, CN, AMP	1	—	—	—	—
P, CIP FOX, AMP	1	1	—	—	—
P, CIP, FOX, CN	—	—	1	—	—
P, CIP, FOX, GEN	1	—	—	—	—
P, CIP, FOX, AMP	1	—	—	—	1
CIP, FOX, TET, E	1	—	—	—	—
CIP, , FOX, CN, AMP	1	—	—	—	—
P, CIP, FOX, CN, E	1	—	—	—	—
CIP, FOX, AMP, E	1	—	—	—	—
P, CIP, FOX, TET, E	1	—	—	—	—
P,CIP , FOX, CN, TET	1	—	—	—	—
P, CIP, FOX, AMP, TET	1	—	—	1	—
P, CIP, FOX, TET, E,	1	—	—	—	—

5.4 Discussion

Since *S. aureus* and CNS may both be detected in raw milk without raising SCC and are also found on the udders of cows, they are commonly linked to intramammary infection (IMI) (Andrade *et al.*, 2021). Our CMT (33.13%) and SCC (54%) results were relatively lower than those previously documented in other parts of Africa, i.e., Uganda, Kenya, and Ethiopia, at 86.2%, 64%, and 59.2%, respectively (Mureithi and Njuguna, 2016; Mugabi, 2018; Fazal *et al.*, 2023). Further analysis showed that every sample collected had *Staphylococcus* spp. after microbiological culturing. MALDI-TOF MS and 16S rRNA gene sequencing revealed that *S. aureus* and other CNS are species occurring in the current study's samples. This investigation supports the claims of Braga *et al.*, (2018) that MALDI-TOF MS is a better method compared to traditional biochemical testing for classifying *Staphylococcus* isolates. Nevertheless, the same author Braga *et al.*, (2018) further narrated that some staphylococci species, such as *M. Sciuri*, *S. xylosus*, and *S. equorum*, cannot be identified at the species level using MALDI-TOF MS but can instead be identified using other methods, such as DNA-based techniques. This was in line with multiple studies that reported on this restriction of MALDI-TOF MS technology (Cameron *et al.*, 2017; Cameron *et al.*, 2018). Furthermore, *S. aureus* was the dominant species, followed by *S. chromogenes*, *S. agnetis*, *S. argenteus*, and *S. devriesei*, according to the current study's 16S rRNA Sanger sequencing data; this method has been used in multiple studies to identify *S. aureus* isolates from various sources (Li *et al.*, 2012; Petersson-Wolfe *et al.*, 2010). When the gene encoding a 16S rRNA gene was amplified, all of the *S. aureus* isolates under investigation had an amplicon size of more than 1250 bp, which validates findings from other studies (Fitranda *et al.*, 2023; Singhal *et al.*, 2023).

In the current study, the two analytical tools (MALDI-TOF MS and 16S rRNA sequencing) produced similar results for most identified organisms; however, some isolates were identified as mixed cultures by MALDI-TOF MS. This is due to the fact that not all *Staphylococcus* species were tested for coagulase and *S. aureus* can be coagulase-negative, amongst other reasons (Simojoki *et al.*, 2012; Magro, 2018). Additionally, it has been demonstrated that the MALDI-TOF MS identification is influenced by variables including cell wall rigidity, growth phase, and culture conditions, including selective media that may have impact on the observed protein expression and cell concentration, where a cut-off below 2.0 enhances misidentification of envisaged pathogens (Banach *et al.*, 2016). These are some of the limitations incurred by

biochemical techniques. For all comparison tests, we utilized one pure colony for both MALDI-TOF MS analysis and the 16S rRNA sequencing. This was an effort to rule out the idea that several microorganisms could be separated, which would appear to cause test disagreement. The current study might have had problems with the detection of nonviable bacteria; however, MALDI-TOF MS and 16S rRNA sequencing methods can also detect nonviable organisms, which can be problematic when trying to diagnose active cases of mastitis (Andrade *et al.*, 2021; Abebe *et al.*, 2016). *Staphylococcus aureus* is a well-known bacterium that poses as a hazard to both human and animal health by generating potentially serious infections. More pathogenic strains of *S. aureus* are thought to be able to use the antibiotic resistance genes that may be present in CNS (Lianou *et al.*, 2021; Banach *et al.*, 2016; Moussaoui *et al.*, 2010; Fitranda *et al.*, 2023). The isolates have shown resistance to penicillin 43/50 (86%), followed by ciprofloxacin 40/50 (80%), and ceftiofur 26/50 (52%). The observed resistance against gentamicin, ampicillin, tetracycline, and erythromycin was 18/50 (36%), 14/50 (28%), 9/50 (18%), and 9/50 (18%), respectively. The results of the current study are somewhat similar to those of Sundareshan *et al.*, (2017), who reported that there were more staphylococci with penicillin resistance in subclinical mastitis (63%) in dairy cows of India.

Our findings, however, did not support those of Schmidt *et al.*, (2018), who discovered that 48% of *S. aureus* isolates in the KwaZulu-Natal province of South Africa were betalactam-resistant. Moreover, due to its use in treating MRSA cases, our analysis found that gentamicin resistance also occurred in the isolates, which was consistent with a study by Martins *et al.*, (2016) that found *S. aureus* to have 12.50% resistance to gentamicin while CNS had just 3.45% resistance in Brazil. Additional research on AMR in CNS is required due to the potential emergence of new resistance mechanisms, which poses a problem for the management of bovine mastitis cases. The acquisition of the staphylococcal cassette chromosome *mec*, a mobile genetic element, contributed to the development of methicillin resistance in *S. aureus*. According to Turner *et al.*, (2019), this cassette contains the *mecA* gene, which controls the development of low-affinity penicillin-binding protein 2a (PBP2a) and gives pathogens resistance to beta-lactamase antibiotics. In our investigation, *mecA* was found in 16% of *S. aureus* isolates linked to mastitis in cattle. The *mecA* gene was detected in 35.70% and 74.08% of *S. aureus* isolates in China and India, respectively, according to Xu *et al.*, (2015) and Patel *et al.*, (2017). Furthermore, Castro *et al.*, (2020) and Monistero *et al.*, (2018) all reported lower levels of the *mecA* gene compared to other ARGs in Brazil and South Africa, respectively. To ascertain the pathogenicity of the species, we also checked for the occurrence of virulence

genes. The gangrenous type of mastitis, which involves a restriction in blood flow to mammary tissues and subsequent injury to smooth muscles, is known to be brought on by hla expression, which is linked to the toxin α -haemolysin (Castro *et al.*, 2020). The *hly* gene is associated with the neutral sphingomyelinase toxin β -haemolysin, which has been shown by Singh *et al.*, (2014) and Neelam *et al.*, (2022) to be responsible for the breakdown of sphingomyelin in the cell membranes of erythrocytes, leukocytes, neurons, and other tissue cells. β -haemolysin promotes the development of biofilms (Schmidt *et al.*, 2018), increases *S. aureus* adhesion to the epithelium of the bovine mammary gland, and increases resistance to antimicrobials (Monistero *et al.*, 2018). The presence of hla and hly genes at 38% in *S. aureus* isolates in the present study are in close agreement with previous findings in Egypt (34.4% and 43.75%) (Singh *et al.*, 2014), Brazil (38% and 58%) (Muhlberg *et al.*, 2020), and China (57% and 36%) (Wang *et al.*, 2016). However, they are comparatively lower than several studies conducted in China, where the hla and hly genes were both detected in over 80% of the isolates (Elsayed *et al.*, 2015; Rodrigues *et al.*, 2017).

Enterotoxin production contributes to the pathophysiology of many human diseases, including toxic shock syndrome, pneumonia, sepsis, and food poisoning epidemics; hence, *S. aureus* strains are also regarded as major foodborne pathogens. Although it is unclear how enterotoxins contribute to the aetiology of bovine mastitis, their presence in milk can be a severe public health problem. Even after milk has been pasteurized, enterotoxins maintain their biological activity because they are stable at high temperatures. Zschock *et al.*, (2005) further alluded to those enterotoxins, which can cause diarrhoea and other difficulties in humans and are produced by enterotoxigenic staphylococci-infected animals' udders, which are then consumed as milk. More than 90% of food poisoning outbreaks linked to *S. aureus* were associated with traditional staphylococcal enterotoxins (Elsayed *et al.*, 2015; Rodrigues *et al.*, 2017). The current study investigated the occurrence of the staphylococcal enterotoxin sea gene in each *S. aureus* isolate, and it was detected in 10% of the isolates. Our observations are in agreement with those of previous studies, which reported the sea gene in 7.10%, 10.9%, and 19.4% of samples in China, Brazil, and Czech Republic (Xu *et al.*, 2015; Grispoldi *et al.*, 2019; Monistero *et al.*, 2020); however, these were much lower than those detected in South Africa (35.29%), northern Egypt (52%), and Italy (65.60%) from raw meats in retail markets (Schmidt *et al.*, 2018; Wang *et al.*, 2016; Zschock *et al.*, 2005).

Staphylococcus aureus attaches to the surface of the host cell to begin the colonization process via adhesins that the bacteria have on their surface (Yılmaz and Seker, 2022). The majority of the adhesins present in *S. aureus* are protein A proteins in the X-region and Lg G-binding regions that are found in cell peptidoglycan (*spa*) (Sharma *et al.*, 2016). Due to its ability to attach to molecules and agglutinate bacteria against particular bacterial antigens, protein A is utilized as a crucial reagent in immunology and diagnostic laboratory technology because it can attach to molecules and agglutinate bacteria against a particular antigen (Atalla, 2010). The *spa* gene produces protein A as a result of this process. The investigation indicated that 20 isolates were positive for the presence of the *spa* (X-region) gene. The isolates containing the *spa* gene were found to form bands of various sequence base pairs such as 130 bp, 200 bp, 290 bp, and 310 bp. As a result, it was discovered that 40% of the isolates possess the *spa* gene; however, there are gene variations. Furthermore, these genes have been detected in numerous studies focusing on bovine mastitis, which may entail the frequent presence of these genes in *S. aureus* (Pérez *et al.*, 2020).

5.5 Conclusions

Rapid and intense alteration of the socio-ecology is impacting more severely on humans and animal health than ever before. The emerging threat posed by the increasing rates of antimicrobial resistance among infectious bacteria is worrisome and demands urgent attention. The development of resistance against medically important antibiotics can be largely attributed to the indiscriminate use of antibiotics in humans and their administration as part of animal husbandry. In this study, bacterial isolates showed a trend towards developing methicillin resistance as well as resistance to a number of other antibiotics. It also demonstrated possible pathogenetic strains of isolates by detecting some virulence genes in the detected isolates. The results of this study highlight the necessity to follow the ‘One Health’ approach for preventing and controlling staphylococcal infections in the Thabo Mofutsanyane district of the Free State Province. To address and curb the threat exposed by the findings, the study urges the involvement and collaboration of a variety of sectors and role-players, including dairy farmers, veterinarians, healthcare professionals, scientists, and relevant government departments.

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CHAPTER SIX

COMPREHENSIVE WHOLE GENOME ANALYSIS OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM DAIRY COWS WITH SUBCLINICAL MASTITIS⁴

ABSTRACT

Staphylococcus aureus is a major bacterium which is a frequent cause of bovine mastitis. Understanding the frequency of virulence traits and antimicrobial resistance would shed light on the molecular epidemiology of *S. aureus* strains linked to mastitis. In the current investigation, 38 mastitis-associated *S. aureus* isolates obtained from the milk of dairy cows on small-scale farms in the Free State Province, South Africa, were subjected to whole-genome sequencing and comparative genomic analysis. The 38 isolates were grouped by *in silico* multi-locus sequencing types (MLST) into seven sequence types (STs); that is: (ST97, ST352, ST152, and ST243) and three new STs (ST8495, ST8500, and ST8501). The average GC content of the isolates genomes was 32.7. Thirty-three strains were divided into seven core SNP clusters. The *spa*-type t2883 accounted for most isolates at a frequency of 12 (31.57%), followed by t416 at 11 (28.94%) and t2844 at 5 (13.15%) among the nine distinct *spa*-types that were found. The data also revealed the identification of four plasmid replicons, with *Rep_N* (rep20) accounting for the majority of the isolates at a frequency of 17 (44.73%), followed by Inc18 (repUS5) at 2 (5.26%). These isolates included 11 distinct antimicrobial resistance genes (ARGs) and 23 genes linked to bacterial virulence. Remarkably, no methicillin resistance was predicted in these isolates. Due to the virulence traits of *S. aureus* that play a role in the mastitis disease process, characterization is essential for risk assessment. The results of this study on genes associated with virulence and antibiotic resistance are relevant due to the implications they pose for public health.

Keywords: Subclinical mastitis, *Staphylococcus aureus*, virulence factors, antimicrobial resistance, whole-genome sequencing

⁴Part of this Chapter has been published as genome announcement to journal: *Microbiology Resources Announcement*

Another Part of this Chapter is under review at *Frontiers in Microbiology*

6.1 Introduction

Staphylococcus species are known to cause acute to chronic infections/diseases that are related to increased morbidity among infected human and animal hosts (Taponen *et al.*, 2017; Song *et al.*, 2018). There are about 53 species with 28 subspecies within the *Staphylococcus* genus, with *S. aureus* being one species that causes persistent clinical and subclinical intramammary infections (IMI) (Barkema *et al.*, 2006). Several researchers have studied the pathogenesis of *S. aureus*, outlining that it begins with teat colonization which spreads through the intramammary space by either progressive colonization or changes in intramammary pressure caused by milking machines (Atalla, 2010; Sivakumar *et al.*, 2023). In the mammary alveolus, *S. aureus* adheres to and enters mammary epithelial cells. These cells serve as the site for multiplication, eventually resulting in a chronic IMI (Sutra and Poutrel, 1994; Gogoi-Tiwari *et al.*, 2017). The molecular mechanisms underlying *S. aureus* IMI still need to be fully deciphered. Generally, bacteria sense host signals and adapt gene expression to match environmental conditions thereby causing infection (Marechal *et al.*, 2011). In addition, several studies have recognised biofilm formation as one important mechanism playing a role in *S. aureus*-related IMI in dairy cows (Bardiau *et al.*, 2013; Grunert *et al.*, 2018). Numerous virulence factors (VFs) involved in the adhesion, invasion, and host defense evasion are known and have been well studied in *S. aureus*, but not in CNS. These VFs are either found in bacterial genomes or are within the transmissible genetic elements in a bacterium (El Bayomi *et al.*, 2016).

The major concern regarding the control of mastitis infection has to do with the mastitis-causing agent's resistance to antimicrobials. The extensive use of antimicrobials in the dairy industry to treat mastitis through the dry cow therapy has put pressure on *S. aureus*, resulting in the development of antimicrobial resistance and the spread of the bacterium (Shi *et al.*, 2010; McDougall *et al.*, 2014). Of more importance is the emergence of multiple-drug resistant bacteria, which poses a significant challenge in treating bovine mastitis as a matter of public health concern. Various studies have documented the emergence of methicillin-resistant *S. aureus* (MRSA) to human and veterinary medicines (Ismail *et al.*, 2017). Moreover, Hogeveen *et al.* (2011) and Goncalves *et al.* (2018) both documented resistance of antibiotics such as tetracyclines, trimethoprim/sulfamethoxazole, and orally administered penicillin to combat *S. aureus*.

Over the last few decades, the molecular epidemiology of staphylococcal species involved in IMI of dairy cattle has focused on the use of multilocus enzyme electrophoresis (MLEE) (Stanley *et al.*, 2001), pulsed-field gel electrophoresis (PFGE) (Herschleb *et al.*, 2007), sequence-based typing schemes (such as multiple-locus sequence typing [MLST]) (Saunders and Holmes, 2007), multiple-locus VNTR (variable number of tandem repeats) analysis (MLVA) (Schouls *et al.*, 2009), random amplification of polymorphic DNA (RAPD) analysis (Mobasherizadeh *et al.*, 2015), and staphylococcal Protein A (*spa*-typing) (Fasihi *et al.*, 2017). Although these methods were helpful in genetic analyses, they failed to reveal the finer details of genetic differences between strains. However, whole-genome sequencing (WGS) of bacterial genomes has become the preferred method to understand microevolution, phylogenies, and inter- and intraspecies differences (Adeolu *et al.*, 2016; Naushad *et al.*, 2016; Gupta *et al.*, 2016). It is for these reasons that the current study utilized WGS to characterise and understand the virulence and antimicrobial resistance statuses of *S. aureus* isolates in the milk of dairy cows reared on small-scale farms in the Free State Province.

The study area, study design, sampling method, and the screening and identification of *S. aureus* isolates and DNA extraction were described and discussed in Chapters four, five, and six. This chapter focuses on whole-genome analysis of *Staphylococcus aureus* isolates from the sampled milk of dairy cows with subclinical mastitis.

6.2 Materials and methods

6.2.1 Whole-genome sequencing

Multiplexed, paired-end libraries (2 x 150bp) were prepared using the Illumina DNA Prep kit (Illumina, San Diego, USA), followed by sequencing on the Illumina NextSeq 550 platform (Illumina, San Diego, USA) with 100x coverage at the National Institute of Communicable Diseases (NICD) Sequencing Core Facility, South Africa.

6.2.2 Quality control and *de novo* assembly

Illumina paired-end reads were analyzed using the JEKESA bioinformatics pipeline v0.1 (Kwenda *et al.*, 2020), which incorporates multiple bioinformatics analysis tools. Briefly, these tools were set to their default options, unless otherwise stated below. Quality control and read filtering of raw paired-end reads were performed using FastQC v0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and TrimGalore v0.6.2 (Krueger, 2020) set to Q>30 and length >= 50 bp. Species identification and closest reference

detection were performed using BactInspector v0.1.3 (<https://gitlab.com/antunderwood/bactinspector>). Contamination checks were performed using ConFindr v0.7.4 (Low *et al.*, 2019) and Kraken2 v 2.0.8-beta (Wood *et al.*, 2019). *De novo* assembly was performed using SKESA v2.3.0 (Souvorov *et al.*, 2018), and the assemblies were optimized using Shovill v1.1.0, with depth and minimum contig length set to 100 and 200 respectively (<https://github.com/tseemann/shovill>). Assembly metrics were assessed using QUAST v5.0.2 (Alexey *et al.*, 2013). The assembled genomes were further investigated using multilocus sequence typing (MLST) which was performed using mlst v2.19.0 (--legacy – scheme saureus) (<https://github.com/tseemann/mlst>) based on traditional PubMLST-typing schemes (<https://pubmlst.org/>).

6.2.3 Antimicrobial resistance prediction

Detection of antimicrobial resistance determinants was performed using a combination of three popular tools: AMRFinderPlus (Feldgarden *et al.*, 2021), ABRicate (<https://github.com/tseemann/abricate>), and staramr (<https://github.com/phac-nml/staramr>). The assembled contigs were scanned against ResFinder (Bortolaia *et al.*, 2020), PointFinder, (<http://www.genomicepidemiology.org/services/>), and AMRFinderPlus databases. The outputs from these tools were summarized using HAMRonization (<https://github.com/pha4ge/hAMRonization>).

6.2.4 Phylogenetic analysis

Core genome-based phylogenetic analysis was performed by initially annotating the assembled genomes using prokka (Seemann, 2014) and performing pan-genome analysis using panaroo (Tonkin-Hill *et al.*, 2020). Variable sites were obtained from the resulting core gene alignment file using snp-sites (v2.5.1) (Page *et al.*, 2016). IQ-TREE v2.0.3 (Minh *et al.*, 2020) was then used to generate a maximum-likelihood phylogenetic tree using the GTR+F+ASC+R4 with 1,000 bootstrap approximations, particularly UFBoot2 (Hoang *et al.*, 2018). The phylogenetic tree was visualized and annotated using Microreact (<https://microreact.org/>).

6.2.5 Core SNP cluster analysis and transmission network reconstruction

Pairwise SNP distances were calculated using snp-dist (v0.8.2; <https://github.com/tseemann/snp-dists>) while only the final alignment file based on variable sites was used. A normalized pairwise SNP distance matrix was used as input for the cluster analysis performed using the R software environment (version 4.2.1). Briefly, K-means clustering was done using default parameters in the eclust function from the factoextra package

(v1.0.7) (Kassambara and Mundt, 2020), but bootstrapping was set to 500, and only core SNP clusters with a silhouette score ≥ 0.5 and SNP cut-off ≤ 25 were considered. Visualization of cluster heat maps was performed using the ComplexHeatmap package (v.14.0) (Gu, 2022). A simple dendrogram showing the SNP clusters was generated using the fviz_dend function from the factoextra package. The minimum spanning tree was generated using the ape package in R (Paradis *et al.*, 2004) based on pairwise SNP distances. Visualization of the minimum spanning tree was performed using the visNetwork package (v2.2.2; <https://datastorm-open.github.io/visNetwork/>).

6.3 Results

6.3.1 Phenotypic characterisation of antimicrobial resistance

Of the tested *S. aureus* isolates, 43 (86%), 40 (80%), 39 (76%), and 26 (52%) were resistant to Penicillin, Ciprofloxacin, Vancomycin, and Cefoxitin respectively. Furthermore, antibiotic resistance to Amoxycillin, Gentamycin, Ampicillin, Tetracycline, and Erythromycin was detected in 25 (50%), 18 (36%), 14 (28%), 9 (18%), and 9 (18%) respectively (see Chapter six).

6.3.2 Genomes of mastitis-associated *S. aureus* strains

In the current study, whole genomes of 38 *S. aureus* associated with SCM were sequenced using the NextSeq Illumina platform. A 100X mean sequence coverage depth was generated from most of the isolates. After the read processing and the assembly of the high-quality reads, the draft genomes were obtained from 31 to 564 contigs. The mean genome size of the isolates was 2.7 Mbp, and the average total GC content was 32.7%. A summary of genome sequences is presented in **Table 6.1**.

Table 6. 1: General features of the core genome of *S. aureus*

Isolate no.	Strain Name	Bio sample accession no.	MLST	No of raw reads	No. of Contigs	Coverage depth	N ₅₀ value (bp)	Genome length (bp)	Total no. of genes	Spa types	Plasmid
1-1	Pf01	SAMN37008033	97	2455396	31	143	193876	2750926		t2883	<i>Rep_N (rep20)</i>
1-1o	Pf02	SAMN37008034	97	2995734	48	157	156217	2829526		t2883	-
1-3	Pf03	SAMN37008035	352	2869374	31	153	190332	2768936		t730	<i>Rep_N (rep20)</i>
1-5	Pf0gunf01	SAMN35674906	8500		40	151	164369	2802669	2,823	t416	-
				2803950							
1-6	Pf04	SAMN37008036	97	2986512	564	152	7640	2802283		t2844	-
1-7	Phofung02	SAMN35674907	8501		33	172	145639	2781918	2,803	t189	<i>Inc18 (repUS5)</i>
				3269844							
1-8	Pf05	SAMN37008037	97	2918854	26	159	341053	2751292		t2883	<i>Rep_N (rep20)</i>
1-9	Pf06	SAMN37008038	352	1838580	33	98	157760	2767856		t730	-
2-1o	Pf07	SAMN37008039	97	2671564	46	142	156085	2827631		t2883	-
2-2	Pf08	SAMN37008040	97	2526344	43	135	189485	2828626		t2883	-
2-3	Pf09	SAMN37008041	352	2546842	30	139	190412	2712076		t2844	-
2-5	Phofung03	SAMN35674908	8501		34	142	145649	2780593	2,801	t189	<i>Inc18 (repUS5)</i>
				2612746							
2-7	Pf10	SAMN37008042	97	1545888	49	83	141706	2825946		t2883	-
2-9	Pf11	SAMN37008043	97	2110296	51	113	140217	2829158		t2883	-
3-1	setsoto01	SAMN35674909	8500		42	124	122277	2799261	2,808	t416	<i>Rep_N (rep20)</i>
				2908804							
3-1o	setsoto02	SAMN35674910	8500		41	157	176781	2799455	2,809	t416	<i>Rep_N (rep20)</i>
				3126264							
3-4	setsoto03	SAMN35674911	8500		31	149	181801	2757166	2,771	t416	<i>Rep_N (rep20)</i>
				2755396							
3-5	setsoto04	SAMN35674912	8500	2196012	32	116	341110	2788663	2,806	t189	<i>Inc18 (repUS5)</i>

3-6	setsoto05	SAMN35674913	8500		37	139	118071	2813862	2,831	t416	Rep_N (rep20)
				2709558							
3-7	setsoto06	SAMN35674914	8500		34	116	184814	2802742	2,818	t416	Rep_N (rep20)
				2204122							
3-8	Se01	SAMN37008044	97	2468030	48	131	156161	2828107		t2883	-
3-9	setsoto07	SAMN35674915	8500		32	146	156101	2756154	2,760	t416	Rep_N (rep20)
				2783710							
4-1o	mantropa01	SAMN35674916	8500		31	149	210386	2802954	2,808	t416	Rep_N (rep20)
				2801174							
4-2	Ma01	SAMN37008045	352	3351448	32	152	186379	2710881		t2844	-
4-3	Ma02	SAMN37008046	352	2782766	27	65	190324	2710723		t2844	-
4-4	mantropa02	SAMN35674917	8500		56	65	106797	2813544	2,832	t4164	Rep_N (rep20)
				1201376							
4-5	Ma03	SAMN37008047	352	2367580	27	129	190402	2710065		t4558	-
4-6	Ma04	SAMN37008048	97	2549736	30	139	341053	2750257		t2883	Rep_N (rep20)
4-7	mantropa03	SAMN35674918	8500		39	140	225079	2815611	2,834	t416	Rep_N (rep20)
				2630238							
4-8	Ma05	SAMN37008049	97	2838244	48	150	156085	2827977		t2883	-
5-1	mantropa04	SAMN35674919	8500		38	89	181707	2802171	2,821	t416	Rep_N (rep20)
				1768784							
5-1o	mantropa05	SAMN35674920	8500		35	164	184780	2803046	2,820	t4164	Rep_N (rep20)
				3095854							
5-2	Ma06	SAMN37008050	97			78				t2883	Rep_N (rep20)
				1423060	36	110	216120	2750150			
5-3	Ma07	SAMN37008051	97	2069772	46		189566	2828411		t2883	-



5-4	mantropa06	SAMN35674921	8500				181811	2756261	2,761	t416	Rep_N (rep20)
				2474622							
5-5	Ma08	SAMN37008052	152		44	82	91272	2710178		t355	Inc18 (rep16) & Rep3 (rep5a)
				1505912							
5-7	mantropa07	SAMN35674922	8495		35	142	185000	2717325	2,725	t2844	-
				2589764							
5-9	Ma09	SAMN37008053	243	3657408	36	201	140036	2729677		t21	-

6.3.3 Distribution of sequencing types (STs), *spa*-types, and plasmids amongst all *S. aureus* isolates in SCM bovine milk

The *in silico* MLST clustered the 38 strains into seven sequence types (STs) (ST 97, ST352, ST152, ST243) and three novel STs (ST8495, ST8500, and ST8501) (**Figure 6.1**). Most were assigned to the ST8 500 (n=14; 36.8%) sequencing type, followed by ST97 (n=12; 31.5%) and ST352 (n=7; 18.4%), while the lowest had frequencies of ST8501 (n=2; 5.2%), and ST8495, ST152, and ST243 (all n=1; 2.6%) respectively. The whole-genome sequencing analysis further revealed nine (9) different *spa*-types of *S. aureus* and four (4) types of plasmids from all isolates. Of all the *spa*-types, t2883 accounted for most isolates at n=12 (31.57%), followed by t416 at n=11 (28.94%) and t2844 at n=5 (13.15%). Interestingly, the study found one unknown *spa* type (t21) when the sequences were submitted to the Ridom Spa server (<http://spaserver.ridom.de/>), and it was concluded that this was a novel *spa* type. On the other hand, plasmid *Rep_N* (rep20) was found in most of the isolates at n=17 (44.73%), followed by Inc18 (repUS5) at n=2 (5.26%). One isolate had both plasmid rep16 and rep5a (n=1; 2.65%). Finally, the results revealed an association between sequencing types (ST97 and novel STs), *spa* types (t2883 and t416), and plasmids (*Rep_N* and Inc18) (**Table 6.1**).



Sequence.Types

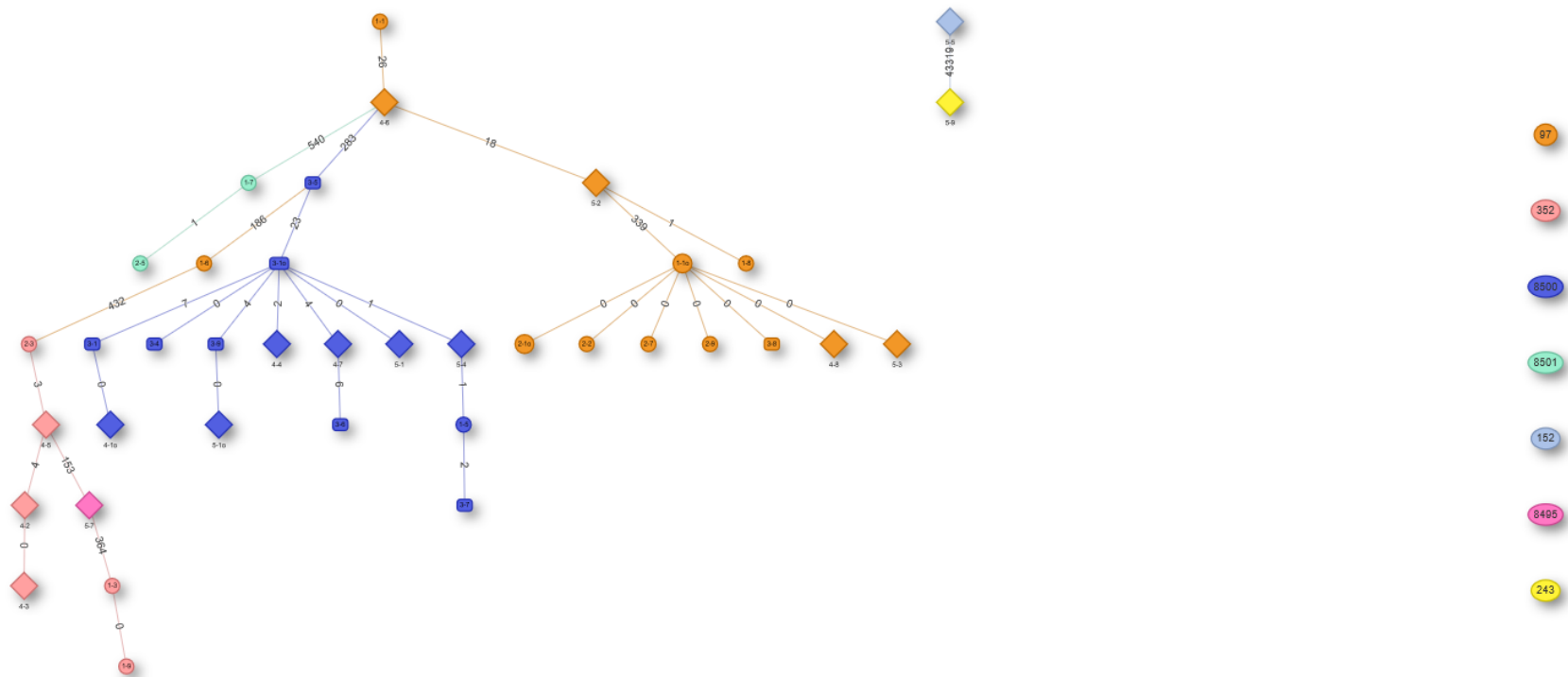


Figure 6.1: Minimum spanning tree (MST) showing sequencing types (STs) from 38 *S. aureus* isolates.

Phylogenetic analysis based on core genes analysis differentiated the 38 *S. aureus* isolates into seven (7) core clusters (**Figure 6.1**). Most isolates were grouped in cluster 2 (13 isolates), followed by cluster 5 (8 isolates), cluster 6 (4 isolates), and cluster 7 (3 isolates). The remaining clusters (1 and 4) had only two isolates each, while cluster 3 had 1 isolate.

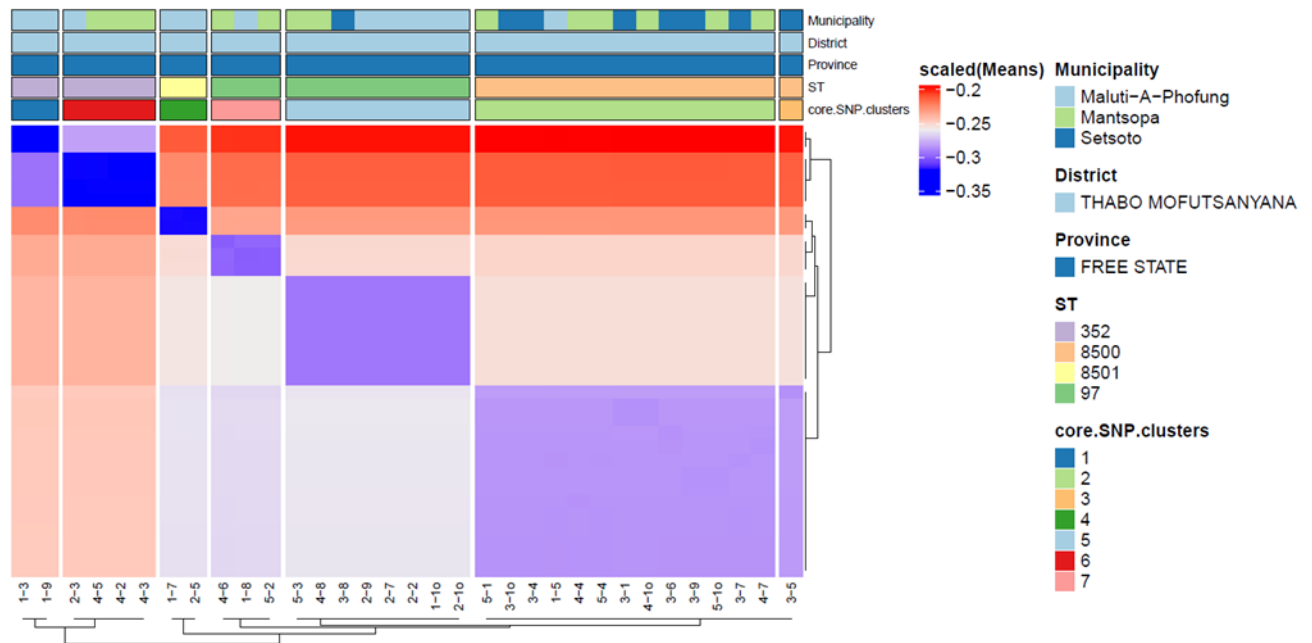


Figure 6.2: Core genome SNP clusters

6.3.4 Distribution of ARG and VFs in *Staphylococcus* species

All isolates belonging to ST8500, ST97, ST352, and ST8501 were resistant to macrolide/phenicol and tetracycline, which was due to the *lmrS*, *mepA* and *tet* (38) genes. On the other hand, 18 (47.36%) of the isolates belonging to ST8500 (31.57%), ST97 (10.52%), and ST152 (2.63%) carried the *cadD* gene encoding for cadmium. Finally, the Fosfomycin (*murA*) encoding gene was carried by two (2) (5.26%) isolates belonging to ST152 and ST243 respectively. The Beta-lactam, ampicillin (*blaI*, *blaR1* & *blaZ*), trimethoprim (*dfrG* & *dfrG_1*), and quinolone (*parE*) genes were carried by one isolate each (2.63%) belonging to ST152 (**Figure 6.3** and **Appendix T4**).

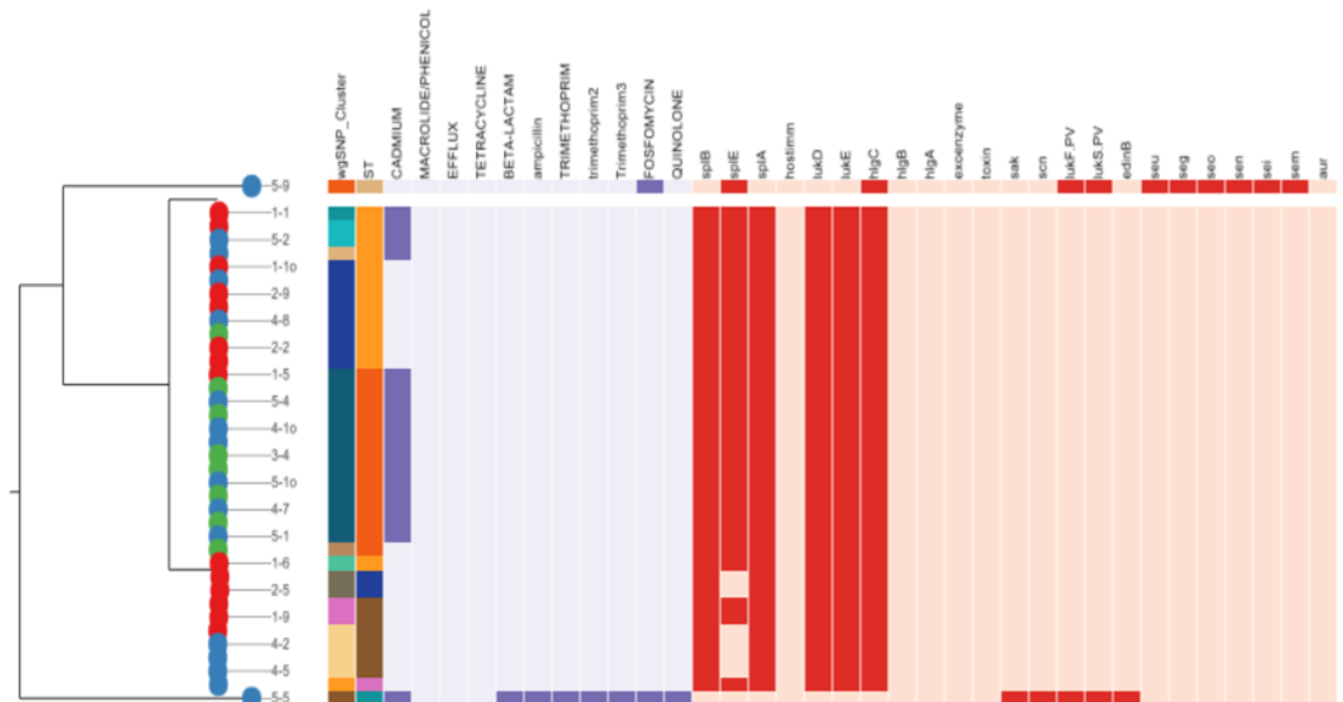


Figure 6.3: Distribution of antimicrobial resistance and virulence genes amongst *S. aureus* isolates

The study further showed that all isolates (100%) belonging to ST8500, ST97, ST352, ST8501, ST243, ST152, and ST8495 carried *aur*, *HlgA*&*B* genes encoding for aureolysin factor, bi-component gamma-hemolysin *HlgAB* subunit A and B respectively, while 36 (94.7%) of the isolates belonging to ST8500, ST97, ST352, ST8500 and ST8495 carried the *LukD*&*E* gene encoding for bi-component leukocidin LukE and D subunit D and E respectively. Furthermore, *splB*, *splE*, and *splA* genes encoding for serine protease A,B & E were carried by 36 (94.7%), 36 (94.7%), and 31 (81.5%) of all the isolates belonging to ST8500, ST97, ST152 and ST8495 respectively. Finally, in two (2) (5.26%) of the isolates, the *LukF.PV* and *LukS.PV* genes encoding for Pantón-Valentine leucocidin were detected only in two isolates belonging to ST152 and ST 243, while the *seu*, *seg*, *seo*, *sen*, *sei*, *sem* and *edinB* genes encoding for enterotoxins were detected in one (1) (2.63%) of the isolates belonging to ST243 (**Figure 6.3** and **Appendix T4**).

6.4 Discussion

Mastitis poses a serious threat to the dairy sector and causes massive economic losses on a global scale. A significant number of instances of subclinical and clinical mastitis have been linked to *S. aureus*, which is a commensal organism that colonizes nasal mucosa in 25-30% of

the healthy human population (Cole *et al.*, 2018) and is responsible for a wide range of human diseases, including serious nosocomial infections.

Staphylococcus aureus encodes many virulence factors, including the surface Ig-binding Protein A (*spa*) whose function is to capture *IgG* molecules in the inverted orientation and therefore prevent phagocytosis of the bacterial cells by the host immune system (Boero *et al.*, 2022). In this study, nine (9) *spa*-types from *S. aureus* isolates were identified and majority of them were assigned to t2883 (31.57%), followed by t416 (28.94%). A high diversity of *S. aureus spa*-types in samples taken along African dairy chains has been reported in previous studies, including t2883 (Asiimwe *et al.*, 2017; Ben Said *et al.*, 2016; Mekonnen *et al.*, 2010). This *spa*-type has also been found recently in a Zambian dairy chain (Phiri *et al.*, 2022). It is noteworthy that a study by Slama *et al.* (2011) detected the t2883 *spa*-type in human hands that had been in contact with animals, showing the possibility of transmission from human to animal, or vice versa. This current study further detected the t416 *spa*-type as one of the dominant *spa*-types in all 38 *S. aureus* isolates. The frequency that this study found was higher than those of Mora-Hernández *et al.* (2020) and Alseqely *et al.* (2021), who respectively detected t416 *spa*-type in 6.06% and 1% of the isolates which they identified.

The extensive use of antibiotics to cure human infections brought on by *S. aureus* as well as bovine mastitis is seriously threatening public health due to the emergence of AMR from dangerous bacteria. The ability to assess the pathogenic potential of *S. aureus* during mastitis depends on the identification of ARGs. The discovery of genetic elements involved in virulence and antibiotic resistance has been made easier by scanning genome sequences. The current investigation uncovered 23 VFs and 11 ARGs from 38 genomes. It was observed that the majority of isolates across all clusters and STs carried the multidrug resistance genes *lmrS*, *mepA*, and *tet* (38), which encode for resistance against drugs such as macrolide/phenicol, multidrug efflux MATE transporter MepA, and tetracycline. The first investigation to identify cadmium antibiotic resistance genes from bacterial plasmids was conducted by Christon *et al.* (1997), and subsequent studies have found these genes (*cadD*) in various isolates. The current study identified the cadmium (*cadD*) gene in ST97, ST152, and ST8500 at a very low rate (17/38; 44.74%). This study is also one of a very few that identified this gene in raw milk, but an earlier study found it in animal feed in the United Kingdom at the somewhat lower rate of 23% (Sharma *et al.*, 2016). In the current study, *MurA*, a gene that codes for resistance to

fosfomycin was present in sequence type 243. Fosfomycin resistance genes and mutations were discovered to varied degrees and were clearly tied to certain clonal complexes (Leijon *et al.*, 2021). The UDP-N-acetylglucosamine enolpyruvyl transferase, which is involved in cell wall formation and is encoded by the *murA* gene, is the target of the antibiotic fosfomycin (Michalopoulos *et al.*, 2011). It was interesting to note that, except for cadmium, only ST152 had all the antibiotic resistance genes in this study. Moreover, this ST152 included quinolone, beta-lactam, ampicillin (*blaI*, *blaR*, and *blaZ*), and trimethoprim (*dfrG*, *dfrG 1*, and *parE*).

However, none of these genes, with the exception of the *bla* genes (*blaI*, *blaZ*, and *blaR1*) that are associated with penicillin resistance, are believed to be involved in either pathogen virulence or AMR. It has been reported that staphylococci frequently show resistance to beta-lactam antimicrobials. By hydrolyzing the beta-lactam ring and rendering the medication inactive, the *blaZ* gene generates a penicillinase (also known as a beta-lactamase) that confers penicillin resistance (Zhang *et al.*, 2001). The gene is often encoded on a plasmid or a chromosome (Olsen *et al.*, 2006). Van den Borne *et al.* (2010) found few to no beta-lactam or penicillin resistant *Staphylococcus* in their study, and the latter study's findings were therefore not corroborated by the findings of the current investigation. Nevertheless, an investigation by Steele and McDougall (2014) in New Zealand discovered that almost 45% of *S. aureus* isolates were *blaZ* positive and phenotypically penicillin-resistant, and that this genotype/phenotype was connected to a remarkably subpar microbiological recovery following antimicrobial therapy.. In the current investigation, the *mecA* and *mecC* genes were not present in any of the detected strains, which were all methicillin-sensitive *S. aureus* (MSSA). Prior to this study, Li *et al.* (2017) noted that 93.4% of isolates linked to bovine mastitis in eastern China were MSSA. Furthermore, compared to the findings of a study by Haulisah *et al.* (2021) which revealed 80-100% resistance to trimethoprim, this study found relatively little resistance to this antibiotic.

Staphylococcus aureus virulence factors (VFs), host genetic makeup, immunological response, regional impacts, and genetic diversity of bacteria are only a few of the variables that affect the pathogenesis of disease-causing organisms (Mikhaylova, 2022). Therefore, this study supports the notion that knowing and understanding the intricacies of these elements through a thorough genomic study of *S. aureus* strains linked to the occurrence of mastitis in various geographical locations may assist researchers in evaluating the risks posed by infection, illness presentation, and transmission dynamics to enhance treatment approaches, particularly for cattle (Macori *et*

al., 2019; Sivakumar *et al.*, 2023). It is noteworthy that, to find the STs in 38 mastitis-associated *S. aureus* strains, whole-genome sequencing was conducted.

Moreover, the production of toxins like hemolysins, leukotoxins, and enterotoxins, as well as enzymes like serine proteases, cysteine proteases, and lipases that function as effectors during pathogenicity, is what gives *S. aureus* its pathogenic potential (Perez *et al.*, 2020). In the current study, nearly all 38 genomes had several hemolysin genes as well as some enzyme-coding genes. Just a few carefully chosen genomes had the genes for enterotoxins, enterotoxin-like proteins, and exfoliative toxins. The Pantone-Valentine leukocidin genes were only discovered in two STs (ST152 and ST243), but leukocidin D/E were discovered in both ST152 and ST342. Similarly, genes encoding adhesins and hemolysins were more common in all STs, but enterotoxins were less common among *S. aureus* strains linked with the bovine mastitis strains that had been isolated.

Another crucial factor in determining virulence in staphylococci, particularly in *S. aureus*, is toxin synthesis. Inflammation and leukocyte cell death are promoted by these toxins, which include cytotoxins (hemolysins, leukotoxins, and leukocidins) and superantigens (enterotoxins, exfoliative toxins, and toxic shock syndrome toxins [TSSTs]) (Tam and Torres, 2019). All *S. aureus* isolates in all clusters carried the cytotoxins *hlgA*, *hlgB*, and *hlgC* that encode alpha, beta, and hemolysin respectively (Aarestrup *et al.*, 1999; Naushad *et al.*, 2019). All *S. aureus* isolates detected in this investigation included leukocidin genes, such as ST97, ST352, *lukS-PV*, and *lukF-PV* (ST152 and ST243). Similar findings have been consistent in research on bovine *S. aureus* isolates, as many studies have discovered leukocidin and leukotoxin genes in the majority of isolates. It was unexpected to detect the Pantone Valentine Leukocidin (PVL) genes in all the bovine *S. aureus* isolates in this study, because these genes are thought to be exclusive to human strains of *S. aureus* (Vrieling *et al.*, 2016). This result could have shown up as a consequence of the similarity search strategy as it is possible to find genes that are related to the one in question but are not the same gene. This may explain these observations in the current study because many of the *lukS-PV* and *lukF-PV* genes detected by analysis shared the same percentage identity across different *S. aureus* isolates, suggesting that the gene sequences may be comparable to those of the genome even if they are absent. Many enterotoxin genes were also found in *S. aureus* isolates in the current study.

Another staphylococcal complement inhibitor was discovered, namely *scn*. It is crucial to prevent contamination of enterotoxin-producing *S. aureus* isolates throughout the food production chain as they can cause severe to acute food poisoning (Nia *et al.*, 2016). Unpasteurized milk-based cheese and raw milk are well-known dietary sources of *S. aureus* food poisoning. It is believed that the chemotaxis inhibitory protein (*chp* product) and the *scn* product are highly specific for staphylococcal isolates of human origin (van Wamel *et al.*, 2006; Sung *et al.*, 2008; Worthing *et al.*, 2018).

The most typical cause of bovine mastitis is staphylococcal enterotoxin C. (Fang *et al.*, 2019; Homsombat *et al.*, 2021). The primary enterotoxin gene of *S. aureus* isolated from cows with mastitis, according to other scientists, is called *sea*. In earlier studies, the prevalence of this gene ranged from 23.6-50%. (Rall *et al.*, 2014; Fursova *et al.*, 2020). Mastitis in female humans has been linked to the *sec* genes which are found on pathogenicity islands and may be transferred from cows to humans according to Franck *et al.* (2017). Three enterotoxin-like genes with known super-antigenic activity but no emetic qualities, two enterotoxin genes (*seg* and *sei*), and two enterotoxin genes make up the cluster *selo*, *selm*, and *seln*. In this investigation, 91.7% of the *S. aureus* isolates tested positive for the SEs coding genes' classical expression (*sea*, *seb*, *sec*, or *sed*). The exfoliative toxins (*eta* and *etb*), toxic shock syndrome toxin-1 (*tsst-1*), Staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed*, *saw*, *seg*, *seh*, *sei*, and *sej*), and *pvl* are all exoproteins that *S. aureus* is capable of producing (Elsayed *et al.*, 2015; Hoque *et al.*, 2018). On the other hand, it is still unclear how staphylococcal enterotoxins affect mammary epithelial cells. Nineteen (19) serologically unique SEs have recently been found in raw milk (Elsayed *et al.*, 2015; Carfora *et al.*, 2015; Hoque *et al.*, 2018). Moreover, the genes most commonly found in *S. aureus* isolates from dairy cows with mastitis are *sec*, *sed*, *seg*, and *sei* (Carfora *et al.*, 2015; Martins *et al.*, 2015; Wang *et al.*, 2009), and *sea* and *seg* (Hoque *et al.*, 2018; Martins *et al.*, 2015; Zecconi *et al.*, 2006).

6.5 Conclusion

Understanding the epidemiology of *S. aureus* genotypes in dairy animals and herds may aid in developing treatment and control plans to stop illnesses from spreading. Sequence Type 97, virulence genes like leucocidin, hemolysin, and aureolysin, and ARGs like *lmrS*, *mepA*, and *tet* (38) were the most frequently identified genes from *S. aureus* isolates in this study. The capacity of *S. aureus* to colonize and penetrate a host may be influenced by the combination of these genes. Consequently, due to the global distribution of these genes, screening them from

S. aureus isolates may be valuable for aiding in clinical outcome predictions and, specifically, for identifying hazardous strains. The current study also showed that all isolates had a nearly identical genotypic pattern and that certain isolates carried virulence factors such as *PVL*-encoding genes, suggesting that *S. aureus* isolates in animals should be closely monitored to prevent the spread of these genes. It is noteworthy that all isolates tested negative for *mecA* and *mecC*, which supports earlier descriptions of the minimal spread of MRSA among isolates of bovine mastitis (Hendriksen *et al.*, 2008; Luini *et al.*, 2015). Furthermore, the current study revealed an association between ST97, *spa*-types t2883 and t416, and the plasmid *Rep_N*.

Based on the findings, this study argues that there is a wide range of *S. aureus* genotypes occurring in dairy cattle in the Free State Province and that genetic variations are related to the geographic origin of the isolates, which in this case were three local municipality areas in this province that comprised the study area. This suggests that taking the region of interest and the strain virulence into consideration may help to formulate strategies directed to stop the spread of infection and to devise control measures in accordance with detected pathogen and host features. Hence, depending on the description of the circulating strain, the farmer will be able to choose whether to slaughter sick animals or isolate positive cows using sanitary milking practices and an appropriate milking schedule.

6.6 References

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CHAPTER SEVEN

GENERAL CONCLUSION AND RECOMMENDATIONS

7.1 Introduction

In the absence of published data in relation to the aims of the study, microbial communities and *Staphylococcus* species were isolated from subclinical mastitis (SCM) infected dairy cows and characterized. Molecular methods were then used to assess the virulence, antibiotic resistance profiles, and whole genome of *S. aureus* isolates.

7.2 General overview of the study

A comprehensive and meta-analytical study was conducted to determine the prevalence of SCM, bacterial agents, and associated risk factors among cattle in Africa. The literature review focused on SCM-related articles that complied with the study's inclusion criteria and were published between January 2010 and December 2020. The analysis of the data that emerged from the published articles demonstrated a considerable degree of variance in SCM incidence across Africa. The reported prevalence by most literature affirmed the need for improved management techniques to better ensure the health of dairy cow herds. Improved knowledge of risk variables may assist in developing efficient management and control techniques to stop the spread of SCM.

The study further used *16S rRNA* metagenomics to comprehend and compare the core microbiota of dairy cow milk samples with those that had SCM and those that appeared to be in good condition. The study showed that, in the area of interest, there was a high prevalence of SCM as well as a high diversity of microbial communities in both sub-clinically infected dairy cows and those that appeared to be healthy (non-SCM). The results of the investigation affirm that a wide range of causative organisms are in fact connected to mastitis infection.

MALDI-TOF MS, *16S rRNA* Sanger sequencing and polymerase chain reaction of the *tuf* and *nuc* genes were conducted. The study further sought to identify, characterise, and detect *Staphylococcus* species from the milk of SCM cows. It was found that all the applied techniques were able to identify *S. aureus* as the dominant species, followed by *S. chromogenes*. Furthermore, other CNS such as *S. epidermidis*, *S. haemolyticus*, *S. agnetis*, *S. argenteus*, and *S. devriesei* were also identified. The disc diffusion method and PCR were also used to understand the antimicrobial resistance and virulence statuses of the isolates. The AMR

test revealed that the majority of the isolates were resistant to cefoxitin, ciprofloxacin, and penicillin. The bacterial isolates showed a trend in acquiring MDR such as penicillin, ciprofloxacin, vancomycin, and cefoxitin. The detection of the *mecA* gene encoding for methicillin resistance showed that not all isolates carried this gene. To ascertain the pathogenicity of the species, the study also investigated the occurrence of virulence genes. The genomic characterization of virulence genes revealed that majority of the isolates contained the *LgG* gene, followed by the *coa* and *spa* genes. Moreover, the genes for *hla*, *hly*, *sea*, and *bap* were detected in some of the isolates. The findings highlight the necessity for improved early detection and surveillance of *Staphylococcus* species and subclinical mastitis.

Finally, the study undertook a comprehensive and comparative genomic analysis of 38 *S. aureus* isolates retrieved from the milk of SCM-infected dairy cows using whole-genome sequencing. The results of the study showed that isolates were grouped by the *in silico* multi-locus sequencing types (MLST) into seven sequence types (STs) (ST97, ST352, ST152, ST243) and three new types (ST8495, ST8500 and ST8501). Thirty-eight strains were divided into seven (7) core SNP clusters. *Spa*-type t2883 accounted for the majority of isolates, followed by t416 and t2844. The data also revealed the identification of four (4) plasmids, with *Rep_N* (rep20) accounting for the majority of isolates, followed by *Inc18*. These isolates included 11 distinct antimicrobial resistance (ARGs) genes and 23 genes linked to virulence, and all of the isolates used in this investigation were methicillin susceptible.

7.3 Recommendations

The extensive use of antibiotics to address human infections brought on by *S. aureus* as well as bovine mastitis are seriously threatening public health due to the emergence of AMR in dangerous bacteria. The ability to assess *S. aureus* pathogenic potential during mastitis therefore depends on the early detection and identification of AMR genes.

Additional research on AMR in CNS is required due to the potential emergence of new resistance mechanisms that pose a problem for the management of bovine mastitis cases. The discovery of genetic elements involved in virulence and antibiotic resistance has been made easier by scanning genome sequences. It is therefore recommended that further studies be conducted to even better understand the epidemiology of *S. aureus* genotypes in dairy animals and herds as this may aid in developing treatment and control plans to stop the illness from spreading.

Moreover, further epidemiological research is required because of the variations in bacterial species between milk from cows with mastitis and milk from healthy (non-mastitis) cows.

7.4 Conclusion

It was hypothesized that raw milk from subclinical mastitis dairy cows is composed of a variety of microorganisms that show and carry antimicrobial and virulence genes. Based on the data generated in this study, both hypotheses were therefore accepted. The hypotheses were supported by the use of a variety of methods that ranged from traditional culture methods to sophisticated molecular techniques such as 16S rDNA metagenomics and whole-genome sequencing. The study has shown that different microbial populations exist in raw milk from SCM dairy cows in the three sampled municipalities (Maluti-A-Phofung; Mantsopa and Setsoto) of the Free State Province of South Africa. Furthermore, the study revealed that various *Staphylococcus* species are involved in the development of SCM and they express various antimicrobial resistance and virulence genes.

APPENDICES

Appendix T1. The number of farms and presumptive *Staphylococcus* species isolates

Agar plates	Characteristics involved	Number (%) of positive isolates							Total number of isolates (%)
		Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Farm 7	
MSA	<i>S. aureus</i> - yellow colonies with yellow zones.	9 (90%)	5(50%)	7(70%)	8 (80%)	10 (100%)	7 (70%)	5 (50%)	51
		1 (10%)	5 (50%)	3 (30%)	2 (20%)	0 (0%)	3 (30%)	0 (0%)	
	CNS - Colourless or Red colonies with red zones.								14
Catalase		10(100%)	10 (100%)	10(100%)	10(100%)	10(100%)	10(100%)	10(100%)	70

Appendix T2: Accession numbers of detected *Staphylococcal* isolates

Sequence length	16S rRNA results	Accession number
1450	<i>S. aureus</i>	OP341380
1453	<i>S. chromogenes</i>	OP341381
1464	<i>S. aureus</i>	OP341382
1451	<i>S. chromogenes</i>	OP341383
1443	<i>S. aureus</i>	OP341384
1453	<i>S. aureus</i>	OP341385
1456	<i>S. aureus</i>	OP341386
1454	<i>S. aureus</i>	OP341387
1452	<i>S. aureus</i>	OP341388
1455	<i>S. argenteus</i>	OP341389
1455	<i>S. chromogenes</i>	OP341390
1430	<i>S. aureus</i>	OP341391
1438	<i>S. aureus</i>	OP341392
1454	<i>S. aureus</i>	OP341392
1456	<i>S. aureus</i>	OP341393
1454	<i>S. aureus</i>	OP341394
1437	<i>S. aureus</i>	OP341395
1435	<i>S. chromogenes</i>	OP341396
1455	<i>S. argenteus</i>	OP341397
1445	<i>S. aureus</i>	OP341398
1461	<i>S. aureus</i>	OP341399
1329	<i>S. agnetis</i>	OP341400
1445	<i>S. chromogenes</i>	OP341401
1448	<i>S. aureus</i>	OP341402
1418	<i>S. aureus</i>	OP341403
1461	<i>S. aureus</i>	OP341404

1459	<i>S. aureus</i>	OP341405
1454	<i>S. agnetis</i>	OP341406
1450	<i>S. agnetis</i>	OP341407
1451	<i>S. aureus</i>	OP341408
1441	<i>S. aureus</i>	OP341409
1450	<i>S. aureus</i>	OP341410
1434	<i>S. aureus</i>	OP341411
1458	<i>S. aureus</i>	OP341412
1423	<i>S. aureus</i>	OP341412
1458	<i>S. aureus</i>	OP341414
1451	<i>S. aureus</i>	OP341415
1455	<i>S. derviesei</i>	OP341416
1439	<i>S. aureus</i>	OP341417
1425	<i>S. aureus</i>	OP341418
1451	<i>S. aureus</i>	OP341419
1451	<i>S. aureus</i>	OP341420
1423	<i>S. aureus</i>	OP341421
1455	<i>S. aureus</i>	OP341422
1450	<i>S. aureus</i>	OP341423
1456	<i>S. aureus</i>	OP341424
1430	<i>S. aureus</i>	OP341425
871	<i>S. agnetis</i>	OP341426
1448	<i>S. aureus</i>	OP341427
1454	<i>S. aureus</i>	OP341389



Appendix T3: Accession numbers of detected staphylococcal isolates



S. aureus

P Pénicilline G	19.7	I	18 / 29.
E Erythromycine	14.7	R	17 / 22.
TE Tétracycline	19.4	S	17 / 19.
AM Ampicilline	31.9	S	16 / 21.
GEN Gentamicine	0	R	11 / 18.
CIP Ciprofloxacin	15.3	R	22 / 25.
AMC Amoxicilline/ac. clavulanique	9.2	R	16 / 23.
FOX Céfoxitine	19.2	I	15 / 22.

chromogenes

P Pénicilline G	0	R	18 / 29.
E			
Erythromycine	20.3	I	17 / 22.
TE			
Tétracycline	20.7	S	17 / 19.
AM			
Ampicilline	16.6	I	16 / 21.
GEN			
Gentamicine	9	R	11 / 18.
CIP			
Ciprofloxacin	8.2	R	22 / 25.
AMC			
Amoxicilline/a			
c. clavulanique	9.6	R	16 / 23.
FOX			
Céfoxitine	22.1	S	15 / 22.

Ø

(mm S/I/ Min/Ma
) R x

Isolate

Antibiotic

Ø (mm)

S/I/R

Min/Max

Isolate

Antibiotic

S. aureus

P Pénicilline G	9	R	18 / 29.
E Erythromycine	19.4	I	17 / 22.
TE Tétracycline	25.4	S	17 / 19.

S. aureus

P Pénicilline G	9	R	18 / 29.
E			
Erythromycine	17	I	17 / 22.
TE			
Tétracycline	9	R	17 / 19.

AM Ampicilline	14.7	R	16 / 21.	AM Ampicilline	20.9	I	16 / 21.
GEN Gentamicine	9	R	11 / 18.	GEN Gentamicine	9	R	11 / 18.
CIP Ciprofloxacin	17.6	R	22 / 25.	CIP Ciprofloxacin	18	R	22 / 25.
AMC Amoxicilline/ac. clavulanique	20.3	I	16 / 23.	AMC Amoxicilline/a c. clavulanique	15.8	R	16 / 23.
				FOX Céfoxitine	12.3	R	15 / 22.

<i>Isolate</i>	<i>Antibiotic</i>	<i>Ø (mm)</i>	<i>S/I/R</i>	<i>Min/Max</i>	<i>Isolate</i>	<i>Antibiotic</i>	<i>Ø (mm)</i>	<i>S/I/R</i>	<i>Min/Max</i>
<i>S. aureus</i>					<i>S. aureus</i>				
	P Pénicilline G	9.2	R	18 / 29.		P Pénicilline G	12.5	R	18 / 29.
	E Erythromycine	16.4	R	17 / 22.		E Erythromycine	19.2	I	17 / 22.
	TE Tétracycline	20.7	S	17 / 19.		TE Tétracycline	18.2	I	17 / 19.
	AM Ampicilline	10.6	R	16 / 21.		AM Ampicilline	20.7	I	16 / 21.
	GEN Gentamicine	21.9	S	11 / 18.		GEN Gentamicine	13.3	I	11 / 18.
	CIP Ciprofloxacin	17.8	R	22 / 25.		CIP Ciprofloxacin	28.2	S	22 / 25.

					AMC Amoxicilline/a c. clavulanique				
	AMC Amoxicilline/ac. clavulanique	22.3	I	16 / 23.		8.4	R	16 / 23.	
					FOX Céfoxitine				
	FOX Céfoxitine	19.4	I	15 / 22.		10.2	R	15 / 22.	
					Ø				
					(mm S/I/ Min/Ma				
Isolate	Antibiotic	Ø (mm)	S/I/R	Min/Max	Isolate	Antibiotic	) R	x	
<i>S. aureus</i>					<i>S. argenteus</i>				
	P Pénicilline G	39.5	S	18 / 29.		P Pénicilline G	21.9	I	18 / 29.
	E Erythromycine	19.4	I	17 / 22.		E Erythromycine	10.2	R	17 / 22.
	TE Tétracycline	16	R	17 / 19.		TE Tétracycline	19.9	S	17 / 19.
	AM Ampicilline	21.1	S	16 / 21.		AM Ampicilline	20.9	I	16 / 21.
	GEN Gentamicine	33	S	11 / 18.		GEN Gentamicine	11.5	I	11 / 18.
	CIP Ciprofloxacin	20.1	R	22 / 25.		CIP Ciprofloxacin	8.6	R	22 / 25.
	AMC Amoxicilline/ac. clavulanique	17	I	16 / 23.		AMC Amoxicilline/a c. clavulanique	19.9	I	16 / 23.
	FOX Céfoxitine	12.5	R	15 / 22.		FOX Céfoxitine	18	I	15 / 22.



E Erythromycine	19.4	I	17 / 22.	E	Erythromycine	18.6	I	17 / 22.
TE Tétracycline	23.9	S	17 / 19.	TE	Tétracycline	20.9	S	17 / 19.
AM Ampicilline	17.8	I	16 / 21.	AM	Ampicilline	21.1	S	16 / 21.
GEN Gentamicine	12.9	I	11 / 18.	GEN	Gentamicine	9.4	R	11 / 18.
CIP Ciprofloxacin	18.8	R	22 / 25.	CIP	Ciprofloxacin	16	R	22 / 25.
AMC Amoxicilline/ac. clavulanique	29.1	S	16 / 23.	AMC	Amoxicilline/a c. clavulanique	0	R	16 / 23.
FOX Céfoxitine	19.6	I	15 / 22.	FOX	Céfoxitine	20.1	I	15 / 22.

					GEN				
	GEN Gentamicine	9	R	11 / 18.		Gentamicine	9.4	R	11 / 18.
					CIP				
	CIP Ciprofloxacin	18.2	R	22 / 25.		Ciprofloxacin	14.7	R	22 / 25.
					AMC				
	AMC Amoxicilline/ac. clavulanique	9	R	16 / 23.		Amoxicilline/a c. clavulanique	16	I	16 / 23.
					FOX				
	FOX Céfoxitine	20.1	I	15 / 22.		Céfoxitine	8.2	R	15 / 22.
					Ø				
					(mm S/I/ Min/Ma) R x				
Isolate	Antibiotic	Ø (mm)	S/I/R	Min/Max	Isolate	Antibiotic	(mm)	S/I/ R	Min/Ma x
<i>S. aureus</i>					<i>S. chromogenes</i>				
	P Pénicilline G	0	R	18 / 29.		P Pénicilline G	11.1	R	18 / 29.
					E				
	E Erythromycine	20.5	I	17 / 22.		Erythromycine	17	I	17 / 22.
					TE				
	TE Tétracycline	19.6	S	17 / 19.		Tétracycline	19	I	17 / 19.
					AM				
	AM Ampicilline	8.8	R	16 / 21.		Ampicilline	21.7	S	16 / 21.
					GEN				
	GEN Gentamicine	25	S	11 / 18.		Gentamicine	12.1	I	11 / 18.
					CIP				
	CIP Ciprofloxacin	24.4	I	22 / 25.		Ciprofloxacin	19.6	R	22 / 25.
					AMC				
	AMC Amoxicilline/ac. clavulanique	19	I	16 / 23.		Amoxicilline/a c. clavulanique	9	R	16 / 23.

FOX Céfoxitine					FOX Céfoxitine				
11.1 R 15 / 22.					11.3 R 15 / 22.				
					</				

Technology, Free State					Technology, Free State				
E Erythromycine	21.1	I	17 / 22.	E Erythromycine	21.3	I	17 / 22.		
TE Tétracycline	20.3	S	17 / 19.	TE Tétracycline	15.1	R	17 / 19.		
AM Ampicilline	16.8	I	16 / 21.	AM Ampicilline	8.6	R	16 / 21.		
GEN Gentamicine	11.5	I	11 / 18.	GEN Gentamicine	23.3	S	11 / 18.		
CIP Ciprofloxacin	28.2	S	22 / 25.	CIP Ciprofloxacin	12.1	R	22 / 25.		
AMC Amoxicilline/ac. clavulanique	0	R	16 / 23.	AMC Amoxicilline/a c. clavulanique	0	R	16 / 23.		
AM Ampicilline	16	I	16 / 21.	FOX Céfoxitine	18.8	I	15 / 22.		
FOX Céfoxitine	22.1	S	15 / 22.						
Isolate	Antibiotic	Ø (mm)	S/I/R	Min/Max	Isolate	Antibiotic	Ø (mm)	S/I/R	Min/Max
S. agnetis					S. chromogenes				
P Pénicilline G	14.3	R	18 / 29.	P Pénicilline G	9	R	18 / 29.		
E Erythromycine	24.8	S	17 / 22.	E Erythromycine	21.7	I	17 / 22.		
TE Tétracycline	24.2	S	17 / 19.	TE Tétracycline	19.7	S	17 / 19.		

AM Ampicilline	20.7	I	16 / 21.	AM Ampicilline	9	R	16 / 21.
GEN Gentamicine	14.3	I	11 / 18.	GEN Gentamicine	22.1	S	11 / 18.
CIP Ciprofloxacin	17.8	R	22 / 25.	CIP Ciprofloxacin	19.9	R	22 / 25.
AMC Amoxicilline/ac. clavulanique	19.4	I	16 / 23.	AMC Amoxicilline/a c. clavulanique	19.7	I	16 / 23.
FOX Céfoxitine	17.2	I	15 / 22.	FOX Céfoxitine	11.3	R	15 / 22.

					Ø			
					(mm S/I/ Min/Ma			
Isolate	Antibiotic	Ø (mm)	S/I/R	Min/Max	Isolate	Antibiotic	) R x	
<i>S. aureus</i>					<i>S. aureus</i>			
	P Pénicilline G	13.7	R	18 / 29.		P Pénicilline G	10.9	R 18 / 29.
	E Erythromycine	19.7	I	17 / 22.		E Erythromycine	19.7	I 17 / 22.
	TE Tétracycline	11.3	R	17 / 19.		TE Tétracycline	20.3	S 17 / 19.
	AM Ampicilline	25.6	S	16 / 21.		AM Ampicilline	16.8	I 16 / 21.
	CIP Ciprofloxacin	9	R	22 / 25.		GEN Gentamicine	8.6	R 11 / 18.
	AMC Amoxicilline/ac. clavulanique	28.9	S	16 / 23.		CIP Ciprofloxacin	26	S 22 / 25.

					AMC Amoxicilline/a c. clavulanique				
FOX Céfoxitine					18.4 I 16 / 23.				
					Ø				
					(mm S/I/ Min/Ma				
<i>Isolate</i>	<i>Antibiotic</i>	<i>Ø (mm)</i>	<i>S/I/R</i>	<i>Min/Max</i>	<i>Isolate</i>	<i>Antibiotic</i>	<i>)</i>	<i>R</i>	<i>x</i>
<i>S. aureus</i>					<i>S. aureus</i>				
	P Pénicilline G	11.9	R	18 / 29.		P Pénicilline G	8.6	R	18 / 29.
	E Erythromycine	26.8	S	17 / 22.		E Erythromycine	20.7	I	17 / 22.
	TE Tétracycline	19.7	S	17 / 19.		TE Tétracycline	37.7	S	17 / 19.
	AM Ampicilline	21.3	S	16 / 21.		AM Ampicilline	19.7	I	16 / 21.
	GEN Gentamicine	12.9	I	11 / 18.		GEN Gentamicine	20.7	S	11 / 18.
	CIP Ciprofloxacin	17.6	R	22 / 25.		CIP Ciprofloxacin	27.4	S	22 / 25.
	AMC Amoxicilline/ac. clavulanique	8.8	R	16 / 23.		AMC Amoxicilline/a c. clavulanique	10.2	R	16 / 23.
	FOX Céfoxitine	19.2	I	15 / 22.		FOX Céfoxitine	17.8	I	15 / 22.
					Ø				
					(mm S/I/ Min/Ma				
<i>Isolate</i>	<i>Antibiotic</i>	<i>Ø (mm)</i>	<i>S/I/R</i>	<i>Min/Max</i>	<i>Isolate</i>	<i>Antibiotic</i>	<i>)</i>	<i>R</i>	<i>x</i>
<i>S. agnetis</i>					<i>S. agnetis</i>				
	P Pénicilline G	0	R	18 / 29.		P Pénicilline G	9.6	R	18 / 29.

E Erythromycine	15.8	R	17 / 22.
TE Tétracycline	19.6	S	17 / 19.
AM Ampicilline	16.6	I	16 / 21.
CIP Ciprofloxacin	28	S	22 / 25.
AMC Amoxicilline/ac. clavulanique	0	R	16 / 23.
FOX Céfoxitine	8.2	R	15 / 22.

E	Erythromycine	27.2	S	17 / 22.
TE	Tétracycline	18.4	I	17 / 19.
AM	Ampicilline	19.6	I	16 / 21.
GEN	Gentamicine	9	R	11 / 18.
CIP	Ciprofloxacin	15.4	R	22 / 25.
AMC	Amoxicilline/a			
c. clavulanique	FOX	11.5	R	16 / 23.
FOX	Céfoxitine	12.5	R	15 / 22.

<i>Isolate</i>	<i>Antibiotic</i>	<i>Ø (mm)</i>	<i>S/I/R</i>	<i>Min/Max</i>	<i>Isolate</i>
<i>S. aureus</i>					<i>S. aureus</i>
	P Pénicilline G	13.1	R	18 / 29.	
	E Erythromycine	24.2	S	17 / 22.	
	TE Tétracycline	19.7	S	17 / 19.	

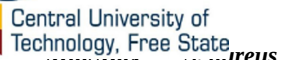
	(mm	S/I/	Min/Ma
Antibiotic	)	R	x
P Pénicilline G	9.6	R	18 / 29.
E			
Erythromycine	21.5	I	17 / 22.
TE			
Tétracycline	19.2	S	17 / 19.
AM			
Ampicilline	23.3	S	16 / 21.

Technology, Free State										
Isolate <i>S. aureus</i>	AM Ampicilline	20.7	I	16 / 21.	Isolate <i>S. aureus</i>	GEN	Gentamicine	20.1	S	11 / 18.
	GEN Gentamicine	11.7	I	11 / 18.		CIP	Ciprofloxacin	9.6	R	22 / 25.
	CIP Ciprofloxacin	31.3	S	22 / 25.		AMC	Amoxicilline/a			
	AMC Amoxicilline/ac. clavulanique	21.1	I	16 / 23.		c. clavulanique	21.5	I	16 / 23.	
	FOX Céfoxitine	29.9	S	15 / 22.		FOX	Céfoxitine	29.9	S	15 / 22.
	P Pénicilline G	12.1	R	18 / 29.						
	E Erythromycine	19.4	I	17 / 22.						
	TE Tétracycline	18.8	I	17 / 19.						
	AM Ampicilline	21.3	S	16 / 21.						
	GEN Gentamicine	10	R	11 / 18.						
CIP Ciprofloxacin	0	R	22 / 25.							
AMC Amoxicilline/ac. clavulanique	19.9	I	16 / 23.							

<i>Isolate</i> <i>S. aureus</i>	<i>Antibiotic</i>	$\emptyset$ (mm)	S/I/R	Min/Max	<i>Isolate</i> <i>S. aureus</i>	<i>Antibiotic</i>	$\emptyset$	S/I/	Min/Ma
							(mm)	R	x
<i>Isolate</i> <i>S. aureus</i>	FOX Céfoxitine	20.5	I	15 / 22.	<i>Isolate</i> <i>S. aureus</i>	P Pénicilline G	9	R	18 / 29.
	P Pénicilline G	9	R	18 / 29.		E Erythromycine	9	R	17 / 22.
	E Erythromycine	18.4	I	17 / 22.		TE Tétracycline	0	R	17 / 19.
	TE Tétracycline	19.4	S	17 / 19.		AM Ampicilline	23.5	S	16 / 21.
	AM Ampicilline	19.4	I	16 / 21.		GEN Gentamicine	39.7	S	11 / 18.
	GEN Gentamicine	10.4	R	11 / 18.		CIP Ciprofloxacine	16.6	R	22 / 25.
	CIP Ciprofloxacine	18.4	R	22 / 25.		AMC Amoxicilline/a			
	AMC Amoxicilline/ac. clavulanique	9.8	R	16 / 23.		c. clavulanique	16.6	I	16 / 23.
						FOX Céfoxitine	9	R	15 / 22.
<i>Isolate</i> <i>S. aureus</i>	<i>Antibiotic</i>	$\emptyset$ (mm)	S/I/R	Min/Max	<i>Isolate</i> <i>S. aureus</i>	<i>Antibiotic</i>	$\emptyset$	S/I/	Min/Ma
							(mm)	R	x
<i>Isolate</i> <i>S. aureus</i>	FOX Céfoxitine	21.5	I	15 / 22.	<i>Isolate</i> <i>S. aureus</i>	P Pénicilline G	9.8	R	18 / 29.
	P Pénicilline G	10.6	R	18 / 29.		E Erythromycine	27.2	S	17 / 22.

Isolate <i>S. aureus</i>	Technology, Free State					TE			
	E Erythromycine	25.2	S	17 / 22.		Tétracycline	23.1	S	17 / 19.
	TE Tétracycline	20.3	S	17 / 19.		AM			
	AM Ampicilline	19.9	I	16 / 21.		Ampicilline	21.7	S	16 / 21.
	GEN Gentamicine	9.8	R	11 / 18.		GEN			
						Gentamicine	11.3	I	11 / 18.
	CIP Ciprofloxacin	18.6	R	22 / 25.		CIP			
						Ciprofloxacin	18.2	R	22 / 25.
	AMC Amoxicilline/ac. clavulanique	9	R	16 / 23.		AMC			
						Amoxicilline/a			
						c. clavulanique	8.8	R	16 / 23.
						FOX			
						Céfoxitine	8	R	15 / 22.
						Ø			
					(mm)	S/I/	Min/Ma		
Antibiotic	Ø (mm)	S/I/R	Min/Max	Isolate	Antibiotic	)	R	x	
					<i>S. devriesei</i>				
					P Pénicilline G	9	R	18 / 29.	
					E				
	P Pénicilline G	11.3	R	18 / 29.	Erythromycine	27.4	S	17 / 22.	
					TE				
	E Erythromycine	17	I	17 / 22.	Tétracycline	17.8	I	17 / 19.	
					AM				
	TE Tétracycline	21.7	S	17 / 19.	Ampicilline	9	R	16 / 21.	
					GEN				
	AM Ampicilline	19.2	I	16 / 21.	Gentamicine	22.5	S	11 / 18.	
					CIP				
	GEN Gentamicine	9	R	11 / 18.	Ciprofloxacin	17.8	R	22 / 25.	

Isolate <i>S. aureus</i>	CIP Ciprofloxacin	16.6	R	22 / 25.	Isolate <i>S. aureus</i>	AMC Amoxicilline/a c. clavulanique	22.3	I	16 / 23.
	AMC Amoxicilline/ac. clavulanique	16.6	I	16 / 23.		FOX Céfoxitine	9.8	R	15 / 22.
							Ø		
	FOX Céfoxitine	8.2	R	15 / 22.		Antibiotic	(mm	S/I/	Min/Ma
		Ø (mm)	S/I/R	Min/Max			)	R	x
	P Pénicilline G	11.5	R	18 / 29.		P Pénicilline G	20.9	I	18 / 29.
	E Erythromycine	28.2	S	17 / 22.		E Erythromycine	14.9	R	17 / 22.
	TE Tétracycline	31.3	S	17 / 19.		TE Tétracycline	9	R	17 / 19.
	AM Ampicilline	31.9	S	16 / 21.		AM Ampicilline	22.9	S	16 / 21.
	GEN Gentamicine	11.9	I	11 / 18.		VA Vancomycine	16	R	17 / 17.
	CIP Ciprofloxacin	17.8	R	22 / 25.		AMC Amoxicilline/a c. clavulanique	31.5	S	16 / 23.
	AMC Amoxicilline/ac. clavulanique	22.3	I	16 / 23.		FOX Céfoxitine	11.3	R	15 / 22.
							Ø		
	FOX Céfoxitine	19.4	I	15 / 22.		Antibiotic	(mm	S/I/	Min/Ma
							)	R	x

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<i>Isolate</i> <i>S. aureus</i>	AM Ampicilline	38.9	S	16 / 21.	<i>Isolate</i> <i>S. aureus</i>	GEN			
	GEN Gentamicine		14.9	I		Gentamicine	9.2	R	11 / 18.
	CIP Ciprofloxacin		26.6	S		CIP			
	AMC Amoxicilline/ac. clavulanique		15.1	R		Ciprofloxacin	17.8	R	22 / 25.
						AMC			
	FOX Céfoxitine		9.8	R		Amoxicilline/a			
						c. clavulanique	22.3	I	16 / 23.
						FOX			
						Céfoxitine	19.4	I	15 / 22.
						Ø			
						(mm	S/I/	Min/Ma	
						)	R	x	
	P Penicillin G		10.2	R		P Pénicilline G	21.9	I	18 / 29.
	E Erythromycine		8.2	R		E			
	TE Tétracycline	22.3		S		Erythromycine	10.4	R	17 / 22.
	AM Ampicilline		11.5	R		TE			
	GEN Gentamicine		8	R		Tétracycline	21.3	S	17 / 19.
	CIP Ciprofloxacin		14.7	R		AM			
						Ampicilline	8.4	R	16 / 21.
						GEN			
						Gentamicine	19.7	S	11 / 18.
						CIP			
						Ciprofloxacin	19.6	R	22 / 25.
						AMC			
						Amoxicilline/a			
						c. clavulanique	9	R	16 / 23.

Isolate <i>S. agnetis</i>	AMC Amoxicilline/ac. clavulanique	16	I	16 / 23.	Isolate <i>S. aureus</i>	FOX			
						Céfoxitine	11.3	R	15 / 22.
							Ø		
							(mm	S/I/	Min/Ma
	FOX Céfoxitine	8.2	R	15 / 22.		Antibiotic	)	R	x
	P Pénicilline G	11.7	R	18 / 29.		P Pénicilline G	11.3	R	18 / 29.
						E			
	E Erythromycine	22.7	S	17 / 22.		Erythromycine	21.3	I	17 / 22.
						TE			
	TE Tétracycline	23.7	S	17 / 19.		Tétracycline	19.4	S	
						AM			
	AM Ampicilline	24.6	S	16 / 21.		Ampicilline	19.9	I	16 / 21.
						GEN			
	GEN Gentamicine	11.1	I	11 / 18.		Gentamicine	11.9	I	11 / 18.
						CIP			
	CIP Ciprofloxacin	28	S	22 / 25.		Ciprofloxacin	10	R	22 / 25.
						AMC			
	AMC Amoxicilline/ac. clavulanique	0	R	16 / 23.		Amoxicilline/a			
	FOX Céfoxitine	8.2	R	15 / 22.		c. clavulanique	31.5	S	16 / 23.
						FOX			
						Céfoxitine	11.3	R	15 / 22.

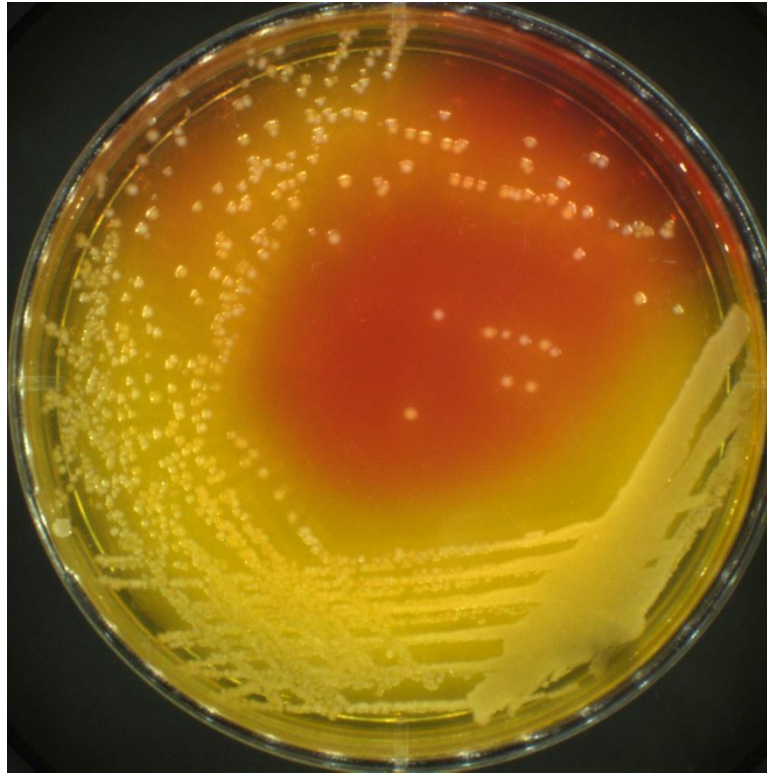


Appendix T4: Predicted antimicrobial resistance and virulence genes using whole-genome sequencing

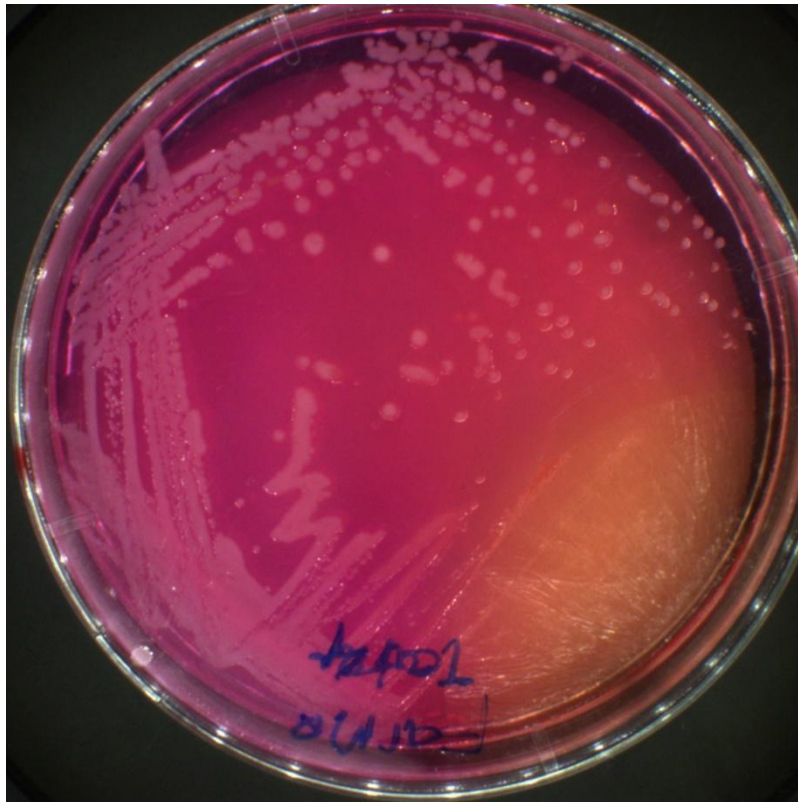
Gene symbol	Gene name	Factor
<i>tet(38)</i>	tetracycline efflux MFS transporter Tet(38)	AMR
<i>aur</i>	zinc metalloproteinase aureolysin	Virulence
<i>cadD</i>	cadmium resistance transporter CadD	AMR
<i>hld</i>	delta-hemolysin	Virulence
<i>hlgA</i>	bi-component gamma-hemolysin HlgAB subunit A	Virulence
<i>hlgB</i>	bi-component gamma-hemolysin HlgAB/HlgCB subunit B	Virulence
<i>hlgC</i>	bi-component gamma-hemolysin HlgCB subunit C	Virulence
<i>icaC</i>	polysaccharide intercellular adhesin biosynthesis/export protein IcaC	Virulence
<i>lmrS</i>	multidrug efflux MFS transporter LmrS	AMR
<i>lukD</i>	bi-component leukocidin LukED subunit D	Virulence
<i>lukE</i>	bi-component leukocidin LukED subunit E	Virulence
<i>mepA</i>	multidrug efflux MATE transporter MepA	AMR
<i>selX</i>	staphylococcal enterotoxin-like toxin X	Virulence
<i>splA</i>	serine protease SplA	Virulence
<i>splB</i>	serine protease SplB	Virulence
<i>splE</i>	serine protease SplE	Virulence
<i>sel26</i>	staphylococcal enterotoxin type 26	Virulence
<i>blaI_of_Z</i>	penicillinase repressor BlaI	AMR

<i>blaR1</i>	beta-lactam sensor/signal transducer <i>BlaR1</i>	AMR
<i>dfrG</i>	trimethoprim-resistant dihydrofolate reductase <i>DfrG</i>	AMR
<i>dfrG_1</i>	<i>dfrG</i>	AMR
<i>blaI</i>	penicillinase repressor <i>BlaI</i>	AMR
<i>blaZ</i>	penicillin-hydrolyzing class A beta-lactamase <i>BlaZ</i>	AMR
<i>ednB</i>	epidermal cell differentiation inhibitor <i>EdnB</i>	Virulence
<i>glpT</i>	<i>Staphylococcus aureus</i> fosfomycin resistant <i>GlpT</i>	AMR
<i>lukF-PV</i>	Panton-Valentine bi-component leukocidin subunit F	Virulence
<i>lukS-PV</i>	Panton-Valentine bi-component leukocidin subunit S	Virulence
<i>murA</i>	<i>Staphylococcus aureus</i> fosfomycin resistant <i>MurA</i>	Virulence
<i>parE</i>	<i>Staphylococcus aureus</i> quinolone resistant <i>ParE</i>	Virulence
<i>sak</i>	staphylokinase	Virulence
<i>scn</i>	complement inhibitor SCIN-A	Virulence
<i>blaZ</i>	<i>blaZ</i>	AMR
<i>dfrG</i>	<i>dfrG</i>	AMR
<i>fosB-Saur</i>	FosB1/FosB3 family fosfomycin resistance bacillithiol transferase	AMR
<i>fosB</i>	FosB1/FosB3 family fosfomycin resistance bacillithiol transferase	AMR
<i>sei</i>	staphylococcal enterotoxin type I	Virulence
<i>sem</i>	staphylococcal enterotoxin type M	Virulence
<i>sen</i>	staphylococcal enterotoxin type N	Virulence
<i>seu</i>	staphylococcal enterotoxin type U	Virulence

Appendix F1: Image showing *S. aureus* on mannitol salt agar



Appendix F2: Image showing other Staphylococci on mannitol salt agar.



Appendix F3: Mueller Hinton Agar plate showing diffusion test for isolates against antibiotic discs

