MILK HYGIENE ASSESSMENT AND CHARACTERISATION OF *STAPHYLOCOCCUS AUREUS* ASSOCIATED WITH THE DAIRY VALUE CHAIN IN WESTERN, LUSAKA AND SOUTHERN PROVINCES OF ZAMBIA

BY

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LUSAKA

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DECLARATION

I, Bruno Stephen July Phiri, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

……. Date……

Bruno Stephen July Phiri

CERTIFICATE OF APPROVAL

This thesis submitted by Bruno Stephen July Phiri, has been approved as fulfilling the requirements for the award of Doctor of Philosophy in Microbiology by The University of Zambia.

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……..……. Date…….....

ABSTRACT

Raw milk provides a good environment for the growth of a wide variety of microorganisms including *Staphylococcus aureus* (*S. aureus*) and can be a vehicle to transmit such pathogens to humans. The study aimed to investigate milk hygiene practices and characterize *S. aureus*/MRSA associated with the Zambian dairy chain in the selected Provinces and reduce the risk to consumers. A total of 1939 samples (of different matrices) were collected from 288 facilities (farms, milk collection centres (MCCs), traders, processing plants, traditional markets, and supermarkets/shops). Microbiological methods were used to analyse the samples with regard to *S. aureus*, including methicillin-resistant *S. aureus* (MRSA). A total of 348 presumptive *S. aureus* isolates (including one presumptive MRSA) were isolated. Molecular characterisation was used to type and characterise isolates of which 295 were confirmed by MALDI-TOF MS and PCR. No MRSA was detected. Raw milk from farms was widely contaminated with *S. aureus* in all the three provinces (Western: 46 percent, Lusaka: 43 percent and Southern: 33 percent) including that from MCCs and traders in Western and Southern provinces. Raw milk and/or sour milk samples from traditional markets and raw milk samples from milk processing plants were contaminated with *S. aureus* in all three provinces. *S. aureus* was less frequently detected from hands and nasal swabs from milkers, milk buckets and mastitis cows. No *S. aureus* was detected from water and commercially processed (heat-treated) dairy products from supermarkets/shops. A total of 36 known *spa* types were detected including seven novel types: t18396, t18397, t18398, t18399, t18400, t18402 and t18416. *Spa* type t355, t267 and t084 were common in all the three provinces. Many isolates from Lusaka province belonged to t189 and t267, while in Southern province t521 was dominant. Diversity and differences in the distribution and pattern of virulence, enterotoxin and antibiotic resistance genes were detected. A total of 29 *S. aureus* strains (10 percent) carried genes that codes for the virulence factor Panton-Valentine leucocidin (PVL). In addition, many of the 77 isolates selected for detailed characterisation by microarray or whole genome sequencing carried the virulence genes *hlgA, hlgB, hlgC, lukD, lukE, lukY, lukX*, and/or *hlb*; some isolates carried at least *SplA, SplB, sak, chp* and/or *scn* while other strains harboured *eta* and *tst1*. Genes for staphylococcal enterotoxins (SEs) were found in only 16 out of 77 isolates. Of the classical SE genes, *sea* was found in two isolates, *seb* and *sec* in three each, and *sed* in one. The predominant antimicrobial resistance in isolates included penicillin (41.7 percent), tetracycline (33.3 percent) and trimethoprim (27.8 percent) and 19.4 percent were multi drug resistant (MDR). Enterotoxigenic, virulent and antibiotic resistant *S. aureus* strains are circulating in the Zambian dairy value chain and represent a public health threat. The possible transmission of *S. aureus* to humans through cow milk is indicated. Therefore, there is a need for improved milk handling and hygienic practices at different stages/levels along the entire Zambian dairy value chain.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACF	Agricultural Consultative Forum
AMR	Antimicrobial Resistance
AST	Antibiotic Susceptibility Test
a_w	Water activity
BfR	Germany Federal Institute for Risk Assessment
BHI	Brain Heart Infusion
Bp	Base pair
BPA	Bair Parker Agar
BPB	Bacto Pepton Bouillon
°C	Degrees Celsius
CA-MRSA	Community Associated MRSA
CASO	Tryptic Soy Broth
CC	Clonal Complex
Cef	Cefoxitin
CFU	Colony Forming Units
CHIPS	Chemotaxis Inhibitory Protein of *S. aureus*
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase Negative Staphylococci
CPS	Coagulase Positive Staphylococci
DNA	Deoxyribonucleic Acid
EUCAST	European Committee on Antibiotic Susceptibility Testing
EFSA	European Food Safety Authority
€	Euro Currency
FAO	Food and Agriculture Organisation
g	Gram
HCCA	a-Cyano-4-hydroxycibnanamic acid
HA-MRSA	Hospital Associated MRSA
ICMSF	International Commission on Microbiological Specifications for Food
ISO	International Organization for Standards
L	Lysostaphin
l	Litre

LA-MRSA	Livestock Associated MRSA
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry
MCC	Milk Collection Centre
MHB	Muller Hinton Broth
Ml	Millilitres
MLST	Multilocus Sequence Typing
MPP	Milk Processing Plant
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
NaCl	Sodium Chloride
NGS	Next Generation Sequencing
NHREB	National Health Research Ethics Board
NRL	National Reference Laboratory
NRL-Staph	National Reference Laboratory for coagulase-positive Staphylococci including. *Staphylococcus aureus*
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PCA	Plate Count Agar
pmol	picomol
PVL	Panton-Valentine Leucocidin
PW	Peptone Water
S. aureus	*Staphylococcus aureus*
SAK	Staphylokinase enzyme
SCIN	Staphylococcal Complement Inhibitor Protein
SE(s)	Staphylococcal Enterotoxin(s)
SFP	Staphylococcal Food Poisoning
SPSS	Statistical Package for the Social Sciences
ST	Sequence Type
TE low buffer	Tris-EDTA Buffer
TPC	Total Plate Count
TSS	Toxic Shock Syndrome

µl	Microlitre
µM	Micromol
UHT	Ultra High Temperature
UNZA	University of Zambia
USA	United States of America
US$	United States Dollars
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Overview of Livestock sector in Zambia

Zambia is a landlocked country located in the Sub-Saharan Africa in the south of the equator. It is bordered by eight countries that include Angola, Botswana, the Democratic Republic of Congo, Malawi, Mozambique, Namibia, Tanzania and Zimbabwe. The population of Zambia is approximately 18, 383, 955 people, and the source of livelihood for the rural communities is mainly agriculture (FASAZ report, 2010). As a result, the Government of the republic of Zambia has recognised agriculture as the main sector that can significantly help in reducing poverty and promote economic diversification (World Bank, 2011; Mumba *et al*., 2013). Agriculture in Zambia is divided into livestock (including beef and dairy), poultry, crop and fish farming. Dairy farming remains one of additional farming activities to crop farming which heavily depends on expensive inputs such as fertilizers, implements and other chemicals especially as Zambian farmers think about diversification of their farming activities. Milk has always been considered as a major component of human diet all over the world. It is an important source of nutrients providing energy, proteins, phosphorus, calcium and other micro nutrients which are vital for growth especially in infants and children (Dror and Allen, 2014; Iannotti, 2018).

1.2 Structure of the dairy sector in Zambia

The dairy sector in Zambia has been identified as a very important sector in driving rural and peri-urban development. It has the potential to expand significantly and contribute to creation of jobs for many Zambians (World Bank, 2011; Mumba, 2012). Small scale dairy farming is a very important component of agriculture for most of the people especially in rural areas as it is a source of food and income. This generally results in people accessing the much needed nutrition and incomes from milk and milk products hence leading to poverty alleviation and food-secure households. The major milk producing provinces in Zambia include Central, Western, Lusaka and Southern provinces (Mumba *et al*., 2013) with the last two provinces being some of the highest

producers because of the large cattle population present. In recent years, Zambia has seen an increase in numbers of people starting dairy enterprises especially retirees who use part of their pension money to invest in dairy farming. In addition, the working class as well are engaged in dairy farming activities. There are about 149,389 households keeping all types of cattle in the study provinces with Lusaka (13,230 households), Western (31,646 households), and Southern (104,513 households) (CSO, 2017). Dairy cattle farmers in Zambia are divided into three categories namely traditional, smallholder and commercial farmers (Figure 1). Traditional dairy farmer means a farmer keeping dairy animals at subsistence level and usually on free range while smallholder dairy farmer means a farmer keeping dairy animals on semi-intensive management system and, commercial dairy farmer is a farmer practicing mainly intensive management system and using conversional animal feeds. Traditional farmers are found in rural areas and mainly keep traditional local (indigenous) breeds of cattle and milk production among them is usually very low as these breeds are not high yielding. The common traditional local breeds used in Zambia are the Barotse, Tonga and Angoni breeds found mainly in Western, Southern and Eastern provinces respectively. These local breeds on average produce about one to three litres of milk per day (ACF, 2012). Smallholder and commercial farmers keep a mixture of cross and pure breeds of dairy cattle such as Friesian and Jersey (ACF, 2012).

Traditional and smallholder dairy cattle farmers mainly supply their milk to milk collection centres (MCCs)/cooperatives (Figure 1) and most of them belong to these structures as members. MCCs are mainly cooperatives or centres which buy milk supplied by members (dairy farmers) where milk is bulked for purposes of selling on behalf of the farmers. MCCs in Zambia have been established with basic cooling facilities where milk is normally cooled in cooling tanks (World Bank report, 2013). Cooling slows down bacterial growth in milk, reducing spoilage and therefore increasing dairy farmer income, and also ensuring that milk is safe for consumers. However, electricity failures remain a potential challenge regarding milk storage (Mweemba *et al*., 2016). On the other hand, commercial dairy farmers mainly sell their milk directly to big milk processors. The existence of MCCs/cooperatives located mainly in milk producing areas has provided the much needed ready market for milk to dairy cattle farmers and traders. Milk from these MCCs/cooperatives is bought

mainly by big milk processing companies. Some of the milk is also sold directly to the general public (Figure 1). However, out of approximately 253 million litres of milk produced per annum in Zambia, only about 44 million litres is estimated to pass through formal markets (ACF, 2012; Mumba, 2012) while the rest find its way into the informal sector or it is consumed by milk producing households directly or indirectly as processed products. In Zambia, traditional fermented product known as mabisi (traditionally processed sour milk) is popular mostly among the people of Western, Southern and Central provinces (Moonga *et al*., 2019). It is made from raw milk fermented at room temperature for approximately 48 hours in a calabash (Figure 2) and make use of bacteria present in the milk. Some of the fermented milk is also sold through the informal market by traders or farmers directly.

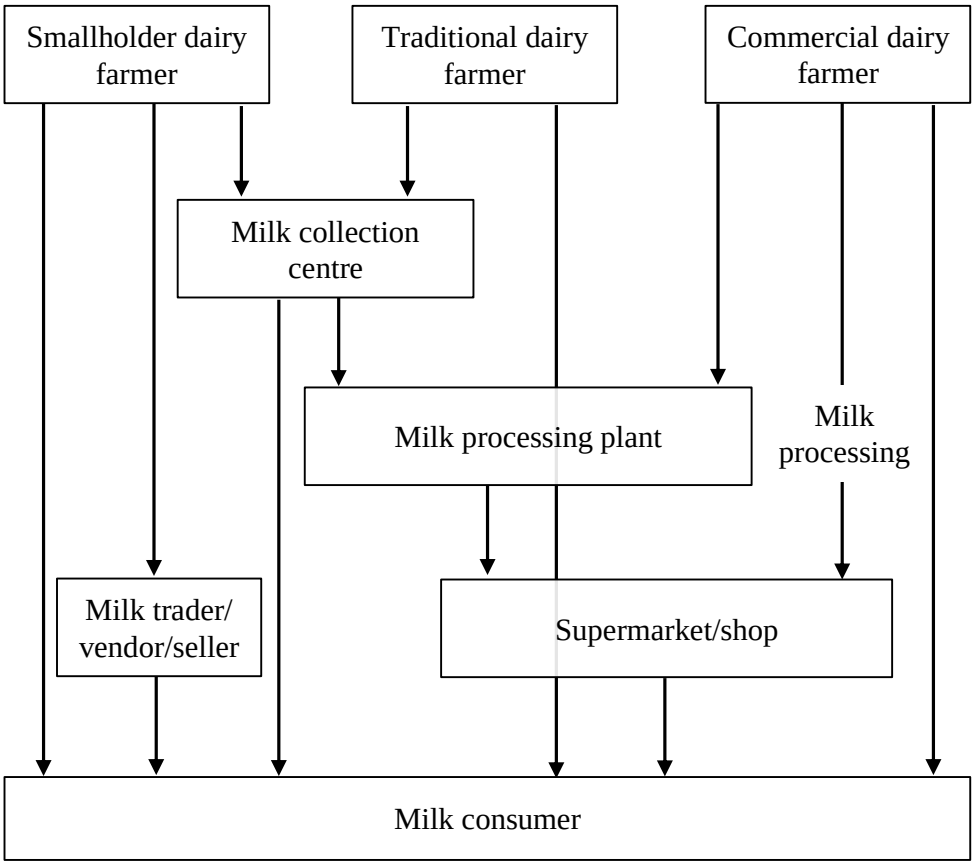


Figure 1: The Zambian dairy value chain structure

Figure 2: A man carrying a calabashi used for processing mabisis in his hands (Moonga *et al*., 2019)

1.3 Zambia dairy value chain as a potential source of pathogens including *S. aureus*

Milk from cow or any animal, due to its high nutritional content, can support a variety of microorganisms naturally present in milk. These microorganisms may enter milk from a variety of sources and, once in milk, can play a number of roles, such as facilitating dairy fermentations (e.g. *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Propionibacterium* and fungal populations), causing spoilage (e.g. *Pseudomonas*, *Clostridium*, *Bacillus* and other spore-forming or thermoduric microorganisms), promoting health (e.g. lactobacilli and bifidobacteria) (Lisa *et al*., 2013). The predominant bacteria in cow's raw milk may include *Lactobacillus lactis* and *Streptococcus thermophilus* (Masoud *et al*., 2012).

Despite all nutritional benefits provided by milk, it can also be a source of disease caused by pathogens present in milk (Currier and Widness, 2018). Milk provides a good environment for the growth of microorganisms and can harbour a variety of microorganisms hence becoming an important source of foodborne pathogens (Oliver *et al*., 2005). Contaminated milk can be a perfect vehicle for transmission of bacterial

pathogens to humans (Dhanashekar *et al*., 2012). If pathogenic microorganisms are involved, they can cause harm to consumers by causing illness and disease (Barros *et al*., 2011). The major challenges of dairy farming in Zambia are livestock diseases such as mastitis, brucellosis and tuberculosis among common diseases (World Bank, 2011). Poor milk handling hygiene at every stage of the Zambian dairy value chain could be one of the sources of contamination of milk with pathogens such as *S. aureus* (Phiri *et al*., 2021). In addition, poor hygiene regarding udder, teat, milkers hands and milk buckets could lead to contamination of milk and may play a role in the maintenance of *S. aureus* in the environment (Sanaa *et al*., 1993; Ndungu *et al*., 2006; Amentie *et al*., 2016; Abunna *et al*., 2019). *S. aureus* can cause foodborne infection due to the ability of enterotoxigenic strains to produce heat stable staphylococcal enterotoxins (SEs) in food (Tranter, 1990; Argudin *et al*., 2010).

The practice of mixing morning and afternoon batches of milk, lack of refrigeration, unhygienic milk handling, poor hand hygiene, use of unclean milking clothes, long distances to the markets or MCCs, unclean containers used in the transportation, storage and preservation including cows with clinical and subclinical mastitis could be responsible for contributing to the possible contamination and/or proliferation of pathogens in milk. People colonized with *S. aureus* asymptomatically, especially those who handle food can also introduce the bacteria into the food chain (Argudin *et al*., 2010). Therefore, good milk handling hygienic practices must be practiced to ensure good milk quality and its safety to consumers.

1.4 The potential risk of *S. aureus* in the Zambian dairy value chain

The consumption of raw milk in Sub-Saharan African countries is still popular among many people (Jans *et al*., 2017) and Zambia is not an exception (Phiri *et al*, 2021). In Zambia, the practice is mainly common among the Tonga and Lozi people of Southern and Western provinces respectively. In these provinces, people also consume traditionally processed sour milk (mabisi) which is produced from raw milk. The consumption of raw milk may put the milk consumer at risk of getting infected with foodborne pathogens such as *S. aureus*. Typically, a staphylococcal food poisoning (SFP) occurs after ingestion of foods that are contaminated with *S. aureus* (through improper handling of food) and storage at elevated temperatures leading to SEs

formation. SFP caused by staphylococcal enterotoxins is among the leading causes of food-borne outbreaks in the European Union (EFSA, 2015). However, there is limited data on foodborne diseases caused by *S. aureus* in Africa probably due to inadequate expertise, poor diagnosis, poor reporting (WHO, 2015), and inadequate specialised laboratories. The dominant symptoms of SFP are vomiting, nausea, diarrhoea and circulatory symptoms. Extremely small amounts of toxin are sufficient to trigger these symptoms. The real incidence of SFP is probably underestimated for a number of reasons, which include unreported minor outbreaks, improper sample collection and misdiagnosis (Argudin *et a*l., 2010). Foods that favour growth of bacteria, especially raw food of animal origin with high protein content such as raw milk and milk products have been frequently implicated in SFP outbreaks (Hennekinne *et al*., 2012).

1.5 Public health implication of *S. aureus*

Milk safety is very important to both dairy farmers and consumers. The occurrence of illnesses and deaths as a result of diseases caused by eating unsafe food worldwide is a growing threat to public health (Kuchenmuller *et al*., 2009). Furthermore, the emergence of antimicrobial resistant strains of *S. aureus* adds another dimension of burden to the effective disease management by aggravating disease outcomes because of treatment failures. Antimicrobial resistance (AMR) among common human zoonotic pathogens is emerging as a major threat to the effectiveness of the healthcare systems worldwide (WHO, 2001). When infected by pathogens resistant to common antibiotics and multiple groups of antibiotics, the patients eventually fail to recover from illnesses despite being treated with antibiotics (Smith and Coast, 2013). In addition, methicillin resistant *S. aureus* (MRSA) has become a public health threat causing treatment failures in a wide range of infections and it is one of the nosocomial pathogens worldwide (Tiemersma *et al*., 2004; Shittu *et al*., 2009). Methicillin resistance is conferred by the *mecA* gene, which encodes a penicillin-binding protein (PBP2A) with decreased affinity for β-lactam antibiotics. Some strains of MRSA contain virulent factors that may enhance their virulence or may enable them to cause particular diseases or clinical syndromes (Gordon and Lowy, 2008). Further, MRSA strains cause severe infections in hospital environment and in the communities (Kesah *et al*., 2003; Falagas *et al*., 2013; Maina *et al*., 2013). To combat AMR, a one-health approach involving medical and veterinary practitioners, microbiologists, pharmacists,

and social scientists is required. There is a wide overlap of antimicrobials used in human and veterinary medicine and as a result, bacteria in food producing animals may harbour resistance determinants for the same antimicrobials as in humans (Marshall and Levy, 2011). Transmission of AMR bacteria from animals to humans is a big challenge to human health (FAO, 2011). There are three main ways through which AMR can be transmitted from animals to humans: This include direct contact of livestock workers with colonized animals (Wulf *et al*., 2006; van Cleef *et al*., 2011), the consumption of food of animal origin contaminated with AMR bacteria (EFSA, 2008), and the spread of AMR bacteria to the environment via exhaust air and slurry (Marshall *et al*., 1990).

1.6 Statement of the problem

Despite all the nutritive beneficial aspects provided by milk, it can also be a source of disease caused by pathogens (Currier and Widness, 2018). Milk provides a good environment for the growth of microorganisms and can harbour a variety of microorganisms hence becoming an important source of foodborne pathogens (Oliver *et al*., 2005). It is usually consumed raw by many people in most communities in Zambia, especially in Western and Southern provinces because of their tradition and culture. Although *S. aureus* is a commensal bacteria found usually on the skin of animals and humans, finding it in food such as milk represent a public health concern. Clinical and subclinical mastitis, poor hygienic practices, unclean milkers' hands and, containers for milk storage and transportation over long distances are potential risk factors for *S. aureus* contaminations of milk in the Zambian dairy value chain (Phiri *et al*., 2021). *S. aureus* is an important foodborne pathogen because of the ability by some strains to produce heat stable enterotoxins (Argudin *et a*l., 2010) and it has been responsible for outbreaks of food poisoning worldwide (Hennekinne *et al*., 2012). There is limited data on the virulence, enterotoxigenic and antimicrobial resistance potential of *S. aureus* in Zambia. Further, the emergence of MRSA in humans and animals is another health threat worldwide. In Zambia, there is growing demand for milk and this has resulted in an increase in livestock production in the recent years. These trends, in combination with high background levels of livestock diseases such as mastitis are driving the usage of antibiotics in developing countries in general to increase rapidly (Van Boeckel *et al*., 2015). The increasing use of antibiotics in

Zambian livestock production (Mainda *et al*., 2015) is a concern that has the potential to contribute to a huge and emerging problem of AMR in human pathogens (Marshall and Levy, 2011) as growing public health threat.

There is very limited data on prevalence of *S. aureus*/MRSA in the dairy value chain in Zambia available. Therefore, it is very important to determine the milk handling, hygiene and safety practices among stakeholders and the presence of virulent, enterotoxigenic and MRSA strains of *S. aureus* along the dairy value chain from farm to fork in order to identify interventions to reduce the risk to milk consumers and farmers including other stakeholders.

1.7 Research questions

1. What are the milk hygiene practices among the stakeholders in the dairy value chain with the potential to increase the possible risk of milk contamination?
2. Are there any enterotoxigenic and virulent strains of *S. aureus* circulating in the dairy value chain in the study provinces?
3. What are the vehicles playing a role in transmission, spread and maintenance of *S. aureus* in the dairy value chain?
4. Are there any antimicrobial resistant strains of *S. aureus* circulating in the Zambian dairy value chain?
5. Are MRSA strains of *S. aureus* circulating in the dairy value chain?

1.8 Objectives

1.8.1 General objective

To assess the milk handling hygiene practices and study the characteristics of *S. aureus* associated with the dairy value chain in Western, Lusaka and Southern provinces of Zambia.

1.8.2 Specific objectives

1. To describe the different milk hygiene practices among stakeholders

2. To characterise *S. aureus* from different matrices associated with the dairy value chain in the three provinces.

4. To evaluate roles of different matrices in transmission, spread and maintenance of *S. aureus* along the dairy value chain?

3. To evaluate antimicrobial resistance genes/profiles of *S. aureus* isolates

4. To analyse the possible presence of MRSA among *S. aureus* isolates

1.9 Significance of the study

Currently, very little research work related to *S. aureus* has been done in Zambia. Pooled milk samples supplied to Zambia Dairy Produce Board (Parmalat now) in Zambia by a number of dairy farmers was screened in 1996 and it revealed gross contamination of milk with *S. aureus* (Pandey *et al.*, 1996). However, this particular study was conducted nearly 23 years ago and it was not comprehensive as it did not employ a dairy value-chain approach and *S. aureus* strains found were not fully characterised. There is scanty information on the prevalence and magnitude of enterotoxigenic *S. aureus*/MRSA in the Zambian dairy value chain. In addition, the potential health risks, caused by *S. aureus*/MRSA associated with livestock to human health in Zambia is not fully understood. Therefore, this study aimed to provide information regarding prevalence of genetic diversity, antibiotic resistant profiles and virulence including enterotoxigenic potential among *S. aureus* isolates in the three study provinces. Further, this information will be important for the Ministries of Fisheries and Livestock, and Health regarding public health policies on milk production and handling hygiene by milk handlers and other stakeholders in the entire Zambian dairy value chain. The current study also highlights the potential risk of *S. aureus* strains found in the three study provinces to consumers. In addition, targets for possible intervention were identified. Policy makers could use this information to direct food safety policies and interventions which may help in the prevention and control targeting the spread of infectious pathogens particularly the virulent, enterotoxigenic and antibiotic resistant *S. aureus* strains. This, coupled with other interventions including boiling of milk, improved milking hygiene and training of stakeholders in hygienic milk handling among many interventions, could eventually lead to a reduction of *S. aureus* possible risk associated with milk to consumers.

CHAPTER TWO

LITERATURE REVIEW

2.1 General overview of *Staphylococcus* in dairy

Staphylococci was first seen in human pus specimen by Robert Kock in 1878. It was then cultivated for the first time in liquid media by Louis Pasteur in 1880 (Wainwright and Lederberg, 1992). Sir Alexander Agoston named the bacteria Staphylococcus after its clustered appearance was evident under a microscope. Bacterial organisms including *S. aureus, S. epidermidis, pseudomonas aeruginosa, Streptococcus agalactiae, Streptococcus uberis, Mycoplasma spp* (including *Mycoplasma bovis*), *Brucellosis melitensis, E. coli, Enterobacter aerogenes, Pasteurella spp* and *Proteus spp* are known to be some of the bacteria causing mastitis in dairy cows. Of these bacterial pathogens, *S aureus* a normal flora, has been known as one of the major causes of mastitis in the dairy industry both in the developed and developing countries (Petzer *et al.*, 2009; Gitau *et al*., 2014). In dairy cows, mastitis plays a major role in reducing milk production (Hagnestam-Nielsen and Ostergaard, 2009).

2.2 Characteristics of the genus *Staphylococcus*

The bacteria belonging to the genus *Staphylococcus* are small, spherical, non-spore forming, non-motile Gram-positive bacteria with a diameter approximately ranging from 0.5 to 1.5 µm (Harris *et al*., 2002). These microorganisms are facultative aero-anaerobic bacteria which makes them able to grow in environments with oxygen and carbon dioxide (CO_2). The genus *Staphylococcus* belongs to the family *Staphylococcaceae* and the order *Bacillales* (Gherardi *et al*., 2018). Staphylococci are usually catalase-positive and this characteristic makes it possible for them to be differentiated from *Streptococcus* as they are catalase-negative. The staphylococci appear in pairs, as short chains or grape-like clusters when viewed through a microscope. There are more than 49 species and 26 subspecies of the genus *Staphylococcus* described to date (Han *et al*., 2015; Kim *et al*., 2018). Staphylococci can exist everywhere including in air, dust, sewage, water and food (including milk) or on food equipment, environmental surfaces and in humans and animals.

The genus *Staphylococcus* is classified into two groups, namely coagulase positive Staphylococci (CPS) and coagulase negative staphylococci (CNS) depending on the ability or inability to produce the coagulase enzyme and to clot blood plasma. Further, the genus has been further classified also based on clinical and epidemiological characteristics (Becker *et al*., 2014). The CPS include *S. aureus*, *S. intermedius*, *S. hyicus* and *S. schleiferi*; CNS include *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. warneri*, *S. capitis*, *S. homis*, *S. pettenkoferi*, *S. carnosus*, *S. felis*, *S. caprae* and *S. lentus* (Becker *et al*., 2014). *S. aureus* is the most important species among the CPS because of its ability to cause severe disease in humans and animals as well as causing food poisoning.

2.3 Growth and survival characteristics of *S. aureus*

Staphylococcus aureus can grow and survive in a wide range of environments characterised by many factors such as temperature, water activity (a_w), pH (Table 1), presence or absence of oxygen and usually, the composition of the food. These physical growth parameters can vary for different strains of *S. aureus* involved (Stewart, 2003). In general, *S. aureus* can grow and multiply at temperature ranging from 7°C to 48°C, with usually an optimum of 30°C to 37°C (ICMSF, 1996; Stewart, 2003). The bacteria have been known to have a relatively high heat resistance (Stewart, 2003). The observed D-value for *S. aureus* (the time after which the initial concentration of viable *S. aureus* cells would be reduced by 1 $\log_{10}$ unit) was between 4.8 to 6.6 minutes at 60°C when heated in broth (Kennedy *et al*., 2005). Further, an extremely heat resistant strain of *S. aureus* was detected from a foodborne outbreak in India with a D-value of approximately 15 to 16 minutes at 60°C (Nema *et al*., 2007). *S. aureus* is also resistant to freezing and can survive well in food stored below -20°C. However, the viability of the bacteria is reduced at temperatures between -10°C to 0°C. Strains of *S. aureus* are readily killed at pasteurization temperature of approximately 71.7°C for about 15 seconds (Stewart, 2003). In addition, the bacterium is able to survive and grow at a pH range between 4 to 10, with optimum range of 6 to 7.5, a_w of > 0.83 with optimum of 0.98 and sodium chloride of up to 15 percent (Stewart, 2003; Montville and Matthews, 2008; Kadariya *et al*., 2014). The ability of *S. aureus* to tolerate all these conditions, allow it to survive in dry environments such as skin, clothes and environmental surfaces (Chaibenjawong and Foster, 2011;

Kadariya et al., 2014). Further, these growth conditions, can also favour the growth of *S. aureus* in many food products (Le Loir *et al*., 2003).

S. aureus is usually sensitive to microbial competition in food. This characteristic has been studied in sour milk. Higher concentration of microorganisms for example in sour milk tends to lower the rate of *S. aureus* growth and Staphylococcal enterotoxin (SE) production (Genigeorgis, 1989). Lactic acid produced by lactic acid bacteria, usually has also a negative growth effect on *S. aureus.* In addition to lowered pH, competition for nutrients with other microorganisms in presence of hydrogen peroxide, and in some cases synthesis of antimicrobial substances such as antibacteriocins produced by other organisms may have negative effect on the growth of *S. aureus* (Le Loir *et al*., 2003).

Some strains of *S. aureus* produce staphylococcal enterotoxins (SEs) usually within the temperature range of 10°C to 48°C, with the optimum temperature of 40°C to 45°C (Table 1). As the temperature decreases, the level of the SE production also decreases. The SEs are known to be highly resistant to heating process and could survive even the processes used to sterilize foods. These toxins will remain stable under freeze storage conditions. Further, SEs production can take place within a pH range of 4.5 to 9.6, with an optimum of 7 to 8 (ICMSF, 1996). In addition, the production of SEs can take place in both anaerobic and aerobic conditions. However, SE production is usually optimum in aerobic environment (Stewart, 2003). SEs are usually produced during the exponential phase of *S. aureus* growth and the quantity is usually strain dependent (Derzelle *et al*., 2009).

Table 1: Limits for growth of *S. aureus* and for enterotoxin production when other conditions are near optimum (ICMSF, 1996)

	Bacterial Growth		**Enterotoxin Production**	
	Optimum	**Range**	**Optimum**	**Range**
Temperature (°C)	37	7 – 48	40 – 45	10 – 48
pH	6 – 7	4 – 10	7 – 8	4.5 – 9.6
Water activity	0.98	0.83 – > 0.99	0.98	0.87 – > 0.99

2.3.1 *S. aureus* pathogenicity and virulence factors

For a bacterium to cause disease, it must possess a number of factors capable of making it survive and establish itself in the host. *S aureus* has in possession a number of virulent factors which help the bacteria attach and invade the host cells/tissue and, evade the host immunity.

2.3.1.1 Cell wall

The cell walls of *S. aureus* is an important cellular component of this Gram positive bacterium and plays a significant role in the infectivity and pathogenicity (Dmitriev *et al*., 2004). The cell wall is mainly composed of peptidoglycan (Weidel and Pelzer, 1964), teichoic acid (Baddiley, 1989) and cell wall-associated surface proteins (Mazmanian *et al*., 2001; Perry *et al*., 2002). About 50 percent by weight of the *S. aureus* cell wall is made of peptidoglycan layers. The *S. aureus* peptidoglycan layers also contain ribitol teichoic acid and lipoteichoic acid (Figure 3). It is believed that differences in the peptidoglycan structure between *S. aureus* strains could contribute to the variation in their capacity to disseminate intravascular coagulation and it is also reported that peptidoglycan has endotoxin-like activity (Kessler *et al*., 1991). Penicillin binding protein (PBP) located in the cytoplasmic membrane is involved in the assembly of the cell wall (Lowy, 1998). Some *S. aureus* strains form capsular exopolysaccharides and these structures play an important role in the pathogenesis and antibiotic resistance ability of the pathogen (Begun *et al*., 2007). About 11 types of microcapsules were found to be produced by more than 90 percent of *Staphylococcus* species (Lee, 1996).

S. aureus cell wall also contains surface protein known as protein A and it is encoded by *spa* gene which can be typed on the basis of base sequence. Protein A (SpA) is a very important virulence factor for *S. aureus* (Foster and Hook, 1998; Palmqvist *et al*., 2002; Fournier and Philpott, 2005; Baum *et al*., 2009). It has been known to be associated with the pathogenesis of *S. aureus* pneumonia (Gomez *et al*., 2004; Gomez *et al*., 2006; Baum *et al*., 2009), invasive diseases (Loughman *et al*., 2009) and has been known to play a role in biofilm formation (Merino *et al*., 2009). SpA is a protein of 42 kDa by weight and comprises of various regions with different functions: The

signal sequence (S region) in the N-terminal part is followed by approximately four to five homologous immunoglobulin G (IgG)-binding domains usually in tandem (the E, D, A, B, and C regions) (Lofdahl *et al*., 1983; Baum *et al*., 2009). The C-terminal region, or X region is divided into two domains namely; (i) the repeat region X_R, consists of variable repeats with mostly octapeptide structures which are used for *spa* typing, and (ii) Xc consisting of a conserved sequence, which has been known to confer anchoring to the cell wall through an LPXTG-binding motif (Schneewind *et al*., 1992; Schneewind *et al*., 1995; Baum *et al*., 2009). The most studied function of SpA includes the interaction with human IgG by binding to the F_c part, which negatively affects the immune system (Foster, 2005; Baum *et al*., 2009). In recent years, *spa* (*S. aureus* protein A) typing use has been on the increase as a typing method (Baum *et al*., 2009).

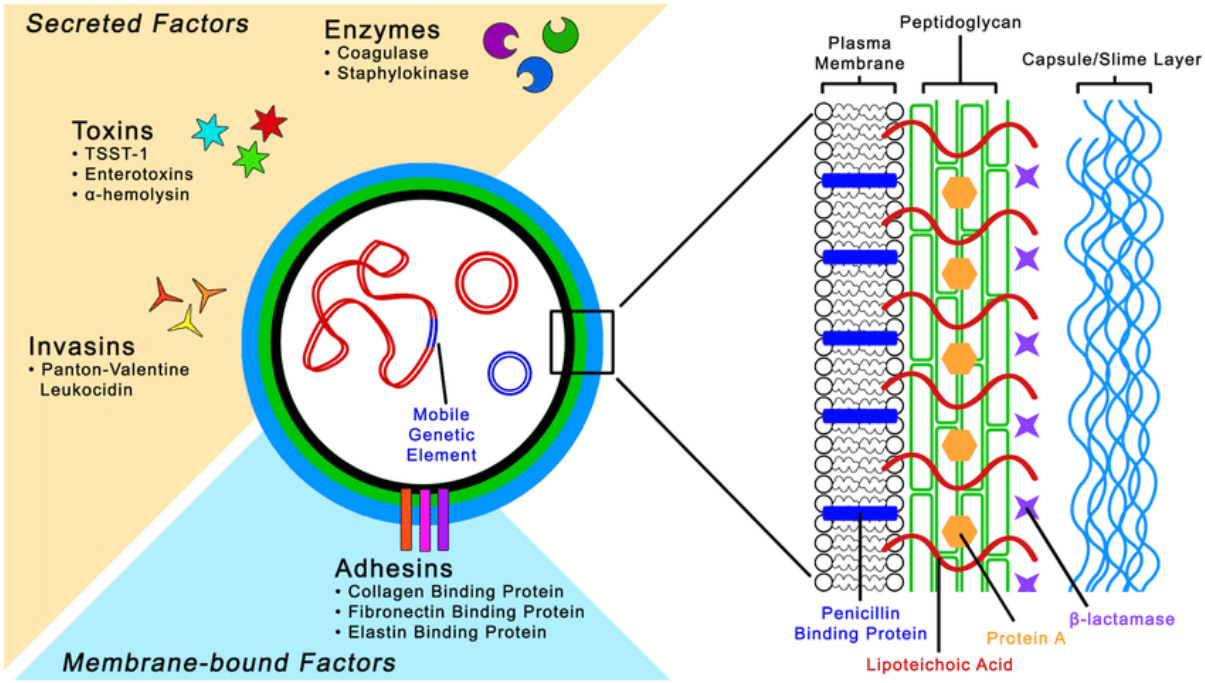


Figure 3: Cell wall structure and virulence factors. Source: (Kong et al., 2016)

2.3.1.2 Virulence factors of *S. aureus*

Staphylococcus aureus is known to possess several genes encoding a wide range of virulence factors that are located on the chromosome or plasmids (Lowy, 1998). These factors are responsible for the host colonisation, invasion of damaged skin and mucosae, transmission throughout the body, and the evasion of the host defence system (Lowy, 1998). Approximately 50 virulence factors expressed by *S. aureus* have been

described (Ferry *et al*., 2005) and these are responsible for many biological activities. *S. aureus* has been implicated as a major cause of several toxin-mediated and suppressive diseases in humans (Ferry *et al*., 2005). It is believed that in staphylococcal scalded-skin syndrome (SSSS) and toxic shock syndrome (TSS) staphylococcal pathogenicity is not due to just one factor. Several factors participate in the pathogenesis of different diseases in a stepwise manner. In each of these steps, one or several virulence factors could be involved (Ferry *et al*., 2005).

Usually, the first step of pathogenesis will start with virulence factors that help *S. aureus* to adhere to the host surface such as protein A and collagen-binding proteins. *S. aureus* protein A which is encoded by the *spa* gene bind to IgG causing an interference in the phagocytosis activity of the immune system of the host (Falugi *et al*., 2013). This allows *S. aureus* to evade host immunity by resisting phagocytosis. Further, some strains of *S. aureus* express collagen-binding proteins known as CAN. The presence of CAN is important and sufficient for the bacteria to adhere to collagenous tissue such as cartilage (Switalski *et al*., 1993). Clumping factor proteins (CIfA and CIfB) mediate adherence of *S. aureus* to fibrinogen and platelets in the presence of fibronectin (Switalski *et al*., 1993; McDevitt *et al*., 1997; Ni Eidhin *et al*., 1998; Risley *et al*., 2007). This results in clumping of the blood plasma. The expression of cell wall-surface proteins (CWA) can be altered by growth conditions. Moreover, some proteins are expressed under iron-limited conditions (Mazmanian *et al*., 2003; Hammer and Skaar, 2011) while some proteins are predominantly expressed during the exponential bacterial growth phase (McAleese *et al*., 2001). CWA are covalently attached to peptidoglycan, and are crucial in the success of *S. aureus* as commensal and pathogenic bacteria (Foster *et al*., 2014).

The second step of *S. aureus* pathogenesis involves the secretion of proteins. These proteins play an active role in disarming the host immunity, disrupting host cells and tissues, and interfering with the host immune mechanisms to release nutrients and to help the bacteria to disseminate (Foster, 2005; Lin and Peterson, 2010; Costa *et al*., 2013). These exotoxins are grouped into four categories namely: superantigens, cytolytic (pore-forming) toxins, various exoenzymes and miscellaneous proteins (Lin and Peterson, 2010). Superantigens are a group of secreted immune-stimulatory

proteins that include staphylococcal enterotoxin (SEs) (as discussed in subsection 2.3.2) and toxic shock syndrome toxin (TSST-1). Further, *S. aureus* secretes a variety of immunomodulatory proteins and virulence factors which mainly target leucocytes (Vandenesch *et al*., 2012; Otto, 2014). Some of the secreted proteins are the bi-component pore-forming leucocidins consisting of an F and S subunit (Alonzo and Torres, 2014; Menestrina *et al*., 2003). The so far known seven bi-component leucocidins of *S. aureus* strains include Panton-Valentine leucocidin (PVL, encoded by *lukS-PV* and *lukF-PV*), LukMF' (a non-human leucocidin encoded by *lukF-PV (P83)* and *lukM*), LukED (encoded by *lukE* and *lukD*), LukXY (also named LukAB or LukGH), gamma-haemolysin AB (HlgAB, encoded by *hlgA* and *hlgB*) and HlgCB (encoded by *hlgC* and *hlgB*). These proteins usually form β-barrel pores in the cytoplasmic membranes of host immune cells and cause leakage of the cell's content at low doses and cell lysis at high doses (Foster, 2005; Lin and Peterson, 2010).

Almost all strains of *S. aureus* secrete extracellular enzymes whose major function is believed to be the disruption of host tissue and, or inactivation of the host antimicrobial mechanisms (including lipids, defensins, antibodies and complement mediators) to acquire nutrients for bacterial growth and help in bacterial dissemination (Dinges *et al*., 2000; Lin and Peterson, 2010; Costa *et al*., 2013). These exoenzymes include lipases, proteases, hyaluronidase, and staphylokinase (Dinges *et al*., 2000; Lin and Peterson, 2010). These enzymes also play a role in destroying the host tissue and help the bacteria to spread to the adjacent tissues leading to bacterial invasion. In addition, *S. aureus* has other specific proteins that can also have a profound impact on the innate and adaptive immune system. These include staphylococcal complement inhibitor, extracellular fibrinogen-binding protein, chemotaxis inhibitor protein, formyl peptide receptor-like-1 inhibitory protein and extracellular adherence protein (Costa *et al*., 2013).

2.3.2 *S. aureus* enterotoxins

SEs are potent gastrointestinal exotoxins synthesized by *S. aureus* throughout the logarithmic phase of growth or during the transitional phase from the exponential to stationary phase (Otero *et al*., 1990; Betley *et al*., 1992; Lowy, 1998; Derzelle *et al*., 2009). SEs, are a family of related secreted proteins in terms of structure, and belong to the pyrogenic toxin superantigen family (Balaban and Rasooly, 2000). These

superantigens usually by-pass the convention antigen recognition probably by cross-linking major histocompatibility complex class II (MHCII) molecules on antigen presenting cells with T-cell receptors. This process, usually result in proliferation of T-cells and cytokine release (which in some cases may result in toxic shock syndrome) (Balaban and Rasooly, 2000). Sufficient amounts SEs must be consumed in food for SFP to occur (Argudin *et al*., 2010). Further, apart from *S. aureus*, there are other *Staphylococcus* species with the ability to produce SEs. These include CPS such as *S. intermedius* (Becker *et al*., 2001) and CNS such as *S. epidermidis*, *S. xylosus*, *S. haemolyticus* and *S. cohnii* (Bautista *et al*., 1988).

The SEs cover proteins with known emetic activity (Lina *et al*., 2004). About 23 types of SEs have been identified mainly based on antigenicity (SEA to SE*l*Y) (Denayer *et al*., 2017; Grispoldi *et al*., 2019). The SEA, SEB, SEC, SED, and SEE are referred to as classical SEs and have been implicated in SFP. Among these enterotoxins, SEA has been the most frequently reported as the common cause of SFP worldwide and involvement of other classical enterotoxins has also been reported (Argudin *et al*., 2010; Kadariya *et al*., 2014; Denayer *et al*., 2017; Grispoldi *et al*., 2019). In addition, out of the new SEs, only SEH has been clearly identified with SFP (Argudin *et al*., 2010). Further, strains of *S. aureus* harbouring new SEs have been widely detected worldwide. However, their role in pathogenicity may be underestimated (Argudin *et al*., 2010).

2.3.3 Expression of *S. aureus* enterotoxins

Enterotoxigenic strains of *S. aureus* have different profiles of staphylococcal enterotoxin (SEs) genes and may harbour several genes simultaneously. All these enterotoxin genes are known to be located on mobile genetic elements such as phages in case of *sea*, *see* and *sep*, on plasmid in case of *sed*, *sej*, *ser*, *ses*, *set*, and *S. aureus* pathogenicity islands (Kuroda *et al*., 2001; Yarwood *et al*., 2002; Omoe *et al*., 2005; Ono *et al*., 2008). The SE genes *ser* and *sej* are usually encoded on two kinds of plasmids along with genes *sed* or *ses* and *set* (Zhang *et al*., 1998; Omoe *et al*., 2003; Ono *et al*., 2008). The enterotoxin genes *sel* and *sec* of *S. aureus* are known to occur together (Fitzgerald *et al*., 2001; Kuroda *et al*., 2001). In addition, the enterotoxin genes *seq* and *sek* also occur together (Yarwood *et al*., 2002; Sumby and Waldor,

2003). The *S. aureus* enterotoxin genes *seg*, *sei*, *sem*, *sen*, *seo*, and sometimes *seu* or *sev* constitute the enterotoxin gene cluster (egc) which is organized as an operon (Thomas *et al*., 2006). The information on the distribution and the prevalence SE genes including enterotoxin-like genes in food-derived staphylococci is on the increase (Borst and Betley, 1994; Bania *et al*., 2006; Chiang *et al*., 2006; Hwang *et al*., 2007; Poli *et al*., 2007; Chiang *et al*., 2008). However, data regarding the individual enterotoxin gene expression and mechanism of gene regulation is still limited, apart from the 5 classical genes. Many differences in the expression may occur among the classical enterotoxin genes regarding their temporal regulation, toxin yield and, mechanism of gene regulation.

Staphylococcus aureus has a very complex network of regulatory pathways to control toxin production (Recsei *et al*., 1986; Fisher *et al*., 2018). Expression of SEs is regulated by multiple and usually overlapping regulatory pathways which are mainly influenced by the environmental factors (Table 1). In addition, regulation of SE expression mainly depends on bacteria phase of growth and changes in the environment (Roux *et al*., 2014). A lot has been known about regulatory systems that control the responses of bacterium to the changes in the environment in the recent past but much still need to be known. In general, proteins responsible for adhesions and invasion of *S. aureus* are usually produced during the exponential bacteria growth phase while SEs including enzymes and exotoxins are synthesised in the post exponential growth phase (Lowy, 1998). These processes are regulated by a complex network of regulatory proteins. The complex regulatory proteins are grouped into two categories: (i) the two-component regulatory systems (TCRSs) and the global regulators of the SarA family (Cheung *et al*., 2004). The TCRSs also include the accessory gene regulator (Agr) and the staphylococcal accessory elements (Sae). The Agr is the main gene regulator system in the expression of virulence factors in *S. aureus*. However, not all SEs are regulated by Agr. For example, in case of SED and SEJ which have similarities in amino acid sequences, only SED is regulated by Agr while SEJ is not (Zhang *et al*., 1998b). It has also been known that the expression of SEA is not regulated by Agr (Tremaine *et al*., 1993). Further, production of SEB and SEC is inhibited in presence of glucose because of the inhibitory effect on *agr* expression due to low pH resulting from glucose metabolism (Regassa *et al*., 1992).

Neutral pH has been documented as the optimum pH for SE production (Table 1). On the other hand, it was noted that all the regulatory networks exert their influence in an interactive manner, not singular to ensure that virulence factors are expressed when required (Costa *et al*., 2013).

2.4 Epidemiology of *S. aureus*

Staphylococcus aureus bacteria are distributed worldwide and are usually found in the nose, groin, axillae, perennial areas (especially in males), mucous membranes, mouth, mammary glands, hair including intestinal, genitourinary and respiratory tracts (Murray *et al*., 2003). Humans, wild and domestic animals are some of the common reservoirs of *S. aureus* (Fitzgerald *et al*., 2001b). The bacteria are spread by direct contact with an infected person, animal or by using contaminated objects, inhaling infected droplets discharged by sneezing and coughing. *S. aureus* is one of the common causes of soft skin infections (Fridkin *et al*., 2005). Skin infections in humans are common, but bacteria can spread through bloodstream. The rates of infection in community settings are increasing (Fridkin *et al*., 2005; David *et al*., 2006). On the other hand, about 20 percent of humans are persistent carriers of *S. aureus*, about 60 percent are intermittent carriers while about 20 percent rarely carry it (Kluytmans *et al*., 1997). Transmission can occur via ingestion of contaminated food with enterotoxins (Le Loir, 2003), infection could be spread from person to person through health care workers or patients (Kluytmans *et al*., 1997) and animals. In addition, *S. aureus* may be divided into methicillin resistant *S. aureus* (MRSA) or methicillin sensitive *S. aureus* MSSA.

2.4.1 MRSA in humans and livestock

There are many antibiotics against *S. aureus* available but some strains have developed antibiotic resistance mechanisms to inhibit them including methicillin resistance mechanism (Lowy, 2003). *S. aureus* had always been known for its ability to adapt to selective pressure from antibiotics. However, the first penicillin-resistant *S. aureus* was reported in 1948 shortly after the introduction of penicillin in the clinic practice (Barber and Rozwadowska-Dowzenko, 1948). Methicillin as a drug of choice in the treatment of infections caused by penicillin-resistant *S. aureus* was introduced in the late 1950s. In 1961, MRSA was identified in UK and it was later known to be a

hospital-associated bacteria worldwide (Jevons *et al*., 1963; DeLeo *et al*., 2010; Otto, 2012).

Methicillin resistant *S. aureus* strains carry the *mecA* gene on the staphylococcal cassette chromosome *mec* (SCC*mec*), which is a mobile genetic element (Katayama *et al*., 2000; Harkins *et al*., 2017). Within the cassette, *mecA* gene is responsible for resistance to β-lactam drugs including methicillin. The product of *mecA* is the peptidoglycan synthesis enzyme penicillin-binding-protein (PBP) 2a which is involved in the crosslinking of peptidoglycan in the bacterial cell wall (Hartman and Tomasz, 1984; Matthews and Tomasz, 1990; Fishovitz *et al*., 2014; Harkins *et al*., 2017). PBP2a usually shows lower affinity for β-lactam antibiotics such as penicillin and methicillin than the native Penicillin-Binding-Proteins encoded in the core genome of *S. aureus* (Hartman and Tomasz, 1981; Hartman and Tomasz, 1984; Harkins *et al*., 2017). This allows the bacteria to produce a functional cell wall and to survive β-lactam antibiotic treatment (Hartman and Tomasz, 1981). The structure of SCC*mec* is somehow diverse and is typed according to the combination of the types of the cassette chromosome recombinase gene complex (*ccr*) and mec gene complex (*mec*) (Luong *et al*., 2002; Ma *et al*., 2002; Ito *et al*., 2004; Chongtrakool *et al*., 2006; Oliveira *et al*., 2006). The *ccr* gene complex is responsible for mobility of the element. Apart from *ccr* and *mec* gene complexes, there are some non-essential regions referred to as junkyard regions, which are also normally included in SCC*mec* typing (Kondo *et al*., 2007). SCC*mec* may also include other antibiotic resistant genes apart from *mecA*, which encode resistance to other antibiotics and/or heavy metals. These genes are part of integrated copies of plasmids and transposons (Grundmann *et al*., 2006).

In 2011, *mecC* a homologue of *mecA* was identified from *S. aureus* isolates from dairy cattle in the United Kingdom (Garcia-Alvarez *et al*., 2011), which shows about 70% nucleotide identity to *mecA*. The *mecC* gene is located at a novel SCC*mec* named SCC*mec* type XI (Garcia-Alvarez *et al*., 2011; Shore *et al*., 2011). The *mecC* gene has been found from a wide range of multilocus sequence types lineages of *S. aureus* isolates from humans and animal (Cuny *et al*., 2011; Garcia-Alvarez *et al*., 2011; Laurent *et al*., 2012).

2.4.1.1 Hospital-associated and community-associated MRSA

Methicillin resistant *S. aureus* strains are resistant to nearly all β-lactam antibiotics (Fuda *et al*., 2004), including the penicillins, methicillin, ampicillin, amoxicillin, and cephalosporins. There are some antibiotic drugs available that are known to be effective in the treatment of MRSA infections and these include vancomycin and linezolid (Taha *et al*., 2019). However, vancomycin resistance has been reported among MRSA strains (Taha *et al*., 2019). MRSA has emerged as a major cause of health-care and community-associated infections (Klein *et al*., 2007). In the past, MRSA infections worldwide were generally considered to be hospital-associated (HA-MRSA) and it has been known to be a main public health challenge in the last five decades globally (Waness, 2010). However, an increase in the number of infections caused by community-associated MRSA (CA-MRSA) has been reported globally in the past years (DeLeo *et al*., 2010; Waness, 2010; Verkade and Kluytmans, 2014). Infections caused by CA-MRSA are mainly seen in individuals without any recent contacts with health care facilities (Sowash and Uhlemann, 2014).

Staphylococcus aureus strains have been grouped in to Clonal Complexes (CC) based on sequence types. Clonal complexes are groups of sequence types (ST) in which every ST shares at least five to seven identical alleles with at least one other ST in the group *(*Day *et al*., 2001*;* Feil *et al*., 2003). Isolates are usually defined according to the alleles present at the seven loci (allelic profile). Each unique allelic profile is assigned an ST. All isolates with the same ST have identical sequences at all MLST loci and are usually considered to be members of a single clone (Feil *et al*., 2003). The clonal complex (CC) CC1, CC8, CC15 and CC97 represent the largest group of *S. aureus* lineages, comprising about 911 different sequence types (Dabul and Camargo, 2014). Approximately ten multilocus sequence lineages of CA-MRSA strains have been known to be distributed worldwide. These include CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45 and CC51. However, among these lineages, CC1, CC5, CC8, CC22, CC30 and CC45 usually contain the most MRSA strains (Feil *et al*., 2003; Holmes and Zadoks, 2011). Genetically, CA-MRSA strains are different from HA-MRSA (Diep and Otto, 2008). In most cases, HA-MRSA carry the SCC*mec* type IV or V and usually they harbour the genes encoding PVL which is associated with infections of the skin and soft tissues (Vandenesch *et al*., 2003; DeLeo *et al*., 2010).

SCC*mec* type IV or V found in CA-MRSA is much smaller than SCC*mec* type I, II and III usually found in HA-MRSA. These genetic differences between CA-MRSA and HA-MRSA are related to the host environment (Diep and Otto, 2008; David and Daum, 2010).

Most of the studies that have been conducted in Africa on MRSA are more focused on human clinical isolates (Lozano *et al*., 2016). Further, studies in Africa have shown diversity of clonal lineages among MRSA strains with some clones such as CC5 or CC8 being common (Schaumburg *et al*., 2014; Lozano *et al*., 2016). Clonal complexes CC8 (ST239 and ST612), CC5, CC30, CC80, and CC88 have been identified as predominant clonal lineages of CA/HA-MRSA in Africa (Schaumburg *et al*., 2014; Abdulgader *et al*., 2015; Lozano *et al*., 2016). The more dominant clonal lineage (24 percent to 83 percent) detected among MRSA strains in humans is CC88, and it has been referred to as the African clone (Schaumburg *et al*., 2014; Lozano *et al*., 2016). In Zambia, there is no data on the common *S. aureus* clones circulating in humans and animals. A lot is not fully understood regarding *S. aureus* strain types and their lineages in humans and animals in Zambia.

2.4.1.2 Livestock-associated MRSA (LA-MRSA)

Besides HA-MRSA and CA-MRSA, MRSA was found to be associated with livestock. In 1972 MRSA was detected in milk from a cow with mastitis (Devriese *et al*., 1972). At that time, it was believed to be associated with human to animal transmission of an MRSA strains acquired likely by a farmer from hospital environment during hospitalization. In the recent years, LA-MRSA strains have been found in different animals in several countries in Europe, Asia and in the USA (Smith and Pearson, 2011). Typically, human lineages of *S. aureus* are rarely found in animal species, suggesting host range barriers (Sung *et al*., 2008). Further, some studies have indicated that certain LA-MRSA strains are associated with specific animal species. For instance, multilocus sequence types ST71, ST97, ST126, ST133 and ST151 are commonly found in ruminants and are the leading causes of bovine mastitis. On the other hand, ST5 is usually associated with poultry, and ST9, ST433 and ST398 are often associated with pigs (Armand-Lefevre *et al*., 2005; Lowder *et al*., 2009; Nickerson, 2009; Holmes and Zadoks, 2011; Moodley *et al*., 2012). The first LA-

MRSA to be found in pigs belonging to clonal complex CC398/ST398 was reported in 2005 in France (Armand-Lefevre *et al*., 2005). However, it was later discovered that the same clonal complex was widely distributed in pigs in the Netherlands (Voss *et al*., 2005). Sequence type ST398/CC398, shows a broader host range compared to other MRSA strains and has been detected also in cattle, veal calves, horses, poultry, companion animals (dogs and cats) and in humans (Verkade and Kluytmans, 2014). However, the rapidly emerging colonisation of pig farmers with pig-associated MRSA shows that some lineages of *S. aureus* have a broader hosts range (van Loo *et al*., 2007; Wulf and Voss, 2008). The widespread colonisation of pigs (de Neeling *et al*., 2007), and emerging infections in people (Krziwanek *et al*., 2009; Golding *et al*., 2010; Rasigade *et al*., 2010a) indicated that the animal reservoir of *S. aureus* can have potentially serious consequences for human health. Pigs have been implicated as the main reservoir of LA-MRSA belonging to clonal complex, CC398 (Hasman *et al*., 2010).

MRSA infections in dairy cattle have been associated to transmission between human and animals, but the directionality of transmission is not always known (Devriese and Hommez, 1975; Juhasz-Kaszanyitzky *et al*., 2007). Transmission of foodborne pathogens from animals, including dairy cattle to humans has been investigated in a number of different countries (Normanno *et al*., 2007). Because of their large population size, farm animals may constitute a potentially enormous reservoir of any pathogen. Even though the largest reservoir of *S. aureus* is human nostrils, the second largest may as well be cows. A recent description of a novel MRSA variant found in both humans and cows, suggests that ST398/CC398 is not the only lineage with an extended host range (Garcia-Alvarez *et al*., 2011). Moreover, the host range of a pathogen is a dynamic evolving trait. For example, Lowder *et al*. (2009) described a recent host shift of a particular *S. aureus* subtype of a world human ST5 clonal lineage from humans to chickens. The host shift was followed by global spread in chickens (Lowder *et al*., 2009).

In Africa, studies mainly focusing on presence, prevalence and molecular typing of MRSA strains in animals are limited. However, information is available for only few countries (Fall *et al*., 2012; Gharsa *et al*., 2012a; Gharsa *et al*., 2012b; Schaumburg *et*

al., 2012a; Schaumburg *et al*., 2012b; Schaumburg *et al*., 2013; Youn *et al*., 2014; Gharsa *et al*., 2015; Schaumburg *et al*., 2015b; Lozano *et al*., 2016). *S. aureus* strains have been reported in both sick and health animals in at least 12 African countries, Whereas, MRSA strains have been isolated in at least seven African countries including Côte d'Ivoire, Egypt, Nigeria, Senegal, Sudan, South Africa and Tunisia (Lozano *et al*., 2016). In the recent years, more studies done in some African countries on animals has generated more information about the role of animals in the evolution, epidemiology and dissemination of *S. aureus* (Lozano *et al*., 2016). For example, some strains belonging to CC88 (t189) were detected in both humans and sheep in the same village in Côte d'Ivoire (Schaumburg *et al*., 2015b). Moreover, LA-MRSA infections have been reported in relatives of farmers (Lozano *et al*., 2016) while in some cases strains belonging to ST398/CC398 have also been detected in people without contact with animals (Benito *et al*., 2014; Lozano *et al*., 2016). In general, some clonal lineages have been found to better adapt to some animal species (Lozano *et al*., 2016) and, *S. aureus* isolation rate in such animals is likely to be higher. Clonal complex CC130 and CC133 have been identified as highly associated with ruminants in Africa and these clonal lineages have been detected also in other continents (Garcia-Alvarez *et al*., 2011; Lozano *et al*., 2016). Colonisation of animals with MRSA in African countries was found to be very low (0 to 3 percent) (Fall *et al*., 2012; Gharsa *et al*., 2012b; Mai-siyama *et al*., 2014; Lozano *et al*., 2016). However, in Nigeria colonisation rate of 20.7 percent was observed in cattle from abattoir (Mai-siyama *et al*., 2014). MRSA has been detected in variable rates in cattle, sheep, goats, horses and camels on the African continent (Lozano *et al*., 2016). However, in Zambia there is no available data on MRSA in livestock.

2.5 Prevalence of *S. aureus* in raw milk

Few studies on *S. aureus* in raw milk from health looking cows in Africa have been done and the prevalence ranges from 6.3 to 100 percent (Lozano *et al*., 2016). A study in Tanzania reported the prevalence of *S. aureus* in bovine raw milk to be 41 percent (Mohammed *et al*., 2018). Other studies have reported differences in the prevalence of *S. aureus* in raw milk with Kenya having 30.6 percent, Morocco (40 percent) and Ethiopia (48.7 percent) respectively (Shitandi and Sternesjö, 2004; Bendahou *et al*., 2008; Daka *et al*., 2012). In addition, a study in Uganda reported 20.3 percent

prevalence of *S. aureus* in milk samples (Asiimwe *et al*., 2017b). A study in South Africa reported very low prevalence of 4 percent (Pekana and Green, 2018). In some African countries, raw milk is rarely tested for presence of microorganisms such as *S. aureus*. However, there are few African countries such as Rwanda with milk collection centres (MCCs) doing microbial analysis of milk (Doyle *et al*., 2015). Since microbial analysis takes days for results to be provided, some contaminated milk usually finds its way in the bulk tanks at MCCs. At some MCCs in Zambia, few testes are done as described in subsection 2.9. (Phiri *et al*., 2021) and when milk is contaminated it is rejected. Clinical and subclinical mastitis have been identified as one of the sources of contamination of raw milk (Oliver *et al*., 2005). Since the privatisation of the veterinary services in most African countries including Zambia, monitoring for mastitis is done by the individual farmers and not government. Farmers have to pay for the veterinary services and it is a challenge for those who cannot manage due to costs of seeking veterinary services when they have cows with mastitis at their farms.

2.6 Prevalence of mastitis caused by *S. aureus* in dairy cows in Africa

Bovine mastitis has been known to be common in both developed and developing countries and it is an important disease in dairy industry worldwide. Some studies on the prevalence of mastitis in Africa have been conducted in about 30 percent of African countries and the highest prevalence was found in Ethiopia (Motaung *et al*., 2017). *S. aureus* has been known to cause contagious and in most cases chronic mastitis in cattle worldwide (Barkema *et al*., 2006). Mastitis causes direct and indirect losses through decrease in milk production, discarded or rejected milk and costs of disease treatment and management (Halasa *et al*., 2007; Heiniger *et al*., 2014; Leuenberger *et al*., 2019). Mastitis and teat canal infections in South African dairy herds were reported to have increased from eight to 24 percent in 2002, to 15.4 to 30.0 percent in 2006, respectively (Petzer *et al*., 2009). In 2006, *S. aureus* was the second predominant (17 percent) bacterium in the South African dairy lactating cows (Petzer *et al*., 2009). A study done in Rwanda reported 20.6 percent prevalence of mastitis caused by *S. aureus* in cows from 13 smallholder dairy farms (Mpatswenumugabo *et al*., 2017). *S. aureus* prevalence rate reported in this study could be associated with poor milking hygiene and lack of teat dipping (Mpatswenumugabo *et al*., 2017). Further, a study done in Tanzania on 69 smallholder farms with lactating cows reported that the prevalence at

herd level of subclinical mastitis due to *S. aureus* was 20.4 percent (Mdegela *et al*., 2009). However, this study also reported that overall clinical mastitis prevalence on the participating 69 smallholder farms was 21 percent, including mastitis caused by pathogens other than *S. aureus* (Mdegela *et al*., 2009). In addition, a study conducted in Ethiopia around Asella town reported 47 percent prevalence of clinical mastitis due to *S. aureus* and 43 percent prevalence of subclinical mastitis due to the same species *(Seyoum et al*., 2018). A study done in Nigeria reported 23 percent prevalence of clinical mastitis due to *S. aureus* on 27 commercial farms (Junaidu *et al*., 2011). Further, a study done in Kenya in Jammu region on 260 cases of bovine clinical mastitis reported 11.5 percent prevalence and *S. aureus* was the most frequently (61 percent) isolated bacteria among other types of bacteria *(*Bhat *et al*., 2017). Another study done in Kenya on the prevalence of bovine mastitis, its therapeutics and control in Tatton Agriculture Park in Inyoro district reported that *S. aureus* was the most prevalent (59 percent) bacteria (Ondeik *et al*., 2013).

In Zambia, a study on the prevalence of subclinical mastitis and udder pathogens on small scale farms in the different areas of Southern province reported that *S. aureus* was the second most prevalent (29 percent) bacterial species (Eriksson *et al*., 2013). There are several factors that could be responsible for influencing geographical distribution and dynamics of *S. aureus* and these may include climatic conditions, cattle nutrition, and movement of cattle (Motaung *et al*., 2017) including milking and milk handling practices, and general farm management. Further, climatic conditions such as seasons have been identified as one of the factors affecting mammary gland pathogens and the composition of milk (Olde Riekerink *et al*., 2007; Zeinhom *et al*., 2016). Therefore, mastitis might be more prevalent in certain seasons such as rainy season than other seasons in African countries such as Zambia.

2.7 Economic impact of *S. aureus* on the dairy industry

Staphylococcus aureus is one of the major causes of clinical mastitis in dairy cows causing huge economic losses worldwide (Lundberg *et al*., 2014; Seyoum *et al*., 2018). Economic losses due to mastitis on global scale are estimated to be approximately US $ 35 billion annually (Wells *et al*., 1998). In the U.S.A, overall costs of mastitis were estimated to be $ 1.5 to 2.0 billion per year, while losses from reduced milk production,

and costs associated with high somatic cell counts (SCC) due to subclinical mastitis were estimated at US $ 960 million (Wells *et al*., 1998). The average costs of clinical mastitis per cow and per year in high-yielding dairy cows, based on data from 5 large herds from 2004 to 2006 in New York State, was US $71, with an incidence of 39.7 percent clinical mastitis cases per 100 cow years and an average cost of a clinical mastitis case of US $ 179 (Bar *et al*., 2008). These costs were composed of US $ 115 due to milk yield losses, US $ 14 due to increased mortality, and US $ 50 due to treatment associated costs per case (Bar *et al*., 2008).

In the United Kingdom, the estimated output losses and costs for treatment and prevention of mastitis were approximately £ 179.9 million, £ 79.8 million and £ 9.3 million, respectively (Rainard *et al*., 2018). In Switzerland, the costs of mastitis were estimated to be 129.4 million Francs per year (Heiniger *et al*., 2014). Other studies indicate that the costs of mastitis per year in Northern Ireland were approximately € 14 million in total, in the Republic of Ireland around € 693 for every infected cow per year and in the Netherlands approximately € 164 to € 235 per infected cow per year (Viguier *et al*., 2009). In Ethiopia, production losses associated with subclinical mastitis were estimated at 5.6 percent and losses were highest (9.3 percent) for urban and small-scale farms (6.3 percent) (Mungube *et al*., 2005). The estimates of the financial losses reported in this study, ranged from US$ 29.1 for dairy herds in secondary towns to $ 66.6 for urban dairy farms, and a total loss of $ 38 was estimated for each cow per lactation (Mungube *et al*., 2005). However, economic impact of bovine mastitis in Africa is not well documented and as a result in most cases, economic losses and expenditures associated with mastitis in Africa are usually underestimated or miscalculated (FAO, 2014). There no comprehensive report on the economic losses caused by mastitis in Zambia. Moreover, little research work has been done on mastitis in Zambia.

2.8 Clinical manifestation of *S. aureus*

2.8.1 *S. aureus* in humans

S. aureus causes a wide range of infections in humans and it is the leading cause of bacteraemia, endocarditis as well as skin and soft tissue infections in humans (Tong

et al., 2015). *S. aureus* skin infections in humans can manifest in various forms, including abscesses, impetigo, cellulitis, and more severe invasive soft tissue infections (Tong *et al*., 2015). *S. aureus* is extremely prevalent in persons with dermatitis. The bacteria are mostly found in places including the armpits, hair, and scalp. Large pimples that appear in these areas may exacerbate the infection if lacerated. This can sometimes lead to staphylococcal scalded skin syndrome. A severe form of staphylococcal scalded skin syndrome, Ritter's disease, can be observed in neonates (Curran and Al-Salihi, 1980). In addition, *S. aureus* causes other diseases including osteomyelitis (Torda *et al*., 1995; Sheehy *et al*., 2010), pneumonia (Koulenti *et al*., 2017), meningitis (Schlesinger *et al*., 1987; Pizon *et al*., 2006) and toxic shock syndrome *(DeVries et al*., 2011). Bacteraemia is a common *S aureus* infection among human patients in hospitals.

2.8.1.1 Bacteraemia in humans

Asymptomatic *S. aureus* colonisation has been identified as one of the risk factors for subsequent infections caused by this pathogen. (Kluytmans *et al*., 1997; von Eiff *et al*., 2001; Schaumburg *et al*., 2014). In most developed countries, the incidence of *S. aureus* bacteraemia ranges from 10 to 30 percent per 100,000 person-years (Laupland *et al*., 2013; Tong *et al*., 2015). Studies conducted in Denmark between 1957 and 1990 indicated that the incidence of bacteraemia due to *S. aureus* increased from three cases per 100,000 person-years to 20 cases per 100,000 person-years (Frimodt-Moller *et al*., 1997). Although the incidence of *S. aureus* bacteraemia has remained stable in some countries such as Denmark for the last 20 years, the contribution of MRSA has been fluctuating. The studies done in Quebec, Canada showed that the incidence of MRSA bacteraemia increased between 1991 to 2005 from 0 to 7.4 percent per 100,000 person-years, despite the stable rates of methicillin susceptible *S. aureus* (MSSA) bacteraemia during the same period (Allard *et al*., 2008). Increasing trends of MRSA bacteraemia incidences were also observed in Minnesota from 1998 to 2005 (El Atrouni *et al*., 2009), Calgary, Canada from 2000 to 2006 (Laupland *et al*., 2008) and in Oxfordshire, United Kingdom from 1997 to 2003 (Wyllie *et al*., 2005).

CA-MRSA clones/strains in North America (such as USA 300) have been implicated in the increased incidences of MRSA bacteraemia (Klevens *et al*., 2007; Laupland *et*

al., 2008). However, in the United Kingdom, the clones of MRSA (United Kingdom EMRSA-15 and EMRSA-16) were also implicated in the incidences of bacteraemia (Johnson *et al*., 2005). However, some countries have experienced reduction in the incidences of MRSA bacteraemia since 2005 probably due to improvements in the infection control procedures they use. These reductions were also observed in the United Kingdom from 2004 to 2011 when the incidences MRSA bacteraemia were reduced by half (Johnson *et al*., 2012; Stone *et al*., 2012). The reports of reduction in the incidences of MRSA bacteraemia have also been documented in the United States, Australia and France (Grayson *et al*., 2008; Jarlier *et al*., 2010; Kallen *et al*., 2010).

In Sub-Saharan Africa, *S. aureus* is a common cause of bacteraemia (Bachou *et al*., 2006; Perovic *et al*., 2006; Schaumburg *et al*., 2014). Human immunodeficiency virus (HIV) has been confirmed as one of the risk factors for *S. aureus* colonisation in Africa (Kinabo *et al*., 2013; Schaumburg *et al*., 2014). A study done in South Africa, on children hospitalised in Soweto reported that bacteraemia caused by *S. aureus* was high mainly among children with HIV (Groome *et al*., 2012). Further, a study done in Kampala, Uganda revealed that bacteraemia affected one in every six malnourished children and caused high mortality especially among HIV-positive children (Bachou *et al*., 2006). Another study done in South Africa on patients aged 13 years and above, admitted to a referral hospital reported that 68.4 percent of patients had bacteraemia caused by *S. aureus*. About 20 percent of all cases in this study were caused by MRSA with 83.3 percent of mortality due to bacteraemia (Steinhaus *et al*., 2018). In addition, a study done in Ethiopia reported that bacteraemia was seen in 20.6 percent (35/170) of hospitalised malnourished children and *S. aureus* was the leading Gram-positive bacteria among the 24 Gram-positive isolates out of 35 (Abrha *et al*., 2011). A study done in Tanzania on hospitalised malnourished children in Mwanza reported that a point prevalence of bacteraemia caused by *S. aureus* was 13.9 percent (56/402) and mortality was observed among the severely malnourished children (Ahmed *et al*., 2017). Further, this study also revealed that *S. aureus* was the commonest Gram-positive bacterium detected with 50 percent being MRSA (Ahmed *et al*., 2017). A few studies conducted in Africa have also revealed that there is a higher incidence of *S. aureus* infection in Africa than in industrialised countries. The annual incidence of *S. aureus* bacteraemia in South Africa was 3.28 cases per 1000 hospital admissions while

in Mozambique the incidence per 100,000 child-years, was 1730/10 in children less than one year old, 782/10 in one to four years old and 49/10 in five years old and older children *(Sigauque et al*., 2009; Naidoo *et al*., 2013; Schaumburg *et al*., 2014). In Kenya (Kilifi), the annual incidence of bacteraemia was 27 cases per 100,000 persons-years among children less than five years old (Berkley *et al*., 2005). In general, infections caused by MRSA in most cases have been associated with high morbidity and mortality (Blomberg *et al*., 2007; Kayange *et al*., 2010; Mhada *et al*., 2012; Ahmed *et al*., 2017). In most developing countries, treatment of MRSA is a challenge because it may require antibiotics which are usually not available in these countries (Ahmed *et al*., 2017). In Zambia, MRSA isolates from patients at University Teaching Hospital, showed high multi-drug resistance (Samutela *et al*., 2017) and infections caused by such strains may be difficulty to treat or manage.

2.8.1.2 Staphylococcal food poisoning (SFP)

SFP is an intoxication that results due to consumption of foods containing sufficient amounts of one, or more preformed staphylococcal enterotoxins (SEs) (Dinges *et al*., 2000; Le Loir *et al*., 2003). The SE dose that can cause illness is typically reported to require a *S. aureus* population of 10^5 to 10^8 CFU/g to be present in food (Seo and Bohach 2007; Pinchuk *et al*., 2010). SFP is one of the most common food-borne diseases throughout the world. However, the true incidences of SFP may probably be underestimated for various reasons, such as misdiagnosis, unreported minor outbreaks, improper sample collection and improper laboratory diagnosis (Argudin *et al*., 2010). In Sub-Saharan African countries such as Zambia, data on SFP is limited or not existing probably because of lack of specialised laboratories, sufficient expertise, surveillance programmes and, poor reporting of food related-illness.

Different types of foodstuffs have been responsible for SFP outbreaks globally. Milk and dairy products, meat and meat products, poultry and egg products, bakery products, ready to eat foods, more especially ice cream-filled pastries, cakes and sandwich fillings have been more frequently found involved in SFP (Seo and Bahach, 2007; Argudin *et al*., 2010; Hennekinne *et al*., 2012). Salted foods, including ham have also been involved in SFP (Qi and Miller, 2000). The notable outbreaks of SFP involving milk and milk products include an SFP outbreak which affected over 13,000

people in Japan (Asao *et al*., 2003), an SFP outbreak at a Christening party in Germany where 13 cases were reported (Fetsch *et al*., 2014) and the SFP outbreak at a Swiss boarding school, involving 14 persons who consumed cheese (Johler *et al*., 2015).

The foodborne disease burden caused by *S. aureus* in Africa is difficult to estimate because of limited data. However, considering the poor food handling and hygiene during production and processing of food, unhygienic environments coupled with inadequate or lack of cooling facilities in most African countries, SFP and other foodborne diseases may likely occur. One example for an SFP outbreak in Africa involved 53 Bulawayo City Council employees in Zimbabwe in 2014, after eating contaminated stewed chicken at a workshop (Gumbo *et al*., 2015). The hands and nails of the food handlers were implicated in this SFP outbreak. Further, another SFP outbreak reported in Africa, involved 37 cases after eating contaminated chicken in Tswane district, Gauteng province in South Africa (Ntshiqua *et al*., 2016). A study in Kenya on 42 districts investigated food poisoning outbreaks involving 926 people from 1970 to 1973 and reported 13 confirmed outbreaks by identification of causative agent. Out of these, five outbreaks were confirmed as SFP outbreaks (Ombui *et al*., 2001). This study identified low reporting, poor diagnosis and confirmation of the causative agent of the few reported cases as challenges Kenya faced (Ombui *et al*., 2001). These challenges are also likely to be faced by other African countries as well. Therefore, there is need for African countries to improve on reporting foodborne outbreaks, outbreak investigations and diagnosis of *S. aureus*.

2.8.1.3 Clinical signs of SFP

Staphylococcal Food Poisoning disease is characterized by a short incubation period of about two to eight hours and may include nausea, violent vomiting, and abdominal cramps with or without diarrhoea. The disease is usually self-limiting and may resolve within 24 to 48 hours after onset. In some cases, it can be very severe resulting in hospitalization of the patients especially when infants, elderly or debilitated persons are involved (Murray, 2005). Sometimes, death may occur from severe dehydration and loss of electrolytes.

2.9 Diagnosis of *S. aureus*

There are various methods available for diagnosis of *S. aureus* from human and animal samples which include isolation and morphology (Phenotypic) identification on solid media, Gram stain, catalase and coagulase tests, biochemical tests and molecular characterisation.

2.9.1 Phenotypic identification

2.9.1.1 Morphological identification of *S. aureus*

Conventional identification of *S. aureus* from milk is based on bacteriological culture of samples, including examination of bacterial colony morphology and haemolysis type on blood agar after incubation for 24 hours at 37ºC, Gram stain morphology, catalase and coagulase reaction (Hogan *et al*., 1999). Mannitol salt agar (MSA) and Baird Parker agar (BPA) are some of the recommended selective media by the food safety agencies and international standardisation bodies for isolation of *S. aureus* strains (Hyun-jung and Se-Wook, 2010). Capurro *et al*. (1999) reported that all *S. aureus* isolates in their study were able to ferment mannitol. As a result of fermentation of Mannitol, the colonies appear yellow with yellow zones on MSA plates. MSA with antibiotics such as cefoxitin can be used for isolation of MRSA strains (Tenhagen *et al*., 2014). Tellurite in BPA is reduced by *S. aureus* and this leads to formation of grey-black shinny colonies usually with clear zones around them on BPA plates which are caused by proteolysis (Baird-Parker 1962). In addition, haemolysis is considered an important feature for rapid identification of *S. aureus* in blood cultures, and three types of haemolysis (complete, incomplete, and a combination of these which is known as double haemolysis) can be seen among *S. aureus* mastitis isolates (Hogan *et al*., 1999). On the other hand, Boerlin *et al*. (2003) reported that all 159 *S. aureus* isolates from bovine mastitis demonstrated haemolysis. However, Younis *et al*. (2000) reported that out of 400 *S. aureus* isolates, 62.7% were found to be non-haemolytic. Further, Lam *et al*. (1995) reported that haemolysis had been found to be less effective in identifying *S. aureus* than a combination of other tests. Due to the variations seen in the results of culture methods, such as different colony morphology and haemolysis patterns of different *S. aureus* strains, we cannot rely on these criteria only when identifying *S. aureus*.

2.9.1.2 Biochemical identification of *S. aureus*

Coagulase production is an important feature used also by clinical microbiology laboratories for the identification of *S. aureus* strains isolated from infections (Goh *et al*., 1992). Since *S. aureus* bacterium is known to be coagulase positive, coagulase tests are used to differentiate *S. aureus* from CNS (Becker *et al*., 2014). Coagulase positive isolates are considered presumptive *S. aureus* isolates until they are confirmed. The tube coagulase test for the detection of free coagulase is considered the standard method for differentiating *S. aureus* from CNS. However, this test is time consuming and requires incubation for 4 to 24 hours (van Griethuysen *et al*., 2001). Further, agglutination tests have been developed for a more rapid detection of presumptive *S. aureus* (Essers *et al*., 1980).

2.9.2 Genotypic identification of *S. aureus*

2.9.2.1 Genome of *S. aureus*

The genome of *S. aureus* is about 2.8 to 2.9 Mbp in size with a guanine-plus-cytosine (G+C) content of about 33 percent (Baba *et al*., 2008; Hishinuma *et al*., 2016). Further, comparative studies revealed that most regions of the staphylococcal genome are well conserved, whereas several large sequence blocks display high variability (Baba *et al*., 2008). In addition, a study on two MRSA stains (N315 and Mu50 isolated in 1982 and 1997 respectively) revealed that the *S aureus* genome is composed of a complex mixture of genes (Kuroda *et al*., 2001) which could have been acquired probably through horizontal gene transfer (Baba *et al*., 2008). The study done by Kuroda *et al*. revealed that most of the antibiotic resistant genes were carried by plasmids or mobile genetic elements including a unique resistant island. Further, this study identified three classes of pathogenicity islands in the genome: a toxic-shock-syndrome toxin island, an exotoxin island, and an enterotoxin island (Kuroda *et al*., 2001). In the exotoxin and enterotoxin islands, clusters of exotoxin and enterotoxin genes were found to be closely linked with other gene clusters encoding putative pathogenic factors (Kuroda *et al*., 2001).

2.9.3 Characterisation of the enterotoxigenic potential of *S. aureus*

Isolation, identification and enumeration of *S. aureus* in food are important steps in the diagnosis of SFP. Numbers of at least log 5 CFU/gram^{-1} of *S. aureus* in food is usually confirmatory of SFP (Seo and Bohach 2007; Pinchuk *et al*., 2010). SEs can be detected by biological, immunological and molecular methods. The use of rhesus and cynomologous young monkey has successfully been used to detect SEs (Cauvin *et al*., 2015). Oral administration of SEs in these monkeys will cause emesis which is a biological detection method. However, ethical issues, expenses including resistance to SEs are some of the factors making this method to be a drawback (Bergdoll and Wong, 2006). One of the commonly used immunological methods for detection of SEs is the micro-slide gel double diffusion, reversed passive latex agglutination (RPLA). The sensitivity of this method is approximately 0.01 to 0.02 µg/ml and it is very possible to detect very low enterotoxins hence the application of this sensitive technique to detect SEs during outbreak investigations (Bergdoll and Wong, 2006). For food samples, ELISA are mainly used. Ridascreen kit and vidas methodology is used in Europe mostly and it is possible to detect SEs below the level 0.001 µg/ml of contaminated food (Freed *et al*., 1982). However, the limitations and drawback of immunological methods include the fact that it is difficult and expensive to prepare a highly purified antibodies against the SEs which are required for immunological tests (Hennekinne *et al*., 2011). Further, peroxidase, protein A and other endogenous substances in food may affect the specificity of immunological methods. Only SEA-SEE have been successfully detected with immunological methods since antibodies for other SEs or SEI are still under development (Benett and Hait, 2011). Molecular methods have also been used to extract *S. aureus* DNA from food directly or cultures and then testing for the presence of enterotoxin genes. It is possible to examine many enterotoxin genes simultaneously in a single PCR run using multiplex PCR. However, the presence of enterotoxin genes does not automatically mean that toxins are formed in food and this is one of the drawback of this method. Molecular assays reduce time and gives valuable information about enterotoxigenic potential of *S. aureus* (Morandi *et al*., 2007; Kim *et al*., 2011; Haiti *et al*., 2014). It is also possible to quantify *S. aureus* in the dairy food and other foods by real time PCR (Hein *et al*., 2005; Alavco'n *et al*., 2006). This could be the first step in the diagnosis of SFP.

2.10 Control of *S. aureus* in the dairy value chain

Good hygienic practices at farm level play a significant role in reducing mastitis cases and contamination of raw milk. Washing of milker's hands with soap and cleaning the udder with water or sanitizing solutions and, drying with individual towels, preferably disposable towels, are highly recommended for preventing the spread of *S. aureus* infections between cows (Vestweber *et al*., 1994; Roberson *et al*., 1999; Sears *et al*., 2003). Hygienic handling of milk with respect to quality and safety has received a great concern around the world (Lemma *et al*., 2018). This is especially true in developing countries where production of milk and various milk products usually takes place under unsanitary conditions and poor production practices (Azeze and Tera, 2015). Production of milk and dairy products for consumers requires good hygienic practices along the entire value chain (Getachew, 2003).

There are several efforts by some African countries to try and help dairy farmers to produce good quality and safe milk for customers (FAO, 2011). For African countries such as Zambia with a developing dairy sector, can learn from Kenya regarding milk safety programmes. Certification of farmers through training in milk quality and safety has significantly benefited both the producers and consumers in Kenya (Kaitibie *et al*., 2009). The main dairy regulatory body in Kenya is the Kenya Dairy Board (KDB) which has the responsibility to licence all dairy premises, monitor the quality and safety of dairy foods, and inspects milk processing plants (Jabbar and Grace, 2012). In addition, the KDB initiated training programmes in good manufacturing practices (GMP) at the dairy training institute for all the dairy value chain stakeholders to improve hygiene and safety of milk. Further, milk safety is implemented through food safety standards and regulations for milk and milk products (Kaitibie *et al*., 2009), mainly by law through the dairy industry and public health acts. Other efforts made by Kenya include registration of primary producers, considering permits for transportation of milk from one point to another; giving licences for the sale of milk and milk products; setting specific materials and standards for the dairy equipment used; certifying premises for milk sales by public health officials; inspecting milk handling personnel to ensure they adhere to public health requirements; and licencing of dairy managers after meeting education standard requirement (FAO, 2011).

Milk safety in the Zambian dairy sector is regulated by the laws through the food and drugs act, and public health act among several laws (Laws of Zambia). Under public health act, all food handlers including milk handlers are supposed to undergo medical examination and if they pass are given permit or licence to handle food. In addition, all premises handling food are required by law (public health act) to be inspected and certified fit for the purpose by health inspectors. However, there are challenges in the implementation of these regulations and this has resulted in some food handlers such as milkers and workers at milk collection centres including supermarket not undergoing regular medical examinations in some cases as required by the law and, some premises handling food are not inspected regularly as well. The Zambia Bureau of Standards (ZABS) is a Zambian body responsible for checking that standards of products including food such as milk and milk products to certify that they meet the requirements by law especially for exports (https://www.zabs.org.zm).

After the government of the Republic of Zambia privatised the Dairy Produce Board (DPB) which was responsible for marketing the dairy products in Zambia in 1991, the dairy sector was deregulated. This made the private milk processing companies to establish a new quality-based raw milk pricing. Prices are calculated using complex formulas based on parameter including bacterial counts and butterfat content among others (Neven *et al*., 2006). Milk processors such as Parmalat uses three grades of milk (grade A, B and C) to describe milk standards, and grade A usually attracts good price for farmers meeting this standard. This good price has influenced some famers to improve milk standards and safety as an incentive. In Zambia, grade A milk must have a total plate count (TPC) of 50,000 CFU/ml (Yambayamba and Zulu, 2011). The legal maximum required bacterial colony count limit by law for raw milk in Zambia is 200,000 CFU/ml (Kunda *et al*., 2015). In most countries in Africa including Zambia, milk collection centres and milk processing plants implement raw milk-quality control measures by using quality tests such as lactometer (to check for milk adulteration) and alcohol test (to check for milk fermentation) (Gwandu *et al*., 2018; Phiri *et al*., 2021). In some African countries such as Ethiopia, there are some quality standards for whole milk (composition and bacteriological) (Ethiopian Standard, 2009).

It is recommended that milk and its products should be refrigerated immediately after production to prevent multiplication of bacteria such as *S. aureus*. The ideal refrigeration temperature for *S. aureus* is below 5°C as the bacterium can grow, multiply and even produce SEs at temperatures from 7°C to 45°C (ICMSF, 1996; Stewart, 2003). Pasteurisation or boiling of milk remains the first step in killing the *S. aureus* bacteria in the milk to ensure milk safety. Milk should be boiled at above 60°C to inactivate *S. aureus* since extremely heat resistant strains could even survive at 60°C (Nema *et al*., 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

Observational studies were conducted between 2016 to 2018 in Western, Southern and Lusaka provinces of Zambia (Figure 4). The aim of the current study was to assess the milk handling hygiene practices and study the characteristics of *S. aureus* associated with the dairy value chain mainly supplied by smallholder and traditional dairy farmers.

3.1.1 Study site

The study provinces were selected because of high dairy cattle populations and differences in agro-ecological conditions. Another reason for the choice of these study provinces was that the milk production systems and practices were different in three provinces. In the Western and Southern provinces of Zambia, cattle rearing was predominantly traditional although smallholder and commercial farmers are also found, while Lusaka province had mainly commercial and smallholder dairy farmers who at least followed modern management systems of cattle rearing and keep mainly improved breeds of dairy cows.

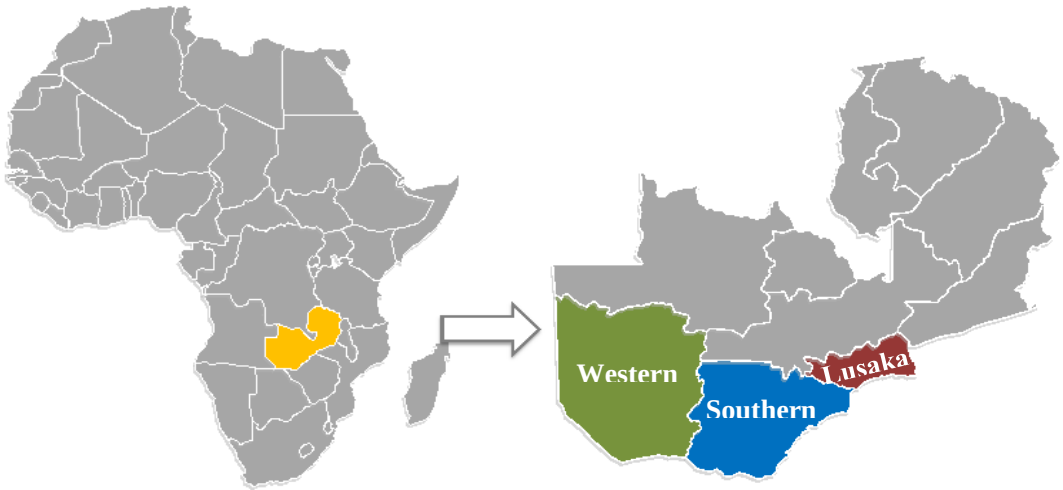


Figure 4: Map of Africa showing position of Zambia and inset map showing study provinces in Zambia. Maps were taken from https://yourfreetemplates.com and were modified.

3.1.2 Sample size calculation

The minimum sample size for the farms sampled was calculated using the following formula as described by Cochran (Cochran, 1963; Bartlett, 2001; Israel, 1992):

$$\boldsymbol{n_0 = \frac{z^2 \times p(1-p)}{e^2}}$$

Where,

- $\boldsymbol{n_0}$ - Sample size, which was estimated
- $\boldsymbol{z^2}$ - Selected critical value of desired level of confidence or risk
- $\boldsymbol{p}$ - Estimated proportion of an attribute that is present in the population or maximum variability of the population
- $\boldsymbol{e}$ - Desired level of precision or margin of error

The following values were used for estimating the sample size:

- $\boldsymbol{z^2}$ - 95% confidence level (the value of (1-α) in standard normal distribution ***z***-table, which is 1.96 for 95%)
- $\boldsymbol{p}$ - 50% variability of the population (which is maximum)
- $\boldsymbol{e}$ - 5% margin of error

Values were added into the given formula:

$$\boldsymbol{n_0 = \frac{(1.96)^2 \times 0.5(1-0.5)}{(0.05)^2} = 384.16}$$

In this study, a minimum of 384 farms in Lusaka, Western and Southern provinces were planned to be visited for sampling. However, this sample size depended on the farm owner's willingness to participate in the study. The assumption was that all the farms targeted would be willing to participate. However, the total number of actual farms visited and sampled was 226. Other facilities visited and sampled were 62 in total and included MCCs (11), Traditional markets (3), Supermarkets (30), milk Processing Plants (3) and informal milk traders (15). These other facilities were selected purposively and the total number of all facilities visited and sampled was 288.

3.1.3 Sampling plan (including interviews)

Milk samples were drawn from various selected smallholder and traditional dairy cattle farms (from cows with clinical mastitis and normal looking health cows), milk

traders and MCCs. Milk and milk products (pre-processed milk/sour milk) were also collected from milk processing plants, traditional markets and supermarkets/shops. Further, environmental water, swabs from milk buckets, nasal and hands of milkers from selected farms were sampled as well.

Sampling was done in selected districts targeting Mongu and Limulunga districts in Western Province, Chilanga, Chongwe and Lusaka districts in Lusaka Province and Choma in Southern province. These districts were selected because of their active dairy farmers and milk collection centres in milk production and handling respectively. Farmers and other stakeholders/facilities along the dairy value chain were identified with the help of Veterinary Officers in the study provinces according to their milk production and handling activities and a list of active farmers and other stakeholders/facilities was generated. Participants were then randomly selected from a list generated based on their willingness to participate in the study. In addition, the inclusion criteria for an individual animal at farm level in the sampling, was evidence of clinical mastitis. A total of 11 MCCs were visited for sampling in the three study provinces (2 in Western, 3 in Lusaka and 6 in Southern) and these MCCs were selected based mainly on the huge volumes of milk they handle, big number of active members and willingness to participate.

Sampling was done as follows:

a) Primary production (farm level)
b) Along the dairy value chain: MCCs, milk processing plants and milk traders
c) Traditional markets (informal markets)
d) Supermarkets/shops (retail level)

In addition to sampling, selected stakeholders along the dairy value chain including farmers, milk collection centres, milk traders, supermarkets and consumers were interviewed using questionnaires.

A

B

C

D

E

F

G

H

I  J 

Figure 5: Interaction with local farmers and field sample collection. A) Typical traditional dairy farm; B) Milking at traditional dairy farm; C) Typical smallholder dairy farm; D) Milking at smallholder dairy farm; E) A milker pooling milk at smallholder farm; F) Collecting milk samples; G) Collecting milk bucket swab; H) Collecting water sample; I) Collecting hand swab from a milker; J) Collecting nasal swab from a milker.

3.2 Sample collection

The inclusion criterion of participants to be included in the study was the willingness to participate in the study.

Table 2: Facilities and types of samples collected in the three study provinces during the first and second field visits

Facility	**Sample type**	**Lusaka**		**Western**		**Southern**	
		1. visit (28-11-2017 to 06-12-2017)	**2. visit** (28-02-2018 to 08-03-2018)	**1. visit** (07-08-2017 to 12-08-2017)	**2. visit** (29-12-2017 to 04-01-2018)	**1. visit** (21-09-2017 to 28-09-2017)	**2. visit** (29-01-2018 to 03-02-2018)
Farms (84, 46, 96 per province, respectively)	Pooled raw milk	84	84	46	46	96	96
	Raw milk from mastitis cows	4	4	0	0	3	3
	Water	84		46		96	
	Nasal swabs	84		46		96	
	Hands swabs	84		46		96	
	Milk bucket swabs	84		46		96	
Milk collection centres (3, 2, 6 per province, respectively)	Bulk raw milk	3	3	2	2	6	6

Milk traders (5 per province)	Pooled raw milk	5	5	5	5	5	5
Milk processing plants (1 per province)	Unprocessed raw milk	1	1	1	1	1	1
	Pre-processed milk	1	1	-	1	-	1
	Sour milk	1	1	-	1	-	1
Traditional markets (1 per province)	Unprocessed raw milk	10	10	-	10	-	10
	Pre-processed milk	10	10	-	10	-	10
	Traditional sour milk	10	10	-	10	-	10
Super-markets/ shops (10 per province)	Processed (Pasteurised) milk (fresh milk)	33	33	-	19	-	20
	UHT-treated (sterilised) milk	28	28	-	12	-	24
	Sour milk	34	34	-	15	-	23
	Cheese	24	24	-	12	-	20
All facilities	**Sub total number of samples**	**584**	**248**	**238**	**144**	**495**	**230**

Table 3: Summary of numbers of facilities visited and total number of samples collected in the three study provinces during the two field visits

Number of facilities sampled	**Total number of samples**	**Lusaka (number of samples)**	**Western (number of samples)**	**Southern (number of samples)**
288	1,939	832	382	725

3.2.1 Farm level

At farm level, the study targeted smallholder and traditional dairy farmers. A total of 226 dairy farmers (Western 46, Southern 96 and Lusaka province 84) were visited to collect samples (Figure 5) in wet and dry seasons. One milker willing to participate in the study was selected among milkers at each participating farm especially those milking mastitis cows. In order to be able to collect samples from milkers, permission (Ethical clearance) was sought from ERES CONVERGE in Zambia (Appendix A1). In addition, consent was also obtained from participating milkers and farmers before

interviewing them and sampling Appendix B). The following samples were collected aseptically from each participating farm in the three provinces during the study period:

1. One sample of pooled raw milk (50 ml).
2. One swab from the milk can/bucket/container.
3. One swab from the hands of one milker.

A sampling area on the palm of the hand of each of the participating milker was selected and swabbed by rubbing and rolling the wet swab firmly several times across the sampling area on the palm. This process was repeated on the other hand using the same swab. Then, the swab was returned into the tube and the lid was tightly closed.

4. One nasal swab from one milker.

From the same milker whose hands were swabbed, a nasal swab was taken. In case the selected milker had excessive mucous because of flu, he was asked to blow his nose to remove excessive nasal secretions to allow the swab to firmly touch the nasal surfaces. Each participating milker was asked to tilt his head back and a swab was inserted approximately 1-2 cm into one nostril and then rotated against the inside of the nostril for approximately three seconds while applying slight pressure with a finger on the outside of the nose to ensure good contact between the swab and the inside of the nose. This procedure was repeated for the second nostril using the same swab, trying not to touch anything but the inside of the nose. Then, the swabs were placed into the plastic transport tube all the way into the bottom of the tube while making sure the cap was seated tightly.

5. One environmental water sample (50 ml) from water used for cleaning the udders of cows, milking vessels and milkers' hands. Water samples were collected from the source, using a sterile 50 ml falcon tube.
6. One sample of milk (50 ml) from individual cows with clinical mastitis where possible (especially if the participating farm had at least one case of mastitis).

Two sampling visits were made to farms in the study provinces during the study period to collect samples. However, the second sampling visit targeted collection of milk samples only. All the samples were transported in cooler boxes packed with ice from the field to Veterinary offices or at veterinary assistant's home or at MCC in the three study provinces with deep freezer where samples were put at -20°C until being

transported to University of Zambia (UNZA) Microbiology laboratory. On the transportation day to UNZA, all the samples were removed from the freezer and immediately again placed in cooler boxes packed with ice packs and transported to the Microbiology laboratory in Lusaka from the three study provinces and put in -20°C freezer until the day for laboratory analysis.

3.2.2 Milk collection centres (MCCs)

MCCs in the study were selected purposively. The inclusion criterion was the willingness of the individual MCC (manager or supervisor) to participate in the study. A total of 11 MCCs (three in Lusaka, two in Western and six in Southern provinces) were visited twice to collect samples (Figure 6). The following samples were collected from each selected MCC in the three study provinces:

1. One sample of pooled raw milk (50 ml) from a bulk tank (with milk from different farms).

Figure 6: Delivery of pooled milk to milk collection centre. A) Farmer delivering milk by bicycle; B) Milk delivery by vehicle; C) Trader delivering milk.

3.2.3 Milk processing plants

A total of three milk processing plants (one plant in each province) which were willing to participate in the study were visited twice during the study period (Figure 7). The following samples were collected from a bulk tank from each selected milk processing plant in the three provinces:

1. One sample of pooled raw milk (50 ml) from bulk tank with raw milk just delivered milk from milk collection centres.
2. One sample of pre-processed milk (50 ml) before a final product mainly sour milk is produced.
3. One packet (different sizes) of sour milk as sample of a finished product.

Figure 7: Milk collection at processing plant

3.2.4 Milk traders

A total of 15 milk traders (five milk traders in each province) in all the three study provinces were visited twice in areas where they normally sale their milk (Figure 8). The following samples were collected from each selected milk trader in the three provinces:

1. One sample of pooled raw milk (50 ml).

Figure 8: Collecting pooled milk from a trader

3.2.5 Traditional markets

A total of three traditional markets in the three provinces (targeting 30 milk sellers with 10 in each province) were visited twice in Lusaka province and once in Western and Southern provinces during the study period (Figure 9, B and C). Traditional markets mean informal open markets where commodities such as food are sold by sellers mainly women. The following samples were collected from each selected seller:

1. One sample of pooled raw milk (50 ml).
2. One sample of pre-processed milk (50 ml), collected at homes of the sellers.
3. One sample of a traditionally processed product (sour milk, 50 ml).

3.2.6 Supermarkets/shops (retail)

A total of 30 supermarkets/shops (10 in each province) were visited to purchase and collect milk and milk products samples during the study period (Figure 9, D). Supermarkets are defined as retail shops where goods or commodities are sold in a formal orgaised way. In Western and Southern provinces, the supermarkets/shops were visited once and in Lusaka province two visits were made to collect samples. The following samples were collected from each selected supermarket/shop:

1. One packet of each processed fresh milk type.
2. One packet of each UHT-treated milk type.
3. One packet of each of sour milk type.
4. One packet of each cheese type.

Figure 9: Buying milk and milk products. A) Buying from a trader; B & C**)** Buying sour milk from traditional markets; D) Buying from a supermarket.

3.3 Survey on milk handling and hygienic practices

A field survey was conducted in parallel with sample collection to receive information about milk handling, hygiene and milk safety practices of stakeholders at the selected facilities along the dairy value chain in Lusaka, Western and Southern provinces of Zambia in order to link to *S. aureus* maintenance, spread, and transmission. A total of 212 individual smallholder and traditional farmers who were willing out of 226 (70 farmers out of 84 in Lusaka , 46 in Western and 96 in Southern provinces) (Figure 10), 15 milk traders (five in each province), 11 MCCs (three in Lusaka, two in Western and six in Southern provinces), 35 supermarkets (15 in Lusaka, 10 in Western, 10 in Southern provinces) and 126 consumers (30 in Lusaka, 42 in Western and 54 in Southern provinces) were interviewed using structured questionnaires Appendix C1-5). The criteria for choosing milk consumers was based on the frequency of buying and consuption of milk including willingness to participate in the interview. Milk processing plants and sellers at traditional markets were not included in the survey.

Figure 10: Traditional farmer answering questionnaire.

3.4 Microbiological methods

Sterile equipment and media (autoclaved) were used and all work was done in a microbiological safety cabinet using aseptic techniques. Field samples were analysed as indicated in the organogram below (Figure 11). Total plate count (TPC) and isolation of presumptive *S. aureus* was performed at the Microbiology Laboratory in the Veterinary School at the University of Zambia (UNZA) in Zambia in the Department of Paraclinical studies.

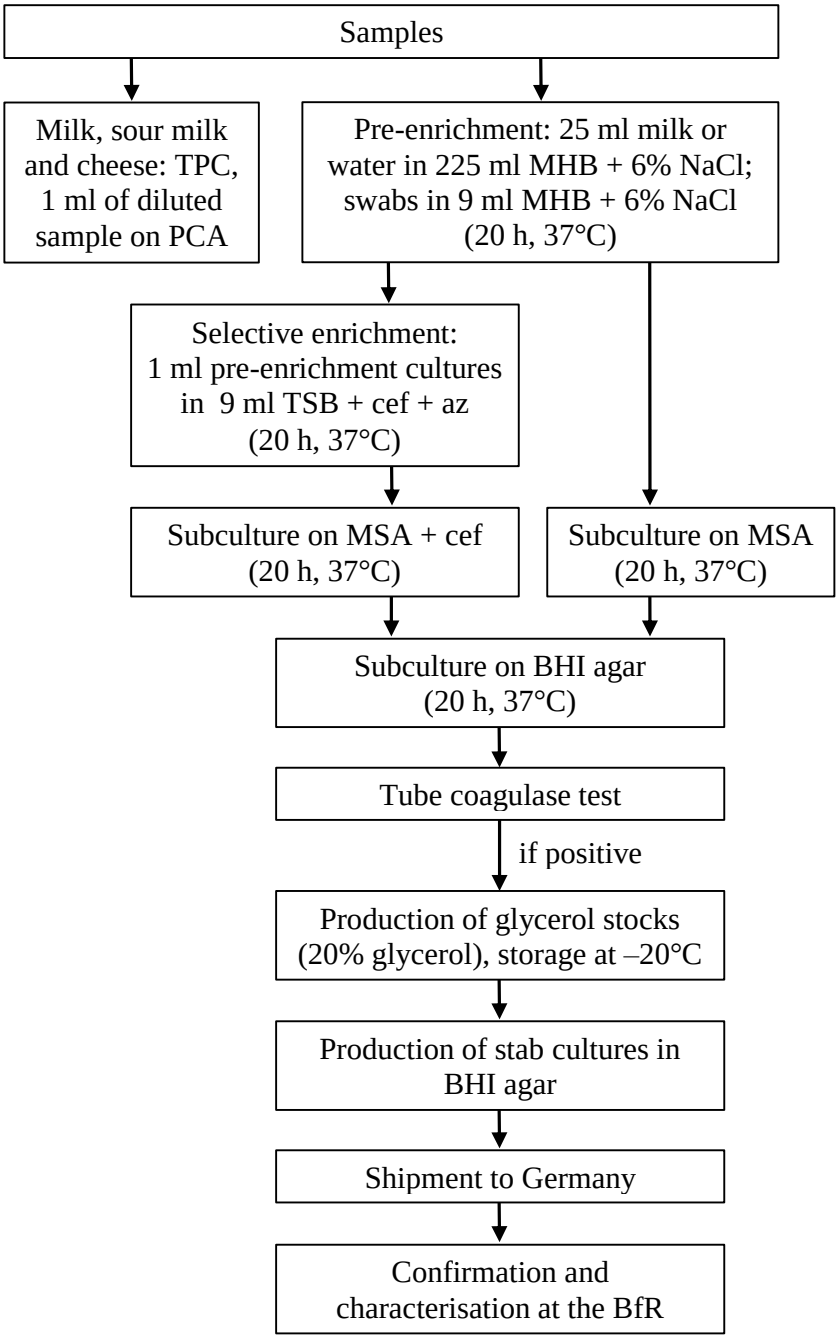


Figure 11: Field sample analysis organogram. Please note that TPC was only done for samples from Lusaka province. PCA: plate count agar; TPC: total plate count; MHB: Muller Hinton broth; TSB: tryptic soy broth; MSA: mannitol salt agar; BHI: brain heart infusion agar; cef: cefoxitin; az: azetreonam.

3.4.1 Total plate count (TPC)

TPC was done according to international standards (ISO 4833-1:2013). Dilutions were made on milk, sour milk, and cheese samples. To do this, 1 ml of each liquid sample (milk, sour milk) and 1 g of solid sample type (cheese) were serially diluted from 10^{-1} to 10^{-7} dilutions by adding 1 ml or 1 g of each sample after vortexing and homogenising

it, respectively, into 9 ml of buffered peptone water (BPW) in a test tube (Appendix D7).

From every dilution of each sample type (liquid and solid), 1 ml was added on a sterile petri dish/plate in duplicates. Then about 15 ml of autoclaved plate count agar (PCA) was cooled at room temperature to approximately 44–47°C and poured on the plates (Appendix D12). The plates were incubated for approximately 72 hours at about 30°C to allow bacteria to grow inside the media. All the colonies from two consecutive dilutions which allow for counting (minimum five colonies and maximum 300 colonies) were counted and the results were recorded. Then the total TPC was calculated using the following formula from ISO 7218: 2014-02.

$$\textbf{Colony forming unit } (\textbf{CFU/ml}) = \frac{\sum \boldsymbol{C}}{\boldsymbol{V} \times (\boldsymbol{n_1} \times \mathbf{1} + \boldsymbol{n_2} \times \mathbf{0.1})} \times \boldsymbol{d}$$

Where:

- $\sum \boldsymbol{C}$ - Sum of all counted colonies identified on all the plates with the two consecutive dilutions
- $\boldsymbol{V}$ - Volume of the sample inoculated (inoculum) on each dish, in ml
- $\boldsymbol{d}$ - Dilution factor counted
- $\boldsymbol{n_1}$ - Number of dishes selected at first dilution
- $\boldsymbol{n_2}$ - Number of dishes selected at second dilution

Only the TPC of samples from Lusaka province were analysed and calculated because sample collection here took only approximately 30 to 40 minutes before samples were put in deep freezer at approximately -20°C and, the average environmental temperature was approximately around 26°C during the sampling period in most cases. Samples from Western and Southern provinces could not be enumerated for TPC because it took at least one hour and 30 minutes or more to have the samples frozen because of long distances from sampling sites and also due to bad roads and floods which made transportation of field samples difficult. The average environmental temperatures in Western and Southern provinces were approximately 38°C or more in some cases during the sampling period. These conditions would favour the rapid

growth and multiplication of bacteria in the milk and its products and as such TPC could not be done in these two provinces.

3.4.2 Isolation of presumptive *S. aureus*

To detect *S. aureus*, each 25 ml of water, milk or sour milk samples (liquid samples) or 25 g of cheese (solid samples) were after vortexing and homogenizing, added into 225 ml of Muller Hinton broth (MHB) with 6% sodium chloride (NaCl), (Appendix D11) representing first pre-enrichment stage. The mixture was thoroughly mixed and incubated for approximately 20 hours at 37°C (Tenhagen *et al*., 2014). For hands, nasal and bucket swabs, each swab was transferred into 10 ml of MHB with 6% NaCl and incubated for about 20 hours at 37°C. After incubation, 1 loop ful (around 10 µl) of each overnight culture was inoculated onto mannitol salt agar (MSA) plates (Appendix D9) and incubated for 20 hours at 37°C. In addition, control strains BfR ST-0129 (*S. aureus*) and BfR ST-1164 (*S. aureus*) (Appendix E1) were also inoculated as a positive and negative control, respectively (Appendix E1) on MSA plates and incubated for 20 hours at 37°C in the Microbiology Laboratory at UNZA. Single colonies of bacteria showing typical characteristics for *S. aureus* and control strains ST-0129 and ST-1164 were inoculated further onto BHI agar (Appendix D5) for stability as the colonies are not stable on MSA after several days and the inoculated BHI agar plates were incubated for approximately 20 hours at 37°C. The colonies of presumptive *S. aureus* isolates from BHI overnight cultures and including control strains were also inoculated on BHI broth (Appendix D6) and Baird Parker Agar (BPA) (Appendix D4) and incubated at 37°C for approximately 20 minutes. Coagulase tube test was performed as described in Subsection 3.4.4 using the overnight BHI cultures. All coagulase positive colonies were considered as presumptive *S. aureus* isolates and were put in 20% glycerol (Appendix D8) and stored at -20°C until shipping to Germany.

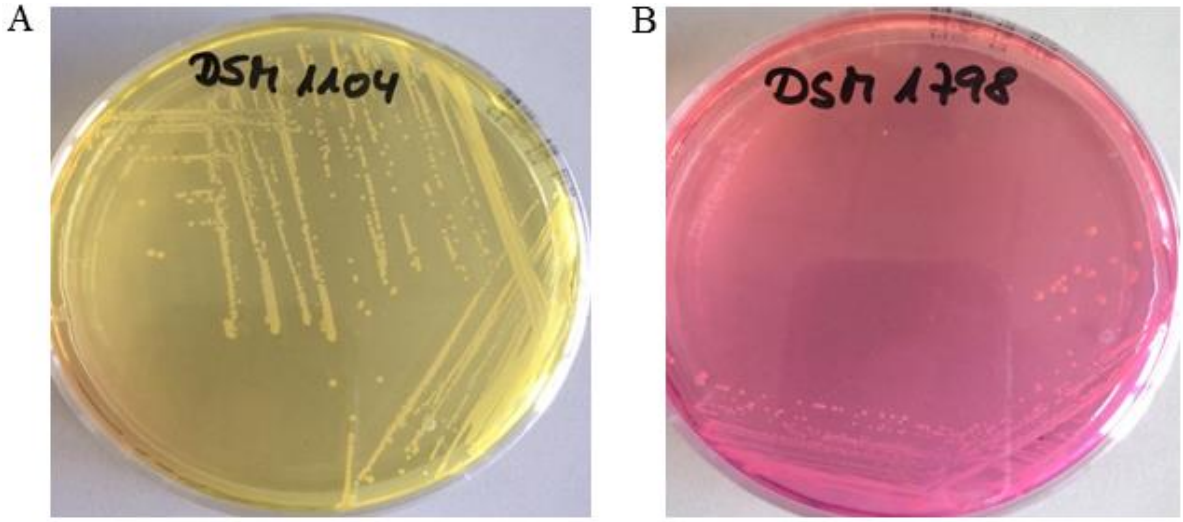


Figure 12: *S. aureus* colonies on MSA plates. A) Mannitol fermentation; B) No mannitol fermentation.

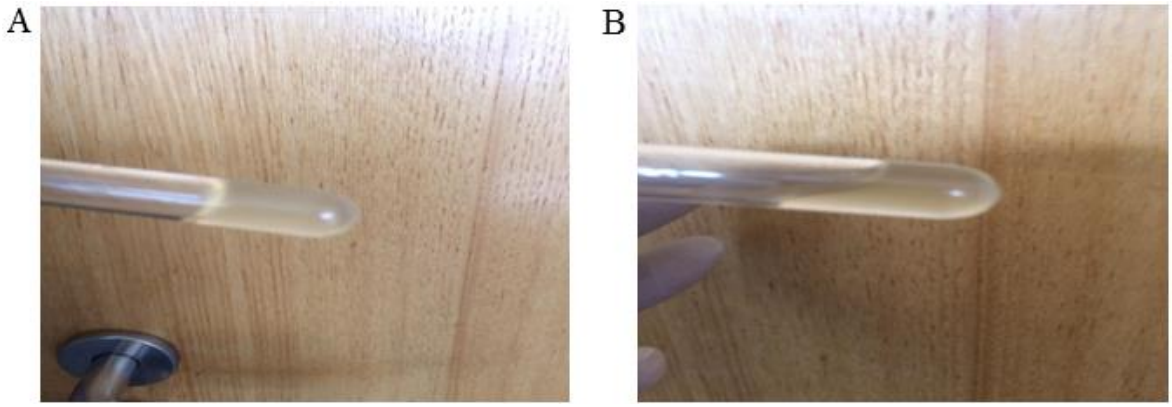


Figure 13: Coagulase tube tests. A) Positive; B) Negative.

3.4.3 Isolation of presumptive methicillin resistant *S. aureus* (MRSA)

The isolation of MRSA was done according to protocol described by Tenhagen *et al*. (2014). To detect MRSA, 1 ml of the first pre-enrichment overnight cultures described above in Subsection 3.4.2 were transferred into individual 9 ml of tryptic soy broth (CASO) (Appendix 13) containing 3.5 mg/L cefoxitin (cef) (Sigma Aldrich) (Appendix D2b) and 75.0 mg/L aztreonam (az) (Sigma Aldrich) (Appendix D2a), second pre-enrichment and incubated for 20 hours at 37°C. Then 1 loopful of each overnight culture was inoculated onto MSA plates with cef (3.5 mg/l) (Appendix 10) and incubated for 20 hours at 37°C. Control strain BfR ST-0167 (MRSA) was used as a positive control and strain BfR ST-0129 (*S. aureus*) was used as negative control (Appendix E2). After incubation, the pure single colonies of bacteria showing typical characteristics for MRSA and of control strain BfR ST-0167 (Appendix E1) were inoculated on BHI agar plates and incubated for about 20 hours at 37°C. The overnight

cultures were inoculated into BHI broth (Appendix D6) and incubated as described in 3.4.2. Then, coagulase tube test was performed as described in Subsection 3.4.4 and all coagulase positive samples were considered as presumptive MRSA isolates and were put in glycerol (Appendix D8) cultures as described in 3.4.2. The cultures were stored at -20°C until shipment.

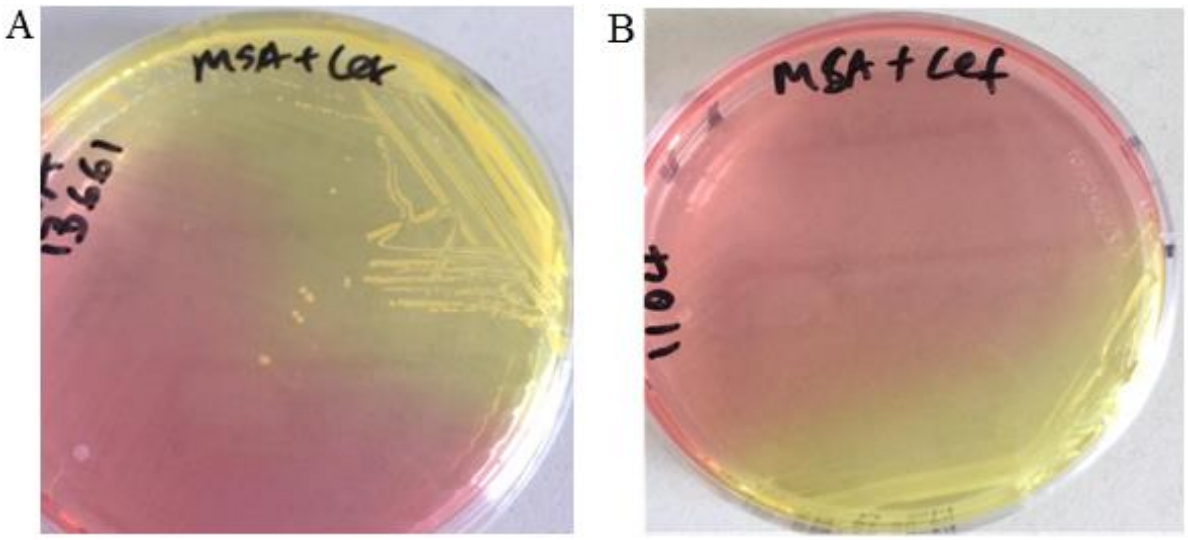


Figure 14: Reference strain for growth on MSA + cef. A) Growth (positive control); B) No growth (negative control).

3.4.4 Coagulase tube test

The coagulase tube test was done according to instructions from the rabbit coagulase plasma supplier (Sigma Aldrich, Germany). Single colonies from overnight BHI agar cultures described above in 3.4.2 and 3.4.3 were inoculated into 9 ml of BHI broth (Appendix D6) and incubated for approximately 20 hours at 37°C. Reference strain BfR ST-0129 (*S. aureus*) as positive control and reference strain BfR ST-0886 (*S. epidermidis*) (Appendix E5) as negative control (coagulase negative) were inoculated in the same way. After incubation, 0.5 ml of each overnight culture was mixed with 0.5 ml of coagulase (rabbit plasma; Sigma Aldrich, Germany). The mixture was vortexed and then incubated at 37°C for 24 hours. The tubes were checked for coagulase positive tubes (clot formation) and negative tubes (no clot, suspension remain homogeneous) after 4 hours and, if negative were checked after 24 hours of incubation.

3.4.5 Preparation of stab cultures for shipment

Autoclaved BHI agar was cooled at room temperature to approximately 65°C and was poured in 1.5 ml cryo vials and allowed to solidify at room temperature. After cooling, the BHI agar filled cryo vials were kept at 4°C until use. Some bacterial material from glycerol cultures of the presumptive *S. aureus* isolates (including presumptive MRSA) were spread out onto BHI agar plates and incubated at 37°C for about 20 hours. After incubation, two to three colonies of the overnight cultures were inserted into the BHI agar. The stab cultures were kept at room temperature for approximately 7 days until shipping day to allow growth of the presumptive *S. aureus* isolates.

3.5. Spectrometric methods

3.5.1 Confirmation of isolates using matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS)

A MALDI-TOF MS (microflex LS, Bruker Daltonik, Germany) was used to do the first confirmation of the presumptive *S. aureus* isolates using instructions from the manufacturer. To do this, all the presumptive *S. aureus* isolates available as stab cultures were inoculated on sheep blood agar and incubated for 20 hours at 37°C. The overnight cultures were checked for growth and single colonies were analysed by MALDI-TOF MS in duplicates. For that, bacterial material was applied on the wells of a MALDI target plate by avoiding spreading the colony material beyond the individual well and not including too much material. Then, a single drop of the matrix a-cyano-4-hydroxycinnamic acid (HCCA, Bruker Daltonik, Germany) was dropped on each well and allowed to dry at room temperature. Mass spectrum data was acquired using the MALDI-TOF system and the Biotyper 3.4 software (Bruker Daltonik, Germany) in *S. aureus* check mode in particular. The score value for a presumptive *S. aureus* isolate to be confirmed as *S. aureus* by MALDI-TOF was ≥ 1.86. All isolates confirmed as *S. aureus* were inoculated on sheep blood agar and incubated for about 20 hours at 37°C. After incubation, single colonies were transferred into a cryo vial containing sterile 800 µl of bacton peptone bouillon (BPB, Becton Dickinson, USA) (Appendix D3). Then, sterile 800 µl of 85% glycerol were added to make a cryo-stock of the isolates. The cryo-stocks were vortexed and stored at -20°C.

Figure 15: *S. aureus* colonies on sheep blood agar showing β haemolysis

3.6 Molecular biological methods

All molecular biological work was performed using sterile and nuclease free equipment, solutions and water. The working benches and DNA work stations were cleaned and disinfected with DNase free solution prior to working on benches to allow working in a sterile environment.

3.6.1 DNA extraction (rapid extraction)

S. aureus isolates from cryo-stock were inoculated on sheep blood agar and incubated for 20 hours at 37°C. The DNA was extracted from about two to six colonies of each *S. aureus* isolate by mixing isolates with 45 µl of Tris-EDTA (TE) low buffer (10 mM Tris-HCl (Merck, Germany) and 1 mM EDTA (VWR-Life Science, USA), pH 8.0) and 5 µl lysostaphin (1 mg/ml; Sigma Aldrich, USA). For negative control, 45 µl of TE low buffer and 5 µl lysostaphin were mixed without adding any bacterial material. The mixtures were incubated for approximately 45 minutes at about 37°C and subsequently put into a hot water bath at 95°C for 12 minutes. Then, the mixture was diluted by adding 200 µl of TE low buffer. All the DNA samples were stored at -23°C until further analysis.

Figure 16: Extracting DNA from *S. aureus*.

3.6.2 Molecular biological confirmation of *S. aureus* and MRSA

An in-house multiplex real time polymerase chain reaction (PCR) was used to screen the presumptive *S. aureus* and MRSA isolates for the presence of *tuf* (a marker gene for *Staphylococcus* species encoding the elongation factor EF-Tu), *nuc* (a marker gene for *S. aureus* encoding a thermonuclease), *mecA* (a marker gene for MRSA mediating methicillin resistance) and *pvl* (encoding the virulence factor Pantone-Valentine leucocidin) as adapted according to Kilic *et al.* (2010) and Kraushaar *et al.* (2017). Sequences of primers and probes used for the PCR assays are listed in Table 4. Multiplex PCR mix was prepared targeting all four genes in a single PCR run. Each single reaction of the multiplex PCR for one *S. aureus* isolate contained a mix of 1 µl of DNA template (DNA sample of individual isolate), 10 µl of 2 x SensiFAST Probe No-ROX Mix (Bioline, United Kingdom), 1.8 µl of nuclease free water (Gibco, United Kingdom), 0.8 µl of tuf-P1 (forward primer, 10 µM), 0.8 µl of tuf-P2 (reverse primer, 10 µM), 0.2 µl of tuf-Probe (10 µM), 0.8 µl of nuc-P1 (forward primer, 10 µM), 0.8 µl of nuc-P2 (reverse primer, 10 µM), 0.2 µl of nuc-Probe (10 µM), 0.8 µl of mecA-P1 (forward primer, 10 µM), 0.8 µl of mecA-P2 (reverse primer, 10 µM), 0.2 µl of mecA-Probe (10 µM), 0.8 µl of pvltaqF (forward primer, 10 µM), 0.8 µl of pvltaqR (reverse primer, 10 µM) and 0.8 µl of pvlTAQT Probe (10 µM). The supplier for the nucleotides was Bioline (United Kingdom). The total volume of the single PCR reaction was 20 µl. DNA template of the reference *S. aureus* strain PC 115-1380 was used as positive control for all target genes. Further, DNase free water (Gibco, United Kingdom) was used as negative control. DNA amplification was done in a CFX96 real time PCR cycler (BioRad, Germany). The cycling conditions were as follows: initial

denaturation at 95°C for 15 minutes was followed by 35 cycles of amplification (denaturation at 95°C for 15 seconds and annealing plus elongation at 60°C for 60 seconds) and finally followed by cooling at 4°C. All the PCR products were kept at 4°C until agarose gel electrophoresis.

Table 4: Primers and probes used for the multiplex detection of *tuf*, *nuc*, *mecA* and *pvl* genes

Target gene	**Primer**	**Sequence (5' – 3')**	**Reference**
tuf	Tuf-P1	AAACAACTGTTACTGGTGTAGAAATG	Kilic *et al*., 2010
	Tuf-P2	AGTACGGAAATAGAATTGTG	
	Tuf Probe (Texas Red)	TCCGTAAATTATTAGACTACGCTGAAGC	
nuc	Nuc-P1	GTTGCTTAGTGTTAACTTTAGTTGTA	Kilic *et al*., 2010
	Nuc-P2	AATGTCGCAGGTTCTTTATGTAATTT	
	Nuc Probe (Cal Gold 540)	AAGTCTAAGTAGCTCAGCAAATGCA	Adapted from Kilic *et al*., 2010
mecA	mecA-P1	AAATATTATTAGCTGATTCAGGTTAC	Kilic *et al*., 2010
	mecA-P2	CGTTAATATTGCCATTATTTCTAAT	
	mecA Probe (FAM)	CAAGGTGAAATACTGATTAACCCAGTA	
pvl	pvltaqF	AATGAAATGTTTTTAGGCTCAAGACA	Fosheim *et al*., 2011
	pvltagR	TGGATAACACTGGCATTTTGTGA	
	pvlTAQ Probe (Cy5)	AGCAACTTAAATGCTGGACAAAACTTCT TGGAA	Adapted from Fosheim *et al*., 2011

3.6.3 Amplification of *spa* by PCR for *spa* typing of *S. aureus* isolates

PCR was applied to amplify the polymorphic X region of the gene for *S. aureus* protein A (*spa*) used for *spa* typing. *Spa* typing was done as described by Shopsin *et al*. (1999) and Kraushaar *et al*. (2017). Single reaction mix contained 10 µl of HS MyTaq Mix (Bioline, United Kingdom), 5 µl of Spa1095F (10 µM; Eurogentec, Belgium or Eurofins, Germany), 5 µl of Spa1517R (10 µM; Metabion, Germany or Eurofins, Germany), 29 µl of nuclease free water (Gibco, United Kingdom) and 1 µl of undiluted DNA (template). Primers used are indicated in Table 5. The total volume of the single PCR reaction was 50 µl (of each *S. aureus* isolate or of reference strain *S. aureus* DSM 1104, as a positive control (*spa* positive) or water as negative control). DNA amplification was done in a 2720 Thermal Cycler (Applied Biosystems, USA). The cycling conditions were as follows: initial denaturation at 94°C for 5 minutes was

followed by 30 cycles of amplification (denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 30 seconds) followed by final elongation at 72°C for 5 minutes and cooling at 4°C. All PCR products were kept at 4°C until gel electrophoresis that was done within the same day.

3.6.4 Agarose gel electrophoresis

PCR products were analysed by electrophoresis on 2% agarose (UltraPure agarose, Invitrogen USA). 5 µl of PCR product mixed with 2 µl of loading buffer (6x Loading Buffer BLUE, EURx, Germany) were loaded per well of the agarose gel (Appendix D1). In addition, 5 µl of DNA ladder (Hyperladder 50 bp, Bioline, United Kingdom) were loaded as marker in a separate well. Then, the gel was placed in an electrophoresis chamber filled with 0.5 M Tris-borate-EDTA (TBE) buffer containing 108 g of Tris (Merck, Germany), 55 g boric acid (Merck, Germany) and 40 ml of EDTA (VWR-Life Science, USA) in 500 ml distilled water. Electrophoresis was performed at 90 volts (V) for 40 minutes for a small gel and 180 V for 60 minutes for a bigger one. The gel was stained in ethidium bromide solution (10 mg/ml; Bio-Rad, USA) for 30 minutes and washed in water for 10 minutes. The Gel Doc XR Imaging System (Bio-Rad, USA) was used for documentation. The PCR products were stored at -21°C in the deep freezer until the day of DNA purification for sequencing.

3.6.5 PCR product purification for *spa* typing

A total of 45 µl of each PCR product were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Health care, Germany) according to the instructions of the manufacturer. The purified PCR products were eluted with 30 µl of water each and were stored at 4°C overnight or for longer period of storage at -21°C until they were prepared for *spa* sequencing.

Table 5: Primers used in *spa* typing PCR

Target gene	**Primer**	**Primer sequences (5' – 3')**	**Amplicon size (bp)**
spa	Spa1095F	AGACGATCCTCCGGTGAGC	variable
	Spa1517R	GCTTTTGCAATGTCATTTACTG	variable

3.6.6 Sequencing of PCR products and *spa* typing

For sequencing of the amplified *spa* fragment, 5 µl of water, 2 µl of forward primer (Spa1095F, 10 µM; Eurogentec) and 8 µl of purified DNA product of each isolate were mixed in 1.5 ml tubes. Sequencing was performed by Eurofins (Germany). Sequences were analysed using the Ridom StaphType software (Ridom GmbH, Germany) and the Ridom *spa* server (http://www.spaserver.ridom.de) to assign the isolates to the various *spa* types (Harmsen *et al*., 2003).

3.6.7 DNA microarray analysis of *S. aureus* isolates

DNA microarray analysis was done using the *S. aureus* Genotyping kit 2.0 from Alere Technologies (Jena, Germany). *S. aureus* isolates were inoculated on Columbia sheep blood agar and incubated at 37°C for 20 hours to prepare them for DNA extraction. After incubation, DNA was extracted from approximately one loop full (loop of 1 mm diameter) of bacterial material using the DNeasy Blood and Tissue Kit (Qiagen, Netherlands) according to the protocol of the Alere Genotyping kit 2.0. The DNA was eluted by adding twice 50 µl of elution buffer (10 mM Tris, 0.5 mM EDTA, pH 9.0; Qiagen). All the DNA samples were stored at -20°C until use.

For the microarray, 5 µl of DNA were labelled with biotin-dUTP according to the instructions of the manufacturer (Jena, Germany). Preparation of the array strips, hybridisation (at 55°C), incubation with a streptavidin–horseradish peroxidase conjugate and washing were done in an Eppendorf Thermomixer Comfort as described by the microarray manufacturer. The arrays were analysed using ArrayMate reader (Alere Technologies) and the overall pattern was analysed automatically for the presence or absence of target genes, including genes for enterotoxins, virulence factors and antibiotic resistance.

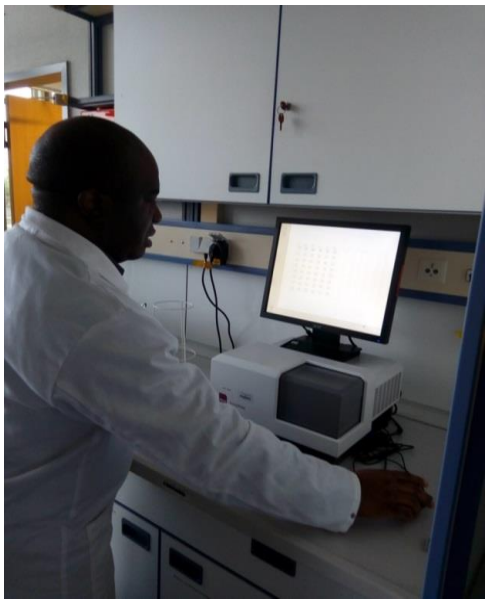


Figure 17: Checking the data on the ArrayMate reader

3.6.8 Next Generation Sequencing (NGS)

DNA from *S. aureus* isolates used in the NGS was prepared as described in Subsection 3.6.7 using the DNeasy Blood and Tissue Kit (Qiagen, Netherlands). Whole genome sequences were obtained by Next Generation Sequencing (NGS) using the Illumina MiSeq sequencing system, paired-end readings, and the Nextera DNA Flex Kit (Illumina) for the library preparation. Raw reads were trimmed using fastp v0.19.5 (Chen *et al*., 2018) and de novo assembled using unicycler v0.4.4 (Wick *et al*., 2017). Phylogenetic trees were constructed using the *S. aureus* cgMLST v1.3 and *S. aureus* MLST v1.1 with Ridom SeqSphere + software (Ridom GmbH, Münster, Germany) as minimum spanning tree (MST) based on 1716 genes of the core genome.

3.7 Determination of the antibiotic resistance profiles of *S. aureus* isolates

Antimicrobial susceptibility test was conducted by microdilution method according to the instructions from Clinical and Laboratory Standards Institute (CLSI) guideline M07-A11 (CLSI, 2018). Resistance against against a total of 19 antibiotics were tested in the antimicrobial susceptibility test and these antibiotics including penicillin, cefoxitin, tetracycline, ciprofloxacin, sulphamethoxazole, trimethoprim, chloramphenicol, clindamycin, erythromycin, fusidate, gentamicin, kanamycin, linezolid, quinupristin/dalfopristin, rifampicin, streptomycin, tiamulin, mupirocin and vancomycin representing 16 groups of antimicrobials (Table 6). The results of antibiotic susceptibility tests were interpreted using the epidemiological cut off

according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org).

Table 6: Classes of antimicrobials used in the susceptibility test

S/N	Class	Antibiotic
1	Penicillins	Benzylpenicillin
2	Cephalosporins	Cefoxitin
3	Phenicoles (Amphenicoles)	Chloramphenicol
4	Quinolones	Ciprofloxacin
5	Lincosamides	Clindamycin
6	Macrolides	Erythromycin
7	Triterpen acid	Fusidic acid
8	Aminoglycosides	Gentamicin
	Aminoglycosides	Kanamycin
	Aminoglycosides	Streptomycin
9	Oxazolidinones	Linezolid
10	Pseudomonin acid	Mupirocin
11	Streptogramines	Quinupristine/Dalfopristine
12	Ansamycines	Rifampicin
13	Inhibitors of tetrahydrofolic acid sythesis	Sulfamethoxazole
	Inhibitors of tetrahydrofolic acid sythesis	Trimethoprim
14	Tetracyclines	Tetracycline
15	Pleuromutilines	Tiamulin
16	Glycopeptides	Vancomycin

3.8 Data analysis

SPSS version 21 (IBM) was used to analyse the data from the questionnaires. Analysis of the other generated data was done using Excel 2010 (Microsoft).

3.9 Source attribution and risk assessment

Source attribution is aimed at assigning a certain proportion of human infections to an animal reservoir (Pires *et al*., 2014). However, the available models require a lot of detailed information on the prevalence of the bacteria and their subtypes in the different animal and human populations. This type of data is typically not sufficiently available in Sub-Saharan African countries such as Zambia and was not fully generated during the study period. The main approach chosen in the current study was based on the patterns of characteristics of enterotoxigenic *S. aureus* found in Zambia as recently

done for different stages of the turkey meat and cattle food chains (Tenhagen *et al*., 2014; Vossenkuhl *et al*., 2014).

3.10 Ethical clearance and export permits

Ethical clearance to conduct research in Zambia was sought from ERES CONVERGE in Zambia (Appendix A1). No names of participants or their facilities has been mentioned in this study. Pictures of participants included in this thesis, were obtained with full consent of the concerned participants. In addition, permission to export the human (milkers) presumptive *S. aureus* isolates to Germany was sought from National Health Research Ethics Board (NHREB) under the Ministry of Health (Appendix A2). Further, permission to export presumptive *S. aureus* isolates from milk and its products, and bucket swabs from the Zambian dairy value chain was sought from the Ministry of Fisheries and Livestock (Department of Veterinary Services) in Zambia (Appendix A3).

CHAPTER FOUR

RESULTS

4.1 Farmers responses

In the current study, a total of 212 dairy farmers out of 226 visited for sampling were interviewed in the three study provinces. The farmers were 70 in Lusaka province, 46 in Western and 96 in Southern. These farmers were segregated according to sex, age, education, income status, family size and herd size per household/farm.

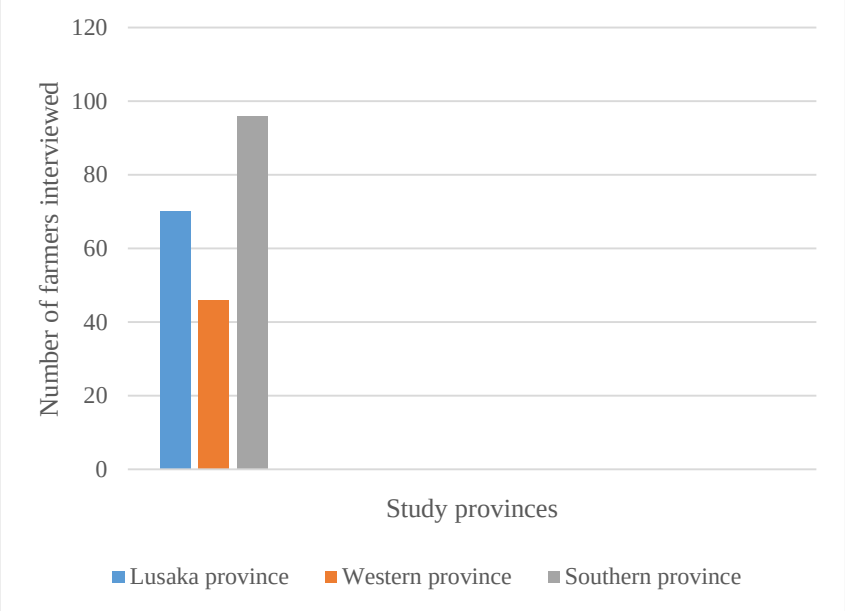


Figure 18: Number of farmers interviewed in the three study provinces

4.1.1 Demographic characteristics of smallholder and traditional dairy farmers' households

4.1.1.1 Age structure of interviewed smallholder and traditional dairy farmers in Lusaka, Western and Southern provinces of Zambia

The age structure of farmers was segregated in age ranges 20 to 29, 30 to 39, 40 to 49, 50 to 69, 60 to 69 and 70 and above. The predominant age distribution of the dairy farmers interviewed in Southern and Lusaka provinces were in the range of 40 to 49 years while in Western province it was 50 to 59 years (Table 7).

Table 7: Age structure of interviewed smallholder and traditional dairy farmers in Lusaka, Western and Southern provinces of Zambia

Age (years)	Lusaka (n=70)	Western (n=46)	Southern (n=96)
20 - 29	3/70 (4.3%)	1/46 (2.2%)	0/96 (0%)
30 - 39	17/70 (24.3%)	3/46 (6.5%)	4/96 (4.2%)
40 - 49	19/70 (27%)	9/46 (19.6%)	42/96 (43.8%)
50 - 59	10/70 (14.3%)	19/46 (41%)	26/96 (27.1%)
60 - 69	14/70 (20%)	14/46 (30%)	22/96 (23%)
70 and above	7/70 (10%)	0/46 (0%)	2/96 (2.1%)

4.1.1.2 Education status of smallholder and traditional dairy farmers interviewed

The education levels for interviewed farmers/milkers were categorised into primary, secondary and tertiary. The study findings indicate that many interviewed farmers in Western and Southern provinces went up to primary. Further, the study findings indicate that Lusaka province had a high number of interviewed farmers who were very educated and tertiary education (attended college or university). The detailed education level of the farmers interviewed is indicated in Table 8.

Table 8: Educational level of the interviewed dairy farmers in the three provinces

Education level	Lusaka (n=70)	Western (n=46)	Southern (n=96)
Primary	6/70 (8.6%)	17/46 (37%)	21/96 (22%)
Secondary	29/70 (41%)	29/46 (63%)	70/96 (73%)
Tertiary	35/70 (50%)	1/46 (2.2%)	5/96 (5.2%)

4.1.1.3 Income status, family and herd size of smallholder and traditional dairy farmers interviewed

Income status, family size and herd size of each household in the three study provinces were determined (Table 9). The average family size in all the three study provinces ranged from about 4 to more than 5 persons/members per family or household. The

findings indicated that Western province had in some cases smallest family size of 4 members per household or family. The study findings revealed that Western province had many interviewed farmers earning 500 Kwacha or less per month as income. Further, the findings indicated that Southern and Lusaka provinces had the highest number of interviewed farmers earning 3000 Kwacha or more as monthly income (Table 9). Lusaka and Southern provinces had average family size of between 5 members to more than five while Western had four to more than five members.

Table 9: Socio-economic characteristics of small scale interviewed dairy farmers in Lusaka, Western and Southern provinces of Zambia

Provinces	**Average family size/number of persons per household**	**Monthly farmers income (kwacha)/per household**	**Number of farms with monthly income**	**Average herd size per farm**	**Number of farmers with herd size categories**
Lusaka	5 to more than 5	≤ 500	11/70 (16%)	1 - 30	48/ 70 (68.6%)
	5 to more than 5	≤ 1000	26/70 (37%)	31 - 60	17/70 (24.3%)
	5 to more than 5	≥ 3000	33/70 (47%)	≥ 61	5/70 (7%)
Western	4 to more than 5	≤ 500	36/46 (78.3%)	1 - 30	17/46 (37%)
	4 to more than 5	≤ 1000	8/46 (17.4%)	31 - 60	26/46 (57%)
	4 to more than 5	≥ 3000	2/46 (4.3%)	≥ 61	3/46 (7%)
Southern	5 to more than 5	≤ 500	0/96 (0%)	1 - 30	83/96 (86.5%)
	5 to more than 5	≤ 1000	32/96 (33%)	31 - 60	11/96 (11.5%)
	5 to more than 5	≥ 3000	64/96 (66.7%)	≥ 61	2/96 (2.1%)

4.1.2 Lactating cows per farm

The number of lactating cows per farmer household in the three study provinces was determined. Overall, interviewed farmers in Western province had fewer lactating cows (mainly 1 to 9) compared to Lusaka and Southern provinces (here, farmers had mostly 3 to 15 lactating cows on average) (Table 10).

Table 10: Number of lactating cows per farm in Lusaka, Western and Southern provinces of Zambia

Number of lactating cows on the interview date	**Lusaka (n=70)**	**Western (n=46)**	**Southern (n=96)**	**Overall percentage (n=212)**
1 to 2	7 (10%)	9 (19.5%)	0 (0%)	16 (7.5%)
3 to 5	19 (27%)	16 (35%)	27 (28%)	62 (29%)
6 to 9	16 (23%)	14 (30%)	44 (46%)	74 (35%)
10 to 15	16 (23%)	5 (11%)	20 (21%)	41 (19%)
16 and above	12 (17%)	2 (4.3%)	5 (5.2%)	15 7.1%)

4.1.3 Management practices

Dairy management practices associated with milk production in the three study provinces were determined. These practices included keeping other livestock and animals such as pigs, dogs and cats, milking outside the milking shed, continuous use of same antibiotics to treat mastitis and, farmers treating cows by themselves without prescription. Other practices included selling milk from cows being treated with antibiotics. The study findings indicate that Southern province had some interviewed farmers keeping pigs (21.9 percent) and dogs (44 percent) as well on their farms. In addition, Western and Southern provinces had a lot of interviewed farmers (98 percent and 90.6 percent, respectively) without milking sheds/parlours and did their milking in kraals, crush pen or outside in the open. Further, Lusaka province had 15.7 percent of interviewed farmers experiencing mastitis on their farms. The study findings also show that in Western and Southern provinces, high number of interviewed farmers (63 percent and 45 percent, respectively) used penicillin, pen-strep (penicillin-streptomycin), megapen (ampicillin and cloxacillin), oxytetracyline and gentamicin to treat mastitis always. In addition, Western and Lusaka provinces had 45.7 percent and 32.9 percent of interviewed farmers, respectively treating cows with antibiotics themselves. Southern province had the highest number (62.5 percent) of interviewed farmers/milkers who did not have knowledge about mastitis. Further, Western and Lusaka provinces had the highest number of interviewed farmers (39.1 percent and 21.4 percent, respectively) who treated their cows with antibiotics for only 1 to 2 days only compared with Southern (Table 11). The study findings show that Western

province had some interviewed farmers (21.7 percent) selling milk from cows being treated with antibiotics and selling milk 1 to 2 days after cows are treated representing 19.6 percent.

Table 11: Potential risk factors for *S. aureus*/ AMR strains at farm level associated with production and management practices in the three study provinces

Potential risk factors	**Lusaka (n=70)**	**Western (n=46)**	**Southern (n=96)**	**Overall (n=212)**
Farmers keeping pigs on their farms	2 (2.9%)	3 (6.5%)	21 (21.9%)	26 (12.3%)
Farmers keeping dogs on their farms	24 (34.3%)	37 (80%)	42 (44%)	103 (48.6%)
Farmers keeping cats on their farms	11 (15.7%)	10 (21.7%)	7 (7.3%)	28 (13.2%)
Farmers having no milking sheds/parlours	5 (7.1%)	45 (98)%	87(90.6%)	137(64.6%)
Farmers/milkers do their milking in the kraals, crush pen or outside in the open	5 (7.1%)	45 (98%)	87 (90.6%)	137 (64.6%)
Farmers experiencing mastitis of their cows in the last 12 months	11 (15.7%)	2 (4.3)%	1 (1%)	14 (6.6%)
Continuous use of penicillin, pen-strep, megapen, oxytetracyline, and/or gentamicin to treat mastitis	14 (20%)	29 (63%)	44 (45%)	87 (41%)
Farmers treating their cows with antibiotics themselves	23 (32.9%)	21 (45.7%)	2 (2.1%)	46 (21.7%)
Farmers/milkers having no knowledge about mastitis	6 (8.6%)	16 (34.8%)	60 (62.5%)	82 (38.7%)
Farmers treating their cows with antibiotics for 1 to 2 days only	15 (21.4%)	18 (39.1%)	3 (3.1%)	36 (17%)
Selling milk from cows during treatment with antibiotics	2 (2.9%)	10 (21.7)%	6 (6.2%)	18 (8.5%)
Selling milk 1 to 2 days after cows are treated with antibiotics	0 (0%)	9 (19.6%)	0 (0%)	9 (4.2%)
Farmers not ensuring safety of milk while selling it to consumers	2 (2.9%)	0 (0%)	2 (2.1%)	4 (1.9%)

4.1.4 Milk handling and hygiene practices

Milk handling and hygiene practices along the dairy value chain in Lusaka, Western and Southern provinces were assessed. The current study described the milk handling

and hygienic practices of farmers/milker in the three study provinces (Table 12). Southern province had the highest number (74 percent) of interviewed farmers mixing milk from cows being treated with antibiotics together with that from healthy cows. In addition, all the three provinces had at least one farmer/milker who did not wash hands before milking among these interviewed. Further, the current study reveals that Southern and Western provinces had highest number of interviewed farmers/milkers (83 percent and 45.7 percent, respectively) who dried their hands after washing before starting milking using cow's tail or their own clothes and did not wash hands between milking different cows during milking session representing (81.3 percent and 63 percent, respectively).

The study findings also indicate that Western province had some interviewed farmers (30.4 percent) who did not filter or strain their milk after milking. Southern province had the highest number (38.5 percent) of interviewed farmers who used a cloth to filter milk after milking compared to the other two provinces. From observation, in all the three provinces the cloth used for filtering milk looked dirty and they were using the same cloth throughout the milking. Western and Southern provinces had many interviewed farmers/milkers (60 percent and 54.2 percent, respectively) who consumed milk directly from the cow teats. Further, some interviewed farmers in Lusaka province (n=40) and Western (n=17) had at least experienced their milk being rejected by their customers or MCCs representing 57 percent and 37 percent, respectively (Table 12). The study findings also showed that Southern and Western provinces had highest number (95 percent and 87 percent, respectively) of interviewed farmers without quality control to check for milk freshness. In addition, the study findings indicated that Western province had a highest number (87 percent) of interviewed farmers who were not able to trace back bad milk to its source than Lusaka and Southern (Table 12).

Table 12: Milk handling practices by farmers/milkers in the three study provinces

Practices	**Lusaka (n=70)**	**Western (n=46)**	**Southern (n=96)**	**Overall (n=212)**
Milking their cows being treated with antibiotics together with the healthy cows (sick cows being milked at the same time as health ones without separating them)	0 (0%)	5 (10.9%)	45 (46.9%)	50 (23.6%)
Farmers/milkers mixing milk from cows being treated with antibiotics with milk from healthy cows	2 (2.9%)	20 (43.5%)	71 (74%)	93 (43.9%)
Farmers/milkers not washing their hands before milking	2 (2.9%)	2 (4.3%)	1 (1%)	5 (2.4%)
Farmers/milkers not drying their hands after washing hands before starting to milk cows	12 (17%)	10 (21.7%)	15 (15.6%)	37 (17.5%)
Farmers/milkers drying their hands after washing before milking using cow's tail or their own clothes	3 (3%)	21 (45.7%)	80 (83%)	104 (49.1%)
Farmers/milkers not washing hands after milking each individual cow during milking sessions	13 (18.6%)	29 (63%)	78 (81.3%)	120 (56.6%)
Farmers/milkers not straining or filtering their milk after milking	9 (12.9%)	14 (30.4%)	5 (5.2%)	28 (13.2%)
Use of a cloth to strain milk	8 (11.4%)	4 (8.7%)	37 (38.5%)	49 (23.1%)
Farmers/milkers consuming milk directly from the teats	15 (21.4%)	28 (60%)	52 (54.2%)	95 (44.8%)
Farmers not taking care of milk safety measures while selling milk on the market or to consumers	2 (2.9%)	0 (0%)	2 (2.1%)	4 (2%)
Farmers having their milk rejected by customers or the cooperatives because of poor quality	40 (57%)	17 (37%)	13 (13.5%)	70 (33%)
Farmers having no quality control checks on their farms to check freshness and water content of milk	34 (49%)	40 (87%)	92 (95%)	166 (78%)
Farmers not able to trace back bad milk to its sources	20 (29%)	40 (87%)	13 (13.5%)	73 (34%)

4.1.5 Farmers/milkers practices associated with milk containers in the three study provinces

Practices associated with milk containers among farmers/milkers were determined in all the study provinces. These practices included the use of plastic, metal and wooden milk containers. The findings of the current study indicate that many interviewed

farmers in Western and Southern Provinces used plastic containers for milking, preserving, storage and selling milk. Further, the study findings showed that some interviewed farmers (54 percent) in Western province used wooden vessels for milking cows and also used cold water only to clean the milking vessels and containers, representing 26.1 percent. Almost all interviewed farmers in Lusaka used metal containers (aluminium/steel) for milking, preservation, storage, transportation and selling milk (Table 13).

Table 13: Farmers/milkers practices associated with milk containers in the three study provinces

Practices	**Lusaka (n=70)**	**Western (n=46)**	**Southern (n=96)**	**Overall (n=212)**
Farmers using metal containers (aluminium/steel) for milking	69 (98.6%)	3 (6.5%)	40 (41.7%)	112 (52.8%)
Farmers using metal containers (aluminium/steel) for making of sour milk (preservation)	66 (94%)	2 (4.3%)	42 (43.8%)	110 (51.9%)
Farmers using metal containers (aluminium/steel) for storage before consumption	68 (97%)	1 (2.2%)	43 (44.8%)	112 (52.8%)
Farmers using metal containers (aluminium/steel) for transportation	69 (98.6%)	2 (2.2%)	43 (44.8%)	114 (53.8%)
Farmers using metal containers (aluminium/steel) for selling milk	65 (92.9%)	3 (6.5%)	43 (44.8%)	111 (52.4%)
Farmers/milkers using wooden vessels for milking cows	1 (1%)	25 (54%)	0 (0%)	26 (12.2%)
Farmers/milkers using plastic containers for milking cows	2 (2.9%)	32 (69.6%)	61 (63.5%)	95 (44.8%)
Farmers/milkers using plastic containers for preserving milk	5 (7.1%)	45 (97%)	57 (59.4%)	107 (50.5%)
Farmers/milkers using plastic containers for storing milk	1 (1%)	46 (100%)	57 (59.4%)	104 (49.1%)
Farmers/milkers using plastic containers for transporting milk	2 (2.9%)	46 (100%)	57 (59.4%)	105 (49.5%)
Farmers/milkers using of plastic containers for selling milk	11 (16%)	44 (95.7%)	58 (60%)	113 (53%)
Farmers/milkers cleaning milk vessels and containers with cold water only	0 (0%)	12 (26.1%)	4 (4%)	16 (7.5%)
Farmers/milkers cleaning milk vessels and containers with hot/warm water only	4 (5.7%)	2 (4%)	8 (8%)	14 (6.6%)
Farmers/milkers not cleaning milk vessels and containers	2 (2.9%)	0 (0%)	2 (2%)	4 (2%)

Some observations regarding milk handling are shown in Figure 19.

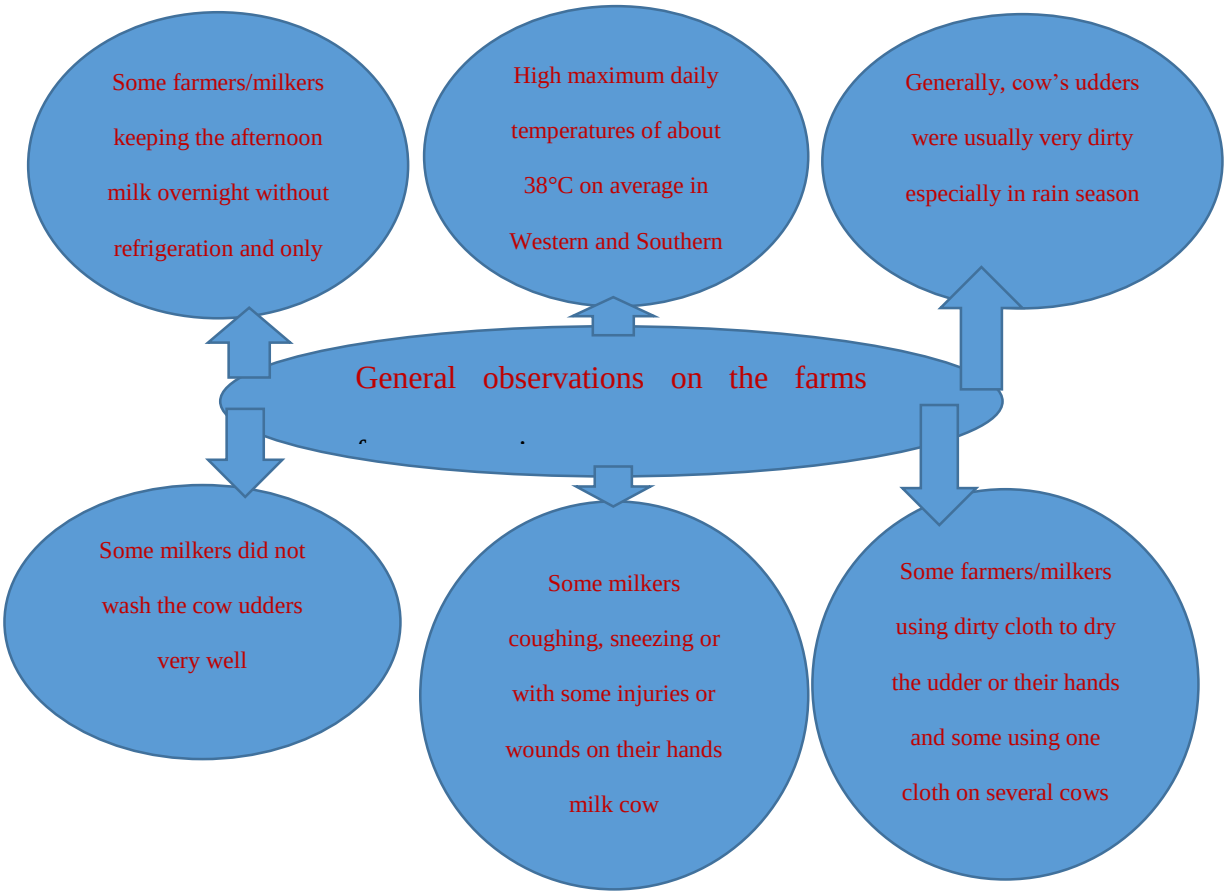


Figure 19: General observations on the farms and environment in the three study provinces

4.1.6 Transportation of milk to the market

Mode of milk transportation in the three study provinces was determined. The study findings indicated that many farmers (54 percent) in Western province transported their milk on foot, and most interviewed farmers (89 percent) in this province covered between 7 to18 km to reach the market (Table 14). In addition, many farmers, (96 percent) each in Lusaka and Southern provinces, transported their milk on bicycles and many covered 1 to 6 km to reach the market. Very few interviewed farmers transported their milk using vehicles with the least being interviewed farmers in Western province.

Table 14: Farmer practices associated with milk transport in the three study provinces

Practices	**Lusaka (n=70)**	**Western (n=46)**	**Southern (n=96)**	**Overall (n=212)**
Farmers transporting milk on foot	0 (0%)	25 (54%)	0 (0%)	25 (11.8%)
Farmers transporting milk on bicycles	67 (96%)	27 (58.7%)	92 (96%)	186 (88%)
Farmers transporting milk using vehicles	3 (4.3%)	1 (2.2%)	4 (4.2%)	8 (3.8%)
Farmers transporting milk using motor bike	0 (0%)	0 (0%)	1 (1%)	1 (0.5%)
Farmers transporting milk using public transport	0 (0%)	0 (0%)	1 (1%)	1 (0.5%)
Farmers covering 1 to 6 km distance to the market	52 (74%)	3 (6.5%)	53 (55%)	108 (51%)
Farmers covering 7 to 12 km distance to the market	18 (26%)	28 (61%)	33 (34%)	79 (37.3)
Farmers covering 13 to 18 km distance to the market	0 (0%)	13 (28.1%)	10 (10%)	23 (10.8%)
Farmers covering 19 to 21 km distance to the market	0 (0%)	2 (4.3%)	0 (0%)	2 (0.9%)

4.1.7 Health related practices and experiences on farms in the three provinces

Health problems associated with milk handling on farms in the three study provinces among farmers, milkers/workers and size of farmer's families/households were described. The current study findings showed that some interviewed farmers in Southern (32.3 percent) and Lusaka (30 percent) provinces had at least experienced skin infections among the people on their farms. In addition, famers in Lusaka (28.6 percent) used antibiotics every time a member or a milker was sick. Further, the study findings indicate that interviewed farmers or people on their farms in Southern (64 percent) and Western (63 percent) provinces took antibiotics without prescriptions. The current study findings also showed that some interviewed farmers and their households (57 percent) in Western province at least experienced a situation where despite taking antibiotics their condition did not improve and 85 percent of people on the farms also consumed raw milk. In addition, the study findings also showed that many interviewed farmers and their households in Southern (84.4 percent) and some farmers in Western provinces (43.5 percent) consumed milk from cows being treated with antibiotics and did not keep any health records on their farms, representing 91

percent and 78.1 percent respectively. It was also observed that some people on farms in the three provinces consume traditionally processed sour milk (Table 15).

Table 15: Health related practices and experiences on farms in the three study provinces

Practices	**Lusaka (n=70)**	**Western (n=46)**	**Southern (n=96)**	**Overall (n=212)**
Farmers experiencing skin infections on their farms among family members and milkers	21 (30%)	11 (23%)	31 (32.3%)	63 (30%)
Farmers using antibiotics every time a family member or milker was sick	20 (28.6)	8 (17.4%)	8 (8%)	36 (17%)
Farmers using antibiotics sometimes when a family member or milker was sick	38 (54%)	30 (65%)	67 (70%)	135 (64%)
People on the farm taking antibiotics without prescriptions	21 (30%)	29 (63%)	61 (64%)	111 (52%)
People on the farm experiencing situation where despite taking antibiotics their condition did not improve	27 (39%)	26 (57%)	9 (9%)	62 (29%)
People on the farm consuming raw milk	18 (26%)	39 (85%)	20 (20%)	77 (36%)
People on the farm consuming milk from cows being treated with antibiotics	2 (2.9%)	20 (43.5%)	81 (84.4%)	103 (48.6%)
People on the farm consuming traditional sour milk	35 (50%)	35 (76%)	63 (66%)	133 (63%)
Farmers not keeping any health records on their farms	20 (29%)	36 (78.1%)	87 (91%)	143 (67.5%)

4.2 Milk collection centre (MCC) responses

In the current study, a total of 11 MCCs were interviewed in the three provinces. The MCCs visited included the three in Lusaka province, two in Western and six in Southern. These MCCs were segregated according to membership, when they started operating and, milk rejection levels at MCCs.

4.2.1 Membership and operations of MCCs in the three provinces

Active membership at MCCs, when MMCs started operations in the three study provinces were described. MCCs in Southern province had the highest number of active members with 91 to 300 members at 5 out of 6 MCCs, followed by Lusaka with

34 to 90 members in the three MCCs while the two MCCs in Western province had the lowest with 12 to 33 members (Table 16).

Table 16: Number of active dairy farmers supplying milk to MCCs in the three provinces

Practices	**Lusaka (n=3)**	**Western (n=2)**	**Southern (n=6)**	**Overall (n=11)**
Number of cooperatives with 12 to 33 active members	0	2	0	2 (18.2%)
Number of cooperatives with 34 to 55 active members	1	0	1	2 (18.2%)
Number of cooperatives with 56 to 70 active members	1	0	0	2 (18.2%)
Number of cooperatives with 71 to 90 active members	1	0	2	3 (27.3%)
Number of cooperatives with 91 to 300 active members	0	0	3	3 (27.3%)

It was observed that the oldest of the interviewed MCCs started about 20 years ago in Lusaka province while the newest MCC started at least 3 years ago in Southern province (Table 17).

Table 17: When the MCCs started operating in the three provinces

Practices	**Lusaka (n=3)**	**Western (n=2)**	**Southern (n=6)**	**Overall (n=11)**
Milk collection centre starting 20 years ago	1	0	0	1 (9%)
Milk collection centre starting 15 years ago	0	0	1	1 (9%)
Milk collection centre starting 13 years ago	0	0	2	2 (18.2%)
Milk collection centre starting 10 years ago	1	2	0	3 (27.3%)
Milk collection centre starting 9 years ago	1	0	2	3 (27.3%)
Milk collection centre starting at least 3 years ago	0	0	1	1 (9%)

4.2.2 Milk rejection due to bad quality at the MCCs in the three provinces

Milk rejection percentages due to the bad quality of milk in all the three study provinces was determined. The study findings revealed that the two interviewed MCCs in Western province had the highest percentage (20 to 30 percent) of milk rejection at reception than Lusaka and Southern provinces (Table 18).

Table 18: Milk rejection levels due to bad quality at the MCCs in the three provinces

Practices/experiences	**Lusaka (n=3)**	**Western (n=2)**	**Southern (n=6)**	**Overall (n=11)**
30% milk rejection	0	1	0	1 (9.1%)
20% milk rejection	0	1	0	1 (9.1%)
5% milk rejection	0	0	4	4 (36.4%)
2% milk rejection	3	0	2	5 (45.5%)
Milk from some farmers was not always of good quality at reception some times	1	1	6	8 (73%)

4.2.3 Milk handling practices at MCCs in the three study provinces

Milk handling practices at MCCs were described and these included receiving milk twice per day, mixing of morning and afternoon batches of milk, milk taking longer before it was collected by processors from MCCs and MCCs not doing microbial analysis. The potential risk factors for possible contamination of milk and multiplication of *S. aureus* were described in Table 19. The major findings of the study show that all the interviewed MCCs in all the three provinces receive milk twice per day and did mix the morning and afternoon milk batches (Table 19). Further, the study also indicated that almost all MCCs in Southern province (5 out of 6), milk sometimes took approximately 48 or more hours before being collected by the processors (Table 19). In addition, almost all the MCCs in all the three provinces did not carry out any microbial analysis and checking antibiotic residues (Table 20). In Western and Southern provinces, milk provided by MCCs to processors was sometimes rejected by the processor (Table 20) and the rejection of was mainly due to milk turning sour.

Table 19: Potential risk factors for possible contamination of milk and multiplication of *S. aureus* at MCCs in the three study provinces

Practices	**Lusaka (n=3)**	**Western (n=2)**	**Southern (n=6)**	**Overall (n=11)**
Mixing morning and afternoon milk	3	2	6	11 (100%)
Some milk received in plastic containers	0	2	3	5 (45.5%)
Having no chilling/cooling facility	0	0	1	1 (9%)
Milk taking 1 to 3 hours before being collected by the processors	0	2	4	7 (63.6%)
Milk taking 24 hours before being collected by the processors	1	0	0	1 (9%)

Milk taking 24 hours to 48 hours before being collected by the processors	2	0	0	2 (18%)
Milk taking 48 hours or more before being collected by the processors	0	0	2	2 (18%)

Table 20: Milk handling practices at MCCs in the three study provinces

Practices	**Lusaka (n=3)**	**Western (n=2)**	**Southern (n=6)**	**Overall (n=11)**
Receiving milk twice per day (morning and afternoon)	3	2	6	11 (100%)
Not checking for antibiotic residues	1	2	5	8 (73%)
Not doing microbiological analyses	2	2	6	10 (91%)
Not able to trace back bad milk to its source	0	1	4	5 (45.5%)
Some milk transported on foot to the MCC	1	2	0	3 (27.3%)
Some milk transported on bicycle to the MCC	3	2	6	11 (100%)
Some milk transported using vehicle to the MCC	3	0	4	7 (63.6%)
Deciding not to sell batch of milk when it turns sour	2	1	3	6 (54.5%)
Processor (Parmalat) being main client buying milk	3	0	2	5 (45.5%)
Community and processor being main client buying milk	0	0	3	3 (27.3%)
Community being main client buying milk	0	2	2	4 (36.4%)
Sometimes milk rejected by the processor	0	2	3	5 (45.5%)
Reason for rejecting milk being turning sour	0	1	3	4 (36.4%)

4.2.4 MCCs workers' health experiences

Health problems related to milk handling experienced by MCC workers in the last 12 months of the interview was determined. The major findings of the study showed that Southern province had the highest number of MCCs with workers who have never received food hygiene and safety education and at least one MCC had workers who experienced skin infections (Table 21).

Table 21: MCCs and their staff health experience

Practices	Lusaka (n=3)	Western (n=2)	Southern (n=6)	Overall (n=11)
Experiencing skin infection among workers	0	0	1	1 (9.1%)
Having no food hygiene and food safety education	0	0	5	5 (45.5%)

Some observations on MCCs milk handling are shown in Figure 20.

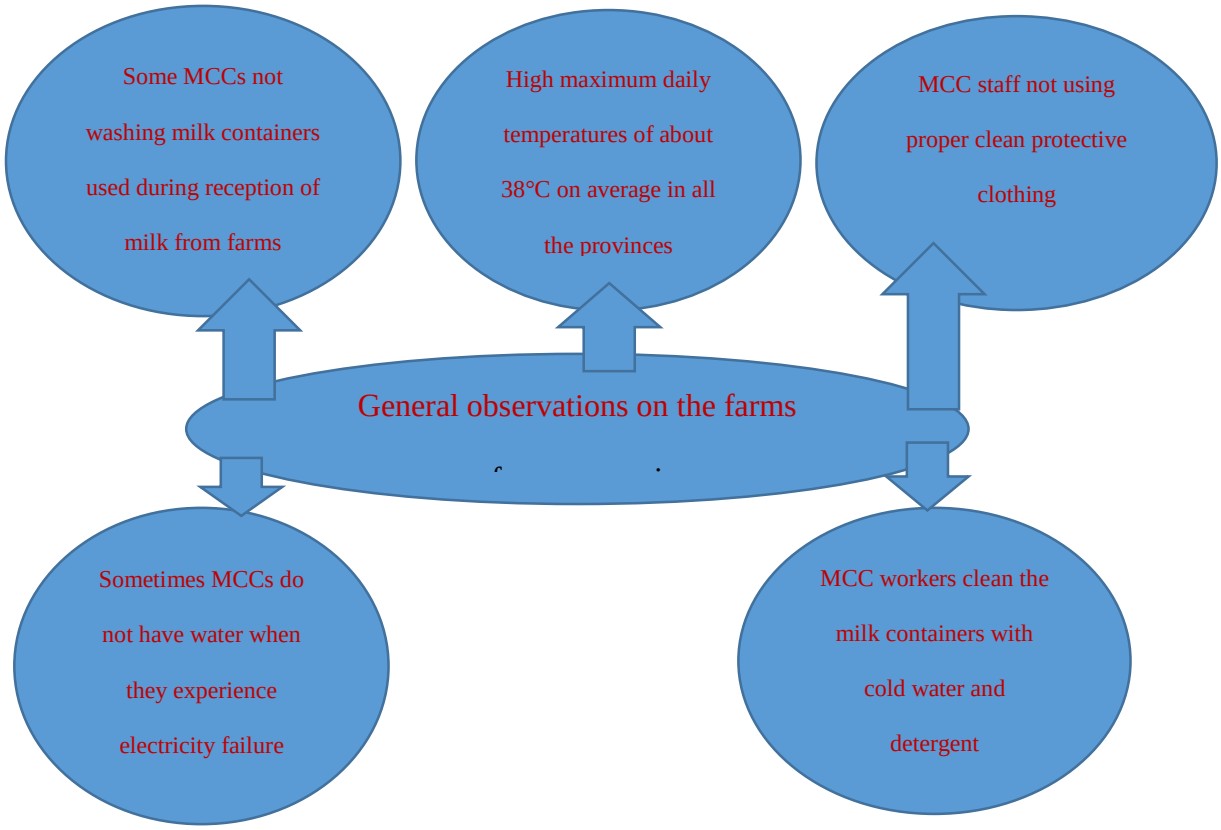


Figure 20: General observations on MCCs environment in the three study provinces

4.3 Milk trader responses

In the current study, a total of 15 milk traders were interviewed in the three provinces. These traders were five in Lusaka, Western and Southern provinces respectively. These milk traders were segregated according to sex, age, family size, education and income status per household/family.

4.3.1 Gender, age and education levels of interviewed milk traders in the three study provinces

Tables 22 and 23, show some general data on sex, age, family size, education levels and income status associated with the interviewed milk traders.

Table 22: Gender, age and education level of interviewed milk traders in the three study provinces

Practices	**Lusaka (n=5)**	**Western (n=5)**	**Southern (n=5)**	**Overall (n=15)**
Female milk trader	2	1	1	4 (26.7%)
Male milk trader	3	4	4	11 (73.3%)
Milk traders between 20 to 29 years old	0	1	1	2 (13.3)
Milk traders between 30 to 39 years old	3	3	1	7 (47%)
Milk traders between 40 to 49 years old	2	0	3	5 (33.3%)
Milk traders between 50 to 59 years old	0	1	0	1 (7%)
Milk traders who attended grade 7 and below	2	2	1	5 (33.3%)
Milk traders who attended grade 8 to 10	1	3	3	7 (47%)
Milk traders who attended grade 12 to College	2	0	1	3 (20%)
Milk traders having no health education in the area of food hygiene and safety	4	5	4	13 (87%)

Table 23: Socio-economic factors associated with interviewed milk traders in the three study provinces

Factors	**Lusaka (n=5)**	**Western (n=5)**	**Southern (n=5)**	**Overall (n=15)**
Having 2 to 3 family members per household	2	2	1	5 (33.3%)
Having 4 to 5 family members per household	2	1	2	5 (33.3%)
Having more than 5 family members per household	1	2	2	5 (33.3%)
Household income ≤ 500 kwacha per month	3	2	2	7 (47%)
Household income ≤ 1000 kwacha per month	0	2	3	5 (33.3%)

Household income ≥ 3000 kwacha per month	2	1	0	3 (20%)
Belonging to cooperative or MCC	2	0	0	2 (13.3%)
Starting the enterprise 1 to 2 years ago	2	1	1	4 (27%)
Starting the enterprise 3 to 4 years ago	1	3	4	8 (53.3%)
Starting the enterprise more than 5 years ago	2	1	0	3 (20%)

4.3.2 Milk handling practices among milk traders in the three provinces

Milk handling practices practiced by milk traders were determined. These included use of plastic containers which are not sufficiently cleaned, sell of raw milk and traditional sour milk, distances covered to reach their market and transportation of milk on foot (Table 24, 25). The current study findings show that most interviewed traders in all the three study provinces bought milk directly from farmers and used plastic containers which are often not sufficiently cleaned (Table 24). The study findings also show that all interviewed milk traders sold raw milk and some also sold traditionally processed sour milk (Table 25). Further, the interviewed milk traders covered up to 9 km and no more than 12 km (Table 25). Most interviewed milk traders did not have cooling facilities (refrigerators) (Table 24).

Table 24: Potential risk factors associated with contamination of milk and promotion of rapid multiplication of *S. aureus* in milk sold by interviewed milk traders in the three provinces

Parameters	**Lusaka (n=5)**	**Western (n=5)**	**Southern (n=5)**	**Overall (n=15)**
MCC as a source of the milk they sell	0	1	0	1 (7%)
Transporting milk on foot to the market or clients	0	0	2	2 (13.3%)
Milk traders without refrigerators	3	4	3	10 (67%)
Using plastic containers to store milk	3	4	5	12 (80%)
Use of wide-necked plastic containers to sell milk	0	1	3	4 (27%)
Milk traders having no quality control to check freshness of milk	5	5	4	14 (93%)
Mixing morning and afternoon milk batches	3	1	0	4 (27%)

Not ensuring general personal hygiene when handling milk	3	2	0	5 (33.3%)
Washing containers with water only	3	3	1	7 (47%)
Milk traders not cleaning milk containers after delivery	3	2	4	9 (60%)
Milk traders using a cup without handle to scoop milk from a storage container	0	1	0	1 (7%)
Milk traders having no ability to trace back bad milk from its source	0	5	0	5 (33.3%)

Table 25: Milk handling practices among interviewed milk traders in the three provinces

Practices	**Lusaka (n=5)**	**Western (n=5)**	**Southern (n=5)**	**Overall (n=15)**
Selling raw milk	3	0	5	8 (53.3%)
Selling at least raw and sour milk	2	5	0	7 (47%)
Family members consuming at least raw and sour milk	4	4	5	13 (87%)
Selling milk mainly to community and market	5	5	5	15 (100%)
Selling milk mainly to cooperatives	2	0	0	2 (13.3%)
Less than 1 Km distance to the market or clients	2	0	1	3 (20%)
1 to 3 Km distance to the market or clients	0	0	3	3 (20%)
4 to 6 Km distance to the market or clients	1	2	1	4 (26.7%)
7 to 9 Km distance to the market or clients	1	3	0	4 (26.7%)
10 to 12 Km distance to the market or clients	1	0	0	1 (6.7%)
Buying milk from farmers as source of milk	5	4	4	13 (87%)
Transporting milk on the bicycle to the market or clients	4	4	4	12 (80%)
Use of narrow-necked plastic containers to sell milk	4	4	2	10 (67%)
Milk traders making sour	2	2	0	4 (27%)

Figure 21: A milk trader using plastic containers for transportation of milk

4.4 Supermarkets responses

In the current study, a total of 35 representatives from supermarkets/shops were interviewed in the three provinces. These were 15 in Lusaka province, 10 in Western and Southern each respectively. Supermarket responses mainly came from managers/supervisors.

4.4.1 Education status of participants from supermarkets/shops

Education levels of participants from supermarkets were determined. Education status was categorised into Grade 7 and below, grade 9, grade 10 to 11, grade 12, college and University (Table 26). Those respondents in the last category were considered very educated. As would be expected, high level of education among participants was observed in Lusaka province compared to the other two study provinces (Table 26).

Table 26: Education levels associated with participants at supermarkets/shops in the three study provinces

Education Level	**Lusaka (n=15)**	**Western (n=10)**	**Southern (n=10)**	**Overall (n=35)**
Primary	0	2	1	3 (8.6%)
Secondary	3	8	9	20 (57%)
Tertiary	12	0	0	12 (34.2%)

4.3.2 Milk handling practices associated with supermarkets/shops in the three provinces

The study findings showed that milk was usually delivered by processors in many interviewed supermarkets/shops in Lusaka province (100 percent) and Western (70 percent) than Southern province (Table 27). Many interviewed supermarkets/shops (86 percent) in Lusaka had at least experienced milk being rejected by customers compared to Western and Southern provinces (Table 27).

Table 27: Milk handling practices/experiences associated with supermarkets/shops in the three study provinces

Practices/experiences identified	**Lusaka (n=15)**	**Western (n=10)**	**Southern (n=10)**	**Overall (n=35)**
Milk being delivered at least by processors to the supermarket/shop	15 (100%)	7 (70%)	5 (50%)	27 (77%)
Milk being delivered at least by an agent to the supermarket/shop	14 (93%)	1 (10%)	2 (20%)	17 (48.6%)
Supermarket/shop collecting milk from processors	1 (6.7%)	3 (30%)	3 (30%)	7 (20%)
0.01 to 1% of milk discarded every week	4 (26.7%)	0 (0%)	1 (10%)	5 (14.3%)
1.51 to 4% of milk discarded every week	5 (33.3%)	0 (0%)	0 (0%)	5 (14.3%)
4.01 to 5% of milk discarded every week	1 (6.7%)	0 (0%)	0 (0%)	1 (0.028%)
Experiencing milk being rejected by customers	13 (86%)	1 (10%)	3 (30%)	17 (48.6%)

4.4.3 Potential risk factors for contamination associated with milk handling practices in supermarkets in the three study provinces

The potential risk factors associated with milk handling in supermarkets/shops in the three study provinces were determined (Table 28). The study findings indicated that some supermarkets/shops in Western (70 percent) and Southern (50 percent) Provinces did mix packed milk sachets, bottles and packs from morning and afternoon batches and also they mixed milk packets from different sources (40 percent and 80 percent, respectively). From observation, milk consumers drink directly from these packets/sachets or bottles even if they may be dirty due spillages of milk. Further, the study findings revealed that many supermarkets/shops (90 percent) in Southern province did not have quality control checks on milk freshness and about 70 percent of the supermarkets/shops are not able to trace back bad milk to its source. In addition,

the study findings showed that many supermarkets (86 percent) in Lusaka province experienced at least handling bad milk (souring and smelling). Further, almost all the supermarkets/shop in the three provinces did not fumigate their premises. Furthermore, the study findings also revealed that Lusaka had more supermarkets (60 percent) where milk batches took at least seven days in the supermarket/shop before they finished (Table 28).

Table 28: Potential risk factors associated with milk handling practices in supermarkets/shops in the three study provinces

Factors	**Lusaka (n=15)**	**Western (n=10)**	**Southern (n=10)**	**Overall (n=35)**
Supermarkets/shops mixing morning and afternoon milk batches of packed packets/sachets or bottles	1 (6.7%)	7 70%)	5 (50%)	13 (37.1%)
Supermarkets/shops mixing milk from different sources	1 (6.7%)	4 (40%)	8 (80%)	13 (37.1%)
Supermarkets/shops having no quality control checks for milk freshness	1 (6.7%)	10 (100%)	9 (90%)	20 (57.1%)
Supermarkets/shops experiencing handling bad (souring and smelling bad) milk	13 (86.7%)	0 (0%)	2 (20%)	15 (42.9%)
Supermarkets/shops not fumigating their premises	14 (93.3%)	10 (100%)	10 (100%)	34 (97.1%)
Supermarkets/shops not being able to trace back bad milk to its source	0 (0%)	3 (30%)	7 (70%)	10 (28.6%)
Fresh milk taking less than 4 to 8 hours before it finishes in the supermarket/shop	0 (0%)	6 (60%)	10 (100%)	16 (45.7%)
Fresh milk taking one day before it finishes in the supermarket/shop	0 (0%)	2 (20%)	0 (0%)	2 (5.7%)
Fresh milk taking 2 days before it finishes in the supermarket/shop	1 (6.7%)	2 (20%)	3 (30%)	6 (17%)
Fresh milk taking 3 to 4 days before it finishes in the supermarket/shop	2 (13.3%)	0 (0%)	0 (0%)	2 (5.7%)
Fresh milk taking 5 to 6 days before it finishes in the supermarket/shop	3 (20%)	0 (0%)	0 (0%)	3 (8.6%)
Fresh milk taking 7 days before it finishes in the supermarket/shop	9 (60%)	0 (0%)	0 (0%)	9 (25.7%)

4.4.4 Health experience of workers handling milk in the supermarkets/shops in the three study provinces

The health related problems regarding milk handling in the three study provinces was determined and are described in Table 29. The current study findings revealed that

many supermarkets/shop (80 percent) in Southern province had workers who had no health certificates and the workers were not subjected to regular health check-ups. Further, the study findings showed that many supermarkets in Lusaka and Western provinces at least experience complaints from customers such as vomiting or diarrhoea after consumption of milk bought from their facilities (Table 29).

Table 29: Health experience of supermarket/shop workers handling milk in supermarkets in the three study provinces

Factors	**Lusaka (n=15)**	**Western (n=10)**	**Southern (n=10)**	**Overall (n=35)**
Supermarkets/shop workers handling milk having no health certificate	4 (26.7%)	4 (40%)	8 (80%)	16 (45.7%)
Supermarkets/shop not subjecting their workers to regular health check-ups	4 (26.7%)	4 (40%)	8 (80%)	16 (45.7%)
Supermarkets/shop experiencing some skin conditions among workers handling milk	1 (6.7%)	0 (0%)	0 (0%)	1 (2.9%)
Supermarkets/shops experiencing customers complaining of vomiting or diarrhoea after consuming milk bought	13 (86.7%)	7 (70%)	5 (50%)	25 (71.4%)

General observations on supermarkets made during the study in the three provinces are shown in Figure 22.

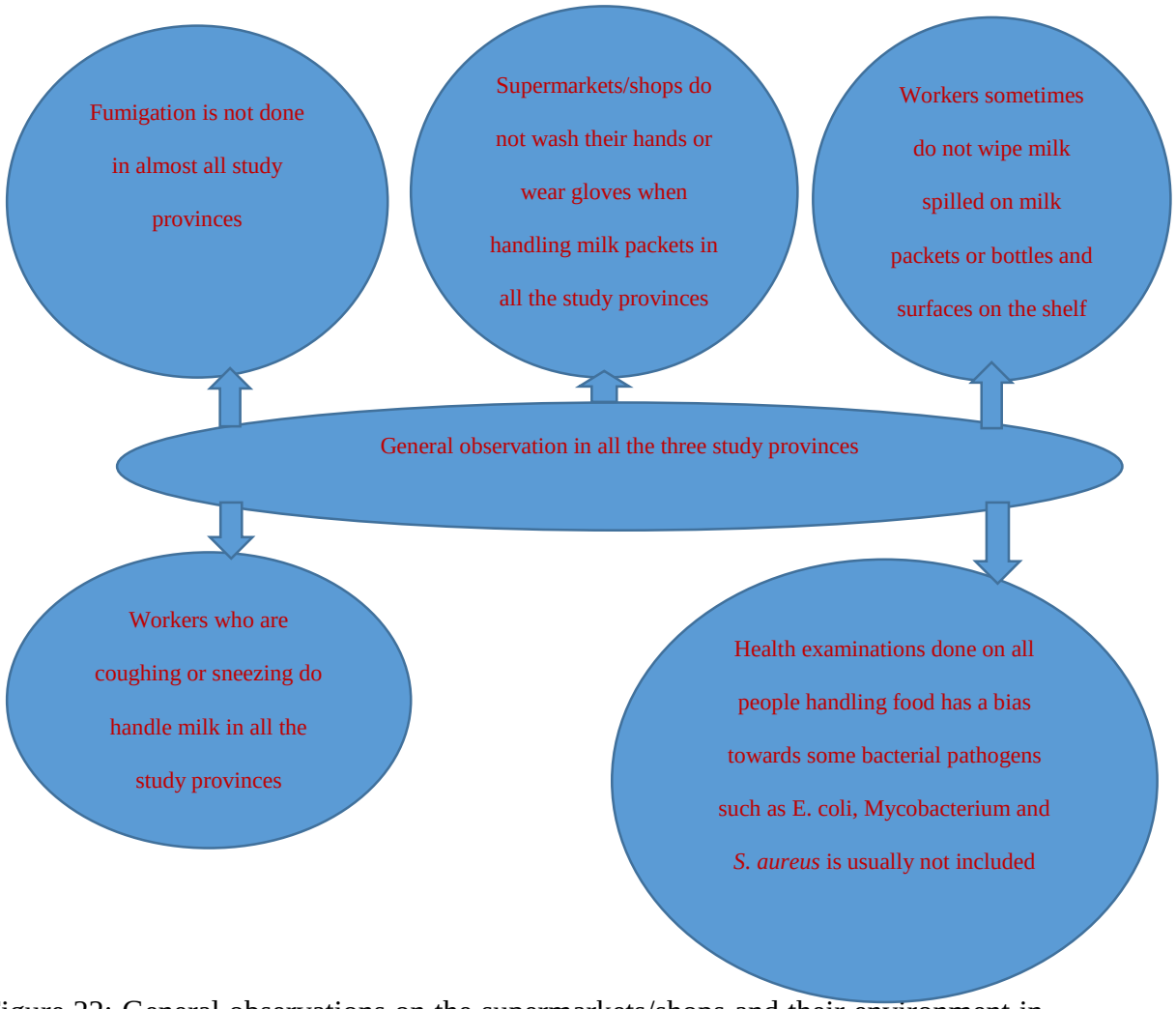


Figure 22: General observations on the supermarkets/shops and their environment in the three study provinces

4.5 Consumers responses

In the current study, a total of 126 milk consumers were interviewed in the three provinces. These were 30 in Lusaka province, 42 in Western and 54 in Southern. These milk consumers were segregated according to sex, age, education level, occupation, income status and family size per household.

4.5.1 Age structure of milk consumers in the three provinces

The study findings show that the majority of the interviewed milk consumers in the three provinces belonged to the age ranges, 30 to 39 and 40 to 49 (Table 30).

Table 30: Age structure of milk consumers in the three provinces

Parameter	**Lusaka (n=30)**	**Western (n=42)**	**Southern (n=54)**	**Overall (n=126)**
20 to 29 years old	5 (17%)	6 (14.3%)	9 (17%)	20 (16%)
30 to 39 years old	12 (40%)	13 (31%)	15 (28%)	40 (32%)
40 to 49 years old	11 (37%)	17 (40.5%)	24 (44%)	52 (41.3%)
50 to 59 years old	2 (7%)	5 (12%)	3 (6%)	10 (8%)
60 to 69 years old	0 (0%)	1 (2.4%)	3 (6%)	4 (3.2%)

4.5.2 Education levels of milk consumers

The study indicates that Southern province (41 percent) had the highest number of interviewed milk consumers who were less educated than the two other study provinces (only attended primary level). Further, the study findings revealed that Lusaka province (76 percent) had the highest number of very educated interviewed milk consumers (attended college or university) compared to Western and Southern provinces. In addition, the findings of the study showed that Lusaka (93 percent) and Western (83 percent) provinces had the highest number of interviewed milk consumers who did not receive hygiene trainings (Table 31).

Table 31: Education levels attended by milk consumers in the three study provinces

Education level/knowledge of food hygiene	**Lusaka (n=30)**	**Western (n=42)**	**Southern (n=54)**	**Overall (n=126)**
Primary	0 (0%)	12 (28.6%)	22 (41%)	34 (27%)
Secondary	7 (23%)	26 (61%)	29 (54%)	62 (49%)
Tertiary	23 (76.7%)	4 (9.5%)	3 (5.5%)	30 (24%)
No knowledge of food hygiene	6 (20%)	10 (24%)	20 (37%)	36 (28.6%)
Consumers thinking food hygiene is not important (Poor attitude towards hygiene)	3 (10%)	3 (7%)	1 (1.9%)	7 (5.6%)

Milk consumers who have not received any hygiene trainings	28 (93%)	35 (83%)	20 (37%)	83 (66%)

4.5.3 Other socio-economic characteristics of milk consumers in the three provinces

Other socio-economic characteristics of milk consumers were categorised into family size, occupation and income. The major findings of the study show that Lusaka (60 percent) and Western (52.5 percent) provinces had the highest number of interviewed milk consumers with more than 5 family members (big family) per household on average. In addition, Southern (61 percent) and Western (60 percent) provinces had highest number of interviewed milk consumers with farming as an occupation. In addition, Lusaka province had the highest number of interviewed milk consumers who were very educated and had higher income of ≥ 3000 kwacha per month**.** Further**,** Western and Southern provinces had the highest number of milk consumers with lower incomes of ≤ 1000 kwacha per month per household (Table 32).

Table 32: Other socio-economic characteristics of milk consumers in the three study provinces

Factors	**Lusaka (n=30)**	**Western (n=42)**	**Southern (n=54)**	**Overall (n=126)**
2 to 3 family members per household (small family)	6 (20%)	0 (0%)	13 (24%)	19 (15.1%)
4 to 5 family members per household (medium sized family)	6 (20%)	20 (48%)	23 (43%)	49 (39%)
More than 5 members per household (large family)	18 (60%)	22 (52.4%)	18 (33%)	58 (46%)
Farming as an occupation	2 (7%)	25 (60%)	33 (61%)	60 (47%)
At least business as an occupation	1 (3%)	7 (17%)	8 (15%)	16 (12.7%)
Self-employment as an occupation	1 (3%)	4 (10%)	9 (17%)	14 (11%)
Working (employment) as an occupation	25 (83%)	6 (14%)	3 (6%)	34 (27%)
Unemployed/ house wife as an occupation	1 (3%)	0 (0%)	1 (1.9%)	2 (1.6%)
Household income ≤ 500 kwacha per month	0 (0%)	5 (12%)	13 (24.1%)	18 (14.3%)
Household income ≤ 1000 kwacha per month	7 (23%)	23 (55%)	31 (57%)	61 (48%)
Household income ≥ 3000 kwacha per month	23 (77%)	14 (33%)	10 (19%)	47 (37%)

4.5.4 Milk handling practices by milk consumers associated with milk source in the three provinces

Milk handling practices of interviewed milk consumers have been described in Table 33. These included the type of milk consumers buy, source of their milk and the distance the milk consumers cover to reach the milk source. The study findings indicated that many interviewed milk consumers in Lusaka province cover 4.1 to 5 km (13 percent) and above 5 km (33 percent) from their homes to the milk source (mainly MCCs). In addition, the study findings revealed that many milk consumers in Western (57 percent) and Southern (44 percent) provinces covered 1 to 4 km to reach the milk source and many bought milk from traders/vendors besides MCCs (Table 33).

Table 33: Milk handling practices by milk consumers associated with milk source in the three provinces

Practices	**Lusaka (n=30)**	**Western (n=42)**	**Southern (n=54)**	**Overall (n=126)**
Always buying at least raw milk	26 (86.7%)	34 (81%)	47 (87%)	107 (85%)
Always buying at least traditional sour milk	8 (27%)	11 (26.2%)	7 (13%)	26 (20.6%)
Consumers buying milk from milk collection centres	24 (80%)	29 (69%)	22 (41%)	75 (60%)
Consumers buying milk from dairy farmers	3 (10%)	7 (16.7)	3 (5.6%)	13 (10%)
Consumers buying milk from traders/vendors	1 (3%)	19 (45%)	27 (50%)	47 (37%)
Consumers buying milk from supermarket/shop	2 (6.7%)	0 (0%)	3 (5.6%)	5 (4%)
0 to 1 km distance from home to the milk source	7 (23%)	16 (38.1%)	30 (55.5%)	53 (42.1%)
1.1 to 4 km distance from home to the milk source	9 (30%)	24 (57%)	24 (44%)	57 (45%)
4.1 to 5 km distance from home to the milk source	4 (13%)	1 (2.4%)	0 (0%)	5 (4%)
Covering above 5 km distance from home to the milk source	10 (33%)	1 (2.4%)	0 (0%)	11 (8.7%)

4.5.5 Potential risk factors for transmission/infection of *S. aureus* associated with milk handling practices among milk consumers in the three study provinces

The potential risk factors associated with consumer milk handling have been described in Table 34. The study findings indicated that some interviewed milk consumers in Lusaka and Western provinces (30 percent and 21 percent, respectively) consumed raw milk and some (47 percent and 43 percent, respectively) did not wash hands before handling milk. In addition, many milk consumers in all the three study provinces consumed sour milk. The study findings also revealed that several interviewed milk consumers (24 percent) in Western province had at least experienced bad quality of milk purchased in the afternoon. Further, many milk consumers in all the provinces used plastic containers to buy, preserve and store milk. In addition, many milk consumers in Western (71 percent) and Southern (70.4 percent) provinces used warm water only to clean their milk containers. Furthermore, many milk consumers in Western province (81 percent) did not filter their milk. In addition, many milk consumers in Southern (26 percent) and Western (24 percent) provinces kept their milk at room temperature (Table 34). Some consumers mainly in Lusaka were not aware of adulterated milk sold by milk traders/vendors.

Table 34: Potential risk factors for transmission/infection of *S. aureus* associated with milk handling practices among milk consumers in the three study provinces

Practices	**Lusaka (n=30)**	**Western (n=42)**	**Southern (n=54)**	**Overall (n=126)**
Consuming at least raw milk	9 (30%)	9 (21%)	4 (7%)	22 (17.5%)
Consuming at least sour milk	15 (50%)	23 (55%)	21 (39%)	59 (47%)
Consumers experiencing the bad quality of milk purchased in the afternoon	0 (0%)	10 (24%)	7 (13%)	17 (13.5%)
Consumers not aware of adulterated milk sold by milk traders/vendors	15 (50%)	0 (0%)	1 (1.9%)	16 (13%)
Consumers not washing hands before handling milk or consumption	14 (47%)	18 (43%)	3 (5.6%)	35 (28%)
Consumers using plastic containers to buy, preserve and store milk	28 (93%)	39 (92%)	51 (94%)	118 (94%)
Consumers using warm water only to clean milk containers	13 (43%)	30 (71%)	38 (70.4%)	81 (64.3%)
Consumers not straining/filtering milk	17 (57%)	34 (81%)	10 (18.5%)	61 (48.4%)

Storing milk at room temperature	0 (0%)	10 (24%)	14 (26%)	24 (21%)
Having no quality control checks on milk freshness	25 (83%)	17 (40.5%)	13 (24.1%)	55 (44%)
Consumers at least rejecting milk because of poor quality	7 (23%)	17 (40.5%)	19 (35.2%)	43 (34%)

The study findings also indicated that milk was more preferred among children in Southern province than other provinces (Table 35).

Table 35: Preference of milk consumption among children and adults in the three provinces

Practices	**Lusaka (n=30)**	**Western (n=42)**	**Southern (n=54)**	**Overall (n=126)**
Milk as preferred food among children	9 (30%)	14 (33%)	32 (59.3%)	55 (43.7%)
Milk as preferred food among adults	11 (36.7%)	16 (38.1%)	16 (29.6%)	43 (34%)

4.5.6 Health experiences by milk consumers

Health experiences associated with milk handling among milk consumers were categorised into skin infections. The major study findings showed that different to Western and Southern provinces, several milk consumers in Lusaka province (23 percent) had at least experienced skin infections in their households in the last 12 months of the interview and they associated them to milk handling.

4.6 Microbiological analysis

A total of 1,939 samples of different matrices were collected along the dairy value chain from 288 facilities in the three study provinces during one to two visit(s) per facility (Tables 2, 3).

4.6.1 Total plate count (TPC) for raw and sour milk (n=230) in Lusaka province

The average TPC for selected samples from Lusaka province was determined. The TPC for raw milk from farms in Lusaka province ranged between approximately 3.1 x 10^4 CFU/ml to 9.7 x 10^7 CFU/ml with minimum and maximum range of 3.1 x 10^4 CFU/ml and 9.7 x 10^7 CFU/ml, respectively. The average TPC for milk from mastitis cow was around 3.4 x 10^6 CFU/ml. The average TPC for raw milk from MCCs was around 1.9 x 10^6 CFU/ml. The average TPC for raw milk from traditional market

ranged between 1.1 x 10^5 to 2.4 x 10^5 CFU/ml. The TPC for traditionally processed sour milk from traditional market in Lusaka province ranged between approximately 9.0 x 10^3 CFU/ml to 2.2 x 10^5 CFU/ml. The TPC for raw milk from milk processing plants was 3.1 x 10^6 CFU/ml. The TPC for pre-processed milk (before milk matures into sour milk) from milk processing plants was 2.8 x 10^6 CFU/ml. The TPC for raw milk for Western and Southern provinces were not done because field samples took long time (approximately 1 hour and 30 minutes or more) before they were frozen. Doing TPC on such samples may not represent the true results as the bacteria could have multiplied in the samples after collection.

4.6.2 Prevalence of *S. aureus* in the Zambian dairy value chain

A total of 295 isolates were confirmed as *S. aureus* by MALDI-TOF MS from 348 presumptive *S. aureus* isolates, including only one presumptive MRSA shipped to BfR, Germany (Table 36). This gives a confirmation rate of 85 percent. The prevalence of *S. aureus* in different matrices in the three study provinces has been described in Table 37. The current study findings indicate that overall prevalence of *S. aureus* in raw milk from farms in the three study provinces was 39 percent (Lusaka province 43 percent, Western 46 percent and Southern 33 percent). Further, the overall prevalence of *S. aureus* in raw milk from milk traders in all the three provinces was 47 percent (Table 37). In addition, the overall prevalence of *S. aureus* in raw milk from milk collection centres in the study provinces was 45 percent. (Table 37). On the other hand, the overall prevalence of *S. aureus* in bucket swabs was 14 percent, nasal swabs five percent, hand swabs seven percent and 25 percent for mastitis milk in the three study provinces (Table 37). Our study findings show that 71.5 percent (206/288) of facilities visited were positive for *S. aureus* in the three study provinces (Table 36). Southern province had the highest percentage of *S. aureus* positive facilities 78 percent (93/119) followed by Western 67.7 percent (44/65) and Lusaka (66 percent, 69/104). Regarding the number of *S. aureus* positive samples, the overall prevalence of *S. aureus* in the three provinces was 15.2 percent (295/1939). *S. aureus* prevalence in Lusaka province was 6.5 percent (127/1939), followed by Southern 4.8 percent (93/1939) and Western 3.9 percent (75/1939) (Table 36). The overall percentage of *S. aureus* positive samples in the three study provinces was 14.8 percent (287/1939).

Table 36: Number of facilities visited and samples taken including *S. aureus* positive ones

Items	**Lusaka**	**Western**	**Southern**	**Total**
Facilities visited	104	65	119	**288**
Facilities with confirmed *S. aureus* isolates	69 (66%)	44 (67.7%)	93 (78.2%)	**206 (71.5%)**
Total samples collected	832	382	725	**1,939**
Number of presumptive *S. aureus* isolates found	171	84 (including 1 presumptive MRSA isolate)	130	**385**
Number of presumptive *S. aureus* isolates shipped to Germany	145	81	122	**348**
Number of confirmed *S. aureus* isolates	127	75	93	**295**
Samples with confirmed *S. aureus* isolates	125 (15%)	72 (19%)	90 (12%)	**287 (14.8%)**

4.6.2 Level of contamination of raw milk from farms, milk traders and MCCs with *S. ureus* in the three study provinces

Raw milk contamination levels at farm, trader and MCCs level has been described in Table 37. The study findings show that the Lusaka province had the highest (80 percent, 67/84) *S. aureus* positive farms followed by Western 76 percent (35/46) and Southern 57 percent (55/96) (Table 37). Western province had highest percentage (46 percent, 42/92) of contaminated raw milk from farms followed by Lusaka 43 percent (72/168) and Southern 33 percent (63/192) (Table 37). Further, most raw milk from traders and MCCs were contaminated with *S. aureus* especially in Western and Southern provinces (Table 37).

4.6.3 Contamination of raw and sour milk from traditional market in the three provinces

Contamination levels of raw and sour milk from sellers from traditional markets was described in Table 37. The current study findings showed that some raw milk and traditionally processed sour milk from traditional markets in all the three provinces was contaminated with *S. aureus*.

4.6.4 Nasal colonisation and contamination of milkers hands

Nasal colonisation and contamination of hands among milkers from selected farms in the study provinces was investigated. The study findings indicated that *S. aureus* was less frequently detected in nasal and hand swabs from milkers, and milk bucket swabs (Table 37).

Table 37: Proportion of confirmed *S. aureus*-positive facilities and samples in the three provinces of Zambia including number of isolates carrying a gene for Panton-Valentine leucocidin (indicated as PVL+)

Parameters	**Lusaka province**	**Western province**	**Southern province**	**Grand total**
Farms with *S. aureus*	67/84 (80%)	35/46 (76%)	55/96 (57%)	157/226 (69%)
Other facilities with *S. aureus*	2/20 (10%)	9/19 (47%)	11/23 (48%)	22/62 (35%)
Pooled raw milk from farms	72/168 (43%) PVL+: 3	42/92 (46%) PVL+: 3	63/192 (33%) PVL+: 10	177/452 (39%) PVL+: 16
Raw milk from mastitis cows	1/6 (17%)	0/0 (0%)	2/6 (33%)	3/12 (25%)
Milk bucket swabs from farms	27/84 (32%) PVL+: 4	4/46 (9%)	1/96 (1%) PVL+: 1	32/226 (14%) PVL+: 5
Nasal swabs from milkers	5/84 (6%) PVL+: 2	5/46 (11%)	1/96 (1%)	11/226 (5%) PVL+: 2
Hands swabs from milkers	11/84 (13%)	2/46 (4%) PVL+: 1	3/96 (3%) PVL+: 3	16/226 (7%) PVL+: 4
Farm water	0/84 (0%)	0/46 (0%)	0/96 (0%)	0/226 (0%)
Raw milk from traders	0/10 (0%)	7/10 (70%)	7/10 (70%) PVL+: 1	14/30 (47%) PVL+: 1
Raw milk and sour milk from traditional market	6/60 (10%)	7/30 (23%)	3/30 (10%) PVL+: 1	16/120 (13%) PVL+: 1

Raw milk from milk collection centres	0/6 (0%)	2/4 (50%)	8/12 (67%)	10/22 (45%)
Unprocessed/pre-processed milk from processing plants	3/6 (50%)	3/4 (75%)	2/4 (50%)	8/14 (57%)
Milk and milk products from supermarket	0/240 (0%)	0/58 (0%)	0/87 (0%)	0/385 (0%)

4.6.5 Phenotypic characteristics/characterisation

Phenotypic characteristics of *S. aureus* isolates from the dairy value chain in the three study provinces were determined. Diversity in phenotypic characteristics of *S. aureus* isolates from three study provinces was observed. All the 295 confirmed isolates were gram and coagulase-positive. Further, all the isolates were positive for growth on mannitol agar and majority were positive for mannitol fermentation (yellow colonies, 219 out of 295 *S. aureus* isolates). However, only 76 *S. aureus* isolates were non fermenters (pink colonies) on mannitol salt agar (Thermo Fisher Scientific, Germany/HiMedia, India). In addition, about 95 percent of *S. aureus* isolates were positive for haemolysis on sheep blood agar. The study findings indicate that only one isolate was able to grow on mannitol salt agar (Thermo Fisher Scientific, Germany/HiMedia, India) with cefoxitin (4 mg/l, Sigma Aldrich, Germany). All *S. aureus* isolates were able to grow on Baird Parker agar (Sigma Aldrich, Germany, MERK KGaA, Germany) with some isolates showing grey-blackish colonies surrounded by a clear zone (typical colonies) and some without clear zones (atypical colonies).

4.7 Molecular characterisation of *S. aureus*

4.7.1 Diversity of *spa* types

Diversity of *spa* types among *S. aureus* isolates was investigated and has been described in Table 38, 39, 40 and 41. The current study findings revealed the presence of *S. aureus* belonging to 36 known *spa* types that included novel *spa* type t18396, t18397, t18398, t18399, t18400, t18402 and t18416. This was the first time these *spa* types have been detected. The following *spa* types (known) were detected: t002 , t015,

t064, t084, t189, t237, t267, t318, t355, t359, t454, t458, t521, t527, t1028, t1096, t1201, t1234, t1236, t1299, t2677, t2844, t2883, t3662, t3772, t4198, t4955, t5229 and t6049. The distribution of the *spa* types among the 295 isolates was different in the three provinces with Lusaka province having 23 *spa* types including the novel type t18396 (four isolates), t18397 (one isolate) and t18402 (one isolate) followed by Southern province with 12 *spa* types including novel *spa* type t18396 (12 isolates) and t18416 (one isolate). Also in Western province, 12 known *spa* types were found, including novel *spa* type t18398 (two isolates), t18399 (three isolates) and t18400 (three isolates). In Lusaka province, many isolates belong to *spa* type t189 and t267, while in Southern province *spa* type t521 dominated. *S. aureus* belonging to *spa* type t355, t267 and t084 were common in all the three provinces. Some *S. aureus spa* types were only found in one or two provinces (Table 38, 39, 40, 41 and Figure: 23). Out of the 295 isolates, 55 were untypeable. In addition, many isolates in Western province were not typable. Three of the 55 isolates not typed gave no PCR product for *spa* typing; for the others sequencing was not successful.

Table 38: Diversity of *S. aureus spa* types in the three study provinces in Zambia

***Spa* type**	**Lusaka (127 isolates)**	**Western (75 isolates)**	**Southern (93 isolates)**	**Total number**
t002	1	0	0	1
t015	1	0	0	1
t064	0	3	0	3
t084	3	3	1	7
t189	46	0	1	47
t237	3	0	0	3
t267	25	7	7	39
t318	1 (1 PVL)*	0	0	1 (1 PVL)*
t355	8 (7 PVL)*	10 (4 PVL)*	6 (PVL)*	24 (17 PVL)*
t359	5	0	2	7
t454	1 (1 PVL)*	0	0	1
t458	1	0	0	1
t521	6	0	37	43
t527	1	0	0	1
t1028	1	0	0	1
t1096	0	0	3 (3 PVL)*	3 (3 PVL)*
t1201	1	0	0	1
t1234	1	0	0	1
t1236	1	0	0	1
t1299	0	5	0	5

t2677	0	0	1	1
t2844	4	0	0	4
t2883	5	0	0	5
t3662	0	1	0	1
t3772	0	1	0	1
t4198	0	0	7 (7 PVL)*	7 (7 PVL)*
t4955	0	0	1	1
t5229	1	0	0	1
t6049	0	1	0	1
t18396 (novel)	4	0	12	16
t18397 (novel)	1	0	0	1
t18398 (novel)	0	2	0	2
t18399 (novel)	0	3	0	3
t18400 (novel)	0	3	0	3
t18402 (novel)	1	0	0	1
t18416 (novel)	0	0	1	1
Total known *spa* types	**23 *spa* types**	**12 *spa* types**	**12 *spa* types**	**36 *spa* types**
Untypeable/unidentified isolates	5 isolates	36 isolates	14 isolates	**55 isolates**

* Number of isolates carrying the gene for Panton-Valentine leucocidin is provided in brackets.

* For each *spa* type at least one isolate was selected for the detailed characterisation

Table 39: Lusaka province *spa* type diversity

***Spa* type**	**Number of isolates**	**Total number of isolates**
t002	1	1
t015	1	1
t084	3	3
t189	46	46
t237	3	3
t267	25	25
t318	1 (1 PVL)*	1 (1 PVL)*
t355	8 (7 PVL)*	8 (7 PVL)*
t359	5	5
t454	1 (1 PVL)*	1 (1 PVL)*
t458	1	1
t521	6	6
t527	1	1
t1028	1	1
t1201	1	1
t1234	1	1
t1236	1	1
t2844	4	4
t2883	5	5
t5229	1	1
t18396 (novel)	4	4

t18397 (novel)	1	1
t18402 (novel)	1	1
Total known *spa* types	**23 *spa* types**	**122 isolates**
Untypeable/unidentified isolates	5 isolates	**5 isolates**

* Number of isolates carrying the gene for Panton-Valentine leucocidin (PVL) is provided in brackets

Table 40: Western province *spa* type diversity

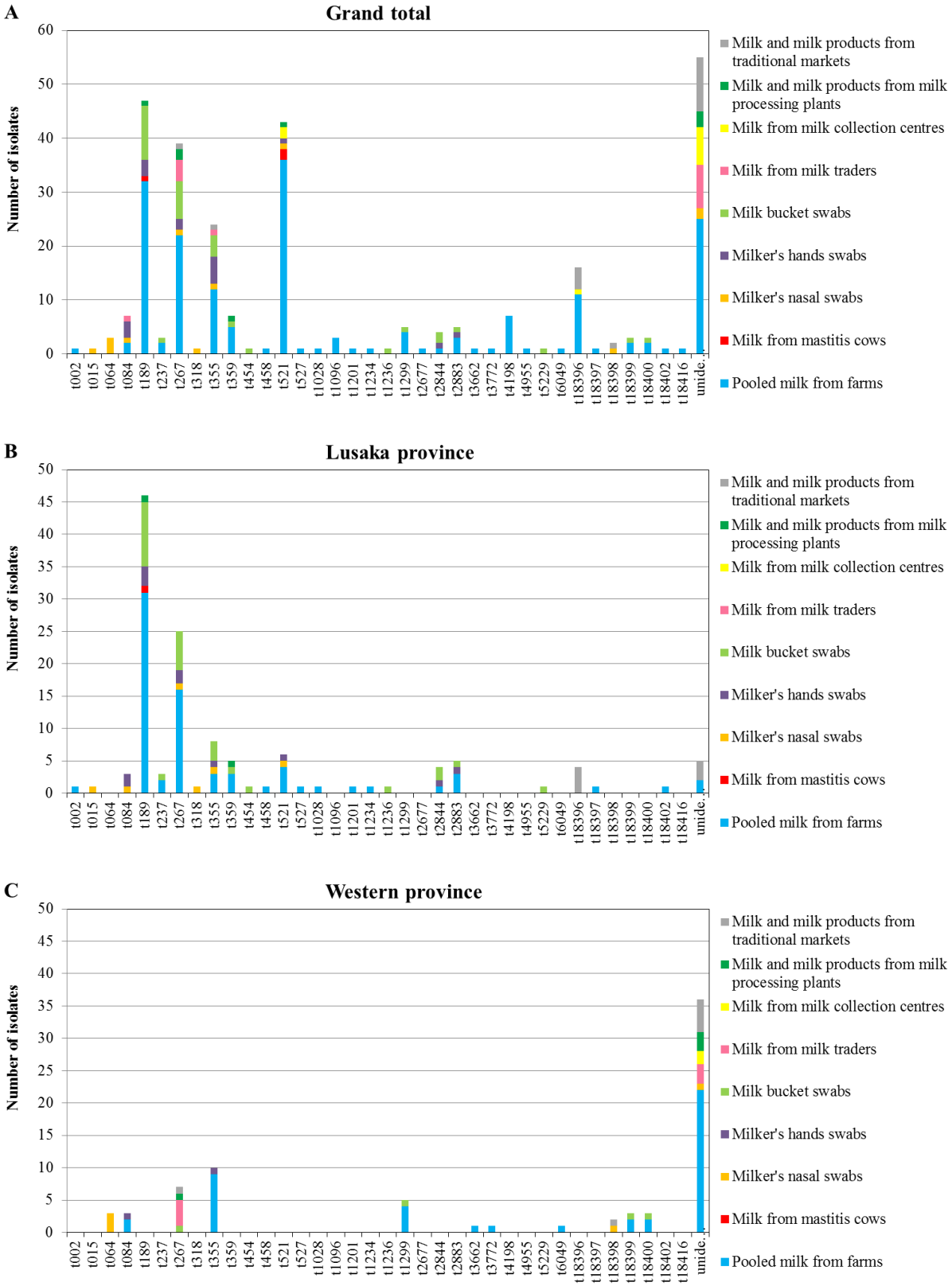
***Spa* type**	**Number of isolates**	**Total number of isolates**
t064	3	3
t084	3	7
t267	7	7
t355	10 (4 PVL)*	10 (4 PVL)*
t1299	5	5
t3662	1	1
t3772	1	1
t6049	1	1
t18398 (novel)	2	2
t18399 (novel)	3	3
t18400 (novel)	3	3
Total known *spa* types	**11 *spa* types**	**39 isolates**
Untypeable/unidentified isolates	36 isolates	**36 isolates**

* Number of isolates carrying the gene for Panton-Valentine leucocidin is provided in brackets

Table 41: Southern province *spa* diversity

***Spa* type**	**Number of isolates**	**Total number of isolates**
t084	1	1
t189	1	1
t267	7	7
t355	6 (PVL)*	6 (PVL)
t359	2	2
t521	37	37
t1096	3 (3 PVL)*	3 (3 PVL)*
t2677	1	1
t4198	7 (7 PVL)*	7 (7 PVL)*
t4955	1	1
t18396 (novel)	12	12
t18416 (novel)	1	1
Total known *spa* types	**12 *spa* types**	**79 isolates**
Untypeable/unidentified isolates	14 isolates	**14 isolates**

* Number of isolates carrying the gene for Panton-Valentine leucocidin is provided in brackets



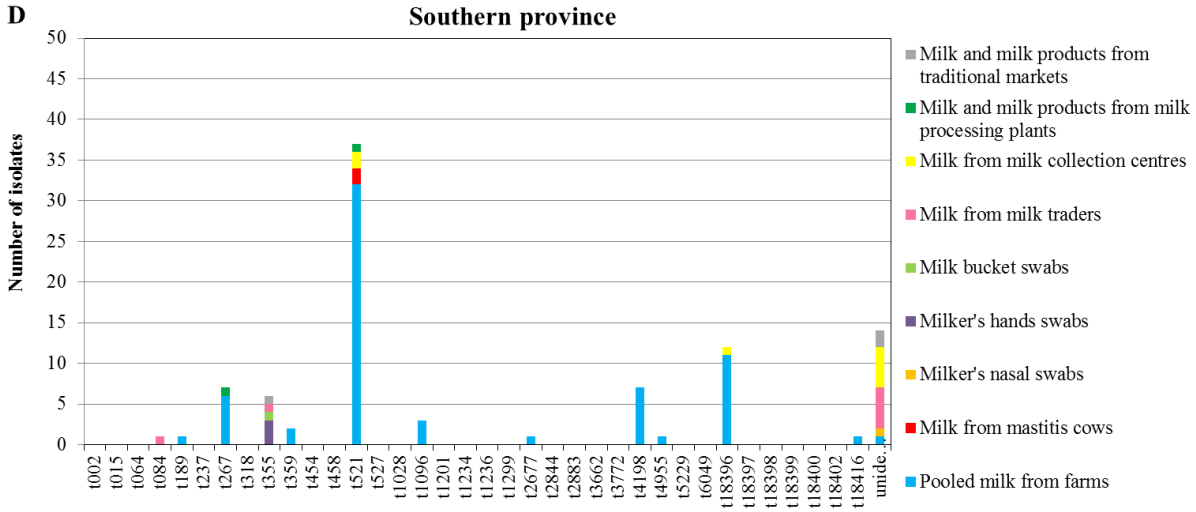


Figure 23: Diversity of *spa* types in various sample types in the three provinces. A) Lusaka, Western and Southern provinces combined; B) Lusaka province; C) Western province; D) Southern province.

4.7.2 Enterotoxigenic and vilurence genes detected in *S. aureus* isolates from Lusaka, Western and Southern provinces

4.7.2.1 Enterotoxin gene patterns

A total of 77 isolates were selected from 295 confirmed *S. aureus* isolates for further characterisation using NGS analysis (40 *S. aureus* isolates) and DNA microarray analysis (37 *S. aureus* isolates). Enterotoxin gene patterns of isolates were investigated. The study findings showed that only 16 out of the 77 isolates (21 percent) were enterotoxigenic strains (harbouring at least one enterotoxin gene). The *sea* gene was found in two isolates, *seb* in three, *sec* in three as well, *sed* in one and *see* in none of the 77 isolates. In addition, *seh* was not harboured by any of the isolates analysed in detail. A total of 11 *S. aureus* isolates (14.3 percent) carried *sei*. In addition, the study findings indicate that only *spa* type t4198 (three isolates were analysed by DNA microarray) harboured two different enterotoxin genes patterns (Table 42). Further, among the 77 isolates selected for detailed characterisation, isolates belonging to five *spa* types (t002, t015, t318, t3772 and t4198) harboured at least six or more enterotoxin genes (Table 42). These isolates were detected in three pooled raw milk samples and from two nasal swabs from milkers.

Table 42: Enterotoxin gene patterns observed among 77 isolates characterised in detail from the three study provinces

***Spa* type**	**Sample type**	**Enterotoxin gene pattern**	**Number of isolates with this pattern and analysed**
t002	Pooled milk-F26-Lusaka-2	*sea, sed, sej, ser, seg, sei, selm, seln, selo, selu*	1 (1 isolate analysed by DNA microarray)
t015	Nasal- F54-Lusaka-1	*sec, sel, seg, sei, selm, seln, selo, selu*	1 (1 isolate analysed by DNA microarray)
t064	Nasal swab -F37-Western-1	*sea*	1 (1 isolate analysed by DNA microarray)
t318	Nasal swab-F64-Lusaka-1	*seg, sei, selm, seln, selo, selu*	1 (1 isolate analysed by DNA microarray)
t355	Traditional market milk (unprocessed)-Southern-2	*sec, sel*	1 (12 isolates analysed by NGS)
t2677	Pooled milk-F91-Southern-2	*sek, seq*	1 (1 isolate analysed by DNA microarray)
t3772	Pooled milk-F46-Western-2	*sec, sel, seg, sei, selm, seln, selo, selu*	1 (1 isolate analysed by DNA microarray)
t4198	Pooled milk-F5-Southern-1 (PVL), pooled milk-F24-Southern-1 (PVL), pooled milk-F70-Southern-1	*seb, seg, sei, selm, seln, selo, selu*	3 (3 isolates analysed by DNA microarray)
Unknown *spa* type	Milk from MCC6-Southern-1, MCC6-Southern-2, unprocessed milk from traditional market-Southern, unprocessed milk from traditional market-Lusaka, pooled milk-F2-Western-2, sour milk from traditional market-Western-2	*sei, sem, sen, seo, seu*	6 (6 isolates analysed by DNA microarray)

*****The sequence quality of the only isolate belonging to t3662 (not listed in the table) was not sufficient for analysis of enterotoxin genes.

4.7.2.2 Virulence gene patterns

Virulence gene patterns of *S. aureus* isolates from the dairy value chain in three study provinces was investigated. Diversity in virulence genes among isolates has been described in Table 43. The current study findings indicate that the overall number of PVL-positive isolates in the three study provinces was 29 out of 295 (9.8 percent) *S. aureus* isolates. In addition, Southern province had highest number of PVL-positive isolates (15/29, 51.7%) followed by Lusaka (9/29, 31%) and Western (4/29, 13.8%) (Table 38, 39, 40, 41). Out of the 77 *S. aureus* isolates characterised in detail, all isolates carried the genes for HlgAB and 55 (71.4 percent) for HlgCB. Furthermore, 54 isolates (70 percent) harboured *lukE* and *lukD* leucocidin genes. Further, 17 isolates (22.8 percent) harboured the PVL genes *lukF-PV* and *lukS-PV*. Of the 37 isolates analysed by DNA microarray, 5 isolates (13.5 percent) carried the bovine variants *lukF-PV (P83)* and *lukM*. Moreover, of these 37 isolates, 36 (97.3 percent) harboured *lukY*, while at least 27 isolates (73 percent) carried *lukX*. For another 11 isolates (29.7 percent) analysed by DNA microarray, the signal for *lukX* was ambiguous. The study findings also showed different *spa* types harbouring different types of leucocidin genes. In addition, isolates belonging to t2883 (three isolates analysed by DNA microarray) carried at least two leucocidin gene patterns. Only the analysed isolates belonging to *spa* type t1299, both analysed by NGS, did not harbour any of the leucocidin genes detected so far by NGS (*hlgA, hlgB, hlgC, lukD, lukE, lukF-PV and lukS-PV*) (Table 43).

Almost all isolates harboured at least two or more genes for other virulence factors, with all 77 isolates characterised in detail carrying *aur* gene (100 percent). In addition, 73 isolates (95 percent) harboured *hlb*, 56 isolates (73 percent) at least harboured *splB*, 53 isolates (69 percent) carried *splA*, 45 isolates (58 percent) carried *splE* and 26 isolates (34 percent) harboured *edinB*. Further, the study findings indicated that isolates belonging to t084 harboured at least two patterns of virulence genes (*aur, etA, hlgA, splA, splB, splE* and *aur, hlgA, splA, splB, splE*). In addition, the current study findings show that all PVL-positive isolates that were characterised in detail carry the *hlb* (β-haemolysin) gene and most of them also carry *edinB* gene (except the characterised isolates belonging to t318 and t4198) (Table 43). The study findings

showed that 12 *S. aureus* isolates out of 77 (15.6 percent) belonging to at least four *spa* types harboured a *tst*1 gene (Table 43).

The study findings showed that 28 *S. aureus* isolates (36.4 percent) out of 77 belonging to 11 *spa* types harboured at least two or three immune evasion cluster (IEC) genes (*sak* + *scn* or *chp* + *scn* or *sak* + *chp* + *scn*). Moreover, one *S. aureus* isolate each, belonging to t015 (one analysed by DNA microarray), t318 (one analysed by DNA microarray) and t3772 (one analysed by DNA microarray), respectively, harboured *sak*, *chp* and *scn* genes. In addition, one isolate each, belonging to t002 (one analysed by DNA microarray), t064 (one analysed by DNA microarray), t454 (one analysed by DNA microarray) and t6049 (one analysed by DNA microarray), respectively, also harboured *sak* and *scn* genes. Further, the study findings also indicated that 12 *S. aureus* isolates belonging to t355 (12 analysed by NGS) and three isolates belonging to t1096 (three analysed by NGS) harboured *sak* and *scn* genes. In addition, four *S. aureus* isolates belonging to t084 and characterised in detail harboured *chp* and *scn* genes (Table 43)

Table 43: Selected virulence genes observed among the *spa* types from the three provinces

***Spa* type**	**Clonal complex**	**Number of analysed isolates**	**Sample type**	**Leucocidin gene pattern**	**Other virulence factors**	**Toxic-shock-syndrome toxin gene**	**Immune evasion cluster gene pattern**
t002	CC5	1 (micro-array)	Pooled milk-F26-Lusaka2	*hlgA, hlgB, hlgC, lukD, lukE, lukY*	*aur*, *etA*, *hlb*,*splA*, *splB*	-	*sak, scn*
t015	CC45	1 (micro-array)	Nasal swab- F54-Lusaka1	*hlgA, hlgB, hlgC, lukX, lukY*	*aur*	*tst*1	*sak, chp, scn*
t064	CC8	1 (micro-array)	Nasal swab-F37-Western1	*hlgA, hlgB, hlgC, lukD, lukE*, (*lukX*)*, *lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	*sak, scn*
t084	CC15	4 (micro-array)	Hands-F49-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE*, (*lukX*)*, *lukY*	*aur*, *etA*, *splA*, *splB*, *splE*	-	*chp, scn*
			Hands-F9-Western1	*As above*	*aur*, *splA*, *splB*, *splE*	-	*chp, scn*
			Pooled milk-F32-Western2, Milk-T3-Southern1	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *splA*, *splB*, *splE*	-	*chp, scn*
t189	Unknown CC (Unknown ST)	5 (NGS)	Pooled milk-F62- Lusaka1, Hands-F62-Lusaka1, Milk bucket-F62-Lusaka1, Mastitis milk-F29-Lusaka1, Pooled milk-F29-Lusaka2	*hlgA, hlgB, hlgC, lukD, lukE*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t237	CC97	1 (micro-array)	Pooled milk-F1-Lusaka2	*hlgA, hlgB, hlgC, lukD, lukE, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t267	Unknown CC (ST97)	5 (NGS)	Pooled milk-F9-Lusaka1, pooled milk-F9-Lusaka2, pooled milk-F61-Lusaka1, milk bucket-F61-Lusaka1,	*hlgA, hlgB, hlgC, lukD, lukE*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-

***Spa* type**	**Clonal complex**	**Number of analysed isolates**	**Sample type**	**Leucocidin gene pattern**	**Other virulence factors**	**Toxic-shock-syndrome toxin gene**	**Immune evasion cluster gene pattern**
			Milk processing plant (pre-processed)-MPP1-Western1				
t318 (PVL)	CC30	1 (micro-array)	Nasal-F64-Lusaka1-	*hlgA, hlgB, hlgC, lukF-PV, lukS-PV,* (*lukX*)*, *lukY*	*aur*, *hlb*, *splE*	-	*sak, chp, scn*
t355 (PVL)	Unknown CC (ST152)	9 (NGS)	Milk bucket-F10-Lusaka1, nasal-F10-Lusaka1, Traditional market milk (unprocessed)-Southern	*hlgA, hlgB, lukF-PV, lukS-PV*	*aur*, *edinB, hlb*	*tst*1	*sak, scn*
			Bucket-F40-Lusaka1, pooled milk-F40-Lusaka2, pooled milk-F30-Western1, hands-F30-Western1, bucket-F32-Southern1, hands-F32-Southern1	*As above*	*aur*, *edinB, hlb*	-	*sak, scn*
t355	Unknown CC (ST152)	3 (NGS)	Hands-F46-Lusaka1, pooled milk-F33-Western1, pooled-F6-Western2	*hlgA, hlgB*	*aur*, *edinB, hlb*	-	*sak, scn*
t359	CC97	3 (micro-array)	Pooled milk-F17-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE,* (*lukX*)*, *lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	*tst*1	-
			Milk bucket-F28-Lusaka1, pooled milk-F48-Southern2	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t454 (PVL)	CC152	1 (micro-array)	Milk bucket-F41-Lusaka1	*hlgA, hlgB, lukF-PV, lukS-PV, lukY*	*aur*, *edinB, hlb*	-	*sak, scn*

***Spa* type**	**Clonal complex**	**Number of analysed isolates**	**Sample type**	**Leucocidin gene pattern**	**Other virulence factors**	**Toxic-shock-syndrome toxin gene**	**Immune evasion cluster gene pattern**
t458	CC97	1 (micro-array)	Pooled milk-F45-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t521	Unknown CC (ST97)	4 (NGS)	Mastitis milk-F70-Southern1, milk collection centre milk-MCC1-Southern1, hands-F50-Lusaka1, pooled milk-F50-Lusaka2	*hlgA, hlgB, hlgC, lukD, lukE*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t527	CC97	1 (micro-array)	Pooled milk-F64-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t1028	CC97	1 (micro-array)	Pooled milk-F69-Lusaka2	*hlgA, hlgB, hlgC, lukD, lukE, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t1096 (PVL)	Unknown CC (ST152)	3 (NGS)	Pooled milk-F3-Southern1, pooled milk-F69-Southern1, pooled milk-F80-Southern1	*hlgA, hlgB, lukF-PV, lukS-PV*	*aur*, *edinB*, *hlb*,	-	*sak, scn*
t1201	CC97	1 (micro-array)	Pooled milk-F37-Lusaka2	*hlgA, hlgB, hlgC, lukF-PV (P83), lukM, lukD, lukE,* (*lukX*)*, *lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t1234	CC97	1 (micro-array)	Pooled milk-F32-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
t1236	CC97	1 (micro-array)	Milk bucket-F25-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE,* (*lukX*)*, *lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-

***Spa* type**	**Clonal complex**	**Number of analysed isolates**	**Sample type**	**Leucocidin gene pattern**	**Other virulence factors**	**Toxic-shock-syndrome toxin gene**	**Immune evasion cluster gene pattern**
t1299	Unknown CC (ST152)	2 (NGS)	Pooled milk-F32-Western1, pooled milk-F32-Western1	*hlgA, hlgB*	*aur*, *edinB, hlb*	-	*sak, scn*
t2677	CC522	1 (micro-array)	Pooled milk-F91-Southern2	*hlgA, hlgB, hlgC, lukF-PV (P83), lukM, (lukX)*, lukY*	*aur*, *hlb*	*tst*1	-
t2844	CC97	3 (micro-array)	Milk bucket-F38-Lusaka1, hands-F47-Lusaka1, pooled milk-F72-Lusaka2	*hlgA, hlgB, hlgC, lukF-PV (P83), lukM, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB, splE*	-	-
t2883	CC97	2 (micro-array)	Pooled milk-F13-Lusaka1, hands-F43-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB, splE*	-	-
t2883	CC97	1 (micro-array)	Milk bucket-F43-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE, lukY*	*aur*, *hlb*, *splA*, *splB, splE*	-	-
t3772	CC25	1 (micro-array)	Pooled milk-F46-Western2	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *etD, edinB, hlb, splA, splB*	-	*sak*, chp, scn
t4198 (PVL)	CC121	3 (micro-array)	Pooled milk-F5-Southern1, pooled milk-F24-Southern1, pooled milk-F70-Southern1	*hlgA, hlgB, hlgC, lukF-PV, lukS-PV, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splB*	-	-
t4955	CC97	1 (micro-array)	Pooled milk-F66-Southern2	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB, splE*	-	-
t5229	CC97	1 (micro-array)	Milk bucket-F42-Lusaka1	*hlgA, hlgB, hlgC, lukD, lukE, (lukX)*, lukY*	*aur*, *hlb*, *splA*, *splB, splE*	-	-
t6049	CC152	1 (micro-array)	Pooled milk-F23-Western1	*hlgA, hlgB, lukY*	*aur*, *edinB, hlb*	-	*sak, scn*

***Spa* type**	**Clonal complex**	**Number of analysed isolates**	**Sample type**	**Leucocidin gene pattern**	**Other virulence factors**	**Toxic-shock-syndrome toxin gene**	**Immune evasion cluster gene pattern**
t18400	Unknown CC	2 (NGS)	Pooled milk-F8-Western1, milk bucket-F8-Western1	*hlgA, hlgB, hlgC, lukD, lukE*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
Un-typed	Unknown (ST3575)	6 (NGS)	Milk from MCC6-Southern1, MCC6-Southern2, unprocessed milk-traditional market-1-Southern-2	*hlgA, hlgB, hlgC, lukD, lukE*	*aur*, *edinB, hlb, splA, splB*	*tst*1	-
			Unprocessed milk-traditional market-1-Lusaka1, (Western) pooled milk-F2-Western2, sour milk-traditional market-Western-2	*As above*	*aur*, *edinB, hlb, splA, splB*	-	-
Un-typed	CC97	1 (micro-array)	Pooled milk-F68-Lusaka2	*hlgA, hlgB, hlgC, lukD, lukE, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-
Un-typed	CC97	1 (micro-array)	Pooled milk-F12-Southern1	*hlgA, hlgB, hlgC, lukD, lukE, lukX, lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*, *sspA*, *sspB*	-	-
Un-typed	CC97	2 (micro-array)	Pooled milk-F92-Southern2, sour milk-Traditional market-1-Lusaka2	*hlgA, hlgB, hlgC, lukD, lukE*, (*lukX*)*, *lukY*	*aur*, *hlb*, *splA*, *splB*, *splE*, *sspA*, *sspB*	-	-
Un-typed	Unknown	2 (NGS)	Pooled milk-F10-Western1, milk bucket-F10-Western1	*hlgA, hlgB, hlgC, lukD, lukE*	*aur*, *hlb*, *splA*, *splB*, *splE*	-	-

[1] The sequence quality of the isolate belonging to t3662 (not listed) was not sufficient for analysis of virulence genes.

[2] MCC stands for milk collection centre, MPP for milk processing plant, F for farms where isolates were detected and T for trader.

[3] Please note that genes *lukF-PV (P83)*, *lukM*, *lukX* and *lukY* were detected with the microarray but so far not by NGS.

* Ambiguous signal in DNA microarray observed in the current study; may mean gene might be present.

4.8 Core-genome MLST analysis

A total of 40 *S. aureus* isolates were analysed by NGS. Core-genome MLST analysis was used to determine the genetic relatedness of selected *S. aureus* isolates belonging to same *spa* types found on same farm or facility during the two sampling visits in the same province or different provinces. The study findings show that the isolates of the same *spa* type isolated from different matrices at the same facilities were genetically related. In addition, the study finding revealed the presence of at least 8 clusters of *S. aureus* (Figure 24). Further, the study finding indicate that isolates belonging to cluster 1, 2, 3, 6, 7 and 8 were related genetically. The current study indicates that isolates from milker hand swab (M42) was closely related to isolate from pooled raw milk (M14) from the same farm obtained during the first sampling visit and these isolates belonged to cluster 2. In addition, isolates from pooled raw milk and milk bucket swabs respectively (L071 and L050 from one farm, L083 and L131 from another farm and, L093 and L069 from another farm), isolated during same sampling period were genetically closely related. Isolates L049 from mastitis a cow and L126 from pooled raw milk collected from the same farm during the same sampling period were genetically related.

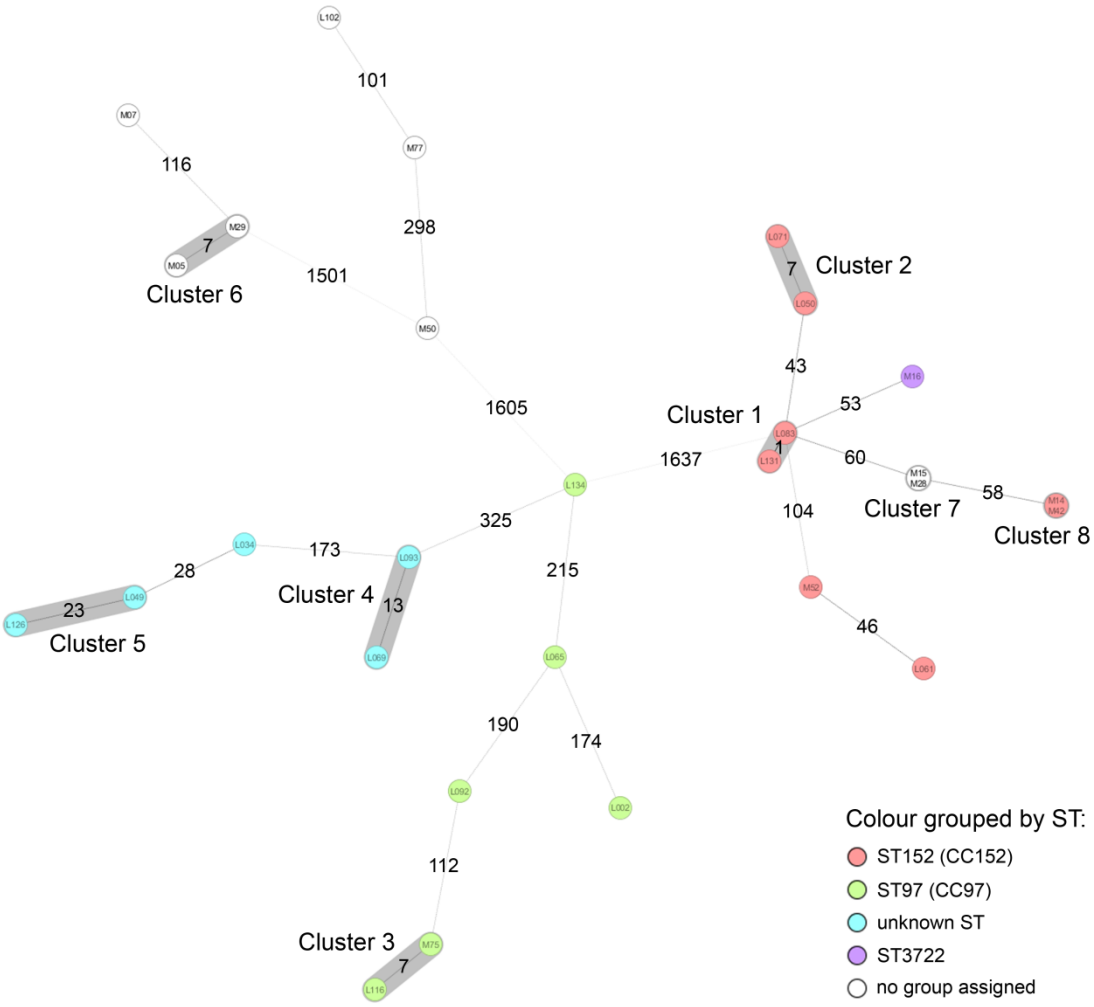


Figure 24: Phylogenetic tree showing genetic relatedness of isolates. The phylogenetic analysis was performed using the cgMLST V1.3 *S. aureus* MLST v1.1. The cluster distance threshold was 24. Clusters of interest included: Cluster 7 (consisting of isolates from pooled milk samples from same farm), Cluster 8 (consisting isolates from pooled milk sample and milker hand from same farm), cluster 1 (consists isolates from pooled milk and bucket swab from same farm), Cluster 2 (consists isolates from nasal swab from milker and milk bucket swab from same farm) and Cluster 3 (consist isolates from pooled milk from a farm and pre-processed milk from milk processing plant).

4.9 Antimicrobial resistance genes of *S. aureus* isolates

For 77 isolates, information on genes mediating antimicrobial resistance was collected and for 72 of these isolates, the patterns were analysed by antimicrobial susceptibility test. Of the 77 isolates, selected for detailed characterisation at least 38 isolates (49.4 percent) harboured *norA-like* (for fluoroquinolone resistance). In addition, 36 *S. aureus* isolates (46.8 percent) harboured the penicillin resistance gene *blaZ* or *blaZ-like* and 25 isolates (32.5 percent) the tetracycline resistance gene *tetK* or *tetK-like*. Other identified antimicrobial resistance genes

detected among isolates included at least: 11 isolates (14.3 percent) with *dfrG* (trimethoprim resistance), eight with *fosB* (fosfomycin resistance), four with *erm(C)* or *erm(C)-like*, one with *mph(C)* (macrolide resistance), one with *msr(A)-like* (macrolide–lincosamide–streptogramin B resistance) and/or one with *cat* (chloramphenicol resistance). Further, 31 *S. aureus* isolates (40.3 percent) out of 77 harboured at least one antibiotic resistance gene, whereas 37 isolates (48 percent) carried at least 2-4 genes as indicated in Table 44.

There was wide diversity of genetic characteristics of *S. aureus* isolates belonging to *spa* type t355 (of which 12 has been analysed in detail) and harboured at least 5 different antimicrobial resistance gene patterns. The study findings also indicated that *S. aureus* isolates belonging to t267 (five isolates characterised) harboured at least four different antimicrobial resistance gene patterns. In addition, at least three different antimicrobial resistance gene patterns are harboured by isolates belonging to t189 (five isolates characterised), t521 (four isolates characterised) and t084 (four isolates characterised) each. Furthermore, *spa* type t2883 (four isolates characterised) and t18400 (two isolates characterised) carried at least two different antimicrobial resistance gene patterns each as shown in Table 44.

4.10 Antimicrobial resistance profiles of *S. aureus* isolates

Of the 72 *S. aureus* isolates subjected to antimicrobial susceptibility tests, 30 (41.7 percent) were phenotypically resistant to at least penicillin (Lusaka province 16 isolates, Western 8 and Southern 6), 24 (33.3 percent) were resistant to at least tetracycline (Lusaka province 15 isolates, Western 5 and Southern 4) while about 20 (27.8 percent) were resistant to at least trimethoprim (Lusaka province 6 isolates, Western 9 and Southern 5). In addition, 28 isolates out of 72 (38.9 percent) were resistant to at least one antibiotic (penicillin/streptomycin/tetracycline or trimethoprim. Further, 24 isolates out of 72 (33.3 percent) were resistant to at least two or more antibiotics. In addition, 14 isolates out of 72 (19.4 percent) subjected to antimicrobial susceptibility test were resistant to three or more antibiotics (multiple or multidrug resistance) (Table 44). Tetracycline (13 isolates), penicillin (12 isolates) and trimethoprim (six isolates) were frequently observed among multiple antibiotic resistant patterns. Other antibiotic resistance profiles observed include resistance to streptomycin in 8 isolates (all from Lusaka province), Erythromycin in six isolates (three from Southern province, two isolates from Lusaka and one isolate from Western), Sulfamethoxazole

in three isolates (all in Western province), Gentamycin, Kanamycin and chloramphenicol in one isolate respectively (all from Lusaka province).

One isolate from pooled raw milk belonging to t267 (six isolates analysed, five isolated from milk and one from bucket swab) was resistant to five antibiotics, gentamicin, kanamycin, penicillin, streptomycin and tetracycline. Further, three *S. aureus* isolates belonging to the same *spa* type were resistant to penicillin and, the other two isolates were resistant to streptomycin. In addition, *S. aureus* isolate belonging to *spa* type t064 (one isolate analysed) isolated from nasal swab was resistant to four antibiotics including penicillin, sulfamethoxazole, tetracycline and trimethoprim. In addition, *S. aureus* isolate belonging to *spa* type t3772 (one isolate analysed) isolated from milk was resistant to four antibiotics including penicillin, sulfamethoxazole, tetracycline and trimethoprim. On the other hand, *S. aureus* isolates belonging to t355 (11 isolates analysed) showed four antibiotic resistance patterns. Further, three isolates (from hand, bucket swab and unprocessed milk from traditional market) were resistant to penicillin, tetracycline and erythromycin and another three isolates (from milk and hands) were resistant to penicillin, tetracycline and trimethoprim while four isolates (from hands and milk) were resistant to penicillin and trimethoprim and, one isolate (from milk) was resistant to tetracycline. *S. aureus* isolates belonging to t084 (four isolates analysed) showed three antibiotic resistance patterns. In addition, one isolate (from hand swab) was resistant to penicillin, tetracycline and trimethoprim and another isolate (from milk) was resistant to penicillin and trimethoprim while two isolates (from hands and milk) were resistant to trimethoprim.

S. aureus isolates belonging to t359 (three isolates analysed) showed two antibiotic resistance patterns. In addition, one isolate (from bucket swab) was resistant to penicillin, tetracycline and streptomycin and, another isolate (from milk) was resistant to tetracycline only while one isolate (from milk) was susceptible (sensitive) to all the 19 antibiotic used. Further, *S. aureus* isolates belonging to t189 (five isolates analysed) also showed two antibiotic resistance patterns. In addition, two isolates (from hands and bucket swab) were resistant to erythromycin, tetracycline and streptomycin and another isolate (from milk from mastitis cow) was resistant to tetracycline while two isolates (from milk) were susceptible (sensitive) to all 19 antibiotics. One isolate from milk belonging to t458 (one isolate analysed) was resistant to penicillin,

chloramphenicol and tetracycline (Table 44). Further, study findings showed that one *S. aureus* isolate from milk in Western province belonging to a novel *spa* type t18400 (two isolates analysed) was resistant to tetracycline while the other isolate from bucket swab at the same farm was susceptible to all 19 antibiotics. In addition, some isolates belonging to unknown *spa* types were resistant to streptomycin and tetracycline while some were resistant to tetracycline only and some were susceptible to all 19 antibiotics used. Further, at least one or more isolate belonging to t189, t359, t521, t1201, t2677, t2844, t2883, t4955 were susceptible to all 19 antibiotics. A total of 20 isolates out of 73 (27.4 percent) were susceptible to all 19 antibiotics used in the antimicrobial susceptibility test (Table 44). A total of 14 isolates were resistant to three or more antibiotics (multidrug resistance) and these were detected in nasal and hand swabs from milkers, in raw milk from farms, raw milk from traditional market and bucket swabs.

Table 44: Phenotypic antibiotic resistance patterns observed among the isolates from the three provinces

***Spa* type**	**Clonal complex**	**Number of analysed isolates**	**Sample type**	**Resistance gene pattern**	**Antibiotic resistance pattern [2]**
t002	CC5	1 (micro-array)	Pooled milk-F26-Lusaka2	*blaZ, fosB*	PEN, TMP
t015	CC45	1 (micro-array)	Nasal-F54-Lusaka1	*blaZ*	PEN
t064	CC8	1 (micro-array)	Nasal-F37-Western1	*blaZ, tetK, fosB*	PEN, SMX, TET, TMP
t084	CC15	4 (micro-array)	Nands-F49-Lusaka1,	*blaZ, tetK, fosB*	PEN, TET, TMP
			pooled milkF32-Western2	*blaZ, fosB*	PEN, TMP
			Hands-F9-Western1, milk from trader milk 3-Southern1	*fosB*	TMP
t189	Unknown CC/unknown ST	5 (NGS)	Hands-F62-Lusaka1, bucket-F62-Lusaka1	*norA-like, tet(K), mph(C), msr(A)-like, ant(6)-la-like*	ERY, STR, TET

			Mastitis milk-F29-Lusaka1	*norA-like, tet(K)-like*	TET
			Pooled milk-F62,Lusaka1, pooled milk-F29-Lusaka2	*norA-like*	Susceptible to all 19 antibiotics tested
t237	Unknown CC (ST97)	1 (micro-array)	Pooled milk-F1-Lusaka2	*tet(K)*	TET
t267	Unknown CC (ST97)	5 (NGS)	Pooled milk-F9-Lusaka1	*norA-like, blaZ-like, tet(K)-like, aac(6')-aph(2"), str-like*	GEN, KAN, PEN, STR, TET
			Pooled milk-F61-Lusaka1	*norA-like, ant(6)-la-like, str-like*	STR
			Milk bucket-F61-Lusaka1	*norA-like, ant(6)-la-like*	STR
			Pooled milk-F9-Lusaka2, milk from processing plant (pre-processed)-MPP1-Lusaka1	*norA-like, blaZ*	PEN
t318 (PVL)	CC30	1 (micro-array)	Nasal-F64-Lusaka1	*blaZ, fosB*	PEN
t355 (PVL)	Unknown CC (ST152)	9 (NGS)	Hands-F32-Southern1, bucket-F32-Southern1, traditional market milk (unprocessed) Southern	*norA-like, blaZ-like, tet(K), erm(C)/ erm(C)-like*	ERY, PEN, TET
			Pooled milk-F40-Lusaka2, (Western) pooled milk-F30-Western1, hands-F30-Western1	*norA-like, blaZ-like, dfrG, tet(K)*	PEN, TET, TMP
			Nasal-F10-Lusaka1, bucket-F10-Lusaka1, hands-F46-Lusaka1, Pooled milk-F6-Western2	*norA-like, blaZ-like, dfrG*	PEN, TMP

t355	Unknown CC (ST152)	1 (NGS)	Pooled milk-F33-Western1	*norA-like, tet(K)-like*	TET
t359	CC97	3 (micro-array)	Milk bucket-F28-Lusaka1	*blaZ, tet(K)*	PEN, STR, TET
			Pooled milk-F48-Southern2	-	TET
			Pooled milk-F17-Lusaka1	-	Susceptible to all 19 antibiotics tested
t454	CC152	1 (micro-array)	(PVL) Bucket-F41-Lusaka1	*blaZ*	PEN, TMP
t458	CC97	1 (micro-array)	Pooled-F45-Lusaka1	*blaZ, tet(K), cat*	CHL, PEN, TET
t521	Unknown CC (ST97)	4 (NGS)	Milk collection centre milk-MC1-Southern1	*norA-like, blaZ-like, str-like*	PEN
			Pooled milk-F50-Lusaka2	*norA-like, blaZ-like*	PEN
			Mastitis milk-F70-Southern1, (Lusaka) hands-F50-Lusaka1	*norA-like*	Susceptible to all 19 antibiotics tested
t527	CC97	1 (micro-array)	Pooled milk-F64-Lusaka1	-	Susceptible to all 19 antibiotics tested
t1028	CC97	1 (micro-array)	Pooled milk-F69-Lusaka2	-	STR
t1096 (PVL)	Unknown CC (ST152)	1 (NGS)	(PVL) Pooled milk-F3-Southern1	*norA-like, blaZ-like, dfrG*	PEN, TMP
t1201	CC97	1 (micro-array)	Pooled milk-F37-Lusaka2	-	Susceptible to all 19 antibiotics tested
t1234	CC97	1 (micro-array)	Pooled milk-F32-Lusaka1	*blaZ*	PEN
t1236	CC97	1 (micro-array)	Milk bucket-F25-Lusaka1	*blaZ*	PEN
t1299	Unknown CC (ST152)	1 (NGS)	Pooled milk-F32-Western1	*norA-like, blaZ-like*	PEN
t2677	CC522	1 (micro-array)	Pooled milk-F91-Southern2	-	Susceptible to all 19 antibiotics tested

t2844	CC97	3 (micro-array)	Hands-F47-Lusaka1	*tet(K)*	TET
			Milk bucket-F38-Lusaka1, pooled milk-F72-Lusaka2	-	Susceptible to all 19 antibiotics tested
t2883	CC97	3 (micro-array)	Pooled milk-F13-Lusaka1	*tet(K)*	TET
			Hands-F43-Lusaka1, bucket-F43-Lusaka1	*blaZ*	Susceptible to all 19 antibiotics tested
t3662	CC97	1 (micro-array)	Pooled milk-F4-Western2	see footnote [3]	SMX, TMP
t3772	CC25	1 (micro-array)	Pooled milk-F46-Western2	*blaZ*, *erm (C)*, *fosB*	ERY, PEN, SMX, TMP
t4198 (PVL)	CC121	3 (micro-array)	(PVL) Pooled milk-F5-Southern1, pooled milk-F24-Southern1, pooled milk-F70-Southern1	*fosB*	TMP
t4955	CC97	1 (micro-array)	Pooled milk-F66-Southern2	*blaZ*, *tet(K)*	Susceptible to all 19 antibiotics tested
t5229	CC97	1 (micro-array)	Milk bucket-F42-Lusaka1	*tet(K)*	TET
t6049	CC152	1 (micro-array)	Pooled milk-F23-Western1	-	TMP
t18400	Unknown CC	2 (NGS)	Pooled milk-F8-Western1	*norA-like*, *tet(K)*	TET
			Milk bucket-F8-Western1	*norA-like*	Susceptible to all 19 antibiotics tested
Un-known	Unknown CC	1 (micro-array)	Pooled milk –F68-Lusaka2	*tetK*	STR, TET
Un-known	Unknown CC	1 (micro-array)	Sour milk from traditional market - Lusaka2	*tetK*	TET
Un-known	Unknown (ST3575)	3 (NGS)	Unprocessed milk-traditional market-Lusaka1, Sour milk-traditional market-Western2,	*norA-like*	Susceptible to all 19 antibiotics tested

			Milk-MCC- 6-Southern1		
Un-known	Unknown CC	1 (NGS)	Pooled milk-F10-Western1	*norA-like*	
Un-known	Unknown CC	2 (micro-array)	Pooled milk F–12-Southern1, pooled milk F- 92-Southern1	-	

[1] MCC: stands for milk collection centre, MPP stands for milk processing plant, F stands for farm:

[2] 16 classes (groups) of antimicrobials (Table 6). CLI: clindamycin, PEN: penicillin, TET: tetracycline, SYN: quinupristine/ dalfopristin, CHL: chloramphenicol, CIP: ciproflaxacin, RIF: rifampicin, KAN: kanamycin, STR: streptomycin, TIA: tiamulin, GEN: gentamicin, FUS: fusidic acid, SMX: sulfamethaxazole, MUP: mupirocin, TMP: trimethoprim, VAN: vancomycin, ERY: erythromycin, FOX: cefoxitin and LZD: linezolid.

[3] The sequence quality of the isolate belonging to t3662 was not sufficient for analysis of virulence genes.

CHAPTER FIVE

DISCUSSION

The current study focused on understanding the milk handling, hygiene and safety practices among stakeholders and determination of the presence of virulent, enterotoxigenic and antibiotic resistant strains of *S. aureus* along the dairy value chain in Lusaka, Western and Southern provinces of Zambia in order to identify interventions to reduce the risk to milk consumers, farmers and other stakeholders.

5.1. Structures, management and milk handling/hygienic practices of stakeholders and potential contribution to contaminations with *S. aureus* in the Zambian dairy chain

5.1.1 Socio-economic status of participants in the three provinces

Only smallholder and traditional dairy farmers were targeted in the current study. Commercial farmers were not visited and were not in focus of the current study. Southern and Lusaka provinces had a highest percentage of farmers in the age range 40 to 49 while in Western province many farmers were in the age range 50 to 59 years (Table 7). In addition, Lusaka and Southern provinces had many farmers earning 3000 Zambian Kwacha or more as monthly income (Table 9) and these probably belonged to the working class hence the highest income. In Western province, many farmers could be mainly retirees and this explains why the province has many farmers earning 500 Kwacha or less per month as income. Further, the other reason for Lusaka and Southern province earning highest monthly incomes might be that some of interviewed farmers had many lactating cows per farm which probably contributed to the monthly income. Many farmers in Western province had very few lactating cows per farm and this may also be contributing less to the monthly income (Table 9, 10). The low monthly incomes in Western province could be the reason why most farmers failed to build milking sheds or buy proper milking equipment such as metal containers.

Western (37 percent) and Southern (22 percent) provinces had the highest number of farmers/milkers who were not very educated and attended only primary level (Table 8). Some interviewed farmers did not take care of milk safety in Lusaka and Southern provinces while selling milk to consumers or communities (Table 12) probably due to ignorance as a result of

low level of education. These findings are consistent with that of a study in Ethiopia on pastoral community with the aim of assessing milk handling practices among farmers which reported low levels of education among the farmers (Worku *et al*., 2014). Further, Lusaka had many very educated farmers who attended tertiary level (Table 8) compared to Western and Southern provinces. In addition, education to a greater extent could play a key role in influencing household incomes, hygienic practices, health and socio-economic status of ordinary families.

MCCs in Southern province had active members between 71-300 followed by Lusaka with 34-90 members and Western province had the lowest with 12 to 33 members (Table 16). The membership had a direct relationship with the volume of milk handled by the MCC. From observation, in Southern province, most of the MCCs handled larger volumes of milk on average compared to Lusaka and Western provinces (Phiri *et al*., 2021).

The majority of the interviewed milk traders were in the age ranges of 30 to 39 and 40 to 49 years and had a monthly income of less or equal to 500 Zambian kwacha. However, only 60 percent (3/5) of milk traders in Lusaka province and 80 percent (4/5) in Western had a monthly income of 500 to 1000 compared to Southern province with one hundred percent (5/5) (Table 23). Further, the income level of milk traders might also have an influence on the amount of milk handled, containers used and hygienic practices. High monthly incomes could make the milk traders to buy and sell more milk. Only two out of 15 interviewed milk traders belonged to the MCCs in Lusaka (Phiri *et al*., 2021) and this mean that they had ready market for their milk as they can sell to the MCCs.

Many milk traders like farmers/milkers had no or little knowledge about milk hygiene and safety due to low level of education (Table 22). Such individuals may practice very poor milk handling and safety practices unknowingly and this may put the consumer at a risk. In addition, most of the milk traders were not very educated in all the three provinces and mainly attended primary and secondary levels (Table 22). The current finding is also consistent with a study in Ethiopia which found that majority (90 percent to 92.5 percent) of milk farmers and traders (77.5 to 90.7 percent) in Boran pastoral communities were illiterate (Amentie *et al*., 2016).
It was observed that majority of the participants in the supermarkets were managers or supervisors in charge of milk and its products sales and orders. Western and Southern provinces

had some supermarkets/shops with managers/supervisors having very low education (grade seven and below) (Table 26). Therefore, the level of education among managers and supervisors could influence the milk handling and hygienic practices in the supermarket or shop. Managers and supervisors with low education such as those in Western and Southern have less knowledge regarding hygiene and food safety compared to the educated counterparts. Such managers or supervisors might allow poor milk handling and hygienic practices among the workers they supervise due to mainly their ignorance.

Lusaka province had a higher number of educated milk consumers with higher monthly incomes than Western and Southern provinces (Table 31, 32). High monthly income had influence on the amount of milk they bought and consumed with their families every month. Further, majority of milk consumers in Southern (57 percent) and Western (55 percent) provinces had lower incomes than those in Lusaka (≤ 1000 kwacha) per month per household (Table 32) (Phiri *et al*., 2021). There is more money circulating in Lusaka province and it is expected that on average, every category of persons will earn more income than those in other provinces. Low levels of income in Western and Southern was probably because most of the milk consumers in these provinces were not in employment. Their low household economic status, coupled with lower education level could have an influence on their milk hygienic practices. Low levels of incomes could make them look for cheaper milk (poor quality) and this could expose them to health risks. Increased family incomes could have an influence on the capacity of consumers to buy more milk. In addition, the majority of milk consumers in Lusaka province (60 percent) and Western (52.5 percent) had more than 5 family members per household (big families). The family size of the milk consumer could also influence the volumes of milk bought and handled on a daily basis. Milk consumers who handle large volumes of milk may have challenges in handling and storage leading to possible compromises in the hygiene and safety of milk. On the other hand, majority of milk consumers in Southern (61 percent) and Western (60 percent) provinces declared that farming was their main occupation and this was there source of livelihood.

Lusaka province had a high percentage (76 percent) of milk consumers who were very educated (at least attending college and university) than Western and Southern provinces (Table 31). However, all the three provinces had some milk consumers with no knowledge of food hygiene and, some of milk consumers thought food hygiene was not important probably due to ignorance on food safety issues, especially in Lusaka and Western provinces. Lusaka province had a higher number of educated milk consumers with higher monthly income than Western and Southern provinces (Table 31, 32). However, education did not seem to improve the milk handling hygiene and safety practices. Food safety and hygiene ignorance can expose the milk consumers to high risk of consumption of contaminated milk. A study in Ethiopia reported that the majority of milk consumers were illiterate and also that milk handling activities along the dairy value chain were unhygienic, probably because of lack of knowledge on hygienic milk handling, and this definitely would affect milk safety (Amentiie *et al*., 2016).

5.1.2 Milk production and management practices of participants

According to the observations made, most farms in Lusaka province were managed by workers instead of family members of farm owners, probably because most owners stayed with their families in the towns (urban areas) and only regularly visited their farms. It is tempting to speculate, that the workers were not very educated and that poor hygienic practices may be applied widely during milking and milk handling (making contamination of milk more likely), because farm owners may not be there to monitor the practices on such farms.

Most of the interviewed farmers in Western (98 percent) and Southern (90.6 percent) provinces did not have milking sheds/parlours (Table 11). These farmers/milkers did their milking outside in the open including in the kraals and crush pens. When cows are milked in the open air, milk might be exposed to environmental contaminants that come from the environment (Godfrey *et al*., 2003; Abunna *et al*., 2019). From the observations in these provinces, milk is usually contaminated with dirt and dust which could harbour *S. aureus* and other bacteria. For example, a study on the survival of *S. aureus* in dust from swine farms found that *S. aureus* and LA-MRSA were able to survive in dust, especially at high initial concentrations of the bacteria, and this study further described dust as a potential vehicle for transmission of *S. aureus* and LA-MRSA to humans (Feld *et al.*, 2018). Therefore, milking in a clean milking shed might reduce possible contamination (Zalalem, 2010; Abunna *et al*., 2019).

Some interviewed farmers/milkers in Western province (30.4 percent) did not filter or strain their milk after milking (Table 12). From observation, milk from farms had some visible dirt including cow's hair and dung. Such milk may be contaminated with a variety of microorganisms including pathogens that can cause diseases in humans. This finding is consistent with a study conducted in Western province of Zambia which found that five out of nine farms had some visible dirt in milk (Knight-Jones *et al.*, 2016). These farmers depended on the MCCs who sieved or filtered the milk before buying it.

Among the three study provinces, Lusaka province had the high number of farmers (15.7 percent) experiencing mastitis on their farms (Table 11). Mastitis is linked to animal breeds and milk yield and it is common in high yielding breeds such as Friesian cows (Litwińczuk *et al*., 2015). This situation is expected in Lusaka Province where farmers are rearing mainly exotic breeds of cows which in most cases are high-milk yielding and usually susceptible to mastitis. Southern and Western had farmers milking traditional breeds of cows which are low-yielding cows and mastitis in such animals may be very low. Mastitis in cows could be probably due to infected cows, contaminated hands of milkers and udder cloth as a source of pathogens such as *S. aureus* to cows including poor mastitis prevention and management strategies. It was observed that Lusaka and Southern provinces had a lot of active dairy farms producing milk. *S. aureus* has been implicated as one of the leading causes of mastitis in dairy cows (Dufour *et al.*, 2012) and mastitis has been identified as a threat to dairy farmers in Sub-Saharan African countries such as Zambia (FAO, 2014). *S. aureus* was less frequently isolated from milk from cows with clinical mastitis in the three provinces with an overall prevalence of 25 percent (3/12) and only two samples from Southern province (n=2) and only one from Lusaka (n=1) was confirmed as *S. aureus*-positive mastitis samples. This could probably be due to the fact that mastitis cases on farms in the three provinces were very low and no mastitis cases were identified on farms in Western province during the study period probably because farmers did not report mastitis cases. However, this finding is not always the case because during some seasons, especially in rain seasons (from November to March) farmers generally record many cases of clinical mastitis in Zambia. In many African countries, the climate is divided into a rainy season and a dry season. A study done in Ethiopia reported a higher prevalence of mastitis during the rainy season compared to the dry season, probably due to its association with poor

sanitation (Biffa *et al*., 2005). It is usually easier to keep the cows clean during the dry season than in the rainy. In the rainy season, the ground will be muddy which will likely promote the proliferation and transmission of mastitis pathogens. In a study by Harouna *et al*. (2009) in Niger, significantly greater numbers of microorganisms, particularly environmental microorganisms, were isolated during the rainy season than in the dry season.

Many interviewed farmers in Western (63 percent) province used penicillin, pen-strep (penicillin + streptomycin), megapen (penicillin + cloxacillin), oxytetracyline and/or gentamicin to treat mastitis compared to Lusaka and Southern provinces (Table 11). The continuous use of same antibiotics to treat mastitis could be a potential risk for development of antimicrobial resistance (AMR) in *S. aureus* (Phiri *et al*., 2021) and other microorganisms as seen in the current study in all the three study provinces (Table 11). A study in India reported that the usage of antibiotics on food producing animals was identified as the potential major driver for AMR development and dissemination (Moudgil *et al*, 2018). Further, some interviewed farmers in Western (39.1 percent) and Lusaka (21.4 percent) provinces treated their cows with antibiotics for 1 to 2 days only probably due to ignorance on antibiotic use (Table 11). Ignorance on drugs can be detrimental to animal, farmer and consumer's health. Treatment of cows by farmers themselves without professional veterinary advice was common among some interviewed farmers in Western and Lusaka provinces (Phiri *et al*., 2021) (Table 11). Self-prescription of drugs such as antibiotics by farmers lead to non-prudent use of antibiotics including wrong-dosing, administering of expired drugs or use of wrong drugs or using wrong route of administration all together (Shitandi, 2004; Kivaria *et al*., 2006; Gwandu *et al*., 2018;) and this could increase the chance of AMR development.

Most of the farmers in Southern (91 percent) and Western (78.1 percent) provinces did not keep any health records about medical treatments on their farms (Table 15). From observation, it was difficult for some farmers to remember some of the health related events and treatments they were given such as antibiotics or other drugs. Further, some farmers mainly in Lusaka province (28.6 percent), used antibiotics every time a family member or a milker was sick. In addition, similar to treatment of farm animals, many farmers or people on their farms in Southern (64 percent) and Western (63 percent) provinces took antibiotics without prescriptions (Table 15). Use of antibiotics without prescription could result in people under

dosing or overdosing themselves or their relatives or workers. In most African countries, antibiotics are sometimes taken without prescription (Lansang *et al*., 1990). This practice is more common in developing countries where there are no regulations about the sale and use of medicines (Auta *et al*., 2012). However, taking medication without prescription in a long run could increase the chances of AMR development in pathogens when lower doses are usually taken, or toxicity and allergic reactions in case of overdose (Llor and Bjerrum, 2014). Many interviewed farmers and their households had at least experienced a situation which made them not to recover despite taking antibiotics in Western province (57 percent) compared to the other two provinces. This might indicate the possibility of microorganisms becoming resistant to types of antibiotics used if these microorganisms are involved.

5.1.3 Hygienic practices of stakeholders in the Zambian dairy value chain

Good hygienic practices are important for the good quality and safety of milk to consumers. This covers personal hygiene among all stakeholders, udder hygiene as well as hygiene of workspace and equipment. However, there are no specific hygiene standards from observation followed by smallholder and traditional dairy farmers during milk production and handling process in Zambia. However, the Codex Alimentarius describes the process for hygienic milk production (Duhem and Bendali, 2006). In addition, the hygienic practices may be different from farm to farm, even among other milk handlers at different facilities such as MCCs and among milk traders (Phiri et al., 2021) which may be influenced by their traditions and culture (Mattiello *et al*., 2018), management system and financial status (Phiri *et al*., 2021).

5.1.3.1 Personal hygiene

Personal hygiene of all stakeholders along the dairy value chain is important and it also requires that there are no carriers of relevant pathogens among them. Some observations made during the current study, showed that some milkers who were coughing, sneezing or with injuries or wounds on their hands in all the three provinces also did milk cows. Those milkers coughing and also sneezing could contaminate their hands and even milk with *S. aureus* as the bacteria can be in their nasal passages as normal flora (Kluytmans *et al.,* 1997; Cole *et al.,* 2001) and may be a source of milk contaminations. Persons with symptoms of any disease such as diarrhoea, coughing and sneezing should not be allowed to take part in the milk handling process (Musa and Akande, 2003; Gwida and EL-Gohary, 2013; Amentie *et al* 2016). Some

interviewed farmers in Southern and Lusaka provinces had at least experienced skin infections among the people on their farms in the last 12 months of the interview (Table 15). Further, some interviewed milk consumers in Lusaka (23%) experienced skin infections in their households in the above mentioned period. These sick people are likely to shed pathogens such as *S. aureus* when sneezing, coughing, scratching (in case of skin infections), vomiting, urinating, defaecating and, such persons could be a source of contamination to milk, milking utensils, and other milking materials.

Nasal colonisation of milkers with *S. aureus* was also detected in the three provinces and the overall prevalence was five percent (11/226) with Western province being the highest at 11 percent (5/46) followed by Lusaka province at six percent (5/84) and Southern being the least at one percent (1/96). People colonised with *S. aureus* asymptomatically, can introduce the bacteria into the food chain (Argudin *et al.*, 2010). Colonised milkers in the current study could be one of the sources of *S. aureus* to contaminate milk. Medical examination of milkers is very important to determine if they are carriers of *S. aureus*. Any milker colonised by *S. aureus* should not be allowed to handle milk as he or she is likely to shed the bacteria with the possibility of contaminating milk. Therefore, a colonised milker can act as a vehicle for *S. aureus* transmission, maintenance and spread (Kluytmans *et al*., 1997; Sakr *et a*l., 2018), and may remain a potential source of contamination to milk in the three Zambian provinces.

All the three study provinces had at least one farmer who did not wash hands before milking cows among the interviewed farmers/milkers. This finding has been described by other studies in Western province of Zambia (Knight-Jones *et al.*, 2016), in Kenya (Ndungu *et al*., 2016) and in Ethiopia (Worku *et al*., 2014; Abunna *et al*., 2019). Although the numbers of farmers/milkers who did not wash hands before milking in all the study provinces may seem to be low in the current study, some farmers/milkers might not have told the truth in the interviews on this matter. In addition, it was observed that majority of milk traders do not wash their hands before handling milk in all the three provinces and such traders could remain a source of *S. aureus* to contaminate milk. Unclean hands could be a source of contamination of milk with *S. aureus* and other bacteria. Further, the current finding is also consistent with that of a study in Ethiopia which found that none of the interviewed milk farmers, other stakeholders including vendors (traders) were washing their hands before milk handling and after every

exposure to risk factors (Amentie *et al*., 2016). In addition, some interviewed milk consumers in Lusaka (47 percent) and Western provinces (43 percent) did not wash their hands before handling milk (Table 34). Amentie *et al*. (2016) reported that none of milk consumer washed hands after every milk handling and exposure to risk factors in a study done in Ethiopia. This practice could be due to lack of knowledge on the dangers of handling food with unclean hands probably as a result of low education levels among the milk handlers.

Although most of the interviewed farmers/milkers washed their hands before milking in all provinces, it was observed that most of them did not wash properly. In addition, some of them use cold water only without detergent/soap and some used cold water with detergent but did not wash properly in clean water (Phiri *et al*., 2021). This finding is consistent with that of a study in Sebeta in Ethiopia which also found that none of the farmers did wash their hands before milking using soap (Ayele *et al.*, 2017). In addition, the current finding is also consistent with a study in Nakuru and Nyandarua counties in Kenya which found that soap was not used at some farms (Ndungu *et al.,* 2016). Similarly, some of the interviewed milk consumers who washed their hands did it with only cold water. This practice makes the unclean hands of farmers/milker, milk traders, consumers, and any milk handlers as a potential source of contamination to milk. Washing hands with water only without detergent/soap results in insufficient washing to remove dirt and germs and it can serve as a source of microbial contamination of milk (Efsun *et al*., 2008; Amentie *et al*., 2016).

Some of the interviewed farmers/milkers in Lusaka, Western and Southern provinces, did not dry their hands after washing and before milking (Phiri *et al*., 2021). If hands are insufficiently cleaned, water and dirt could easily enter the milk during milking/handling and contaminate it with pathogenic bacteria such as *S. aureus*. Moreover, many interviewed farmers in Southern province (83 percent) and some farmers in Western (45.7 percent) used cow's tail, and their own clothes to dry their hands after washing before milking (Table 12) (Phiri *et al*., 2021). This finding is consistent with that of a study done on Borana pastoral community in Ethiopia which reported that milkers dried their hands using their own clothes (Worku *et al*., 2014). The possibility of contamination of milk during milking process and milk handling could be high as a result of using unclean materials and water for washing hands and, personnel not washing hands with soap and potable water (Almaz *et al*., 2001; Worku *et al*., 2014).

It was also observed that some workers in some of the visited MCCs did not wear protective clothing or wear clean clothes. In addition, some of the milk traders did not ensure personal hygiene when selling milk, especially in Lusaka and Western provinces. From observation, milk traders in all the provinces did not wear coats or aprons. This finding is consistent with that of a study done in Eastern Ethiopia which reported that none of the observed milk producers, collectors and transporters, vendors and customers in the study area dressed in clean outer garments while working with milk (Amentie *et al*., 2016). Dirt clothes could be a source of contamination to milk with *S. aureus*. *S. aureus* is found everywhere in the environment and can survive in dry environments such as clothes and environmental surfaces (Chaibenjawong and Foster, 2011; Kadariya *et al.*, 2014; Leuenberger *et al.*, 2019). To summarise, it is highly recommended that all milk handlers should be healthy, wearing clean clothes, washing their hands with warm water and detergents/soap and dry them properly with clean disposable towels before beginning to handle milk (Zelalem, 2012; Al Suwaidi *et al*., 2015; Amentie *et al*., 2016).

Majority of supermarkets/shops (80 percent) in Southern province had workers who had no health certificates and are usually not subjected to regular health check-ups (Table 29). However, all food handlers in Zambia are required by law to undergo medical examination at least every 6 months and if they pass, are given health certificates or food handlers permits to be allowed to handle food. This practice is important to ensure food handlers are free from diseases. In addition, any food handler showing symptoms of a disease should not be allowed to handle food such as milk and its products (Musa and Akande, 2003: Amentie *et al* 2016). Therefore, regular medical examinations of all milk handlers could help in reducing the potential contamination of milk and its products with *S. aureus* and other pathogens. It was also observed that some supermarkets/shops had workers who were coughing or sneezing but continued handling milk in all the provinces and these people could be a potential source of contamination to milk.

5.1.3.2 Udder hygiene

Mastitis is still a big challenge to small scale dairy farmers in in developing countries such as Zambia and it contributes to a reduction in milk production at farm level (FAO, 2014) and causes milk rejections at the MCCs (Phiri *et al*., 2021). Therefore, good milking hygiene and

management of cow's udder health are very important in reducing possible contamination of milk with *S. aureus* (Younan, 2004; Kurwijila, 2006; Barkema *et al*., 2006; Barkema et al., 2009). Further, cows with clinical or subclinical mastitis caused by *S. aureus* could be one of the vehicles for transmission, maintenance (Ndahetuye *et al*., 2019) and spread of some strains into the Zambian dairy value chain, particularly if farmers/milkers have no knowledge about mastitis as found to be the case for most farmers/milkers in Southern province in the current study (Table 11) (Phiri *et al*., 2021).

From general observation, findings indicate that the udders and teats of cows were usually very dirty especially in rainy season in all the three provinces. Further, it was observed that some milkers in Western province did not wash the cow's udders and teats and all milkers who washed udders and teats in all the provinces, did not wash very well as they used cold water only instead of warm water (Phiri *et al*., 2021). This finding is consistent a study done in Ethiopia which described that most farmers did not wash the cow's udders and teats (Ayele *et al*., 2017). Proper cleaning of the udder of cows before milking is one of the important hygienic practices for clean milk production. This is vital since the udder and teats of cows could have direct contact with contaminated ground, dung, dust and environment, and increase bacterial counts (Kurwijila, 2006).

Majority of the interviewed farmers in Southern (81.3 percent) and Western (63 percent) provinces did not wash their hands after milking each individual cow during milking session compared to Lusaka province (Table 12). This finding is consistent with that of a study done in Sub cities of Addis Ababa in Ethiopia which found that 30.6 percent of dairy farmers did not wash their hands between milking individual cows (Abunna *et al*., 2019). In case of cows having clinical or subclinical mastitis, this practice can facilitate the spread and transmission of *S. aureus* and other pathogens from one animal to the other and could lead to contamination of milk. Humans and dairy cows have been identified as some of the main carriers of *S. aureus* (Cuny *et al.,* 2013). The udder of an infected cow or the hands of milkers could be responsible for passing on the bacteria into milk (Dhanashekar *et al.,* 2012).

5.1.3.3 Hygiene of equipment/materials

Generally, it was observed that some farmers/milkers used the same udder cloth to dry the udders on several cows during milking sessions in all the three provinces and in most cases the udder cloth looked dirty. This finding is consistent with that of a study done in Ethiopia which found that 47 percent of dairy farmers used common towel for udder drying (Abunna *et al*., 2019). This practice could facilitate the spread of *S. aureus* from one cow to the other and may contaminate milk. Use of the clean individual towel and following essential hygienic practices is very important for good milk quality and safety. In addition, it was also observed that a cloth was used to filter dirt at some farms. The use of dirty cloth to filter milk could be another source of contamination of milk with *S. aureus* especially among farmers/milkers.

Majority of farmers/milkers in Western and Southern provinces used plastic containers for milking, sour making (preserving), storage, selling and transportation of milk (Table 13) (Phiri *et al*., 2021). This finding is consistent with that of Amentie *et al*., (2016) who reported that the majority of dairy farmers (87.5 to 97.5 percent) in Eastern Ethiopia used plastic containers. Further, some MCCs milk were supplied milk in plastic containers in Western and Southern provinces (Table 19) (Phiri *et al*., 2021). This finding is consistent with that of a study conducted in Central Ethiopia which found 32% of MCCs used plastic containers (Tigabu *et al*., 2015). Other studies in Africa have also reported the use of plastic containers by dairy farmers and MCCs in Ethiopia (Ayele *et al*., 2017) and in Western province of Zambia (Knight-Jones *et al*., 2016). In addition, majority of milk traders used mainly plastic containers for selling and storing of milk (Table 24) (Phiri *et al*., 2021). The study findings also show that many milk consumers in all the three provinces used plastic containers to buy, preserve and store milk. Plastic containers are usually difficulty to clean because of the nature of their design and if not properly washed they could be a source of contamination to milk (Gilmour, 1999; Worku *et al*., 2014). The use of plastic containers contributes to the spoilage of milk since they are almost impossible to clean, especially if they have handles which are not accessible during cleaning (Mwangi *et al*., 2000; Ndungu *et al*., 2016). Re-use of insufficiently cleaned plastic containers can cause contamination of milk (Ombui *et al*., 1995).

Non-food grade plastic containers, buckets and jars may not be recommended for clean milk production (Kurwijila, 2006; Abunna *et al*., 2019). Aluminium or stainless steel cans are highly

recommended for milking, storage and transportation of milk because they do not have adhesive properties and are easy to clean compared to plastic containers (Karuga, 2009; Bereda *et al*., 2012; Zalalem, 2012; Abunna *et al*., 2019). Therefore, it is important for milk handlers to choose the right type of containers to ensure quality and safety of milk to consumers. On the other hand, the majority of farmers/milkers (54 percent) in Western province also used wooden vessels for milking (Table 13). Wooden milking vessels usually had cracks and rough surfaces which made them also difficulty to clean and thus can be a source of contamination of milk like plastic containers.

Some farmers/milkers in Western province (26.1 percent) used cold water only to clean the milking vessels and milk containers (Table 13) (Phiri *et al*., 2021). Washing, with cold water alone is not sufficient because of the possibility of some *S. aureus* strains producing biofilms and attaching on the milk container surfaces (Cui *et al*., 2015). However, some farmers/milkers in all the three provinces used hot/warm water only or cold water and soap to clean milk containers (Phiri *et al*., 2021). A study done in Ethiopia reported that 76 percent of dairy farmers used cold water and soap to wash milk containers (Abunna *et al*., 2019). About 32 percent (27/84) of milk buckets from farms in Lusaka province were contaminated with *S. aureus* followed by Western with nine percent (4/46) and Southern was the least with one percent (1/96) (Table 38). The overall prevalence of *S. aureus* in milk buckets swabs in the three provinces was 14 percent (32/226). This finding was consistent with that of a study conducted in Sebeta, central Oromia in Ethiopia which reported that the prevalence of *S. aureus* from milk bucket swabs was 11 percent (3/27) (Ayele *et al.*, 2017). The current study finding probably was due to the fact that buckets were not washed at all or not properly washed and, such buckets when used could be the sources of contamination to milk with *S. aureus*. Therefore, milk containers/cans could be one of the vehicles for *S. aureus* transmission and spread into milk along the dairy value chain in the three Zambian study provinces.

Some interviewed milk traders mainly in Lusaka and Western provinces did wash milk containers with cold water only while some did not wash after delivery and these practices could be a source of contamination of milk with *S. aureus*. General observation was that most of the MCCs workers did not wash milk cans they used between receiving milk from each individual farmer and they used cold water and detergent to clean the milk cans resulting in

partially clean or uncleaned milk cans. Further, these practices might result in contamination of milk in the bulk tanks at MCCs if milk from some of the farmers is contaminated. In addition, majority of interviewed milk consumers in Western (71 percent) and Southern (70.4 percent) provinces used warm water only to clean their milk containers. Washing with warm water only may not remove all the dirt and germs. In addition, cleaning containers with cold water only may not be sufficient as described above. Such containers when used can also be a source of contamination to milk.

It was also observed that sometimes, MCCs did not have water, especially whenever they had power failure and this situation made cleaning of containers not possible in some cases. In addition, the use of cold water alone without soap to clean milk containers is also not sufficient to remove milk residues and the partially cleaned containers could likely be sources of contamination to milk with *S. aureus*. A study in central Ethiopia reported that they found that farms that involved washing milk containers with detergent and hot/warm water had reduced the risk of contamination of milk with *S. aureus* by 66 percent (Tigabu *et al*., 2015). Therefore, this emphasizes the importance of using hot/warm water with detergent in the cleaning of milk containers to remove dirt and bacteria.

5.1.3.4 ***Hygiene and hygienic milk handling***

Majority of milk consumers in Western province (81 percent) did not filter their milk (Table 34) (Phiri *et al*., 2021). Raw milk from cows may have some physical contaminants (Kumar *et al*., 2018) and filtering it could help to remove most of the dirt (Phiri *et al*., 2021. These physical contaminants have the potential to cause harm to consumers. The dirt in the milk could be another potential source of *S. aureus* contamination. Milk contamination could lead to milk spoilage. The main reason for milk rejection at MCCs or by customers was mainly due to mastitis and milk spoilage (Phiri *et al*., 2021) caused by bacteria including lactobacilli.

Majority of milk traders in Southern and Lusaka provinces sold raw milk to the consumers (Table 25). If milk is not handled in a hygienic manner by milk traders, it could be contaminated with *S. aureus* and it can be a risk to consumers. In addition, most of the interviewed milk traders in Western and Lusaka provinces also sold traditionally processed sour milk (Table 25). Hygienic processing is very important to reduce possible contamination of the final product

and this might result in a safer product for consumers (Pal, 2018). If poor hygienic practices are applied at any stage of the dairy value chain, contamination of milk with microorganisms such as *S. aureus* and could occur, affecting the final product (Pal, 2018). Hygienic milk handling at MCCs is also very important considering that milk comes from different sources practicing different hygienic practices.

It was also observed that in all the three study provinces, packages of commercially produced milk or milk products had spilled milk from damaged packets. Dirt surfaces of packages of milk and its products were in most cases not cleaned or wiped, and in some cases the wiping was done with a dirt cloth. Spilled milk on milk packages and containers could in some case pose a risk to consumers who like to consume milk directly from the uncleaned milk packets/sachets. Therefore, cleaning of spilled milk is vital in reducing the risk of contaminations of the surfaces of milk packets and containers.

Some interviewed farmers in the three study provinces in the current study did not ensure that milk was safe while selling to consumers (Table 11) probably due to inadequate knowledge about milk handling hygiene and food safety. Almost all MCCs in Southern province in the current study had workers who have never received food hygiene and safety education (Table 21). In addition, milk traders and consumers in all the three study provinces also did not receive any food hygiene and safety trainings (Table 22, 31). This finding agrees with that of a study done in Eastern Ethiopia which reported that none of respondents (milk handlers) across the milk supply chain in the study area got formal training on hygienic milk handling practices (Amentie *et al*., 2016). Knowledge of hygienic milk handling practices and milk safety is very important for workers at MCCs to ensure milk hygiene and safety. This finding is consistent with Amentie *et al*. (2016) who reported that none of respondents (milk handlers) across the milk supply chain in the study area in Ethiopia did get formal training on hygienic milk handling practices. The current finding is also consistent with that of a study in Tanzania in 2012 which reported that out of 225 respondents, only 122 (54 percent) had received hygiene and food safety training (Häsler *et al*., 2019). Therefore, knowledge about food safety and hygiene among these milk traders could be very low, especially due to lower education levels. Milk traders with low knowledge on food safety and hygiene could be practicing poor hygienic practices. Therefore, training of the milk traders in food safety and hygiene is likely to improve

their knowledge and eventually result in good hygienic practices. However, there are studies that indicate that having knowledge on hygiene does not always results in change of practice or attitude (Ariyarathne *et al*., 2013; Phiri *et al*., 2021;). A study in India reported that training in intervention was associated with a clear improvement of knowledge (Lindahl *et al*., 2019). The high proportion of illiteracy, coupled with absence of formal training on hygienic milk handling practices and food safety knowledge among the milk traders in this study could affect the quality and safety of milk sold to consumers. Training in milk handling hygiene and safety to consumers, could probably also increase their knowledge on hygienic practices and this may help in reducing the potentials risk of exposure to *S. aureus*.

It was observed that some farmers and milk traders processed sour milk traditionally. Processing of traditional sour milk depends on unknown microorganisms to ferment milk (Moonga *et al*., 2019). However, if pathogenic bacteria such as *S. aureus* are involved, the final product may not be safe to consumers. In the current study, *S. aureus* strains were isolated from sour milk from traditional markets in the three study provinces. This finding agrees with that of a study done in South Western Uganda which reported isolation of *S. aureus* from sour milk from households (Asiimwe *et al.,* 2017b). Therefore, processing of traditional sour milk should be done in hygienic environments and should involve good hygienic milk handling practices to ensure good quality and safe sour milk. Further, the milk used in the production should be from a clean and hygienic source to ensure a safe product.

Almost all supermarkets/shops in the three provinces did not fumigate their premises (Table 28) (Phiri *et al*., 2021). It is important for supermarkets to fumigate their premises regularly in order to control pests that could be carrying pathogens such as rodents, cockroaches, houseflies and others pests. These pests could carry pathogens that can cause diseases in humans. Wild rodents have been known to be colonized by *S. aureus* strains (Mrochen *et al.,* 2018). Further, houseflies carry many pathogens including bacteria and most of them cause diseases in humans (Nayduch *et al.,* 2013; Khamesipour *et al.,* 2018). Therefore, proper control of these pests coupled with general cleanliness of the environment, good personal and handling hygienic practices and knowledge on food safety could reduce the risk of contamination of milk and other food products in supermarkets/shops.

5.1.3.5 Storage and transportation of milk

From the observations, the majority of interviewed farmers in all the three provinces kept the afternoon milk overnight without refrigeration. This practice may provide a perfect environment to allow *S. aureus* to multiply in the milk. Lack of adequate cooling facilities in rural areas have been identified to account for the loss of large volumes of milk in developing countries (Ndambi *et al*., 2007). In this study, all visited MCCs in all the three provinces receive milk twice per day. It was observed that at some MCCs, the morning and afternoon milk was usually mixed. Further, some interviewed milk traders mainly in Lusaka also mixed morning and afternoon milk batches (Table 24). This practice could result into contamination of milk with *S. aureus* if one of the milk batch is contaminated. Such milk may end up being sold to unsuspecting consumers who may be at risk of consuming raw milk contaminated with *S. aureus*. More investments at MCCs may be needed to address this challenge by increasing the number of bulk tanks. Even though all MCCs have cooling facilities for milk, failures in constant electricity supply were usually experienced affecting the cooling facilities. Further, the current study findings indicate that in Lusaka province milk and milk products usually take long to finish from visited supermarket probably because they order large volumes and because of high prices to consumers compared with milk from farms and MCCs.

Some interviewed milk consumers in Southern (26 percent) and Western (24 percent) provinces keep their milk at room temperature (Table 34). Keeping milk at room temperature may allow the bacteria to multiply and could reach optimal levels to start producing SEs (ICMSF, 1996; Stewart, 2003). The consumption of milk with SEs could pose a risk of SFP (Argudin *et al*., 2010; Hennekinne *et al*., 2012). Keeping milk under the cooling facilities slows down the growth and multiplication of *S. aureus* bacteria and this may reduce the chance of SEs production (ICMSF, 1996; Stewart, 2003). Further, long period of storage coupled with inappropriate conditions including cooling which is not effective, might affect the quality of milk and its products at MCCs or in the Supermarkets and households of consumers. In addition, if enterotoxins (SEs) are already produced in milk, cooking or boiling does not help because SEs are heat-stable (Balaban and Rasooly, 2000). Therefore, boiling milk may kill most of the pathogens making boiled milk safer to consume (Metwally *et al*., 2011), provided it is done immediately after milk is produced or purchased to avoid production of SEs. Distance, depending on the mode of transport could influence the shelf life of raw milk,

especially if cooling facilities are not available or not working effectively. This situation coupled with type of a container, high environmental temperatures and poor milk handling practises may be the perfect conditions for growth and multiplication of *S. aureus* and other pathogens in milk.

Like in other rural African regions (Debela, 2015), most of the farmers in Western province (54 percent) transported their milk on foot. Some farmers (28.1 percent) covered distances of between 7 to18 Km to reach to the market or MCC (Table 14) and it took approximately 120 minutes (Phiri *et al*., 2021). Thus, the milk will be exposed for a long time to ambient temperature without refrigeration. This finding is consistent with the finding of a study done in Western province of Zambia which found that farmers took approximately 30 to 120 minutes to reach MCCs (Knight-Jones *et al*., 2016). Further, majority of interviewed milk traders (53.3%) covered one to nine km while the rest cover less or more to reach the clients (Table 27). The distance from milk source to the market or MCCs determines the time taken to transport milk. Time taken from milking to the time when milk is refrigerated is important for milk quality and safety. The longer milk takes to reach the MCCs or clients/market where it can be refrigerated, the more the bacteria such as *S. aureus* will grow and multiply (Swai and Schoonman, 2011; Knight-Jones *et al*., 2016; Paraffin *et al*., 2018; Phiri *et al*., 2021). This increases the chance of milk becoming spoiled and eventually being rejected at the MCCs.

The types and cleanliness of containers used for milk transportation may also determine the quality of milk at MCCs or market or with milk trader. Good milk storage and transportation help to ensure the good quality of milk during processing (Walstra *et al*., 1999). Therefore, all stakeholders should ensure that they use appropriate and clean milk containers for storing and transporting milk. From observation, some stakeholders, especially farmers in all the provinces transport and store milk in metal cans and plastic containers. A study in Ethiopia reported that most of the farmers used plastic containers during milking and transporting milk to vending shops (Abunna *et al*., 2019). The re-use of insufficiently cleaned plastic containers and the lack of cooling during storage and transportation favour *S. aureus* contamination and rapid bacterial growth/multiplication, respectively (Swai and Schoonman, 2011). In addition, some interviewed farmers in Western province also used bicycles to transport their milk to cooperatives or MCCs while others transported milk on foot (Phiri *et al*., 2021). This finding

is consistent with that of a study conducted in Western province of Zambia (Knight-Jones *et al*., 2016). Further, in Lusaka (96 percent) and Southern (96 percent) provinces, the majority of farmers transported their milk on bicycles and many covered only short distances to reach the market (Phiri *et al*., 2021). Some milk consumers in Lusaka province covered short distances and while others covered longer distances from their homes to the milk source. The distance covered to reach the source of milk determines the time taken for milk to be collected from source to home. In addition, majority of milk traders in all the study provinces transported their milk on bicycles to the market or directly to consumers (Table 25). It seems the bicycle is the commonest mode of transport for farmers and milk traders in the three study provinces (Phiri *et al*., 2021). The time taken from milking to the time when milk is refrigerated is also important for milk quality and safety. Milk handling hygiene is very important starting from production at farm level, during transportation, processing and storage. Therefore, good milk storage and transportation could help in ensuring good quality during processing (Walstra *et al*., 1999).

5.1.3.6 Milk delivery at MCCs by farmers and collection by processors

Farmers sold their milk directly to MCCs in the three study provinces. In all the three study provinces, milk was collected from MCCs by big processors (Mumba *et al*., 2013) using refrigerated tankers as these were the main buyers of milk. The major processing company was based in Lusaka province. In addition, almost all the milk collected at MCCs in Western province was usually processed as sour milk mainly within the province by one of the processors. In addition, milk at MCCs, sometimes took long to be collected by processors (48 to 50 hours) in Southern and Lusaka provinces (Table 20) and this coupled with constant interruption of power, may likely make cooling facilities not to work well. This situation may promote multiplication of bacteria such as *S. aureus* with the possibility of production of enterotoxins in the milk, especially when environmental factors are suitable (ICMSF, 1996; Stewart, 2003). From observation, when processors delay to collect milk, all the bulk tanks will be full and the MCC workers will start putting milk in metal cans which will be kept at room temperature until it is sold to customers or collected by processors especially in Lusaka province. Keeping milk at room temperature is not a good practice as milk is a perishable commodity and can easily be spoiled or turn sour.

5.1.3.7 Quality testing, rejecting and back-tracing of milk

MCCs are important facilities for ensuring a certain level of milk quality. Microbial and antibiotic residues analysis of milk received at MCCs remains very important activities to check for presence of pathogens (Doyle *et al*., 2015) and antibiotics residues (Sachi *et al*., 2019) in the milk before being consumed. However, in the current study, these were not done in many MCCs in the study provinces (Phiri *et al*., 2021). Further, Western province among the three provinces had experienced highest percentage (20-30%) of milk rejection from MCCs (Table 18) mainly because of milk poor quality and turning sour (Phiri *et al*., 2021) and this could also mainly be due to poor milk handling hygiene and long distances covered (Phiri *et al*., 2021). In addition, MCCs in Southern province experienced situations of milk not being of good quality at reception. At MCCs, poor quality milk was usually detected by alcohol test. This finding is consistent with that of a study conducted in Central Ethiopia which reported used of alcohol test in 20 MCCs out of 22 (Tigabu *et al*., 2015). A study in Ethiopia reported the use of lactometer reading and alcohol test for checking milk freshness at MCCs (Ayele *et al.,* 2017). At MCCs, milk that fails the test was usually rejected and the milk rejection was usually a big loss to a farmer and his or her household (Phiri *et al*., 2021). About 50 percent of MCCs in Southern province had experienced milk being rejected by processors and this was mainly due to poor quality (milk turning sour) (Phiri *et al*., 2021). Good hygienic handling of milk at MCCs plays an important role in determining the quality of milk sold to consumers and processors

Majority of milk traders also did not have quality control activities to check for milk freshness just like famers in the current study. (Table 24). Further, majority of supermarkets/shops (90 percent) in Southern province did not have quality control checks as well (Table 28). This practice may make the farmers, milk traders, MCCs and supermarkets to sell bad quality milk to unsuspecting consumers. In addition, majority of supermarkets (86 percent) in Lusaka province had experienced milk being rejected by customers (Table 27). The milk rejection was mainly due to milk spoilage due to microbial contamination. Majority of supermarkets (86 percent) in Lusaka province had at least experienced handling bad milk (Table 28). This situation could be as a result of contamination of milk with bacteria as milk is a good growth medium for bacteria (Dhanashekar *et al.,* 2012; Hennekinne *et al.,* 2012). Therefore, testing milk for freshness at all levels could be an ideal situation to ensure that only good quality milk

is sold. In addition, majority of MCCs and supermarkets/shops in Southern provinces and milk traders in Western province were also not able to trace back bad milk to its source (Table 20, 28). This practice may make it difficult to trace the source of milk in case of an illness among consumers. A source of good quality milk and products is very important to ensure that only safe milk and its products are sold to consumers. Therefore, identification of the source of bad milk could play an important role to avoid buying milk from such a source/facility and to prevent further occurrences.

Majority of interviewed milk consumers mainly in Lusaka did not check for freshness of milk (Table 34). Such practice may result in unsuspecting milk consumers not able to identify bad milk and instead they may consume it. Therefore, quality control checks for freshness of milk not usually done by consumers might be important as it could help them to know if milk is fresh or not. However, some interviewed milk consumers had the experience of rejecting milk because of its bad quality in all the three provinces. Poor quality milk could be a source of pathogens such as *S. aureus* as milk is a perfect growth medium for microorganisms (Dhanashekar *et al.*, 2012; Hennekinne *et al.*, 2012) and could be a perfect vehicle for transmission of pathogens to humans (Oliver *et al.*, 2005).

5.1.3.8 Milk consumption

The majority of farmers and their families mainly in Western province (85 percent) consumed raw milk than in Lusaka and Southern provinces (Table 15). In addition, Lusaka and Southern provinces had some farmers and their families (26 percent and 20 percent, respectively) who consume raw milk (Table 15). A study in Sebeta, central Oromia in Ethiopia reported that 35 percent of respondents (farmers) consumed raw milk (Ayele et al., 2017). Ayele *et al*. (2017) also reported that consumption of raw milk was relatively higher among uneducated farmers (38.5 percent, 5/13) than those who read and write (30 percent, 3/10). This finding is consistent with that of the current study since Western province (37 percent) had a high percentage of interviewed farmers who were not very educated compared to Lusaka (8.6 percent) and Southern (22 percent) provinces (Table 8) (Phiri *et al*., 2021). Further, some milk consumers in Lusaka (30%) and Western (21%) provinces consumed raw milk and also sour milk (50% and 55% respectively) (Table 34). This finding is consistent with that of a study conducted in central Oromia in Ethiopia which reported that 28 percent of consumers consumed milk in

form of raw milk and raw milk products (Ayele *et al.,* 2017). Consumption of raw milk exposes the consumers to health risks as milk could be contaminated with *S. aureus* or other pathogens (Angulo *et al*., 2009; Oliver *et al*., 2009). Ayele *et al*. (2017) reported that 85 percent of farmers in Ethiopia were not aware of food borne diseases associated with consumption of raw milk. This emphasises the need for increased awareness on dangers, regarding consuming raw milk through trainings in the three study provinces to reduce the risk of *S. aureus* to consumers.

Majority of the interviewed farmers and their families in the three study provinces consumed traditionally processed sour milk (Table 15). Further, many milk consumers in all the three provinces consumed traditionally processed sour milk (Table 34). Poor hygienic practices during milk handling from the farm, transportation, processing and storage could be the source of contamination to traditionally processed sour milk and consuming it may be a potential risk of *S. aureus* and SEs. Therefore, good hygiene from milking to processing is very important to ensure good milk quality and safety. In addition**,** majority of interviewed milk consumers in Southern province indicated that milk is preferred among children than any other province (Table 35). This category of people may be at high risk of consuming bad milk without knowing. Therefore, parents could play a very critical role to help children in identifying good quality milk for their children to reduce the risk of infections.

Majority of milkers in Western (60 percent) and Southern (54.2 percent) provinces consumed milk directly from the teats of cows (Table 12). This practice could be a potential risk to milkers of infections with *S. aureus* as the bacteria is also found on the skin of animals, particularly on the teats and udder (Chaibenjawong and Foster, 2011; Kadariya *et al*., 2014; Leuenberger *et al*., 2019). A study in Switzerland found *S. aureus* on the teat surface of cows (Leuenberger *et al.,* 2019).

In general, it seems farmers probably did not request for much help or advice from veterinary officials both in government and private, mainly because of costs implications. Further, due to limited knowledge on the possible effects of antibiotic residues on consumers, farmers may supply antibiotic-contaminated food of animal origin to consumers (Nonga *et al.,* 2010; Bukuku *et al.,* 2015; Gwandu *et al.,* 2018). In addition, many famers may not understand the withholding periods for milk after intra-mammary treatment with antibiotics. This could result

in high levels of the drug residues in food such as milk and this may cause health problems including drug toxicity, allergic reactions and cancer (Petrović *et al*., 2008; Layada *et al*., 2016: Gwandu *et al*., 2018). The current study finding is consistent with that of a study in Kenya which found that about 50 percent of farmers were not aware of the effects of antibiotic residues on milk quality, the withdrawal period required for various antibiotics and their effects on human health (Ndungu *et al*., 2016). Most of the interviewed farmers mainly in Southern province (74 percent) mixed milk from cows being treated with antibiotics with milk from health cows (Table 12). In addition, majority of interviewed farmers and their households in Southern (84.4 percent) and some farmers in Western (43.5 percent) provinces consumed milk from cows being treated with antibiotics (Table 15) probably due ignorance. This practice may put the consumers at risk of consuming antibiotic residues. The presence of antibiotics in milk is a public health concern (Kumar *et al*., 2013; Singh *et al*., 2014).

Consumption of food such as milk contaminants with antibiotic residues over a long period of time could increase the possibility of development of AMR in microorganisms in the gut and, the transmission and spread of antimicrobial resistant pathogens (Aalipour *et al*., 2013; Layada *et al*., 2016; Granados-Chinchila, 2017; Gwendu *et al*., 2018). Further, presence of antibiotics in milk could affect the process of fermentation in the production of sour milk and other milk products. When antibiotics (penicillin, streptomycin-penicillin and oxytetracycline) were given to sheep by intramammary injection, the antibiotics were excreted into milk in concentrations harmful for yogurt production (Anifantakis, 1982). Therefore, educating the farmers on the dangers of antibiotic residues in milk to consumers and milk processing is vital in dealing with the risks associated with antibiotic residuals. Majority of supermarkets/shops in Lusaka and Western provinces and half of the interviewed supermarkets/shops in Southern at least experienced complaints from customers of vomiting or diarrhoea after consumption of milk bought from their facilities (Table 29). Occurrence of such symptoms following consumption of milk could probably indicate SFP. Staphylococcal Food Poisoning occurs due to consumption of foods containing sufficient amounts of one or more preformed SEs in food (Dinges *et al*., 2000; Le Loir *et al*., 2003).

5.2 Detection and isolation of *S. aureus*

5.2.1 *S. aureus*-positive facilities and overall prevalence

Majority of visited facilities (71.5 percent) in all the three study provinces were positive for *S. aureus* with Southern province being the highest at 78 percent followed by Western (67 percent) and Lusaka (66 percent). This finding could be due to poor milk handling practices in Western and Southern provinces compared to Lusaka province as indicated from interviews of participants in the study provinces. However, a lower proportion of *S. aureus*-positive facilities in the dairy value chain in Lusaka province was only detected at post-farm level. In general, the high proportion of *S. aureus*-positive facilities could be due to infected cows with (subclinical/clinical) mastitis and colonised milkers at farm level, poor hygiene and milk handling practices along the entire dairy value chain (Bonfoh *et al*., 2003).

The overall prevalence of methicillin-sensitive *S. aureus* (MSSA) in the samples taken along the dairy value chain in three provinces was 15.2 percent (295/1939) (Tables 36, 37). Prevalence of *S. aureus* in samples from Western province was 19.6 percent (75/382), followed by Lusaka 15.3 percent (127/832) and Southern 12.8 percent (93/725) provinces. MRSA was not identified in all the samples from all the three study provinces. The current finding agrees with that of the MSSA prevalence data in different studies conducted in the African continent which reported that prevalence ranges between 3 percent to 58 percent (Lozano *et al.*, 2016). The MSSA prevalence in Africa has been known to be highly variable probably due to mainly the different methods that have been used (Lozano *et al.*, 2016) . Therefore, many studies on prevalence of *S. aureus* in a number of African countries have found differences in the prevalence. In addition, the current study finding also indicate that the overall *S. aureus* prevalence in raw pooled milk from farms in the three provinces was 39 percent (177/452) with Western province at 46 percent (42/92) followed by Lusaka at 43 percent (72/168) and Southern at 33 percent (63/192). This finding is consistent with that of a study in Tanzania which reported the prevalence of *S. aureus* in raw milk to be 41 percent (Mohammed *et al.*, 2018). Other studies have reported the different frequencies of *S. aureus* contamination of milk including in Morocco (40 percent), Ethiopia (48.7 percent) and Kenya (30.6 percent) (Shitandi and Sternesjö, 2004; Bendahou *et al*., 2008; Daka *et al*., 2012). In addition, other studies in

Uganda have reported that 20.3 percent of raw milk samples were contaminated with *S. aureus* (Asiimwe *et al.*, 2017b).

The overall prevalence of *S. aureus* in raw milk from milk traders in Western and Southern provinces was 47 percent (14/30). However, in Lusaka province *S. aureus*-positive raw milk samples from traders were not found. A study in Ghana reported 14.6 percent prevalence of *S. aureus* in informally marketed raw milk (Donkor *et al.*, 2007). Other studies have reported high *S. aureus* prevalence of 74.5 percent in India (Sharker *et al*., 2014) and 61.7 percent Madurai region of South India (Lingathurai and Vellathurai, 2011). Further, the overall prevalence of *S. aureus* in milk and sour milk from traditional markets was 13 percent (16/120). Other studies have reported 33.3 percent prevalence of *S. aureus* in cow's raw milk at selling point in Ethiopia (Reta *et al.*, 2016) and 9 percent in Iran on traditional dairy products (Rahimi, 2013).

The overall prevalence of *S. aureus* in milk from MCCs in the three study provinces was 45 percent (10/22). This finding agrees with that reported by Addis *et al*. who reported that 46 percent of bulk milk samples from MCCs in Ethiopia were positive for *S. aureus* (Addis *et al*., 2011). Other studies have also have found higher prevalence of *S. aureus* in raw bulk milk from MCCs, including 75 percent (Desissa *et al*., 2012) and 72 percent (Wubete, 2004) respectively. However, the current study findings indicate that despite Lusaka province being the second highest province regarding prevalence of *S. aureus* in raw milk at farm level, MCCs did not have any isolate confirmed as *S. aureus* although at MCCs no milk treatment is done. This could be probably due to the strict measures in the testing of milk freshness in determining the good acceptable milk quality from different farms. Milk which does not pass at MCCs is rejected and not allowed to be mixed with the other batches. Further, another reason might be due to the fact that big milk processors are based in Lusaka province and milk is collected almost daily from MCCs. This may suggest that milk batches sampled at farms level were not the same as those sampled at MCCs during the sampling visits, and also that all the MCCs might have better cooling facilities and may supress multiplication of *S. aureus* in bulk milk. The overall prevalence of *S. aureus* in raw and/or pre-processed milk from milk processing plants was 57 percent (8/14) with milk from Western province processing plant being the most contaminated at 75 percent (3/4) followed by Lusaka province (50 percent; 3/6) and Southern

province (50 percent; 2/4). This might have been due to infected cows at farm level or compromise in milk hygiene including poor milk handling from farms to MCCs.

In the current study, no *S. aureus* strains were isolated from samples of environmental water used for cleaning milk utensils such as milk containers/cans and for cleaning cows' udders and milkers' hands on the farms in the three study provinces. Although it cannot be concluded that the farm water is always free of *S. aureus*, it is suggested that the water could not be one of the major sources of *S. aureus* to contaminate milk. However, contamination of water might also vary depending on the season.

5.2.2 Total plate count (TPC) for raw, sour and pre-processed milk in Lusaka province

The study finding indicated that the average TPC for raw milk from farms in Lusaka province ranged between 3.1 x 10^4 CFU/ml to 9.7 x 10^7 CFU/ml with the majority of raw milk being in the range of 1.0 x 10^5 to 3.8 x 10^6 CFU/ml. The maximum TPC of raw milk from farms in Lusaka in the current study was higher than those reported in other studies in Zambia of 100,000 (1.0 x 10^5) CFU/ml (Yambayamba *et al.,* 2011; Kunda *et al*., 2015). However, the minimum TPC of raw milk samples from farms in Lusaka province in the current study was 3.1 x 10^4 CFU/ml and it was lower than that reported ones by other studies in Zambia (Yambayamba *et al.,* 2011; Kunda *et al*., 2015). The average TPC for milk from mastitis cow was around 3.4 x 10^6 CFU/ml. A study done in Zambia found higher TPC in cows associated with greater yields and with increased udder infections (Pandey *et al*., 1996). The TPC for raw milk from MCCs was around 1.9 x 10^6 CFU/ml in the current study. Other studies on smallholder farmers in Sub-Saharan countries such as Rwanda, Kenya and Cameroon have reported lower TPC of 1.0 x 10^6 to 1.0 x 10^8 CFU/ml for raw milk at MCCs (Belli *et al*., 2013; Adugna and Asresie, 2015; Doyle *et al*., 2015; Ndungu *et al*., 2016). The average TPC for raw milk from traditional market ranged between 1.1 x 10^5 to 2.4 x 10^5 CFU/ml while the TPC for raw milk from milk processing plants was 3.1 x 10^6 CFU/ml. The average TPC for pre-processed milk from milk processing plants was 2.8 x 10^6 CFU/ml. Therefore, some milk samples from Lusaka province in the current study would have not qualify for grade A milk which is the highest raw milk grade bought by big processing plants. In Zambia, grade A raw milk must have a TPC of 50,000 CFU/ml (Yambayamba and Zulu, 2011) and this milk grade is usually bought at higher price than other grades. This may also suggest that milk samples

with higher TPC probably were not produced and/or handled under good hygienic practices. Further, the milk samples could have been coming from infected cows.

The average TPC for traditionally processed sour milk from traditional market in Lusaka province ranged between approximately 9.0 x 10^3 CFU/ml to 2.2 x 10^5 CFU/ml. A study conducted in Western province of Zambia reported a higher TPC in sour milk of 1.5 x 10^6 CFU/ml (Knight-Jones *et al*., 2016). Sour milk microbiological quality was slightly better than fresh raw milk in the current study. This situation may not represent better hygiene but probably due the acidic conditions of sour milk. Acidic conditions in sour milk tend to inhibit many pathogens (Lund *et al*., 2000). The TPC for commercially processed milk and sour milk including cheese were not included in the current study because no *S. aureus* isolates were isolated from these matrices.

5.2.3 *S. aureus* on farms

The study finding showed that Lusaka province had the highest proportion (80 percent, 67/84) of *S. aureus*-positive farms than Western (76 percent, 35/46) and Southern (57 percent, 55/96) provinces. One possible reason for the high proportion of *S. aureus*-positive farms in Lusaka might be that most farmers in Lusaka province had high yielding cows, especially the Friesian breed and that farmers could be practicing different management styles compared to the farmers in Western and Southern provinces since some keep cows under semi-intensive management. High volumes of milk may make hygienic milk handling a challenge for farmers/milkers in Lusaka province. In addition, the current study finding indicate that contamination could be related to poor hygiene in milk handling during milking including milker's hand and poor udder hygiene. It is also possible that the cow's health may contribute to the contamination of milk as well.

5.2.4 *S. aureus* in raw milk from milk traders

Most of the raw milk samples from milk traders taken in Western and Southern provinces, with each 70 percent (7/10), were contaminated with *S. aureus*. The traders stated that they receive the milk mainly or exclusively from farms. Hence, contamination might have already taken place at farm level. The pooling of milk in some cases from different milk sources among traders and absence of pasteurization could further increase the chance of milk contamination

and the risk of *S. aureus* (Donkor *et al.*, 2007). Therefore, improving hygienic practices and milk handling might help in reducing contamination of raw milk among milk traders.

5.2.5 *S. aureus* in raw and sour milk from traditional markets

Analysed raw milk and traditionally processed sour milk from the traditional market was contaminated with *S. aureus* with samples from traditional market in Western province being the most contaminated at 23 percent followed by Lusaka (10 percent) and Southern (10 percent). However, it should be noted that not all presumptive *S. aureus* isolates generated from samples from traditional markets in Lusaka province were analysed for confirmation (due to an error in sample shipment). Thus, the number of *S. aureus*-positive samples might even be higher. The occurrence of *S. aureus* in the traditional dairy products could be due to contamination at farm level or during subsequent handling of milk. The use of unpasteurized milk for the traditional production of sour milk could be a source of contamination to sour milk with *S. aureus*.

5.2.6 *S. aureus* in bulk milk from milk collection centres

Most raw milk samples taken from MCCs in Southern province were contaminated with *S. aureus* (67 percent; 8/12) followed by Western province with 50 percent (2/4). On the other hand, no *S. aureus* isolate was isolated from raw milk samples from MCCs in Lusaka province. A study in Sebeta, central Oromia in Ethiopia reported that 80 percent of bulk milk samples from MCCs were found to be contaminated with *S. aureus* (Ayele *et al*., 2017). The detection of *S. aureus*-contaminated milk at MCCs in the current study might be due to contamination of milk at farm level during milking, transportation from farms to MCCs and/or at MCCs during handling (Addis *et al*., 2011). Mixing of batches of milk observed at all MCCs in the three provinces could, besides other factors, be responsible for contamination of raw bulk milk with *S. aureus* especially if some farms are contaminated. MCC workers who do not wash their hands before handling milk, wear dirty clothes, and have symptoms such as coughing and sneezing may be another potential source for contamination with *S. aureus* to milk. Longer duration of transportation of milk observed especially in Western province and storage coupled with inefficient or lack of cooling facilities, would promote bacterial multiplication (usually indicated by TPC). This could lead to production of SEs in milk. If SEs are already produced

in milk, such milk may not be safe to be consumed even after boiling because SEs are heat-stable.

5.2.7 *S. aureus* in unprocessed milk from milk processing plants

Most of samples of unprocessed and/or pre-processed milk from selected milk processing plants were contaminated with *S. aureus*. The contamination of unprocessed/pre-processed milk with *S. aureus* was probably because of contaminated raw milk coming from individual farms/MCCs.

5.2.8 Absence of *S. aureus* in commercially processed milk and milk products

No samples of processed milk products from milk processing plants tested positive for *S. aureus*. This could suggest the efficacy of processing which employs heat treatment of milk. Therefore, pasteurisation remains the best way of eliminating pathogens such as *S. aureus* in milk and could safeguard the lives of milk consumers. However, subsequent contamination with *S. aureus* or other pathogens after pasteurisation must be prevented. *S. aureus* was not detected in samples of commercially processed milk and milk products from supermarket/shops in all the three provinces. This finding agrees with that of a study in Ethiopia which reported that no *S. aureus* was detected in pasteurized milk samples (Ayele *et al.*, 2017). In addition, the current study also agrees with that of a study in Iran which reported similar findings (Rahimi, 2013). This suggests that *S. aureus* is killed during pasteurization process and that no post pasteurization contamination took place (Havelaar *et al.,* 2015). The current finding also indicates the efficacy of pasteurization (heat treatment) in eliminating *S. aureus* in the milk and its products. However, good milk handling hygiene is very important at retail level to minimize possible post processing contamination and spoilage.

5.3 Molecular characterisation of *S. aureus* isolates

5.3.1 Diversity of *spa* types in the Zambian dairy value chain

The study findings revealed the presence of *S. aureus* strains belonging to 36 different known *spa* types including seven novel *spa* types detected mainly in raw milk, indicating a high diversity of isolates from the Zambian dairy value chain. Besides, 55 *S. aureus* isolates, mainly from Western province, were untypeable (Table 38, 39, 40, 41, Figure 23). This was probably

due to deficiency in Staphylococcal Protein A (spa) or the isolates were mutants (Votintseva *et al*., 2014). The distribution and diversity of the *spa* types was different in the three provinces. For example, it seems that the novel *spa* type t18396 is common in Lusaka and Southern provinces, while the novel types t18398, t18399 and t183400 were only found in Western province. This may suggest that strains belonging to these *spa* types could be circulating only in Western province of Zambia. In addition, this study also revealed a wide distribution of some *S. aureus spa* types circulating in the Zambian dairy food chain in two or all of the three study provinces. This finding may also indicate that these *spa* types could be present in other parts of Zambia as well.

In the current study, the isolates belonging to *spa* types t189, t521, t267 and t355 were the most prevalent in the Zambian dairy value chain. Considering the *spa* types, the study finding indicates that t355, t267 and t084 were present in all the three provinces and this indicated that strains belonging to these *spa* types were circulating in the three study provinces. Other studies in Gabon on asymptomatic carriers and diseased persons, reported that t355 was the second most prevalent *spa* type after t084 *(Schaumburg et al*., 2011b). However, in the current study t355 was the fourth prevalent *spa* type after t189. A study in Zambia at a referral hospital reported finding MSRA strains belonging to t355 in pus and blood of human patients (Samutela *et al*., 2017). The referral hospital is based in Lusaka province and usually admits patients from different provinces of Zambia including Western and Southern provinces. Isolates belonging to t355 were the most detected *spa* type among MRSA strains from clinical isolates from studies conducted in Ghana (Egyir *et al*, 2014b; Samutela *et al*., 2017; Donkor *et al*., 2018). Further, MRSA strains belonging to t355 have also been reported from clinical isolates in Nigeria and Uganda (Shittu *et al*., 2011; Seni *et al*., 2013; Samutela *et al*., 2017), and MSSA belonging to t355 from nasal samples of abattoir workers in Nigeria (Odetokun *et al*., 2018).

Other studies have reported detecting MRSA isolates belonging to t355 in nasal cavity from carriers in Ghana (Donkor *et al*., 2018), in clinical samples in 5 countries in Africa (Asadollahi *et al*., 2018) including Kenya, South Africa, Uganda, Gabon, and Nigeria mostly as MRSA except in Gabon and South Africa, who reported detection of some MSSA strains and in Gabon on asymptomatic carriers and diseased persons (Schaumburg *et al*., 2011b). A study in Angola, reported the detection of isolates belonging to t355 in patients and health workers (Conceicao

et al., 2015). This *spa* type was also found in human isolates in Côte d'lvoire (Schaumburg *et al*., 2015b). In Côte d'lvoire, *S. aureus* isolates belonging to t355 were also detected in bovine milk samples (Jans *et al*., 2017). In addition, a study in North-West Ethiopia reported detection of t355 in milk samples from clinical mastitis cows and health cow's quarters (Mekonnen *et al*., 2018).

In the current study, isolates belonging to t355 were detected in nasal and hands swabs from milkers, swabs from milk buckets, and in raw milk from farms, milk traders and traditional markets. Considering the data from the literature, t355 circulating in the Zambian dairy value chain could be human MSSA strains. Moreover, cows with clinical or subclinical mastitis may be another source of contamination of milk with *S. aureus* belonging to t355. Presence of *S. aureus* in milker' nasal/hand swabs and in raw milk suggests a strong possibility of transmission of these strains from humans (milker) to cows/milk or vice-versa. However, the direction of transmission is not known.

The second *spa* type common in all three provinces, t084, was also detected in nasal and hands swabs from milkers and in raw milk from farms and traders. This study finding is consistent with that of a study in Tunisia which reported detecting *S. aureus* strains belonging to t084 in bovine bulk tank milk samples from various regions of Tunisia (Ben Said *et al*., 2016). Other studies have reported detecting *S. aureus* isolates belonging to t084 in bovine fermented milk samples in Côte d'lvoire (Jans *et al*., 2017), in clinical isolates from hospitals in some African countries (Asadollahi *et al*., 2018) and t084 in humans from communities, domestic animals (goats and sheep) and wildlife (monkey) in the Democratic Republic of Congo, Gabon and Côte d'lvoire (Schaumburg *et al*., 2015b). Other African studies have also reported the detection of t084 in nasal samples of abattoir workers and in cattle and goats (Odetokun *et al*., 2018) as well as in clinical, nasal (carrier) and environmental samples in Ghana (Donkor *et al*., 2018). A study in Gabon found *S. aureus* strains belonging to t084 to be the second predominant strains recovered from carriers (nose, groin and axilla) and infected people (Schaumburg *et al*., 2011b). In addition, another study in Angola reported detecting isolates belonging to t084 in patients and health workers (Conceicao *et al*., 2015). These findings could suggest a possibility that *S. aureus* strains belonging to t084 could be of human origin.

Therefore, their presence in pooled raw milk in the current study could indicate the possible transmission of these bacterial strains between humans and cows/milk.

In the current study, *S. aureus* isolates belonging to t267 (the third *spa* type detected in all three study provinces) were detected in nasal/hand swabs from two milkers and in milk bucket swabs, raw milk from farms, milk traders and milk processing plants, pre-processed milk from milk processing plants and traditionally processed sour milk. This finding agrees with that of a study in Kenya which reported detecting isolates belonging to t267 in bovine milk samples (Jans *et al*., 2017). In addition, the current finding also agrees with that of a study in India which reported detection of MSSA isolates belonging to t267 in milk from cows with clinical mastitis (Mistry *et al*., 2016). This *spa* type has further been reported in sheep in Tunisia (Gharsa *et al*., 2012b) and in milk samples from sheep with intramammary infections in Switzerland (Merz *et al*., 2016). These studies may suggest that *S. aureus* strains belonging to t267 could be livestock associated MSSA (LA-MSSA) and a possible cause of intramammary infections in domestic livestock. Possible transmission of strains belonging to t267 between cows and humans is likely to occur.

In the current study, the only isolate belonging to t064 was isolated from nasal swab of a milker. This finding is consistent with that of a study done in Kwa-Zulu-Natal in South Africa which reported detecting *S. aureus* strains belonging to t064 in bovine milk from mastitis cows and nasal swab samples from humans in the dairy environment (Schmidt *et al*., 2017). The current study finding also agrees with that of a study done in Namibia and Gabon which reported isolating isolates belonging to t064 (MRSA) among colonising strains and clinical isolates (Nurjadi *et al*., 2014). Other studies have reported the detection of MRSA strains belonging to t064 from clinical samples in South Africa (Moodley *et al*., 2010) and in patients and health workers in Angola (Conceicao *et al*., 2015). These studies may confirm that *S. aureus* isolate belonging to t064 detected in the Zambian dairy value chain could be of human origin. Therefore, finding these human associated *S. aureus* strains in milkers' nasal sample strongly suggest that a milker could be a potential source of contamination to milk. The possible MSSA and MRSA human-to-animal transmission has been suggested in some other studies in Africa (Schaumburg *et al*., 2012b; Schaumburg *et al*., 2015b; Lozano *et al*., 2016). In Europe, *S. aureus* strains belonging to t064 were commonly detected and were known to be human strains

(Kock *et al*., 2013; Kinnevey *et al*., 2014; Lozano *et al*., 2016; Tegegne *et al*., 2017). In Africa, *spa* type t084 (CC15) and t064 (CC8) were among the more prevalent MSSA strains among the clinical isolates while t002 (CC5) was one of the most prevalent types among clinical isolates in Europe, Asia and America (Asadollahi *et al*., 2018). Detection of *S. aureus* strains of human origin in animals has become very common (Tegegne *et al*., 2017). Therefore, contamination of milk with human *S. aureus* strains, represent a growing public health concern.

The current findings also showed that most *S. aureus* isolates from samples taken in Lusaka province belonged to *spa* type t189 (n=46) and t267 (n=25), while in the Southern province t521 (n=37) and in the Western province t355 (n=10) were the dominant types identified. *S. aureus* isolates belonging to t189 were isolated from few hand swabs from milkers, but mainly from raw milk from farms (including few mastitis milk samples) and from milk bucket swabs. Further, one sample of pre-processed milk from a processing plant was positive for isolate belonging to t189. A study done in Kwa-Zulu-Natal in South Africa reported isolating *S. aureus* strains belonging to t189 in bovine milk from mastitis cows and nasal swab samples from humans in the dairy environment (Schmidt *et al.,* 2017). This finding could also suggest the involvement of *S. aureus* strains belonging to t189 in clinical and subclinical mastitis in cows and it could be one of the *spa* types causing mastitis in Lusaka province as it was mainly detected in this province in the current study. The current finding could also suggest that cows with intramammary infections could be a source of contamination of raw milk with *S. aureus* strains. Further, milker's hands could also be another source of contamination to milk with *S. aureus*, and a source of infections to cows in a herd. Other studies have reported detecting isolates belonging to t189 in humans and sheep in Côte d'lvoire (Schaumburg *et al*., 2015b; Lozano *et al*., 2016) and from carriers (nasal, groin and axilla) and in diseased cases in Gabon (Schaumburg *et al*., 2011b). Therefore, these findings may also suggest the possible transmission of strains belonging to t189 between humans and cows with possible contamination of milk.

The current finding revealed that *S. aureus* isolates belonging to t521 were isolated from pooled raw milk from farms, milk from mastitis cow, bulk milk from milk collection centre, unprocessed milk from processing plant, nasal and hand swabs from milkers. Previously isolates belonging to t521 were detected in milk from bulk tanks on Minnesota dairy farms

(Haran *et al*., 2012), in individual cow's milk from dairy farms in Tunisia (Ben Said *et al*., 2016) and in milk from buffaloes with mastitis in Iran (Panahi and Saei, 2019). These studies could suggest that some *S. aureus* strains belonging to t521 may also be involved in causing mastitis in cows on the Zambia farms. Therefore, proper identification, prevention and control of mastitis might help in ensuring good quality and safe milk production and, minimise contamination of milk along the Zambian dairy value chain. In addition, the sequence type ST97 lineage (where t521 found in this study belong) is among the lineages mostly detected frequently in bovine milk worldwide (Mistry *et al*., 2016). Other studies have reported detecting t521 from blood cultures from patients in South Africa (Orth *et al*., 2013) and in human nasal swabs from human carriers in a community in the Amazonia forest of French Guiana in South America (Ruimy *et al*., 2010). These findings could also suggest the possibility of transmission of *S. aureus* between cows and humans. However, the direction of transmission is not known.

The current study finding also showed that some *S. aureus spa* types were only found in one or two provinces (Table 38, 39,40, 41). This may suggest that these strains could be specifically localised in these provinces. However, because of movement of people and livestock between provinces, transmission and spread to other provinces could be possible. On the other hand, *S. aureus* isolates belonging to t2677 are rare in Africa and data is limited. However, some strains belonging to t2677 have been isolated in Germany and included in the Ridom *spa* database (Harmsen *et al*., 2003). Much is not known about strains belonging to t2677 in Africa and this could be the first time t2677 is being reported to be detected in milk in Africa. In addition, *S. aureus* isolates belonging to t6049 are also rare in Africa and data is not available. However, the Ridom *spa* database, show that t6049 has been isolated before. Furthermore, the association and country of origin is not known (Harmsen *et al*., 2003). This could be the first time it has been reported in Africa from milk samples.

At clonal level, prevalence was highest for CC97 with 127 isolates and CC/ST152 with 34 isolates. CC15 and CC121 had 7 isolates each; the other 1–3 isolates. This finding contrasts with that of other studies which reported that CC5, CC15 and CC30 were the most prevalent clonal complexes in Africa (Ruimy *et al*., 2008; Conceicao *et al*., 2015; Ruffing *et al*., 2017)

with C121 being the most prevalent clonal complex in Sub-Saharan Africa (Schaumburg *et al*., 2014; Ruffing *et al*., 2017). All the isolates in the current study were methicillin sensitive *S. aureus* (MSSA). Other studies have reported a high diversity of MSSA among the strains isolated from animals in Africa with about 23 clonal complexes and many different *spa* types have been identified (Gharsa *et al*., 2012a; Gharsa *et al*., 2012b; Lozano *et al*., 2016). MSSA strains belonging to CC1, CC5, CC8, CC9, CC30, CC97 and CC121 have been detected in animals in several countries in Africa and have also been found in other livestock animals in other continents (Lozano *et al*., 2016). The CC97 is mainly related to ruminants (Gharsa *et al*., 2012b) and *S. aureus* strains belonging to clonal complex CC97 have been known to cause mastitis worldwide *(*Moser *et al.*, 2013*;* Luzzago *et al*., 2014; Luini *et al*., 2015; Wang *et al*., 2015). CC97 has also been found in pigs *(*Battisti *et al*., 2010; Gomez-Sanz *et al*., 2010).

On the other hand, CC8 and CC121 have been detected in other continents in cattle, horses, pigs, and rabbits (Vancraeynest *et al*., 2006; Smyth *et al*., 2009; Walther *et al*., 2009; Gomez-Sanz *et al*., 2010; Sakwinska *et al*., 2011; Lozano *et al*., 2016). Furthermore, besides CC97, Fall *et al*. (2012) reported the detection of CC1, CC5, CC8, CC15, CC72, CC121 and CC152 in pigs . In addition, CC5 has been associated with MSSA and MRSA detected in pigs, dogs and goats in other studies in some countries in Africa (Youn *et al*., 2014; Schaumburg *et al*., 2015b; Lozano *et al*., 2016). In addition, CC30 has been found in MSSA strains from other animal species, excluding pigs in Zambia, Uganda, Tunisia and Côte d'lvoire (Schaumburg *et al*., 2012a; Schaumburg *et al*., 2012b; Schaumburg *et al*., 2013; Gharsa *et al*., 2015; Lozano *et al*., 2016). Besides occurrence in ruminants and other animals, *S. aureus* strains belonging to CC97 are also considered to be circulating among humans (Lozano *et al*., 2011; Egyir *et al*., 2014a). In the current study, isolates belonging to CC97 were detected mainly in pooled milk on farms (including milk from mastitis cows) and a few in milk buckets and from hand and nasal swabs from milkers. This may suggest that clinical and subclinical mastitis or milkers/buckets could be some of the sources of contamination to milk with *S. aureus* strains belonging to these *spa* types.

5.3.2 Diversity of enterotoxin gene patterns of *S. aureus* isolates isolated from the three study provinces

The current study findings showed that only 16 *S. aureus* isolates (21 percent) out of 77 isolates analysed by NGS or DNA microarray and six unidentified/unknown types were enterotoxigenic strains. A study done in Ethiopia reported the detection of enterotoxin genes among isolates from bovine milk (Seyoum *et al*., 2016). In addition, the study findings show that all the three analysed isolates belonging to *spa* type t4198 harboured at least two different enterotoxin genes patterns and that isolates belonging to t355 harboured enterotoxin genes or none (Table 42). Seyoum *et al*. (2016) reported that 26 strains of *S. aureus* from bovine milk also harboured more than one enterotoxin genes. In the current study, only two isolates out of 77 analysed isolates were positive for at least one gene of the group *sea* and only eight harboured at least *sea* to *see* genes encoding the classical SEs. This finding disagrees with the study done by Normanno *et al*. who described that *sea* gene was the most frequent enterotoxin gene among the enterotoxigenic strains of *S. aureus* (Normanno et al., 2005). In addition, Seyoum *et al*. (2016) also reported that sea gene was the most prevalent among isolates from bovine milk. The current study finding revealed that only isolates belonging to t002 and t064 (only one isolate analysed by DNA microarray each) harbour *sea* gene. This study finding agrees with that of a study in South-West Uganda which reported that only two out of about 41 isolates harboured enterotoxin gene *sea* (Asiimwe *et al*., 2017b).

The SEA enterotoxin encoded by *sea* has been known to be the most common cause of food poisoning (FP) worldwide (Argudin *et al*., 2010). However, other SEs have also been implicated in FP outbreaks including SEH which is encoded by *seh* (Argudin *et al*., 2010). In the current study, enterotoxin gene *seh* was not detected among the *S. aureus* isolates. Further, the predominant enterotoxin gene in the current study was *sei* (11/77, 14.3 percent), which is part of the *egc* cluster (see below). This finding disagrees with that of other studies which have reported that the predominant enterotoxin gene in African isolates was *seb* (Schaumburg *et al.*, 2011a; Ruffing *et al*., 2017). Only three isolates harboured *seb* (together with the *sec* gene) in the current study. This could suggest enterotoxin gene diversity in the strains of *S. aureus* from the dairy value chain in African countries.

The actual capability of these *S. aureus* strains to produce SEs was not analysed and may depend on many factors. These factors may include population density of *S. aureus*, density of competitive microflora, storage environment, storage temperature, pH, salt concentration, water activity (a_w) and presence of oxygen (Akkaya *et al*., 2014). *S. aureus* could be capable of producing enterotoxins after reaching concentrations of about 10^5 to 10^6 CFU/g of food (Untermann and Muller, 1992; Akkaya *et al*., 2014). However, lower concentration of *S. aureus* might be sufficient to produce enterotoxins depending on the strain involved and environment. Therefore, good milk handling hygiene and keeping milk under cooling facilities (fridges) could help in reducing the growth and multiplication of *S. aureus* and possible production of enterotoxins in milk. This could further reduce the risk to consumers. Pasteurization or boiling of raw milk and prevention of subsequent contaminations remains the golden standard in eliminating such as *S. aureus* in raw milk (Claeys *et al*., 2013; Forrokh *et al*., 2013).

The study findings indicate that out of the selected 77 *S. aureus* isolates, 13 isolates harboured at least five or more enterotoxin genes, including those of the enterotoxin gene cluster *egc* (Table 42). The *egc* operon encodes the enterotoxins SEG, SEI, SEL, SEM, SEN and SEO (Jarraud *et al*., 2001; van Belkum *et al*., 2006). The *egc* cluster was detected in isolates belonging to t002 (one isolate), t015 (one isolate), t318 (one isolate), t3772 (one isolate), and t4198 (three isolates) including in six isolates of unknown/unidentified *spa* type belonging to ST3575. However, these six isolates belonging to unknown/unidentified *spa* type seem to lack the *seg* gene. The absence of usually one or more genes in the *egc* cluster has been previously reported in other studies (Lozano *et al*., 2011). The isolates not assigned to a particular *spa* type were from different samples of unprocessed and processed (sour) milk and, the isolates with enterotoxin gene of *spa* type t002, t3772 and t4198 were from pooled raw milk. On the other hand, isolates positive for enterotoxin genes belonging to t015 and t318 were isolated from nasal swabs from milkers. This suggests that milkers could be the source of contamination to milk with enterotoxigenic strains of *S. aureus*.

A study in South Africa on milk from infected cows and samples from nasal swabs from humans in contact with animals, reported the detection of isolates belonging to t015, with strains from bovine not carrying any enterotoxin genes while those from humans harboured *sec*, *seg*, *sei*, *sel*, *selm* and *selo* (Schmidt *et al*., 2017). A study in Mozambique on admitted

children less than 15 years old reported that isolates belonging to t015 from blood harboured no enterotoxins (Vubil *et al*., 2017). In addition, Shittu *et al*. (2011) reported that isolates belonging to t002 and t3772 from clinical and non-clinical sample did not harbour any enterotoxin genes. Further, Vubil *et al*. (2017) reported that isolates belonging to t002 from blood harboured enterotoxin gene *sea*. Other studies have reported that isolates belonging to t002 from nasal swabs from human carriers that harboured only *seb* or *sec* (Ruimy *et al*., 2010). A study done in Nigeria reported the detection of isolates belonging to t318 harbouring enterotoxin genes *seo, seg* and *seu* (Ayeni *et al*., 2018). Further, the current study finding revealed that isolate belonging to t318 at least harboured six enterotoxin genes. Ruimy *et al*. (2010) reported detecting isolates belonging to t318 from nasal swabs from human carriers harbouring only the gene *seb* or no enterotoxin genes. These findings further indicate diversity of *S. aureus* strains circulating in Africa, which could to some extent be dependent on the geographical locations.

The current study findings also showed that only one isolate (PVL-positive) from unprocessed milk from traditional market in Southern province, out of 12 *S. aureus* isolates isolated belonging to t355 harboured enterotoxin genes (Table 42). The enterotoxin genes *sec* and *sel* were detected in the isolate described above. In addition, three of the 77 selected isolates for detailed analysis by NGS belonging to t355 were PVL-negative. A study conducted in Côte d'lvoire reported detection of isolates belonging to t355 from milk and fermented milk samples and these isolates did not carry any enterotoxin genes (Jans *et al*., 2017). Another study done in Nigeria reported detection of PVL-positive isolates belonging to t355 from two locations among clinical isolates from nasal swabs including from carriers in communities and these isolates did not harbour any enterotoxin genes (Ayeni *et al*., 2018). However, other studies have reported detecting some isolates belonging to HA-MSSA and CA-MSSA t355 strains harbouring enterotoxin *sem* (Breurec *et al*., 2011). This finding could also suggest the diversity of strains belonging to t355 in Africa which may be associated with regions or countries.

The current study finding also showed that the analysed isolates belonging to t084 (4 selected isolates analysed in detail out of even isolates generated in the study) and t267 (all the five isolates belonging to t267 analysed in detail) circulating in the three provinces did not carry any enterotoxin genes. However, since only these five of the isolates belonging to t267 out of

39 were analysed in detail for the presence of SE genes among the generated isolates, other isolates probably were enterotoxigenic although they were not analysed in detail. Previous studies on some isolates of t084 and t267 (including isolates from human and milk/sour milk) showed absence of SE genes (Jans *et al*., 2017; Vubil *et al*., 2017), while other isolates carry SE gene(s). Presence of *sea* in isolates belonging to t084 has been reported in isolates from milk and fermented milk samples in Côte d'lvoire (Jans *et al*., 2017) and among HA-MRSA and CA-MSSA isolates from patients in hospitals and communities in five towns of Africa (Breurec *et al*., 2011). Moreover, *S. aureus* isolates from raw meat of sheep and belonging to t084 and chicken belonging to t267 were found to carry the enterotoxin gene *sen* (Chairat *et al*., 2015). However, in the current study the isolates belonging to t084 not analysed in detail cannot be ruled out as carrying enterotoxins.

The contamination of raw milk in the Zambian dairy value chain with enterotoxigenic strains of *S. aureus*, represents a public health concern because of the ability of some strains of *S. aureus* strains to produce heat-stable enterotoxins in milk (Argudin *et al*., 2010). This could be a risk to consumers especially if milk is kept at elevated temperatures as *S. aureus* could grow and multiply rapidly, and may produce enough enterotoxins in milk to cause food poisoning (Argudin *et al*., 2010). Therefore, lack of cooling facilities and high environmental temperatures would provide perfect conditions for enterotoxin production. The potential production of enterotoxins by enterotoxigenic *S. aureus* strains in milk, also represent a potential threat to public health as the ingestion of preformed toxins in food is the major cause of FP worldwide (Le Loir *et al*., 2003; Srinivasan *et al*., 2006; Schmidt *et al*., 2017). Therefore, the detection of enterotoxigenic *S. aureus* strains in the Zambian dairy value is a public health concern. The detailed characterisation of selected isolates showed a diversity of *S. aureus* strains isolated from the Zambian diary chain and the potential of some of these strains to produce SE(s) in milk.

5.3.3 Presence of PVL-positive *S. aureus* in the Zambian dairy value chain

PVL-positive MSSA isolates have been known to be endemic in Africa (Schaumburg *et al*., 2014; Stegger *et al*., 2014; Lozano *et al*., 2016) with a high prevalence of 17–74 percent (Breurec *et al.,* 2011; Schaumburg *et al.,* 2014). However, the prevalence of PVL-positive MSSA strains in Europe is very low and rang between 0.9–1.4 percent (von Eiff *et al*., 2004;

Schaumburg *et al*., 2014). The current study findings showed that a total of 29 methicillin sensitive *S. aureus* (MSSA) isolates were PVL-positive (9.8 percent, 29/295) in the three provinces with Lusaka, Western and Southern having nine, four and 16 PVL-positive isolates respectively (Table 38, 39, 40, 41). *S. aureus* strains carrying PVL are frequently isolated in Africa (Egyir *et al.,* 2014b). Studies in West and East Africa have reported 40 percent prevalence of PVL-positive MSSA among clinical isolates (Breurec *et al.,* 2011; Schaumburg *et al.,* 2011b; Shittu *et al.,* 2011; Egyir *et al.,* 2014a; Abdulgader *et al.,* 2015). PVL is a virulence factor described for *S. aureus* isolates from human samples (Dinges *et al*., 2000; Zecconi *et al*., 2006; Vrieling *et al*., 2016; Mekonnen *et al*., 2018).

In the current study, most PVL-positive isolates belong to CC152 and ST152 (t355, t454, t1096). Besides, PVL was found in isolates of CC121 (new *spa* type t14198) and in one isolate of CC30. This finding agrees with other studies conducted in Central and West Africa which reported that CC/ST152, CC/ST121 and CC/ST30 are some of the major clones that show high proportions of PVL-positive isolates (Schaumburg *et al.,* 2014). Further, PVL has been detected mainly in some MRSA strains isolated from animals in several African countries including Democratic Republic of Congo, Senegal, Tunisia and Côte d'lvoire (Fall *et al*., 2012; Gharsa *et al*., 2012b; Schaumburg *et al*., 2015b; Lozano *et al*., 2016). These findings may indicate the diversity of the population structure of *S. aureus* strains in Africa. It has been well established in Africa that the population structure of MSSA stains is much diverse than MRSA (Breurec *et al*., 2011) and it has been suggested that MRSA probably originated from a few epidemic lineages of MSSA through transfer of SCC*mec* (Deurenberg and Stobberingh, 2008). The high number of PVL-positive MSSA strains could lead to emergence and possible spread of successful and highly virulent PVL-positive MRSA and this happening has already been observed in Africa (Breurec *et al*., 2011).

The current study findings indicated that 71 percent (17/24) of the *S. aureus* isolates belonging to t355 were PVL-positive, with presence in all three provinces. The isolates were recovered from nasal and hand swabs of milkers, pooled raw milk and milk buckets from farms, and from one unprocessed milk sample from traditional markets. Other studies have also reported detection of PVL-positive isolates belonging to t355 (3/79, 4 percent) in milk samples (Mekonnen *et al.,* 2018), in MRSA strains from nasal swabs of asymptomatic carriers and

patients (Ruimy *et al*., 2008; Asiimwe *et al.*, 2017a; Schaumburg *et al.*, 2011b; Breurec *et al.*, 2011). In addition, a study conducted by Ayeni *et al.* (2018) in Nigeria reported the detection of PVL-positive MSSA strains belonging to t355 from clinical human sample. PVL-positive isolates from milk in the current study could be from milkers.

In the current study, all of the identified *S. aureus* isolates belonging to t1096 (n=3) were PVL-positive and were isolated from pooled raw milk samples from farms in the Southern province. Also for this *spa* type, PVL-positive isolates were previously detected in human samples in Africa. A study in 5 African countries (Madagascar, Morocco, Senegal, Niger, and Cameroon) on patients suspected of staphylococcal infection reported isolates belonging to t1096 to be PVL-positive MSSA (HA-MSSA and CA-MSSA) (Breurec *et al*., 2011). Further, a study in Ghana in the Ashanti region reported isolation of PVL-positive *S. aureus* belonging to t1096 from nasal swabs from children admitted to the Agogo Presbyterian hospital (Eibach *et al.*, 2017). In addition, a study in Haiti also reported detection of PVL-positive MSSA of t1096 (CC152) in osteomyelitis infection from patients (Schaumburg *et al.*, 2015a). The current study finding also reveals that all of the *S. aureus* isolates belonging to t4198 (n=3) characterised in detail, were PVL-positive and were isolated from pooled milk from farms in the Southern province. Schaumburg *et al.* (2015a) also reported the detection of PVL-positive t4198 (CC121) MSSA in abscesses from patients. It has been suggested that *S. aureus* strains from Haiti could be related to clones which might have probably originated from Africa during slave trade or later due to African diaspora (Schaumburg *et al.*, 2015a). However, there is limited data on PVL-positive MSSA strains belonging to t4198 in Africa.

The only isolate analysed belonging to PVL-positive t318 (MSSA) was isolated from nasal swab from a milker. PVL-positive isolates belonging to t318 were also detected in humans and goats in Côte d'lvoire (Schaumburg *et al.*, 2015b). Further, PVL-positive isolates belonging to t318 were detected and identified as CA-MSSA and HA-MSSA from samples of patients taken from hospitals in 5 different African countries (Breurec *et al.*, 2011). In a study on healthcare institutions in Kenya on patients, isolates belonging to MSSA t318 were detected in tracheal aspirate and MRSA t318 in nasal swabs of patients (Omuse *et al.*, 2016). Omuse *et al*. suggested that there was a possibility of *S. aureus* strains belonging to t318 *spa* type acquiring the SCC*mec* element locally. However, with the possibility of local acquisition of SCC*mec*

element, the CA-/HA-MSSA isolates could become CA-/HA-MRSA. The possible dissemination of PVL-positive MRSA could present a significant challenge to disease management and infection control to most African countries (Abdulgader *et al.,* 2015). This might also be true for the dairy value chain in Zambia, considering a potential transmission of such isolates from humans to cows/milk.

The current study finding also revealed that the one identified *S. aureus* isolate belonging to t454, isolated from a milk bucket swab, was PVL-positive. This *spa* type was rarely found in Africa, but a study in Ghana reported detecting PVL-positive isolates belonging to t454 in patients from hospitals (Egyir *et al*., 2014b). These findings strongly suggest that PVL-positive MSSA isolates could be of human origin and their presence in milk could indicate the possible human to cow transmission but contaminate the milk. Finding *S. aureus* isolates belonging to t454 in the milk bucket swab could also suggest that milk buckets were not properly cleaned. Therefore, unclean milk containers could be vehicles for transmission and spreading *S. aureus* in the Zambian dairy value chain.

The current study did not detect any *luk-PV* genes among the isolates belonging to t064. This finding disagrees with that of a study in five towns of Africa which reported detection of some isolates belonging to t064 among HA-MRSA and CA-MSSA isolates from patients in the hospitals and carriers in communities harbouring *luk-PV* genes (Breurec *et al*., 2011). These findings could indicate the diversity in virulence among isolates belonging to t064 in Africa. In addition, the *luk-PV* genes (*lukF-PV* and *lukS-PV*) encode for PVL toxin and this toxin has been implicated in deep abscess, severe necrotizing pneumonia and severe bone and joint infections in humans (Lina *et al*., 1999; Gillet *et al*., 2002; Breurec *et al*., 2011). However, the current study finding indicates that isolates belonging to t064 did not harbour any *lukF-PV* and *lukS-PV* genes. PVL is a highly cytotoxic and pore forming toxin produced by some strains of *S. aureus* and which usually targets the human immune system cells including the neutrophils, monocytes, and macrophages (Szmigielski *et al*., 1998; Breurec *et al*., 2011). The pores caused by PVL toxin leads to cytokine release depending on the concentration of toxin, and cell death mainly due to necrosis or apoptosis (Genestier *et al*., 2005; Breurec *et al*., 2011). PVL toxin has been associated with the highly aggressive and fatal form of community acquired infections (Stryjewski and Chambers, 2008).

In the current study, all the PVL-positive isolates were MSSA strains and some of them belonging to the pandemic sequence type ST30 (t318, one isolate isolated) and ST121 (t4198, seven isolates isolated). This finding agrees with that of a study done in Nigeria which reported detecting MSSA strains belonging to t318 harbouring *lukS-PV* and *lukF-PV* genes (Ghebremedhin *et al*., 2009). The current study finding also agrees with that of other studies that have reported that PVL-positive ST30 and ST152 are more common in Africa than any other continent (Ruimy *et al*., 2008; Rasigade *et al*., 2010b; Schaumburg *et al*., 2011b). These studies could suggest that *S. aureus* strains belonging to these sequence types circulating in the Zambian dairy value chain, could be reservoirs for PVL-positive MRSA as previously suggested by other studies (Rasigade *et al*., 2010b; Schaumburg *et al*., 2011b).

5.3.4 Diversity of leucocidin and other virulence genes of *S. aureus* isolates isolated from the three study provinces

MSSA strains carry different virulent factors that help the bacteria to increase its ability for invasion and survival (Lowy, 1998; Ferry *et al*., 2005). To do this, *S. aureus* is able to express different virulence factors that promote survival and pathogenesis inside the host (Malachowa *et al*., 2012). Besides PVL, presence of leucocidin genes was determined by NGS or DNA microarray analysis. The current study findings revealed that almost all *S. aureus* isolates analysed in detail harboured at least leucocidin genes. However, only for the selected isolates of *spa* type t1299 (two isolates were analysed by NGS) no leucocidin genes were identified (Table 43). In addition, all 77 (100 percent) isolates analysed in detail (NGS and DNA microarray) carry the gene *HlgAB* and these encode bi-component leucocidins which play a significant role in phagocytosis of *S. aureus*. These toxins kill the polymorphonuclear phagocytes (PMNS), disrupt epithelial and endothelial barriers, and lyse erythrocytes to provide iron for bacterial growth (Venkatasubramaniam *et al*., 2018). The current study finding agrees with that of a study done in Nigeria by Ghebremedhin *et al*. (2009) which reported detecting MSSA strains belonging to PVL-positive t318, t064 harbouring *hlgA* and *hlgB*. In the current study, a total of 55 isolates out of 77 (71.4 percent) harboured *HlgCB* gene. These genes are responsible for encoding bi-component-leucocidin toxins which are primarily known for their lytic activity towards polymorphonuclear phagocytes (Spaan *et al*., 2017). This finding is consistent with that of a study done in South Africa which reported that 75 percent of bovine

and 69 percent of human *S. aureus* isolates were positive for leucocidin (*hlg)* genes while 86 percent from cattle and 25 percent from humans were positive for *hlb* genes (Schmidt *et al*., 2017). A study on *S. aureus* in Democratic Republic of Congo reported that the prevalence of *hlgA, hlgB, hlgC* virulence genes was 79.6 percent among isolates while for *hlb* virulence genes it was 90 percent (Lebughe *et al*., 2017). In addition, 73 isolates out of 77 (95 percent) in the current study harboured *hlb* (β-haemolysin) and this finding is consistent with a study done in Democratic Republic of Congo (Lebughe *et al*., 2017).

The study findings showed that about 55 *S. aureus* isolates (55/77, 70 percent) at least harbour *lukE* and *lukD* leucocidin genes and were the third most prevalent genes after *HlgAB* and *HlgCB.* The presence of *lukD* and *lukE* genes have been observed in cases of impetigo (Gravet *et al*., 2001) and in patients with antibiotic-associated diarrhoea (Gravet *et al*., 1999). The genes *lukE and lukD* encode the leucocidin toxin LukED. Like other leucocidins, LukED toxin targets and kills human neutrophils (Vasquez *et al*., 2020). In the current study, *lukE* and *lukD* genes were present in 22 out of 36 *spa* types (t002, t064, t084, t189, t237, t267, t359, t454, t458, t521, t527, t1096, t1201, t1234, t1236, t2844, t2883, t 3772, t4198, t4955, t5229 and t18400). A study done in South Africa, reported detection of *lukE* and *lukD* in all bovine isolates and in human isolates (Schmidt *et al*., 2017). A study on *S. aureus* in Democratic Republic of Congo reported that *lukE* and *lukD* genes were the second prevalent leucocidin genes detected (Lebughe *et al*., 2017). Another study in Africa reported detecting a higher prevalence of *lukE* and *lukD* genes in milk and fermented milk products (Jans *et al*., 2017). However, the current finding disagrees with that of other studies in other countries that have reported that leucocidin *lukD* and *lukE* are less frequently detected (Gravet *et al*., 1998; Becker *et al*., 2003; von Eiff *et al*., 2004; Schaumburg *et al*., 2011b). These toxins, especially in combination with other different virulence factors could contribute to the incidence and probably severity of infection.

The current study findings also revealed that isolates belonging to t2883 (three isolates submitted for DNA microarray analysis) harboured three leucocidin gene patterns while isolates belonging to t084 (four isolates submitted for DNA microarray analysis) carry two leucocidin gene patterns (Table 43). A study in Tunisia reported that isolates belonging to t2883 from bovine milk from bulk tanks and individual cows harbour *lukD*, *lukE* and *lukM* leucocidin genes *(Ben Said et al*., 2016). However, in the current study *lukM* leucocidin gene was not

detected in isolates belonging to t2883. In addition, the current study finding agrees with that of a study in Tunisia which reported that all isolates belonging to t2844 from bovine milk harbour at least *lukM* gene (Ben Said *et al*., 2016). This may also suggest diversity in virulence among strains belonging to t2883 and t2844 circulating in Africa. On the other hand, the current finding also agrees with that of a study in Nigeria on clinical samples from inpatients upon being admitted to two hospitals in South western Nigeria which reported detecting MSSA strains belonging to t084 harbouring *lukD and lukE* genes (Ghebremedhin *et al*., 2009). Other studies have reported that all isolates belonging to t084 from bovine milk harbour at least *lukD* and *lukE* genes (Ben Said *et al*., 2016). A study done in Tunisia reported the detection of MSSA isolates belonging to t084 from veal and sheep carrying no any leucocidin genes (Chairat *et al*., 2015). Further, these findings may indicate differences in the strains belonging to t084 in Africa. Another study done in Tunisia reported that isolates belonging to t359, t521, t2844 and t2883 from bovine milk also harbour *lukD* and *lukE* genes (Ben Said *et al*., 2016). Chairat *et al*. (2015) reported detecting MSSA isolates belonging to t189 strains from sheep harbouring *lukE* in Tunisia. This could suggest diversity in virulence and role in pathogenesis among strains belonging to t189.

A total of 36 out of 77 *S. aureus* isolates (47 percent) harboured *lukY*, while at least 27 isolates (35.1 percent) carried *lukX* genes. Jans *et al*. (2017) reported a higher prevalence of *lukY* and *lukX* genes in milk and fermented milk products. This could suggest that there was diversity in virulence of strains of *S. aureus* circulating in the Zambian dairy value chain. The current study findings also show that isolates belonging to four *spa* types out of 36 (t1201, t1236, t2677 and t2844) harbour *lukM* leucocidin gene and five isolates carry the bovine variants *lukF-PV (P83)* and *lukM*. A study done in South Africa detected *lukF-PV (P83)* and *lukM* in 56 percent of bovine isolates and only one in human isolates (Schmidt *et al*., 2017). The *lukF-PV (P83)/lukM* genes, which encode a bi-component leucotoxin that is highly active against bovine neutrophils, was present in only 5 isolates out of 37 analysed by DNA microarray. These genes were only detected in isolates belonging to clonal complex CC97 (t1201) from pooled raw milk and t2844 from pooled raw milk, nasal and bucket swab and CC522 (t2677) from pooled raw milk. In addition, *lukF-PV (P83)/lukM* genes have been reported previously to be common in cattle (Monecke *et al*., 2008; Jamrozy *et al*., 2012). The findings strongly suggest that isolates

belonging to t1201, t2677 and t2844 are from bovine (cattle). Therefore, the cows could be a source of these strains into milk.

Moreover, almost all *S. aureus* isolates analysed in detail harboured the *aur* gene. Further, a total of 56 isolates (73 percent) at least harboured *splB*, 53 isolates (69 percent) *splA*, 45 isolates (45/78, 58 percent) *splE*, 26 isolates (34 percent) *edinB*. The *splA*, *splB* and *splE* genes encode spl proteases. The epithelial cell differentiation inhibitor EDIN is encoded by *edinA*, *edinB* and *edinC* genes (Munro *et al*., 2010). The study findings also indicated that 26 isolates belonging to t355 (12 isolates), t454 (one isolate), t1096 (3 isolates), t1299 (two isolates), t3772 (one isolate), t6049 (one isolate) and six isolates belonging to unknown spa type (ST3575) harboured the *edinB* gene. Further, the study findings indicated that isolates belonging to *spa* type t084 (four isolates analysed by DNA microarray analysis) harboured two patterns of virulence genes with one isolate harbouring at least *aur*, *eta*, *hlgA*, *splA*, *splB*, *splE* genes and three isolates carrying at least *aur*, *hlgA*, *splA*, *splB*, *splE* genes. A study in Mozambique on admitted children less than 15 years old reported isolating isolates belonging to t084 and t064 from blood harbouring at least *splA* and *splB* (Vubil *et al*., 2017). A frequent occurrence of the proteases genes *splA* and *splB* in African *S. aureus* isolates was previously described (Ruffing *et al*., 2017). Besides, *splE* gene was more prevalent in Germany isolates (Ruffing *et al*., 2017).

The current study findings agree with that of a study in five towns of Africa which reported detection of *eta* gene (exfoliatin A) among HA-MRSA and CA-MSSA isolates belonging to t084 from patients from hospitals and communities (Breurec *et al*., 2011). These studies may suggest that strains belonging to t084 circulating in the Zambian dairy value chain could be very virulent with the potential to cause infections in both humans and animals. Further, the virulence gene *eta* was only found in two isolates out of the 77 selected isolates, belonging to t084 (from nasal swab of a milker) and t002 (from pooled raw milk) in the current study. This finding is consistent with that of a study in Gabon which reported low prevalence of *eta* genes among human clinical and carrier isolates (Schaumburg *et al*., 2011b). In addition, the current study disagrees with the studies in Nigeria which reported that *eta* is more prevalent in 80 percent of *S. aureus* (Adesiyun *et al*., 1991). Chairat *et al*. (2015) reported that *S. aureus* isolates from raw meat of veal and sheep belonging to t084 did not carry *eta*. However, this study also reported that *S. aureus* isolates from sheep belonging to t189 carried *eta* (Chairat *et*

al., 2015). The genes *eta*, *etb* and *etd* encode the exfoliative toxins (ETA, ETB and ETD) (Lee *et al*., 1987; Ladhani, 2001). These are interesting virulence factors of *S. aureus* and they have been known to be directly responsible for the clinical manifestation of staphylococcal scalded skin syndrome (SSSS) which is characterised by skin exfoliation (Ladhani, 2001). Further, exfoliative toxins like other staphylococcal toxins represent a risk to humans and they have been associated with severe infections (Lina *et al*., 1999; Jarraud *et al*., 2002; Ben Said *et al*., 2016). In addition, only one isolate belonging to t3772 analysed by DNA microarray, carried *etd* gene. This finding agrees with a study done by Becker *et al*. which described that *etd* is less common than *eta* and *etb* (Becker *et al*., 2003). However, the current study did not detect *etb* gene in any isolate among the 77 *S. aureus* isolates analysed by NGS or DNA microarray.

The genes *hla* (alpha-haemolysin gene) and *hld* (delta-haemolysin gene) were present in almost all *S. aureus* isolates analysed by DNA microarray or NGS. A study done in South America reported detecting *hla and hld* genes in all *S. aureus* isolates from nasal swabs from human carriers (Ruimy *et al*., 2010). Further, a study in Tunisia reported that MSSA isolates belonging to t267 from chicken, t189 from sheep and t084 from veal and sheep harbour *hla* and *hld* (Chairat *et al*., 2015). A study done in Mozambique on admission of children reported isolating isolates belonging to t3772 from blood harbouring at least *hla* and *hld* (Vubil *et al*., 2017). Moreover, in the current study, the β-haemolysin gene *hlb* was frequently detected in the isolates selected for detailed characterisation. The *hlb*-positive isolates belong to 31 of the 33 analysed *spa* types, including those with PVL (Table 43). This finding agrees with that of a study done in five towns of Africa which reported detection of some isolates belonging to PVL-positive t318, t355 and t1096 among HA-MRSA and CA-MSSA strains from patients in the hospitals and carriers in communities harbouring *hlb* genes (Breurec *et al*., 2011). Breurec *et al*. (2011) also reported detection of some isolates belonging to t064 among HA-MRSA and CA-MSSA isolates from patients in the hospitals and carriers in communities that harbour the *hlb* gene.

Out of the 77 isolates studied in detail in the current study, 23 harboured both *hlb* genes and genes of the immune evasion cluster IEC. This finding agrees with that of other studies that have reported detection of both *hlb* and IEC genes, since the IEC is located on the β-haemolysin converting bacteriophages (van Wamel *et al*., 2006; Schmidt *et al*., 2017). However, isolates

belonging to t084 (four analysed by DNA microarray) and t015 (one isolate analysed by DNA microarray) did not harbour both *hlb* and IEC genes, but only IEC genes.

5.3.5 Beta-haemolysin-converting bacteriophages (HLG-Conv-Phages) virulence genes observed among the isolates from the three study provinces

The current study findings showed that 28 *S. aureus* isolates (belonging to 11 *spa* types) out of 77 isolates selected for detail characterisation harboured two or three immune evasion genes (*sak, scn* or *chp scn* or *sak, chp* and *scn*). The *sak, chip* and *scn* genes encode the staphylokinase (SAK) enzyme, chemotaxis inhibitory protein of *S. aureus* (CHIPS), and the staphylococcal complement inhibitor protein (SCIN) respectively (Kumagai *et al*., 2007). The three human specific innate immune modulators SAK, CHIPS, SCIN and SEA (Schmidt *et al*., 2017) are considered to form immune evasion cluster (IEC) that may easily be transferable between strains of *S. aureus* by a diverse group of beta-haemolysin-converting bacteriophages (van Wamel *et al*., 2006). Further, the current study findings revealed that one *S. aureus* isolate each, belonging to t015, t318 and t3772 harbour *sak, chp* and *scn* genes while 002, t064, t355, t454, t1096, t1299 and t6049 respectively, harbour *sak* and *scn* genes only. A study done in South Africa on dairy farms reported detecting *S. aureus* isolates belonging to t015 from both bovine and humans (Schmidt *et al*., 2017). However, in this study only the human isolates were reported as harbouring *chp and sac* genes while the bovine isolates did not harbour any immune evasion genes (Schmidt *et al*., 2017). This finding is consistent with that of a study in Democratic Republic of Congo which reported detecting *S. aureus* isolates belonging to t002, t064, t355 and t1096 from patients with bloodstream infections harbouring immune evasion genes (Vandendriessche *et al*., 2017). In addition, the current study findings also show that four *S. aureus* isolates belonging to t084 harboured *chp* and *scn* genes (Table 43). This finding agrees with that of a study in Tunisia which reported that isolates belonging to t084 from bovine milk harbour *chp* and *scn* genes (Ben Said *et al*., 2016). The current finding also agrees with that of a study in Democratic Republic of Congo which reported detecting *S. aureus* isolates belonging to t084 from patients with bloodstream infections harbour immune evasion genes (Vandendriessche *et al*., 2017). Therefore, this could strongly indicate that isolates belonging to t002, t084, t355, t1096, t1299, t3772, t6049 found in the raw milk and isolate belonging to t454 from bucket swab harbouring immune evasion genes (Table 43) in the current study could

be of human origin. Therefore, colonised milker could be a source of contamination to milk. However, not all human isolates have been found with immune evasion genes (Verkaik *et al*., 2011; Schmidt *et al*., 2017).

5.3.6 Diversity of antimicrobial resistance gene patterns and antibiotic resistance profiles of *S. aureus* isolates isolated from the three study provinces

A total of 38 *S. aureus* isolates (49 percent) out of 77 analysed by NGS or DNA microarray) at least harboured *norA-like* gene (for fluoroquinolone resistance). This may suggest that the resistance against fluoroquinolones could be due to fluoroquinolone drugs commonly used in animals, poultry and humans. Some of the drugs in this group such as ciprofloxacin have been used mainly to treat some infections such as sepsis, typhoid, skin and soft tissue infections (Wisemann and Balfour, 1994). Further, ofloxacin has been used to treat urinary tract infections (Kromann-Andersen and Krøyer Nielsen, 1990) while nalidixic acid has been used for treating dysentery (Bennish and Salem, 1992). Currently ceftriaxone (a new third-generation cephalosporin) is being used for treatment of gonorrhoea in hospitals and clinics because of fluoroquinolone resistance in some bacteria (Lamb *et al*., 2002). Further, a study in a secondary health care hospital in Zambia reported a high fluoroquinolone resistance among Enterobacteriaceae isolated from outpatients and health clinic attendants studied (Nagelkerke *et al*., 2017). In addition, a study done in Sub-Saharan Africa reported fluoroquinolone resistance mainly among *Salmonella* and *Escherichia coli* (Chattaway *et al*., 2016). However, this studies did not report on detection of fluoroquinolone resistance genes. The fluoroquinolone resistance genes could also be as a result of the presence of the drug residues in the environment or resistance genes coming from other biological systems such as fungi and other bacteria.

Penicillin and tetracycline were the second and third most detected resistance genes after fluoroquinolone resistance denes in the current study. A study in South Africa on *S. aureus* from meat carcasses and bovine milk from abattoirs and dairy farms of Eastern Cape reported that *blaZ* gene was detected in 14.9 percent of *S. aureus* isolates (Pekana and Green, 2018). Other studies done in Ghana on *S. aureus* isolates from patients and carriers reported that all isolates harboured *blaZ* gene while prevalence of the *tetK* gene was low (Donkor *et al*., 2018). It seems penicillin and tetracycline are some of the common drugs especially used in treatment

of mastitis by farmers in dairy cows (Mainda *et al*., 2015). However, the proportions of genes may also be due to natural resistance genes in the natural environment. Further, evidence shows clearly that some organisms and environment could harbour antibiotic resistance genes regardless of the level of human use of antibiotics (Allen *et al*., 2010).

The current study findings also showed that 31 *S. aureus* isolates (40.3 percent) harboured at least one resistance gene, and 37 (48.1 percent) isolates carried 2-4 antibiotic resistance genes (Table 44). This could suggest diversity of antimicrobial resistance among the *S. aureus* isolates in the Zambian dairy value chain. In addition, the study findings revealed wide diversity of genetic characteristics of MSSA isolates belonging to *spa* type t355 (Table 38, 39, 40, 41) and isolates from this *spa* type harboured at least four different antibiotic resistance gene patterns. All 12 isolates selected for t355 for detailed characterisation by NGS harboured one or more antibiotic resistance genes. A study done in Côte d'lvoire reported the detection of MSSA isolates belonging t355 from milk and fermented milk samples not harbouring any resistance genes (Jans *et al*., 2017).

S. aureus isolates belonging to t267 (five isolates belonging to t267 analysed by NGS) harboured four different antibiotic resistance gene patterns (Table 44). Jans *et al*. (2017) also reported the detection of tetracycline and penicillin resistance genes in raw milk and fermented milk in Côte d'lvoire in isolates belonging to t267. Further, the study findings also showed that three different antibiotics resistance gene patterns are harboured by isolates belonging to t189 (five isolates analysed by NGS), t521 (four isolates were analysed by NGS) and t084 (four isolates analysed by DNA microarray) each respectively. However, in contrast, Jans *et al*. reported that isolates belonging to t084 from milk and fermented milk samples in Côte d'lvoire harboured only one pattern of penicillin , tetracycline and fosfomycin (*blaZ*, *tetK* and *fosB*) resistance genes (Jans *et al*., 2017). Further, a study in Tunisia reported detecting isolates belonging to t084 from nasal swabs from health humans with no animal contact but in contact with hospitalised patients harbouring only one antibiotic resistance gene, *tetK* while some did not harbour any (Ben Slama *et al*., 2011). Other studies also in Tunisia reported that some *S. aureus* isolates from raw meat of sheep belonging to t084 carried *tetK* gene only while others from veal did not carry any gene and those from sheep belonging to t189 also carried *tetK* gene only (Chairat *et al*., 2015). The current study finding disagrees with a study in South Africa on

milk from infected cows and health humans in contact with animals which reported detecting isolates belonging to t189 but did not detect any antimicrobial resistance genes among bovine and human isolates (Schmidt *et al*., 2017). The current study finding also indicates that *spa* type t2883 (four isolates were analysed by DNA microarray) and t18400 (two isolates were analysed by NGS) harboured at least two different antibiotic resistance gene patterns each respectively. Ben Slama *et al*. (2011) reported detecting isolates belonging to t2883 from nasal swabs from health humans with no animal contact but in contact with hospitalised patients without antibiotic resistance genes.

Other detected antibiotic resistance genes in the current study included *dfrG* (trimethoprim resistance), *erm(C), erm(C)-like* or *mph(C)* (macrolide resistance), *msr(A)-like* (macrolide–lincosamide–streptogramin B resistance), *cat* (chloramphenicol resistance) and *fosB* (fosfomycin resistance) genes. Other studies in Tunisia reported that *S. aureus* (MSSA) isolates from raw meat samples also carried antibiotic resistant genes for erythromycin/macrolide (14.3 percent), kanamycin (9.5 percent) and tobramycin (2.4 percent) (Chairat *et al.*, 2015). Some of these drugs may be directly administered into the mammary gland to treat mastitis. Although several antibiotic resistant genes were detected, these could also be due to drugs present in the environment from other biological systems such as fungi and soil bacteria (Clardy *et al*., 2009). The current study findings also show that a total of 52 out of 72 analysed isolates showed that they were resistant to several drugs, with resistance against benzylpenicillin, tetracycline and trimethoprim being most frequent. In accordance with the genetic characterisation, resistance to the cephalosporin and cefoxitin (used as marker for methicillin resistance) was not identified.

A total of 14 isolates out of 72 (19.4 percent) in the current study were found to be resistant to three of the considered antibiotic classes (multiple or multidrug resistance) (Table 44). Tetracycline (13 isolates, 18 percent), penicillin (12 isolates, 16.7 percent) and trimethoprim (six isolates, 8.3 percent) were frequently observed among the antibiotic resistant patterns identified, including among the multiple resistant MSSA strains (Table 44). A study done in South Africa on *S. aureus* from meat carcasses and bovine milk from abattoirs and dairy farms of Eastern Cape reported that multidrug resistance patterns were observed in 18 isolates from 200 raw milk samples out of 87 isolates from meat and milk samples and antibiotic resistance against two to nine antibiotics was observed (Pekana and Green, 2018). Another study

conducted in Tanzania on milk from smallholder dairy farmers reported that penicillin and tetracycline were frequently observed multiple antibiotic resistance patterns among *S. aureus* isolates (Massawe *et al*., 2019). The current study findings also agree with that of a study in South Africa on *S. aureus* from meat carcasses and bovine milk which reported that multidrug resistance among all isolates was noted for penicillin, oxacillin, erythromycin and tetracycline (Pekana and Green, 2018). In addition, a study in Zambia conducted on companion animals at the veterinary teaching hospital reported that the resistant rate among *S. aureus* isolates against penicillin was (63.6 percent) followed by trimethoprim-sulfamethoxazole (36.4 percent) (Youn *et al*., 2014). Further, other studies in Tanzania on milk from smallholder dairy farmers in Mbeya region have reported that 57.2 percent of *S. aureus* isolates were resistant to penicillin and 19 percent to tetracycline (Massawe *et al*., 2019) and in South Africa, *S. aureus* resistance against penicillin was 71.6 percent and tetracycline 39.2 percent (Pekana and Green, 2018).

Other studies in Africa have also reported absolute penicillin resistance and high percentage of resistance to other antibiotics in isolates from milk (Kasozi *et al*., 2014), clinical cases (Egyir *et al*., 2014b) and nasal colonisation in human carriers (Lebughe *et al*., 2017). Further, a study done in the Democratic Republic of Congo reported that resistance against tetracycline was also high (74.4 percent) in isolates from nasal colonisation in human carriers and in infection (Lebughe *et al*., 2017).

In the current study, isolates belonging to t267 analysed in detail showed three antibiotic resistance patterns. One isolate was resistant to five antibiotics; gentamicin, kanamycin, penicillin, streptomycin and tetracycline (which belong to three antimicrobial classes). Three of the five isolates were resistant to penicillin, while the other two were resistant to streptomycin only. A study in Tunisia reported that MSSA isolates belonging to t267 from chicken were resistant to only one antibiotic each and showed two resistance patterns (penicillin or tetracycline) (Chairat *et al*., 2015). Laxminarayan and Heymann, (2012) reported that the common antibiotic resistance pattern among isolates (inclusive of those belonging to t267) from nasal and axillary swabs were penicillin, tetracycline and sulfamethoxazole in Kenya. The only *S. aureus* isolate belonging to *spa* type t064 (isolated from nasal swab from a milker) was resistant to one pattern of four antibiotics including penicillin, sulfamethoxazole, tetracycline and trimethoprim (multidrug resistance). A study done in Nigeria on clinical

samples from inpatients admitted to two hospitals in South-Western Nigeria reported that MSSA strains belonging to t064 showed two antibiotic resistance patterns; penicillin, sulfamethoxazole, tetracycline, ciprofloxacin and gentamicin and another pattern with penicillin, sulfamethoxazole and tetracycline (Ghebremedhin *et al*., 2009). *S. aureus* isolate belonging to spa type t3772 (one analysed that was isolated from pooled milk) was resistant to four antibiotics including penicillin, sulfamethoxazole, tetracycline and trimethoprim. Another study done in Nigeria reported that *S. aureus* isolates belonging to t3772 (MSSA) from clinical and non-clinical sources showed one antibiotic resistance pattern covering penicillin and sulfamethoxazole only (Shittu *et al*., 2011).

Staphylococcus aureus isolates belonging to t355 (12 isolates analysed) showed four antibiotic resistance patterns. Three isolates (from hand, bucket swab and unprocessed milk from traditional market) were resistant to penicillin, tetracycline and erythromycin and another three isolates (from milk and hands) were resistant to penicillin, tetracycline and trimethoprim, while four isolates (from hands and milk) were resistant to penicillin and trimethoprim, and one isolate (from milk) was resistant to tetracycline only. Shittu *et al*. (2011) also reported that *S. aureus* isolates belonging to t355 (MSSA) from clinical and non-clinical sources in Nigeria showed three antibiotic resistance patterns; penicillin only, penicillin and ciprofloxacin, and penicillin, tetracycline and trimethoprim/sulfamethoxazole. This finding could suggest that strains belong to t355 circulating in the Zambian dairy value chain may be genetically diverse as indicated by antibiotic resistance patterns.

Staphylococcus. aureus isolates belonging to t084 (four isolates analysed) showed three antibiotic resistance gene patterns. One isolates (from hand swab) was resistant to penicillin, tetracycline and trimethoprim and another isolate (from milk) was resistant to penicillin and trimethoprim, while two isolates (from hand swab and milk) were resistant to trimethoprim. A study done in Tunisia reported that MSSA isolates belonging to t084 from raw meat from veal and sheep were resistant to two patterns; penicillin, tetracycline and tobramycin, and penicillin only (Chairat *et al*., 2015). In Kenya, a study on 85 inpatients reported that the common antibiotic resistance among MSSA isolates (inclusive of those belonging to t267, t002, t015, t064, t084, t318, t355, t359 and t3772) from nasal and axillary swabs from Thika level 5 hospital, was against penicillin, tetracycline and sulfamethoxazole (Laxminarayan and

Heymann, 2012). Ghebremedhin *et al*. (2009) reported detecting MSSA strains belonging to t084 with the same antibiotic resistance pattern (penicillin, tetracycline and sulfamethoxazole)in isolates from inpatients in Nigeria. Another study done in Tunisia reported that all isolates belonging to t084 from bovine milk were susceptible to all antibiotics tested (Ben Said *et al*., 2016).

The current study finding indicates that *S. aureus* isolates belonging to t359 (3 isolates were analysed) showed two antibiotic resistance patterns. One isolate (from bucket swab) was resistant to penicillin, tetracycline and streptomycin and another isolate (from milk) was resistant to tetracycline only, while one isolate (from milk) was susceptible (sensitive). A study in Nigeria on clinical samples reported detecting MSSA strains belonging to t359 with only one antibiotic resistance pattern (penicillin, sulfamethoxazole and tetracycline) (Ghebremedhin *et al*., 2009). Ben Said *et al*. (2016) also reported that all isolates belonging to t359 from bovine milk were susceptible to all antibiotics tested. These findings show that there are different *S. aureus* strains of t359 with different antibiotic resistance and susceptibility profiles circulation in the Zambian dairy value chain as well as in other African countries. The current findings also show that *S. aureus* isolates belonging to t189 (5 isolates analysed) showed two antibiotic resistance patterns. Two isolates (from hands and bucket swab) were resistant to erythromycin, tetracycline and streptomycin and another isolate (from mastitis milk) was resistant to tetracycline only, while two isolates (from milk) were susceptible (sensitive) to all antibiotics tested. Chairat *et al*. (2015) also reported that MSSA isolates belonging to t189 from sheep raw meat showed two antibiotic resistant patterns; penicillin and tetracycline, and penicillin only. Further, penicillin resistance in isolates belonging to t189 was not observed in the current study.

The study findings also indicated that one isolate from milk belonging to t458 (one isolate analysed) was resistant to penicillin, chloramphenicol and tetracycline. A study in Nigeria reported that *S. aureus* isolates belonging to t458 (MSSA) from clinical and non-clinical sources were resistant to penicillin and tetracycline (Shittu *et al*., 2011). In addition, Ben Said *et al*. (2016) reported that all isolates belonging to t2883 from bovine milk were resistant to penicillin. However, one *S. aureus* isolate (from milk) belonging to t2883 in the current study, was resistant to tetracycline; the other two isolates (one from hand swab of a milker and milk bucket swab) were susceptible to all antibiotics tested. This finding including the outcome of

the survey suggest that penicillin and tetracycline are the most commonly used antibiotics on dairy cattle than any other drugs (Mainda *et al*., 2015). This practice could be due to the fact that penicillin and tetracycline are readily available and easily accessible for everyone. Regulation of sale of veterinary antimicrobials in Sub-Saharan countries such as Zambia is still a challenge as drugs are usually sold in shops and markets without prescriptions (Okeke *et al*., 2005; Mainda *et al*., 2015).

The prevalence of antimicrobial resistance among isolates from the Zambian dairy value chain could be an indicator of the misuse, abuse and/or prolonged (continuous) use of same antibiotics in cows with infections such as mastitis as indicated in the responses from farmers in the current study. However, reservoirs for antimicrobial resistance could also be present in healthy human or animal populations (Okeke *et al*., 2005). Other studies conducted have reported that prolonged usage and misuse of antimicrobials in livestock farming and in treatment of human diseases have resulted in a rapid resistance of many bacteria to several antibiotics of different classes (Valsangiacomo *et al*., 2000; Kitai *et al*., 2005; Umaru *et al*., 2017; Pekana *et al*., 2018). In addition, the consumption of raw milk and milk products without observing withdrawal periods for antibiotics as indicated by the responses from participants (interviewed farmers) in the current study may expose consumers to low doses continuously. This practice could result into resistant strains of bacteria such as *S. aureus* in all settings to survive and persist. Further, resistant strains exert pressure on the veterinary and health professionals including dairy cattle farmers because of the negative impact of these pathogens on the antibiotic treatment success (Abera *et al*., 2010; Massawe *et al*., 2019) and they could also have an impact on the cost of inputs.

Antibiotic resistance remains a growing concern for the developing countries (Laxminarayan and Heymann, 2012). However, the ability of these *S. aureus* strains harbouring antimicrobial resistance genes to express phenotypic resistance to antimicrobials as seen in the current study, is a public health concern. In addition, *S. aureus* can also acquire the staphylococcal cassette chromosome mec which can give rise to MRSA strains (Pantosti, 2012; Ben Said *et al*., 2016). The evidence of LA-MRSA strains colonising and infecting animals and humans in close contact with farm animals remains a concern; however, MRSA were not identified in the current study. In general, antibiotic susceptibility testing remains an important test to prevent

treatment failures both in veterinary medicine and human health. Further, enforcements of regulations and monitoring coupled with public education on the use, sale and dangers of antibiotics is required in Zambia. Therefore, the current study findings show diversity of antibiotic resistance among isolates circulating in the Zambian dairy value chain and in other African countries.

5.3.7 Presence of toxic-shock syndrome toxin (*tst1*) gene in the isolates from the three study provinces

The study findings show that nine *S. aureus* isolates out of 77 isolates analysed in detail harbour a *tst*1 gene. The gene *tst*1 encodes toxic-shock-syndrome-toxin (TSST-1) which is an exotoxin belonging to superantigens family (Dinges *et al*., 2000). The mechanism of action of these superantigens involves the ability to induce a non-specific stimulation of T lymphocyte proliferation and cytokine secretion, leading to clinical signs such as fever, hypotension and shock (Plata *et al*., 2009; Argudin *et al*., 2010; Schmidt *et al*., 2017). The *tst1*-positive isolates belong to the *spa* types t015 (isolate from nasal swab), t355 (three of 12 isolates, derived from raw pooled milk, milk bucket swab, milker nasal swabs), t359 (one of three isolates, derived from raw pooled milk and found to contain a bovine variant of *tst*1), t2677 (isolate from pooled raw milk) and unidentified/unknown *spa* type(s) (three isolates from raw pooled milk). A study in Mozambique at Mahanica district hospital on admitted children less than 15 years old reported isolates belonging to t015 from blood harbouring *tst*1 *(*Vubil *et al*., 2017).

Other studies, in five towns of Africa reported detection of some isolates belonging to t355 among HA-MRSA and CA-MSSA strains from patients in the hospitals and carriers in communities not harbouring any *tst1* gene (Breurec *et al*., 2011). In the current study, all the PVL-positive isolates belonging to t355 harboured *tst1*. Ghebremedhin *et al*. (2009) reported detecting MSSA strains belonging to t359 harbouring *tst1* gene in clinical samples from inpatients in Nigeria. Ben Said *et al*.(2016) reported detecting isolates belonging to t359 from bovine raw milk form bulk tanks and individual cows, which did not harbour any *tst1* gene. The current study shows genetic variation regarding virulence genes including *tst1* among isolates belonging to different *spa* types and also the same *spa* type, identified in Zambia. This confirms the diversity in virulence among the *S. aureus* circulating the Zambian dairy value chain.

5.3.8 Potential transmission of *S. aureus* in the three provinces

Good management practices are vital in the production of good quality and safe milk on farms and the entire dairy value chain. In the current study, some interviewed dairy farmers mainly in Southern province kept pigs (21.9 percent) and dogs (44 percent) on their farms (Table 11) and this could be a potential risk regarding *S. aureus* contamination of raw milk. For example, pigs have been implicated as one of the reservoirs of *S. aureus* (Hasman *et al*., 2010; Velasco *et al*., 2018). Further, animal carriers could pose a potential zoonotic risk to farm workers who also could be the milkers of dairy cows. Research studies in the Netherlands in 2005 reported transmission of LA-MRSA between pigs and pig farmers and their families (Voss *et al*., 2005). *S. aureus* of ST398/CC398 have been identified as the main colonizer of pigs, cattle, horses and poultry, including humans in contact with animals (Witte *et al*., 2007; Golding *et al*., 2010; Mediavilla *et al*., 2012; Cuny *et al*., 2013; Gomez-Sanz *et al*., 2014; Gomez *et al*., 2016). Most human colonisation with *S. aureus* ST398 has been known to be associated with exposure to pigs (Armand-Lefevre *et al*., 2005; Voss *et al*., 2005; Witte *et al*., 2007; Khanna *et al*., 2008). Thus, farm animals such as pigs and dogs can be a source of *S. aureus* to colonize cows or farm workers who then might contaminate milk or cows. Further, a study done in Zambia at a Veterinary hospital found *S. aureus* in dogs (Youn *et al*., 2014). Dogs in the farm environment could be another potential source of *S. aureus* to humans.

A total of 16 *S. aureus* isolates (5.4 percent, 16/295) from raw pooled milk at farm level in the three provinces carried genes for PVL with Southern province having the highest number. Moreover, five PVL-positive *S. aureus* isolates were recovered from milk buckets swabs from Lusaka and Southern province and one from raw milk from a traditional market in Southern province. PVL has been detected mainly in *S. aureus* isolates of human origin (Dinges *et al*., 2000; Zecconi *et al*., 2006; Vrieling *et al*., 2016; Mekonnen *et al*., 2018). The current finding of PVL in *S. aureus* isolates from raw milk may suggest the possible transmission PVL-positive strains of *S. aureus* from human to milk/cow. A study done in South Africa reported transmission of a human associated isolate belonging to sequence type, ST8 from human to a dairy cow, which subsequently caused mastitis and it was speculated that the close contact between dairy cows and humans could have enabled such transmission (Schmidt *et al*., 2017).

A study done on the sanctuary chimpanzees from Zambia, Namibia and Uganda including veterinarians from African countries working in great ape sanctuaries reported that PVL-positive strains were more frequent in isolates from Chimpanzees (28 percent) than from humans (10 percent) (Schaumburg *et al*., 2012b). The high proportion of PVL in isolates from chimpanzees supports the theory of human to animal transmission of human-adapted *S. aureus* in sub-Saharan Africa (Breurec *et al*., 2011; Schaumburg *et al*., 2012b). Similarly, the current study suggests a possible transmission of *S. aureus* strains from humans to cows and even contaminate milk. However, the pathogenic potential of PVL-positive *S. aureus* strains in animals is so far not well understood. In humans, PVL has been known to be associated with severe skin and soft tissue infections and necrotizing disease including pneumonia (Lina *et al*., 1999; Schaumburg *et al*., 2012b). The outcome of Core genome Multi Loci Sequence Typing (cgMLST) analysis in the current study indicated that isolates from a hand swab of a milker (M42) was genetically very closely related to isolate from pooled raw milk (M14) from the same farm obtained during the first sampling visit. Contaminated hands of a milker could be acting as vehicles for *S. aureus* transmission and spread to cow/milk at farm level. Thus, good hygiene and milk handling practices are important in reducing the possible risk of *S. aureus* to reduce potential health threats to farmers/milkers, consumers, and animals (Phiri *et al*., 2021).

The current study findings also show that the isolates of the same *spa* type isolated from different matrices at the same facilities were closely related. For example, isolates belonging to s*pa* type t355 from *S. aureus* found in a human (milker) nasal and milk bucket swabs at the same farm during the first sampling visit to Lusaka province were genetically closely related. This could strongly suggest that a milker is responsible for transmission of *S. aureus* to cow/milk. *S. aureus* strains belonging to sequence type ST1, ST5, ST6, ST8, ST15, ST101, ST121, ST152, ST188, ST567 and ST1472 have been detected in humans, domestic animals and, or in wild life (Schaumburg *et al*., 2015b). However, Schaumburg *et al* assessed the possibility of transmission between humans and animals and reported that transmission of *S. aureus* was more frequent between humans and wildlife (Schaumburg *et al*., 2015b). This study suggested the possible transmission of *S. aureus* between human and animals.

5.3.9 Potential persistence of *S. aureus* in the Zambian dairy chain

In general, a persistence of pathogenic bacteria such as *S. aureus* in facilities, animals, and workers is possible. The current study results partly point to a potential persistence (at least over few months) of *S. aureus* on the farms. Single isolates of the same *spa* type had been identified at two sampling times on the same farm (Appendix G). These *spa* types cover t189, t237, t267, t355 and t521. An under-coverage is possible, as in principle, several *S. aureus* strains can occur in one sample. However, in the current study only one presumptive *S. aureus* isolate was further investigated per sample. Furthermore, in the second sampling period, only raw milk (but not milk buckets, milkers hand and nasal swabs or water were collected) were analysed at farm level.

In this study, selected isolates were further examined by means of NGS and cgMLST. This was done to ascertain the phylogenetic analysis for two isolates (both belonging to *spa* type t355) that were generated from a milk bucket swab and a pooled raw milk sample taken at different times on the same farm showed a clear similarity. Other studies have detected isolates belonging to t355 in humans and in domestic animals in Côte d'lvoire (Schaumburg *et al*., 2015b). The current finding indicates that strains belonging to t355 could be maintained by cows and/or humans within farm environment. Further, this finding could suggest the possibility of persistence within the farms or facilities. In addition, the current finding also suggests that some milkers and cows could be potential carriers of *S. aureus* belonging to t355 during the period they are colonized and may remain potential sources of *S. aureus* strains to contaminate milk. Therefore, since isolates belonging to t355 were also detected on different farms or facilities within the three provinces, this could suggest possible transmission of *S. aureus* strains between farms/facilities as well within or across the provinces due to movement of cows through purchases or gifts from other farms/farmers. Mekonnen *el al*. (2018) described that because the isolates belonging to t355 were found at different farms, there is possibility that contagious transmission would occur not only within, but also between different farms through cattle movement, or from government breeding and heifer distribution centres in Ethiopia (Mekonnen *et al*., 2018).

A potential persistence of isolates might be also indicated in the current study for other *spa* types. For examples, isolates belonging to t189 were found in samples of raw milk and/or bucket swabs taken at seven farms in Lusaka province during both sampling visits (Appendix G). Further, *S. aureus* isolates belonging to t267 were also detected at two farms in Lusaka province during both first and second sampling visits in pooled raw milk and milk buckets swabs. *S. aureus* of t267 has been implicated in causing clinical mastitis in cows (Mistry *et al*., 2016). In addition, the current study findings indicate that isolates belonging to t521 were detected in samples of pooled raw milk at five farms in Southern province during both first and second sampling visits. Moreover, this *spa* type was also identified among isolates from a milker's hand swab and a raw pooled milk sample during first and second sampling at one farm in the Lusaka province (Appendix G). Therefore, infected cows (clinical and subclinical mastitis) could remain the sources of *S. aureus* to contaminate milk hence persistence at farm level.

In general, it appears to be possible that *S. aureus* is maintained (at least for few months) in farm environments. Potential reservoirs of *S. aureus* might be particularly infected individual cows with intramammary infections and/or humans.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to determine milk hygiene practices among stakeholders along the dairy value chain in Lusaka, Western and Southern provinces of Zambia and to study the characteristics of *S. aureus* isolates regarding enterotoxins, virulence, antimicrobial resistance. The finding of this study gave rise to the following conclusions and recommendations.

6.1 Conclusions

1. There was generally poor milk hygiene practices among stakeholders especially in Western and Southern provinces.
2. There was a wide contamination of raw milk with *S. aureus* in all the three study provinces.
3. *S. aureus* was less frequently detected in swabs from milk buckets, milkers hands and nose in all the three provinces.
4. Traditionally processed sour milk was also contaminated with *S. aureus*.
5. Further, commercially processed milk and milk products at retail level were also not contaminated probably indicating the efficacy of industrial processing (heat treatment) in reducing *S. aureus*.
6. Cows, milk, contact materials and milkers could be some of the vehicles for transmission, maintenance and spreading *S. aureus* along the Zambian dairy value chain.
7. Characterisation of *S. aureus* revealed diversity of *spa* types and gene patterns regarding enterotoxins, virulence and antibiotic resistance among *S. aureus* isolates detected in Lusaka, Western and Southern provinces.
8. Enterotoxigenic, virulent and/or antibiotic resistant strains of *S. aureus* including strains belonging to novel *spa* types are circulating in the Zambian dairy value chain.
9. MRSA strains were not found in the three Zambian provinces.
10. The presence of different virulence factors associated with human disease in the MSSA strains from the Zambian dairy value chain emphasises the role of these strains as a potential threat to public health.
11. Possible transmission of *S. aureus* between humans and cows in the study provinces is indicated. However, the direction of transmission is not known.

12. MSSA strains belonging to the pandemic sequence type ST30 and ST121 circulating in the Zambian dairy value chain could serve as reservoirs for potential PVL-positive MRSA strains.
13. Furthermore, farms, milk collection centres, traders, traditional markets and consumers have been identified as targets for interventions to reduce contamination of milk with *S. aureus* and to lower potential health threats to humans.

6.2 Recommendations

Poor milk handling hygiene among stakeholders, contamination of milk with virulent, enterotoxigenic and antibiotic resistant strains of *S. aureus* circulating in the dairy value chain in Lusaka, Western and Southern provinces of Zambia signify that raw milk is not very safe to consume unless it is boiled or pasteurised to kill pathogens and reduce potential health risks. In this case, however, subsequent contaminations with pathogenic microorganisms such as *S. aureus* must be prevented. It is therefore, recommended that:

6.2.1 All stakeholders in the dairy value chain

Conducting training of all stakeholders in general hygiene, hygienic milk handling and food safety, including awareness on heat-stable enterotoxins.

6.2.1.1 Farm level

1. Conducting training of farmers and milkers in personal, milk vessels, milking materials and cow's udder/teat hygiene (theory and practical demonstrations). Further, conduct training of farmers and milkers in milk handling hygiene and food safety.
2. Advising dairy cattle farmers on the prudent use of antibiotics when treating diseased animals to reduce antibiotic resistance in pathogens with special advice to seek guidance and advice from qualified veterinary staff.
3. Encouraging dairy cattle farmers to keep milk in cold chains to prevent rapid growth and multiplication of bacteria such as *S. aureus* to reduce the possibility of SE production.
4. Encouraging farmers/milkers to seek advice from qualified veterinarians whenever they have animal health problems to avoid self-medication of cows with antimicrobials to prevent potential antimicrobial resistance development in microorganisms.
5. Encouraging farmers/milkers to use recommended milk vessels/containers, preferably aluminium metal cans.

6. Advising dairy cattle farmers/milkers to use properly cleaned milk vessels/containers and to wash their hands properly with soap and water when handling milk preferably using warm water.

6.2.1.2 MCC level

1. Providing additional information to active MCCs handling huge volumes of milk like in Lusaka and Southern provinces on the need to invest in additional bulk tanks with cooling facilities to minimise keeping milk at room temperature when current tanks are full.

6.2.1.3 Traditional market level

1. Promoting good personal hygiene among milk sellers.

6.2.1.4 Milk trader level

1. Promoting good personal hygiene among milk traders.
2. Encouraging milk traders to sell milk immediately it is produced to consumers to avoid long periods without refrigeration.
3. Providing advice to milk traders on the need to use proper recommended containers for milk transportation and storage.

6.2.1.5 Milk consumers

1. Educating the public on hygienic milk handling practices and the need to boil milk immediately it is produced or as soon as it is purchased and promote storage of milk in refrigerators to reduce the multiplication of bacteria such as *S. aureus* and possible production of SEs. SEs are heat-stable and cannot be denatured with boiling and preventing their production is the best option. Various messages will be required targeting the milk consumers. Different media platforms could be used to reach out to the general public including meetings, workshops/seminars, drama, posters/cartoons, radio and electronic media.
2. Discouraging milk consumers to consume raw cow's milk.
3. Advising milk consumers to wash hands when handling milk and milk products

6.2.2 Government level

Harmonisation of the roles of the Ministry of Fisheries and Livestock with that of the Ministry of Health to ensure coordination in implementation of milk hygiene and safety activities.

6.2.2.1 Ministry of Health

1. Conducting regular medical examination of all milkers, processors and traders including milk handlers. The examination should also include *S. aureus* as one of the target bacteria, especially on food handler's hands and in the nostrils to check colonisation. Colonised people could be a potential source of *S. aureus* to contaminate food and they should not be allowed to handle milk. In addition, the Ministry of Health in their public health education campaigns should include messages to milk consumers on the need to boil milk immediately to kill pathogens in the milk and prevent production of enterotoxins by microorganisms such as *S. aureus*. In the Ministry of Health food safety awareness messages, statements such as "Raw milk, boil it and if you can't, don't drink it" could be included.
2. Conducting regular inspections of MCCs, processing plants, traditional markets and supermarket and shops handling milk to monitor compliance on maintaining good hygienic practices to ensure milk safety to consumers.

6.2.2.2 Ministry of Fisheries and Livestock

1. Developing milking standards and code of hygiene practices for use at farm level. Further, the Ministry will need to have inspectors who will be looking at hygienic milk production and handling at farm level.
2. Conducting inspections on farms as part of official controls to check compliance on hygienic milk production.
3. Conducting regular monitoring and surveillance on *S. aureus*/MRSA and mastitis in dairy farms
4. Provide extension services on best practices of milk production, udder hygiene, milk safety and, prevention and control of mastitis in dairy cows.

6.2.3 Future research

1. Study the prevalence of enterotoxigenic and antimicrobial resistant *S. aureus* and possible MRSA occurrences at all levels in the Zambian dairy value chain including on commercial farms.

2. Study the role of livestock and other domestic animals and wild animals as potential reservoirs of virulent, enterotoxigenic and antibiotic resistant *S. aureus*/MRSA strains in Zambia.

3. Study the toxin-producing potential of *S. aureus* strains from Zambia dairy value chain.

4. Research and development of cost-efficient and reliable cooling systems including solar-powered cooling systems for small scale dairy farmers in Zambia.

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APPENDICES

Appendix A: Permissions

1. ERES Convergence research approval letter

33 Joseph Mwilwa Road
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Tel: +260 955 155 633
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Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

1st March, 2017

Ref. No. 2016-Dec-003

The Principal Investigator
Dr. Bruno S.J. Phiri
Central Veterinary Research Institute (CVRI)
P.O. Box 33980,
LUSAKA.

Dear Dr. Phiri,

RE: CHARACTERIZATION OF *STAPHYLOCOCCUSAUREUS* ASSOCIATED WITH THE DAIRY VALUE CHAIN IN WESTERN, LUSAKA AND SOUTHERN PROVINCES OF ZAMBIA.

Reference is made to your corrections dated 23rd February, 2017. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2016-Dec-003**
Approval and Expiry Date	Approval Date: 1st March, 2017	Expiry Date: 28th February, 2018
Protocol Version and Date	Version-Nil	28th February, 2018
Information Sheet, Consent Forms and Dates	• English, Lozi, Tonga, Nyanja.	28th February, 2018
Consent form ID and Date	Version – Nil.	28th February, 2018
Recruitment Materials	Nil	28th February, 2018
Other Study Documents	Questionnaires.	28th February, 2018
Number of participants approved for study	-	28th February, 2018

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- ERES Converge IRB does not "stamp" approval letters, consent forms or study documents unless requested for in writing. This is because the approval letter clearly indicates the documents approved by the IRB as well as other elements and conditions of approval.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Prof. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON

33 Joseph Mwilwa Road
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I.R.B. No. 00005948
F.W.A. No. 00011697

20th February, 2018

Ref. No. 2016-Dec-003

The Principal Investigator
Dr. Bruno S.J. Phiri
Central Veterinary Research Institute (CVRI)
P.O. Box 33980,
LUSAKA.

Dear Dr. Phiri,

RE: RENEWAL "CHARACTERIZATION OF *STAPHYLOCOCCUSAUREUS* ASSOCIATED WITH THE DAIRY VALUE CHAIN IN WESTERN, LUSAKA AND SOUTHERN PROVINCES OF ZAMBIA"

We would like to acknowledge receipt of your End of Year report date 30th January, 2018.

1. Further analysis of isolates in Germany needs to be approved by the National Health Research Ethics Committee. Please apply for this Material Transfer Agreement (MTA) approval and keep us posted on outcomes.

2. Please note that this is a late submission – refer to page 2 of your Approval letter. In future please ensure timely reporting.

The study is renewed for another year and the new expiry date is **28th February, 2019.**

Yours faithfully,
ERES CONVERGE IRB

Prof. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON

2. Permission to export human *S. aureus* isolates to Germany

THE NATIONAL HEALTH RESEARCH AUTHORITY
Paediatric Centre of Excellence
University Teaching Hospital
P.O. Box 30075
LUSAKA
T: +260 211 250309 | **E:** nhrasec@gmail.com | www.nhra.org.zm

3rd April, 2018

Dr. Bruno S.J Phiri
Central Veterinary Research Institute
LUSAKA

Re: Request for Biological Material Transfer

The National Health Research Ethics Board is in receipt of your request for authority to export biological material under the under the research titled **"Characterisation of Staphylococcus Aureus Associated with Dairy Value Chain in Western, Southern and Lusaka Provinces".**

The Board has noted your response to the concerns raised and finds it satisfactory. The Biological Material transfer is **approved.**

Yours sincerely,

Prof. Patrick Musonda
Chaiperson,
National Health Research Ethics Board

3. International sanitary certificate for S. aureus isolates from the Zambian dairy value chain

Date: 7/3/2018

ISC Number: 19797

REPUBLIC OF ZAMBIA

MINISTRY OF LIVESTOCK AND FISHERIES DEVELOPMENT

DEPARTMENT OF VETERINARY SERVICES

INTERNATIONAL SANITARY CERTIFICATE

(for animal, animal products and non- animal products linked to animal health)

This is to certify that the items hereunder listed being consigned by:

Dr BRUNO PHIRI

of UNIVERSITY OF ZAMBIA, SCHOOL OF VETERINARY MEDICINE

P.O. BOX 32379, LUSAKA

To GERMANY FEDERAL INSTITUTE FOR RISK ASSESSMENT

of UNIT 44 - NRL for NRL - Staph

DIEDERSDORFER WEG 1, 12277 BERLIN

Has/have been inspected and found to comply with the sanitary requirement(s) for export of the same to (name of Country:- GERMANY

SR No.	Item Description	Quantity	Seal Mark
	BACTERIAL LYSATES	400	

The packaging and sealing of the above named items been supervised by the undersigned and the seal mark on the package(s) container(s) is listed.

Government Veterinary Officer ONLY:

Signature:

Full Name: Dr F. M. CHIKWONDO

Rank: DISTRICT VET OFFICER

Amount paid: K50-00

REPUBLIC OF ZAMBIA
MINISTRY OF FISHERIES & LIVESTOCK
LUSAKA DISTRICT
07 MAR 2018
Official Stamp
DISTRICT VETERINARY OFFICER
P.O. BOX 30041, LUSAKA.

Distribution of copies:-
Original to accompany consignment *Duplicate to consignee* *Triplicate to DVS* *Quadruplicate to be returned*

FOR USE BY THE VETERINARY EXPORT CERTIFICATE OFFICE ONLY

Sub-Number: 1700202 26

Veterinary Export certificate Number: ZM1800032572

Date Issued:

Signature:

7 MAR 2018

NOT VALID WITHOUT AN OFFICIAL DATE STAMP AND VALID FOR ONE MONTH ONLY FROM DATE OF ISSUE.

This certificate saves as a receipt

Appendix B: Information sheet and consent form for participants

INFORMATION SHEET

THE UNIVERSITY OF ZAMBIA

The Department of Paraclinical Studies, School of Veterinary Medicine

CONSENT BY PARTICIPANT FOR PARTICIPATION IN A RESEARCH PROTOCOL

Protocol Name: Nasal and hands swabs

STUDY TITLE: **Characterization of *Staphylococcus aureus* associated with the dairy value chain in Western, Lusaka and Southern Provinces of Zambia**

1. RESPONSIBLE PERSONS AND THEIR CONTACT

Principal investigators Directing Research: Bruno Stephen July Phiri (PhD Student) and Professor Bernard Mudenda Hang'ombe, PhD (Supervisor).

Address: The University of Zambia,
Great East Campus,
Paraclinical Studies Department,
School of Veterinary Medicine,
P.O. Box 32397,
Lusaka,
Zambia.

Telephone Number: +260 211 291515

You are being asked to participate in a research study. A member of the research team will explain what is involved in this study and how it will affect you. This consent form describes the study procedures, the risks and benefits of participation, as well as how your confidentiality will be maintained. Please take your time to ask questions and feel comfortable making a decision whether to participate or not. This process is called informed consent. If you decide to participate in this study, you will be asked to sign this form.

2. WHY IS THIS STUDY BEING DONE?

The purpose of this study is to investigate what kind of Staphylococcus spp (normal bacteria) live in your nostrils and live on your hands and how this changes when it enters the cow's udder and milk. It is known that Staphylococcus is part of normal bacteria that live on our skin. But, it is not well understood how these communities of bacteria develop, or how these

communities change when they enter or contaminate cow's milk and milk products. This research is being done to define these bacterial communities, which may generate insights allowing advances in treating animals, medical diseases, advances in ecological theory, and choice of better antimicrobial substances to use.

3. HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

About 30 people in each study site will take part in this study.

4. WHAT IS INVOLVED IN THE STUDY?

During this study, Bruno Stephen July Phiri and his research team will collect information about you for the purposes of this research. You have been approached to be included in this study because you are milkers of animals. In this study, we will analyse *Staphylococcus* bacteria on hands and in the nose of your body. If you agree to participate in this study, we will ask you to swab your nostrils and the hands once every 3 months for one year. Participants will be swabbed on the palm of their both hands and a nasal swab will be performed for respiratory bacteria (*Staphylococcus spp*).

A member of the research staff will pick up the swab samples and put them in a cooler box and transport them to the microbiology laboratory at the University of Zambia. Each sample will then be analysed for *Staphylococcus* bacteria species they contain.

5. HOW LONG WILL YOU BE IN THE STUDY?

You will be in the study for 3 years and if you decide to withdraw there will be no penalty.

6. WHAT ARE THE RISKS OF THE STUDY?

There are no health risks for participation in this study.

7. ARE THERE ANY BENEFITS TO TAKING PART IN THE STUDY?

If you agree to take part in this study, there will be no direct medical benefit to you. But, we hope the information learned from this study will allow insights into how bacterial communities (*Staphylococcus*) form which may have implications in Livestock sector, animal and medical diseases, and antimicrobial substances.

8. WHAT OTHER OPTIONS ARE THERE?

Instead of being in this study, you may choose not to participate.

9. WHAT ARE THE COSTS?

There will be no costs to you or your insurance company resulting from your participation in this research study.

10. WILL I BE PAID FOR MY PARTICIPATION?

You will not be paid for participation.

11. WHAT ABOUT CONFIDENTIALITY?

Study records will be kept confidentially and no names will be asked or written down. No medical history will be obtained. We will analyse the bacteria on your hands and nostrils. All information obtained or data generated will be strictly confidential and stored in a password protected computer file and will only be accessed by the research team. The data collected in this study will be used for the purpose described in the form. The research team includes the individuals listed on this consent form and other personnel involved in this study at the University of Zambia.

Your records may be reviewed by ERES COVERGE the organization whose responsibility is to protect participants in research. This consent form will be kept by the research team for at least six years.

12. WHAT ARE MY RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. If you choose not to participate in this study, it is your right and no one will force you to do so. You may choose not to participate at any time during the study. You have the right to withdraw anytime if you feel so. You are free to seek clarification anytime during the study.

If you choose to no longer be in the study, you must inform Dr Bruno Stephen July Phiri or Professor Bernard Mudenda Hang'ombe verbally. Bruno Stephen July Phiri or Professor Bernard Mudenda Hang'ombe may still use your information that was collected prior to your written notice.

You will be given a signed copy of this document. This consent form document does not have an expiration date.

13. WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

You have to talk to Dr Bruno Stephen July Phiri or Professor Bernard Mudenda Hang'ombe about this study and you have the opportunity to ask questions concerning any and all aspects of the research. If you have further questions about the study, you may call Bruno Stephen July Phiri or Professor Bernard Mudenda Hang'ombe at +260 211 291515.

14. CONSENT FORM

14.1 Participant

The research project and the procedures associated with it have been explained to me. The experimental procedures have been identified and no guarantee has been given about the possible results. I will receive a signed copy of this consent form for my records.

I agree to participate in this study. My participation is voluntary and I do not have to sign this form if I do not want to be part of this research study.

Signature of Subject: _____

Or

Thumb print: _____

Date: _____ Time: _____ AM/PM (Circle)

15. PERSON OBTAINING CONSENT

I have explained to the participant the nature and purpose of the study and the risks involved. I have answered and will answer all questions to the best of my ability. I will give a signed copy of the consent form to the participant.

Signature of person obtaining consent: _____

Date: _____ Time: _____ AM/PM (Circle)

16. INVESTIGATOR:

Signature of Investigator _____

Date: _____ Time: _____ AM/PM (Circle)

Appendix C: Questionnaires

1. FARMER QUESTIONNAIRE

SECTION 1

A. QUESTIONNAIRE IDENTIFICATION INFORMATION

Questionnaire Identification Number: _____

Interviewer Name: _____ Date completed: _____

Time Interview started: _____

Time Interview ended: _____

Province:

_____

District:

_____

Veterinary Camp:

_____

Village/Farm:

_____

B. DEMOGRAPHIC INFORMATION (ABOUT THE FARMER) Tick where applicable

1 **Age:** _____

2 Sex:

1. Male _____
2. Female _____

3 Marital status:

0 Married____

1 Unmarried ____

4 Total number of family members living in your household?

1. 2 members ____
2. 3 members ____
3. 4 members ____
4. 5 members ____
5. More than 5 members _____

5 Formal education attended:

1. Grade 7 and lower _____
2. Grade 8 _____
3. Grade 9 _____
4. Grade 10 _____
5. Grade 11 _____
6. Grade 12 _____
7. College _____
8. University _____
9. Other please specify _____

6 Other sources of income:

Please specify _____

7. Household income per month?

1. ≤ K 500 _____
2. ≤ K1000 ____
3. ≥ K3000 _____

C. GENERAL INFORMATION

1. Do you belong to a Dairy cooperative?

0. Yes _____

1. No _____

2. When did you start the enterprise?

1. 1 year ago _____

2. 2 years ago _____

3. 3 years ago _____

4. 4 years ago _____

5. 5 years ago

6. More than 5 years ago _____

3. Who takes care of your cattle?

1. Relatives_____

2. Workers_____

a). How many are they? 1. One ____ 2. Two ____ 3. Three ____ 4. Four _____ 5. More than five ___

4. Where do you get water used for cleaning cow's udders, milking buckets and for washing hands for milkers?

1. Borehole _____

2. Well ____

3. Piped water ____

4. River ____

5 Other please specify _____

5. Do you buy any feed for your animals?

0. Yes____

1. No____

D. INFORMATION ABOUT CATTLE HERD AND OTHER LIVESTOCK AND ANIMALS

1. Indicate information about the cattle herd:

Number of cattle in this herd _____

Breed of cows _____

Cows in milk _____

Cows that have had one or more calves

_____

Adult breeding bulls _____

Female less than 1 year _____

Male less than 1 year _____

Female >1 year not yet calved _____

Male >1 year not breeding bulls _____

Total _____

a) Do you have any identification system for each cow?

0. Yes____

1. No ____

2. Indicate information of other livestock and animals kept at the farm/village:

1. Chickens-free range:_____

2. Chickens-housed:_____

3. Ducks:_____

4. Goats:_____

5. Pigs:_____

6. Dogs:_____

7. Cats:_____

8. Other please specify:_____

E. LAST CASES OF DISEASE ON THE FARM IN THE LAST 12 MONTHS

Date	Disease	Group affected	Number affected	Treatment

F. MEDICATION OF CATTLE IN THE HERD

1. Do you perform any vaccinations on your cows per year?

0. Yes ____

1. No ____

a). if Yes (1), please specify

_____

2. Do you administer any medicines or antibiotics to your cows:

0. Yes____

1. No ____

a). If Yes (2), please specify_____

3. Who treat your animals in case of a disease?

1. Veterinary official ____

2. Myself ____

3. Fellow farmer _____

4. Other please specify _____

G. ANTIBIOTIC USE

1. Do you know mastitis?

0. Yes ____

1. No ____

2. If Yes (1), how do you manage mastitis?

_____

a) Do you treat the individual cows 0. Yes_____ 1. No ____

b) If No (a) do you treat all the cows in the herd? 0. Yes _____ 1. No _____

c). What type of drugs do you give your animals?

1. Penicillin ___

2. Gentamicin ____

3. Oxytetracyclin ____

4. Penstrep (Penicillin streptomycin) ___

5. Other please specify

_____

d). How do you give drugs to your cows?

1. Oral____

2. Parental____

3. Local treatment ____

4. Other specify_____

e). Are you treating any of your cows with any drugs now?

0. Yes ___

1. No ___

f). If Yes (e), provide more information;

_____

g). How long do you usually treat your cows with antibiotics?

1. 1 day ___

2. 2 days ___

3. 3 days ___

4. 5 days ____

5. More than 5 days ____

h). If you are treating a cow with antibiotics, do

you sell the milk from that cow at the same time:

0. Yes ____

1. No ____

i). And If No (h), how long do you wait before starting to sell?

1. Day ____

2. 2 days ____

3. 3 days ____

4. 4 days ___

5. 5 days ____

6. 6 days ____

7. 7 days ____

8. according to manufactures

9. More than 7 days _____

j). How do you milk treated cows?

_____

k). Do you mix the milk from the treated cows with the milk from other cows?

0. Yes ___

1. No ___

l). Do you consume milk from treated cows? 0. Yes ___ 1. No ___

m). If No (l), what do you do with it?

1. Discard ___

2. Consume ____

3. Other please specify_____

SECTION 2

H. HANDLING AND STORAGE OF THE MILK

1 Do you normally wash your hands before milking cows?

0. Yes ____

1. No___

a) If Yes (1), do you dry your hands before milking?

0. Yes ___

1. No ____

b) If Yes (1), what do you use to dry your hands?

1. Towel ___

2. Cow's tail ____

3. My clothes ____

4. Other please specify ____

2. Do you wash hands after milking each individual cow?

0. Yes ____

1. No ____

3 Do you have a milking shed at your farm?

0. Yes ___

1. No ___

a) If Yes (3), do you clean the milking shed?

0. Yes ____

1. No____

4 What type of utensils do you use?

a. Milking: 1. Plastic containers ____ 2. Aluminium cans ___ 3. Wooden vessels 4. Other please specify

_____

b. Milk preserving: 1. Plastic containers ____ 2. Aluminium cans ___ 3. Wooden vessels 4. Other please specify

_____

c. Storing: 1. Plastic containers ____2. Aluminium cans ___ 3. Wooden vessels 4. Other please specify

_____

d. Transporting: 1. Plastic containers ____ 2. Aluminium cans ___ 3. Wooden vessels 4. Other please specify

_____

e. Selling: 1. Plastic containers ____ 2. Aluminium cans ___ 3. Wooden vessels 4. Other please specify

_____

5. How do you get the milking utensils and other milk containers cleaned**?**

a. Cold water and soap _____ b. Warm water only ____ c. Hot water and soap _____ d. Others please specify _____

_____

6. How often do you clean these milk utensils?

a. Every after milking ____ b. I do not clean ____ c. I clean before milking ____ d. Other please specify _____

7. Is milk strained or filtered? 0. Yes ____ 1. No ____

a) If Yes (7), what method do you use? 1. Sieve (mesh) _____ 2 Cloth _____ 3 Other please specify _____

8. Where do you do the milking? 1. In the milking parlour ____ 2. In the crush pen ___ 3. In the kraal ____ 4. Outside ____ 5. Other please specify

_____

a) And what preliminary preparations do you do before milking cows? 1. Clean teats of the cow _____ 2. Dry the teats with towel/cloth _____3. Wash hands _____ Other please specify _____

9. What means of transport do you use in distributing milk? 1. Head carrying _____2. Bicycle _____ 3. Public transport _____ 4. Own vehicle ____ 5. Hired vehicle _____ 6. Other, please specify _____

10. What is the distance from home to the milk market in km?

11. Do you process milk? 0. Yes ___ 1. No ___

a) If Yes (11), what milk products do you make? 1. Sour milk ___2. Yoghurt ____ 3. Butter ____ 4. Cheese ____ 5. Others please specify

_____

12. At what time(s) do you milk? 1. Morning ___ 2. Afternoon ____. 3. Other please specify ____

b). Do you consume milk directly from the teats? 0. Yes ____ 1. No ____

13. At what time do you sell your milk? 1. Morning ____ 2. Afternoon ____ 3 Other specify

_____

__

14. Where do you sell your milk? 1. Cooperative ___ 2. Individual customers ___ 3 Market ___. Other please specify ___

15. Do you have trouble to sell all the milk produced each day? 0.Yes ___ 1.No ___

a) And if Yes (15), why?

_____

I. QUALITY CONTROL

1. Do you have any quality control checks? 0. Yes ____ 1. No ____

a) If Yes (1), then what parameters do you monitor?

1. pH __ 2 Specific gravity ___ 3. Clot on boiling ___ 4. Determination of insoluble matter ___ 5. Other please specify

_____

2. How do you handle any deviations?

_____

3. How do you ensure the safety of milk while selling it to the agents and at the market or communities?

_____

4. Have you ever had your milk rejected? 0. Yes ___/1. No ___

5. If yes did you get it rejected?

_____

6.What actions did you take to prevent it from recurring?

_____

J. HEALTH STATUS OF FARMERS AND MILKERS

1. Have you experienced any skin infections/injuries in your household or on your milkers in the last 12 months? 0. Yes ___ 1. No ___

a) If Yes (1), how did you manage these infections/injuries?

_____

2. How frequently have you used antibiotics on any member of the family**?**

a. Anytime one is sick ____ b. Sometimes when one is sick ____ c. We do not use any antibiotics ____ d. Other please specify

_____

3. Have you ever taken antibiotics without prescription from the hospital or clinic? 0. Yes ___ 1. No ____

4. Have you ever experienced a situation where despite treatment with antibiotics the condition did not improve? 0. Yes ____ 1. No ____

5. Usage of milk per day

1. Do you often buy milk from other people? 0.Yes ____ 1. No ____

a) If Yes (1), what type of milk ? 1. Fresh milk ___ 2. Sour milk ___ 3. Other please specify _____

2. How do you prepare milk for home consumption? a. Raw ___ b. Boil ____c. Make sour milk ____ d. Other please specify

_____

3. Who consumes milk in your household? a. Children ____ b. Everyone ____ c. Adults ____

4. How is the milk consumed? a. Raw ___ b. Boilled ____c. Sour milk ____ d. Other please specify_____

_____

K. DOCUMENTATION

1 Do you keep any records of milk produced or sold in your herd? 0. Yes ___ 1. No ___

2 Are you able to trace back bad milk from its source? : 0. Yes ___ 1. No __

3. Do you keep any health records? 0. Yes ____ 1. No ____

2. MILK TRADER QUESTIONNAIRE

SECTION 1

A. QUESTIONNAIRE IDENTIFICATION INFORMATION

Questionnaire Identification Number: _____

Interviewer Name: _____ Date completed:_____

Time Interview started:_____

Time Interview ended: _____

Province:

_____

District:

_____

Trading Area:

_____

B. DEMOGRAPHIC INFORMATION (ABOUT THE TRADER)

Tick where applicable

1. Age**:** _____

2. Sex:

    1. Male _____
    2. Female _____

3. Marital status:

1. Married____
2. Unmarried ____

4. Total number of family members living in your household?

1. 2 members ____
2. 3 members ____
3. 4 members ____
4. 5 members ____
5. More than 5 members _____

5. Formal education attended:

1. Grade 7 and lower _____
2. Grade 8 _____
3. Grade 9 _____
4. Grade 10 _____
5. Grade 11 _____
6. Grade 12 _____
7. College _____
8. University _____
9. Other please specify _____

6. Any other sources of income:

1. Farmer ____
2. Self – employed _____
3. Working_____
4. Other please specify _____

7. Household income per month?

1. ≤ K 500 _____
2. ≤ K1000 ____
3. ≥ K3000 _____

C. GENERAL INFORMATION

1. Do you belong to a Dairy cooperative?

0. Yes _____

1. No _____

2. When did you start your enterprise?

1. 1 year ago _____

2. 2 years ago _____

3. 3 years ago _____

4. 4 years ago _____

5. 5 years ago

6. More than 5 years ago _____

3. What type of milk do you sell?

a. Raw milk ___ b. Boiled milk ___ c. Fermented milk ___ d. Other please specify

_____

4. Does any of your family members consume the milk you buy? 0. Yes ___ 1. No ____

a). If Yes (4) in what form?

_____

5. Who are your main customers?

a. Community ____ b. Market ____ c. Cooperative _____ d. Other please specify

6. How long does it take to reach them in km?

_____

7. Where do you buy the milk for your business?

a. From farmers _____ b. Cooperative/milk collection centre _____ c. Other please specify

_____

SECTION 2

D. HANDLING AND STORAGE OF THE MILK

1. What do you use to transport your milk?

A. On the head _____ b. Bicycle _____ c. A car ____d. Other (Specify)

_____

2. How many litres of milk do you buy per day?

a. ≤ 10 litres ____ b. ≤ 20 ___ c. ≥ 21 litres ___ d. other please specify

_____

3. How much do you buy your milk per litre:

1. Dry season _____ 2. Rain season _____

4. How much do you sell per litre:

1. Dry season _____ 2. Rain season _____

5. What time do you start picking up your milk?

1. Before 09:00 hrs Am _____ 2. 10:00 hrs Am ___ 3. 11:00 hrs Am ____ 4. 14:00hrs Pm ____ 5. 15:00 hrs Pm ____ 6. Other please specify_____

6. What time do you stop selling your milk? 1. 09:00 hrs Am _____ 2. 10:00 hrs Am ___ 3. 11:00 hrs Am ____4. 14:00hrs Pm ____ 5. 15:00 hrs Pm_____6. Other please specify_____

7. Do you have temperature control facilities? 0. Yes ____ 1. No ____

a). If Yes (7), please specify

_____

8. What containers do you use for milk storage?

1. Aluminium ____ 2. Stainless Steel ____ 3. Plastics____ 4. Others please Specify:

_____

9. What type of container(s) do you use for selling milk?

a) Wide necked-aluminium vessels _____

b) Wide necked-plastic vessels _____

c) Narrow necked plastic containers _____

d) Used plastic water bottles _____

e) Other(s) specify_____

10. How do your customers get the milk?

a) I deliver to their places? 0. Yes ___ 1. No _____

b) (specify the means of transport) _____

c) At a special selling point _____

d) Other please Specify_____

11. Do you test for the quality of milk you buy or sell? 0. Yes ___ 1. No ___

a). If Yes (11), what parameters do you test for?

1. pH ____ 2. Specific gravity ____ 3. Clot on boiling ___ 4. Determination of insoluble matter ___ 5. Other please specify

_____

12. Do you sell all the milk that you collect? 0. Yes____ 1. No___

a). If No (12), what do you do with the unsold milk?

_____

13. If you discard unsold milk, what percentage of milk do you discard every week?

_____

14. How do you decide when it is no longer “okay” to sell that batch of milk?

_____

15. Do you preserve the milk in any way? 0. Yes____ 1. No___

a). If Yes (15), how do you do it:

_____

16. Do you ever mix different batches of milk? (eg. Morning and-afternoon): 0. Yes___ 1. No___

17. Do you ensure the general hygiene of yourself before handling the milk: 0. Yes ____ 1. No ____

a). If Yes (17), Please specify:

_____

18. How do you ensure that the equipment/containers are clean before using them?

_____

19. How is your cleaning routine for the milk containers? Please indicate

a) Cleaning just before putting in milk _____

b) Cleaning after delivery of milk _____

c) Twice a day before putting in milk and after delivery of milk ____

d) Other please specify_____

20. Do you ever add anything to your milk for some reason: 0. Yes____ 1. No____

E. PUBLIC HEALTH

1 Have you ever experienced any skin conditions in your household? 0. Yes ___ 1. No ____

a). If Yes (1), how did you manage these infections?

_____

2. Have you ever heard about food hygiene and its importance? 0. Yes ___ 1. No ___

a). If Yes (2), what do you know about food hygiene?

_____

3 In your opinion is food hygiene important to human beings? 0. Yes ___ 1. No ____

F. MILK QUALITY CONTROL

1. Do you have any milk quality control checks: 0. Yes____ 1. No____

a) If Yes (1), how do you handle any deviations? _____

2. How do you ensure the safety of milk while selling it to the consumer/customer?

_____

3. Have you ever had your milk rejected: 0. Yes____ 1. /No____

a). If Yes (3),

why did you get it rejected: _____

4. What actions did you take to prevent it from recurring?

_____

5. What type of container do you use to fetch milk from the holding storage container?

1. A cup with a handle ___

2. A cup without a handle ____

3. Other(s) specify_____

G. EXPERIENCE

1.How long have you been working as a vendor/agent/seller?_____

2. Do you have any education within the area of food hygiene/safety? 0. Yes____ 1. No____

H. DOCUMENTATION

1. Do you keep any records? 0. Yes____ 1. No ____

2. Are you able to trace back bad milk to its source? 0. Yes____ 1. No____

3. MILK COLLECTION CENTRE QUESTIONNAIRE

SECTION 1

A. QUESTIONNAIRE IDENTIFICATION INFORMATION

Questionnaire Identification Number: _____

Interviewer Name: _____ Date completed: _____

Time Interview started:_____

Time Interview ended: _____

Province:

_____

District:

_____

Name of Milk collection Centre

_____

B. DEMOGRAPHIC INFORMATION (Milk collection centre)

Tick where applicable

1. Total number of active members in the cooperative? _____

2. When was the milk collection centre established? _____

C. GENERAL INFORMATION

1. How many litters of milk does the centre receive per day? 1. Dry season _____ 2. Rain season _____

2. How many times does the centre receive milk per day?

a. Once per day ____ b. Twice per day ____ c. Other please specify

_____

3. What parameters are checked in order to accept/reject milk?

a. pH ____ b. Specific gravity ____ c.. Clot on boiling _____ d. Determination of insoluble matter ____ e. Water content ____f. Other please specify

_____

4. How is the acceptance/rejection percentage in general?

_____

5. Is there a chilling/cooling machine? 0. Yes _____ 1. No _____

6. How long does the milk stay before it is transported to the factory/processing plant?

_____

SECTION 2

D. HANDLING AND STORAGE OF THE MILK

1. How is the milk transported to the centre?

a. Bicycle ___ b. Vehicle____ c. On the head _____ Other means (specify)

_____

2. What time is the milk transported to the centre?

a. Morning ____ b. Afternoon ____ c. Other specify

_____

3. How long does it take for milk to be transported to the factory/processing plant in hours?

_____

4. What types of containers are used by farmers to get milk to the collection centres?

1. Plastic containers ____ 2. Aluminium cans ___ 3. Wooden vessels___d. Other please specify _____

5. What type of milk do you receive at the centre? a. Fresh milk ___ b. Sour milk ____

6. Do you process milk? 0. Yes ___ 1. No ___

a). If Yes (6), what type of products do you produce? Pease specify

_____

7. Is the milk always of good quality? 0. Yes ____ 1. No _____

a). If No (7) what do you do with the milk which is not of good quality?

_____

8. How many liters of milk do you sell per day? 1. Dry season _____ 2. Rains season _____

9. How much do you buy your milk per litre: 1. Dry season _____ 2. Rain season _____

10. How much do you sell milk per litre: 1. Dry season _____2. Rain season _____

11. And who are your main clients?

_____

12. Do the clients collect milk they buy themselves? 0. Yes ____ 1. No _____

13. Are there any agents who deliver milk to the clients on your behalf? 0. Yes ____ 1. No ____

a). If Yes (13) how many different agents deliver milk for you?

_____

14. How long do you hold the milk before it usually gets sold?

_____

15. Do you sell all the milk received: 0. Yes ____ 1. No ____

16. If No (15) what do you do with the rest of the milk?

_____

17. If you discard unsold milk, what percentage of milk do you discard every week?

_____

18. How do you decide when it is no longer “okay” to sell that batch of milk?

_____

a). Do you mix the morning and afternoon milk batches? 0. Yes ____ 1. No ____

19. Do you preserve the milk in any way: 0. Yes ____ 1. No____

20. How do you ensure that general hygiene of yourself /staff is observed before handling the milk?

_____

22. How do you ensure that the milk equipment/container is clean before using it?

_____

23. Do you ever add anything to your milk for some reason: 0. Yes ____ 1. No ____

E. QUALITY CONTROL

1. Do you have any quality control checks? 0. Yes____ 1. No ____

a). If yes, what parameters do you monitor?

1. pH ___ 2 Specific gravity ___ 3. Clot on boiling ___ 4. Determination of insoluble matter ___ 5. Other please specify

_____

2 How do you handle any deviations?

_____

3. How do you ensure the safety of milk while selling it to the customer?

_____

4. Have you ever had your milk rejected? 0. Yes ___ 1. No ___

a). If Yes (4), why was it rejected?

_____

5. What actions did you take to prevent it from recurring?

F. PUBLIC HEALTH

1. Do you check or test for antibiotic residues? 0. Yes ____ 1. No____

2. Do you do microbial analysis? 0. Yes ____ 1. No ____

3. Do you have health certificates for staff handling milk? 0. Yes ____ 1. No ___

4. Have you ever experienced skin infections among your staff? 0. Yes ____ 1. No ____

a).If Yes (4), how did you manage it?

G. EXPERIENCE

1. How long has the centre been working as a milk seller? _____

2. Do you have any education within the area of food hygiene/safety among your staff?

0. Yes ____ 1. No ____

H. DOCUMENTATION

1. Do you keep any records? 0. Yes ___ 1. No ____

2. Are you able to trace back bad milk to its source? 0. Yes ___ 1. No ___

4. SUPERMARKET/SHOP QUESTIONNAIRE

SECTION 1

A. QUESTIONNAIRE IDENTIFICATION INFORMATION

Questionnaire identification Number: _____

Interviewer Name: _____ Date completed:

_____

Time Interview started: _____

Time Interview ended: _____

Province:

_____

District:

_____

Supermarket Locality:

_____

B. DEMOGRAPHIC INFORMATION (ABOUT THE SUPERMARKET HEAD)

Tick where applicable

1. Age: _____
2. Sex:
    1. Male _____
    2. Female _____

3. Marital status:
    1. Married____
    2. Unmarried ____

4. Formal education attended:
    1. Grade 7 and lower _____

2. Grade 8 _____

3. Grade 9 _____

4. Grade 10 _____

5. Grade 11 _____

6. Grade 12 _____

7. College _____

8. University _____

9. Other please specify _____

5. How many workers do you have?

1. one ____ 2. Two _____ 3. Three _____ 4. Four _____ 5. More than four _____

C. GENERAL INFORMATION

1. What type of milk product(s) do you sell?

1) Raw milk _____

2) Boiled milk _____

3) Fermented milk _____

4) Other (s) specify_____

2. What time do you get milk/milk products from the producer(s)?

1) Morning hours ____

2) Afternoon hours ____

3) Evening hours _____

4) Other (s) specify_____

3. Where do you get fresh milk from?

1) A recognized vendor(s) in the area _____

2) Famer(s) in the neighbouring village _____

3) Farmer(s) from the same village _____

4) Other(s) specify_____ _____

4. Apart from fresh milk, do you get other milk products elsewhere? 0. Yes ____ 1. No ___

a). If Yes (4), which ones?

1) Sour milk ____

2) Yoghurt _____

3) Pasteurized milk _____

4) Other(s) specify_____

5. How do you get the milk/milk products at the shop/supermarket?

1) Delivered by the producer ____

2) Delivered by agents ____

3) Collect form the producer on foot ____

4) Use own transport to get from the producer ____

5) Other(s) specify_____

6. What type of containers are used to deliver milk to the shop/supermarket?

1) Wide necked-aluminium vessels ____

2) Wide necked-plastic vessels _____

3) Narrow necked plastic containers _____

4) Used plastic water bottles _____

5) Other please specify_____

7. How many litres on average do you receive from suppliers? _____

SECTION 2

D. HANDLING AND STORAGE OF THE MILK

1. Approximately how long in hours does it take for fresh milk to finish? _____

2. Name the main Zambian milk products you sell?

_____

3. How much do you buy your fresh milk per litre?_____

a). and how much do you sell it for? _____

4. How long do you hold the milk before it is usually sold? _____

5. Do you sell all the milk delivered? 0. Yes ____ 1. No ____

6. If No (5), what do you do with the rest?

_____

7. If you discard unsold milk, what percentage of milk do you discard every week?

_____

8. How do you decide when it is no longer “okay” to sell that batch of milk?

_____

9. How do you store the milk?

_____

10. Do you sell sour milk (Mabisi) commercially produced: 0. Yes ____ 1. No ____

11. Do you ever mix different batches of milk? (eg. Morning and afternoon) 0. Yes ____ 1. No _____

12. Do you mix milk from different sources? 0. Yes ____ 1. No. _____

13. How do you ensure the general hygiene of yourself /staff before handling the milk?

_____

E. QUALITY CONTROL

1. Do you have any quality control checks? 0. Yes____ 1. No____

2. How do you ensure the safety of milk while selling it to the customer?

_____

3. Have you ever had your milk rejected by customers? 0. Yes ____ 1. No ____

4. Why did you get it rejected?

_____

5. What actions did you take to prevent it from recurring?

_____

6. Do you do any fumigation in your shop/supermarket? 0. Yes ____ 1. No ____

a). If Yes (6), how often do you do it? _____

F. PUBLIC HEALTH

1. How long have you been operating as a business_____

2. Do you have health certificates for staff handling milk or food products? 0. Yes ____ 1. No ____

3. Have you ever experienced skin infections among workers handling milk? 0. Yes ___ 1. No ____

a) If Yes (3), how did you treat it?

_____

4. Are your workers handling milk subjected to healthy checkups regularly? 0. Yes____ 1. No____

a). If Yes (4) how often? _____

5. When was the last time workers had a health checkup?

_____

6. Have you ever got any complaints from the customer (s) after consumption of milk on the following conditions?

1) Vomiting _____

2) Diarrhea _____

3) Amoebic dysentry _____

4) Malaise_____

5) Other (s) specify_____

G. DOCUMENTATION

1. Do you keep any records of the milk purchases/sells? 0. Yes ____ 1. No ____

2. Are you able to trace back bad milk to the source? 0. Yes ____ 1. No ____

5. MILK CONSUMER QUESTIONNAIRE

SECTION 1

A. QUESTIONNAIRE IDENTIFICATION INFORMATION

Questionnaire Identification Number: _____

Interviewer Name: _____ Date completed:

_____

Time interview started:_____

Time interview ended: _____

Province:

_____

District:

_____

B. DEMOGRAPHIC INFORMATION (MILK CONSUMERS)

Tick where applicable

1. Age: _____

2. Sex:

    1. Male _____
    2. Female _____

3. Marital status:

    1. Married____
    2. Unmarried ____

4. Total number of family members living in your household?

    1. 2 members ____
    2. 3 members ____

3. 4 members ____

4. 5 members ____

5. More than 5 members _____

5. Formal education attended:

1. Grade 7 and lower _____

2. Grade 8 _____

3. Grade 9 _____

4. Grade 10 _____

5. Grade 11 _____

6. Grade 12 _____

7. College _____

8. University _____

9. Other please specify _____

6. Occupation:

1. Farmer ____

2. Businessman _____

3. Self – employed _____

4. Working_____

5. Other please specify _____

7. Household income per month?

1. ≤ K 500 _____

2. ≤ K1000 ____

3. ≥ K3000 _____

C. GENERAL INFORMATION

1. What type of milk do you usually buy?

a. Raw fresh milk ____ b. Sour milk ___ c. Other please specify

_____

a) How do you consume milk or milk products?

1. In porridge ____ 2. In nshima ____ 3. As raw milk ___ 4 As sour milk ____ 5. Other please specify _____

b). Do you boil the milk before consuming or using it? Yes ___ 1. No. ____

2. How often do you buy your milk?

_____

3. How much milk does your family consume in a week?

_____

4. How is the quality of fresh milk purchased?

a) Morning 1. Good _____ 2. Bad _____

b) Evening 1. Good _____ 2. Bad _____

5. What is the price of fresh milk purchased per litre? a. In dry season _____b. In rain season _____

6. Where do you buy your fresh milk?

a) Private vendors/traders ____

b) Milk collection centre (Cooperative) ____

c) Diary Farmers _____

d) Shop/ Supermarket _____

e) Any other (Please Specify) _____

7. Do you purchase milk every day? 0. Yes ____ 1. No. ____

a). If No (7), how often?

_____

8. Please indicate reasons for patronizing the present milk supplier?

a) Regularity in supply _____

b) Time availability _____

c) Reasonable price _____

d) Quality _____

e) Credit facility _____

f) Door Delivery _____

g) Long time association _____

h) No alternative choice _____

i) Children's Choice _____

j) Any other (please Specify) _____

9. Do you ever buy shelf stable milk or processed fresh milk? 0. Yes _____ 1. No _____

a). If Yes (9), how long do you leave it on the shelf?

_____

b). If yes (9), why do you buy shelf stable milk?

1) I like the taste ____

2) I like organic milk and a lot of shelf stable milk comes that way _____

3) I buy it for my kids ____

4) Other (s) specify: _____

10. How do you use milk you buy?

a) Sweet making _____

b) Curd making _____

c) Cake making _____

d) Porridge _____

e) Tea/Coffee etc. _____

f) Drinking as such _____

g) Other Please Specify)_____

11. What Zambian milk Products are consumed in your household monthly?

Please indicate the brand name:

(a) Milk Powder

_____

(b) Butter:

_____

(c) Cheese

_____

(d) Yogurt

_____

(e) Other Products

_____

Please specify

12. State the reason/reasons for your preference of the various milk product brands?

1. Quality___
2. Price ____
3. Taste___
4. Packing____
5. Convenience ____

6. Any other (Please specify)

_____

13. Do you think locally produced milk products are better than those imported outside Zambia? 0. Yes ___ 1. No ___

14. What is your opinion about the price of the products of local manufacturer compared to the outside products? 1. High ____ 2. Low _____

15. Are the locally produced dairy products easily available in the market? 0. Yes ___ 1. No ___

16. Are you aware of milk adulterated being supplied by:

(a) Private milk vendors: 0. Yes ___ 1. No ___

(b) Supermarket: 0. Yes ___ 1. No ___

17. What is the choice of food by adults in order of preference (Rank it as 1,2,3 etc.), 1-ranked first, 2-ranked second, 3- ranked third, 4- ranked fourth etc.

(a) Meat _____

(b) Milk & Milk Products _____

(c) Eggs _____

(d) Fish _____

(e) Other food items (Please specify)

_____

18. Choice of food by children in your family (Rank them as 1,2,3 etc)

(a) Meat _____

(b) Milk & Milk products _____

(c) Eggs _____

(d) Fish _____

(e) Other food items please specify

_____

SECTION 2

D. HANDLING AND STORAGE OF THE MILK

1. Do you normally wash your hands before handling milk? 0. Yes ___ 1. No ___

2. What type of utensils do you use for milk buying, preserving / storing?

1. Plastic container____2. Aluminium cans ___ 3. Other (Specify)

_____

3. How do you get the milk buckets/containers cleaned before and after milk is bought?

1. I use cold water and soap _____ 2. I use warm water ____ 3. I use hot water and soap _____ 4. Others please specify _____

4. Is milk strained or filtered when bought? 0. Yes __ 1. No ___

a). If Yes (4), what method do you use?

1. Sieve (mesh) _____ 2. Cloth (cotton cloth) _____ 3 other please specify

_____

5. Do you boil raw milk when it is bought before drinking or using it? 0. Yes ___ 1. No ___

a) If No (5), do you drink or consume raw milk some times? 0. Yes ____ 1. No _____

6 What means of transport do you use to have your milk in your household?

1. Head carrying _____ 2. Bicycle ___ 3. Public transport _____ 4. Own vehicle ____5. Hired vehicles _____6. Other please specify)

_____

7. What is the distance from home to the milk source in km?

_____

8. Have you received milk hygiene training before? 0. Yes ___ 1. No ___

a). And if Yes (8), who conducted the training?

_____ (eg government, non-governmental organisation etc)

b). And was the training helpful in anyway? 0. Yes ___ 1. No ___

9. What is the average quantity of milk you buy per day? 1 Dry season_____ 2. Rain (Wet) season _____

10. How do you store your milk?

_____

11. Do you process milk? Yes __/ No __

a) If Yes (11), what milk products do you make?

a. Sour milk _____ b. Yoghurt ____ d. Butter ____ e. Cheese ____ f. Others please specify

_____

12. At what time(s) do you buy milk?

_____

13. Is milk delivered to your household sometimes? 0.Yes _____ 1. No ____

a). If Yes (13), what time? _____

b). and how often?

_____

14. Do you have trouble to buy all the milk delivered to you each day? 0. Yes ___ 1. No ___

a). and if Yes (14), why? give more information:

_____

15. What is your unit of measure when buying milk? 1. Litre ____ 2. Cup ____ 3. 2.5 litres plastic container ____ 4. Other please specify

_____

16. Who sets the price of milk?

1. Producer ____ 2. Buyer____ 3. Negotiator _____ 4. Other please specify

_____

E. PUBLIC HEALTH

1 Have you ever experienced any skin conditions in your household? 0. Yes ___ 1. No ____

a). If Yes (1), how did you manage these infections?

_____

2. Have you ever heard about food hygiene and its importance? 0. Yes ___ 1. No ___

a). If Yes (2), what do you know about food hygiene?

_____

3 In your opinion is food hygiene important to human beings? 0. Yes ___ 1. No ____

F. QUALITY CONTROL

1. Do you have any quality control checks? 0. Yes ____ 1. No ____

a). If Yes (1), then what parameters do you monitor?

1. pH ___ 2 Specific gravity ___ 3. Clot on boiling ___ 4. Determination of insoluble matter___ 5. Other please specify _____

2. How do you handle any deviations?

_____

3. How do you ensure the safety of milk before you consume it?:

_____

4. Have you ever rejected milk? 0. Yes ___ 1. No ___

5. Why did you reject it?

_____

6. What did you do with the milk you rejected? _____

7. What actions did you take to prevent it from recurring: _____

Appendix D: Preparation of media and antibiotics

1. Preparation of agarose gel

To prepare 2% Small Gel, 100 ml of TBE was added into a flask with stirring magnet and 2 g of Agarose **(**Sigma Aldrich, Germany**)** was added as well. The flask was then heated in the microwave until all the Agarose was dissolved. Then the heated Agarose was poured in a small Gel chamber to cool and solidify. To prepare 2% Big Gel, 200 ml of TBE was added into a flask with stirring magnet and added 4 g of Agarose and the flask was then heated in the microwave until all the Agarose was dissolved. Then the heated Agarose was poured into a big Gel chamber to cool and solidify. TBE (10x) contained 108g of Tris, 55g Boric acid, 500ml distilled water and 40ml of EDTA (0.5M).

2. Preparations of antibiotics

a) Aztreonam (50 mg/litre)

Weighed 50 mg of aztreonam (Sigma Aldrich, Germany**)** on a weighing balance and put it into a 50 ml tube and added 25 ml of sterile distilled water and vortexed to mix.

b) Cefoxitin (4 mg/litre)

Weighed 100 mg of Cefoxitin (Sigma Aldrich, Germany) and put it into a 50 ml tube and added 25 ml of sterile distilled water and vortexed to mix. Then 1.5 ml sterile empty tubes were filled with one ml of diluted cefoxitin until all the 25 ml of diluted cefoxitin prepared was finished by producing 25 filled tubes and these were stored at -20°C until used.

3. Preparation of bactone peptone bouillon(BPB) broth

Added 15 g of Bacton Peptone Bouillon (broth) powder (BPB, BECTON DICKINSON, USA) in one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions and allowed it to cool at room temperature and it was then stored at 4°C until its use.

4. Preparation of Baird Parker agar (BPA)

Added 58 g of BPA powder (Sigma Aldrich, Germany, MERK KGaA, Germany) in 950 ml of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions and allowed it to cool at room temperature to about 45°C to 50°C and then 50 ml of egg yolk tellurite emulsion (Sigma-Aldrich, Germany) was added. The mixture was mixed by gently shaking and poured the liquid agar onto to the plates before solidification and allowed the agar to solidify.

5. Preparation of brain heart infusion (BHI) agar

Added 52 g of BHI agar powder (Sigma-Aldrich, Germany) in one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions and allowed it to cool at room temperature to 45 to 50°C then poured the liquid agar onto to the plates before solidification and allowed the agar to solidify at room temperature.

6. Preparation of brain heart infusion (BHI) both

Added 37g of BHI *(*Sigma-Aldrich, Germany/Thermo Fisher Scientific, Germany) broth was added into one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions and allowed it to cool at room temperature. Then it was put at 4°C until its use.

7. Preparation of buffered peptone water (BPW) broth

Added 20g of BPW broth powder (HiMedia, India) in one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions and allowed it to cool at room temperature and it was put at 4°C until it was used.

8. Preparation of glycerol cultures

Added 13 g of nutrient broth powder (HiMedia, India) in one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions and allowed it to cool at room temperature and it was put at 4°C until it was used. Then added one ml of nutrient agar in vials and then added 200 µl of 95 percent glycerol and pure colonies were introduced into the mixture. The mixture was vortexed and stored at -20°C until shipping to Germany.

9. Preparation of mannitol salt agar (MSA)

Added 111g of MSA powder (Thermo Fisher Scientific, Germany/HiMedia, India) was added into one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions. Then the liquid agar was allowed to cool at room temperature up to approximately 45 to 50°C and then poured into sterile plates and allowed the agar to solidify at room temperature until plating/culturing. The remaining plates after culturing were then put at 4°C until they were used again.

10. Preparation of mannitol salt agar with cefoxitin (MSA + cef)

Added 111g of MSA powder (Thermo Fisher Scientific, Germany/Himedia, India) into one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions. Then the hot liquid agar was allowed to cool at room temperature up to approximately 45 to 50°C and added one ml of cefoxitin (4mg/l, Sigma Aldrich, Germany).

Then the media was mixed thoroughly by gently shaking the bottle to ensure the antibiotics were mixed and the warm liquid agar was poured into sterile plates before solidifying, then allowed to solidify at room temperature until plating/culturing. The remaining MSA plates after culturing were then put at 4°C until the next bacterial plating.

11. Preparation of Muller Hinton broth (MHB) with 6% sodium chloride

Added 21 g of MHB broth powder (Thermo Fisher Scientific, Germany/HiMedia, India) and 60 g of sodium chloride (NaCl) (to make 6 % NaCl) was added into one litre of distilled water and autoclaved at 121°C for 15 minutes according to manufacturer's instructions. Then the hot mixture was allowed to cool at room temperature. Then it was put at 4°C until use.

12. Preparation of plate count agar

Added 17.5 g of plate count agar powder (Sigma Aldrich, Germany/MERK KGaA, Germany) into one litre of distilled water and stirred to dissolve the powder by shaking the bottle gently and then autoclaved the mixture at 121°C for 15 minutes according to manufacturer's instructions. Then the hot agar was allowed to cool to about 50°C and it was then poured on the sterile plates prepared for total plate count (TPC). The temperature was maintained at approximately 50°C by putting the agar bottle in the hot water bath.

13. Preparation of tryptic soy broth with cefoxitin (4 mg/l) and aztreonam (50 mg/l)

Measured one litre of distilled water using measuring cylinder and transferred it in a one litre autoclavable glass bottle. Then added 30 g (according to instructions from supplier) of powdered tryptic soy broth (Merck KGaA, Germany) into one litre of distilled water in a bottle and dissolved completely by heating on a flame while gently shaking. The mixture was autoclaved at 121°C for 15 minutes according to manufacturer's instructions and allowed to cool to 50°C and then 25 ml of tryptic soy broth was taken out from a one litre bottle and was discarded. Then the one litre bottle of tryptic soy was refilled with 25 ml of prepared aztreonam (50 mg/l, Sigma Aldrich, Germany) and then added 875 µl of prepared cefoxitin (4 mg/l, Sigma Aldrich, Germany). Then the media was mixed thoroughly by gently shaking the bottle to ensure the antibiotics were mixed very well and the broth was allowed to cool at room temperature and put in the refrigerator (4°C) until it was used.

Appendix E: Control strains (positive and negative)

1. BfR-ST 00129 (*S. aureus*) from strain collection of NRL Staph in Berlin, Germany (from mouse) was used as mannitol fermentation positive control and BfR-ST 01164 (*S. aureus*) from strain collection of NRL Staph, Berlin, Germany (from unknown matrix) was used as a mannitol fermentation negative control.
2. BfR-ST 00167 (MRSA) from strain collection of NRL Staph in Berlin, Germany (from chicken) was used as positive control (growing on MSA + cef and fermenting mannitol) and BfR-ST 00129 (*S. aureus*) from strain collection of NRL Staph, Berlin, Germany (from mouse) was used as a negative control (no growth on MSA +cef).
3. BfR-ST 00167 (MRSA) from strain collection of NRL Staph in Berlin, Germany (from chicken) was used as positive control (growing in tryptic soy broth + azetreonam + cef) and BfR-ST 00129 (*S. aureus*) from strain collection of NRL-Staph, Berlin, Germany (from mouse) was used as a negative control (no growth in tryptic soy broth + azetreonam + cef).
4. BfR-ST 00167 (MRSA) and BfR-ST 00129 (*S. aureus*) from strain collection of NRL Staph in Berlin, Germany (from chicken and mouse respectively) were used as positive controls (growing on BPA).
5. BfR-ST 00129 (*S. aureus*) from strain collection of NRL Staph in Berlin, Germany (from mouse) was used as positive control (coagulase positive) and BfR-ST 00886 (*S. epidermidis*) from strain collection of NRL Staph, Berlin, Germany (from cow milk) was used as a negative control (coagulase negative).
6. 11S01380 (MRSA) from strain collection of NRL Staph in Berlin, Germany (from wild boar) was used as positive control for MRSA identification Real Time PCR.
7. DSM 1104 (*S. aureus*) from Leibniz Institute, DSMZ-German Collection of Microorganisms and cell cultures GmbH (from clinical isolate, country of origin is unknown) was used as positive control for *spa* typing PCR.
8. DSM 13661 *S. aureus* from Leibniz Institute, DSMZ-German Collection of Microorganisms and cell cultures GmbH (from human clinical, USA) was used as positive control for DNA preparation.

Appendix F: Bruno S. J. Phiri at the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung) in Berlin, Germany

Appendix G: Potential persistence of *S. aureus* on farms during both sampling periods

Sampling period (1./2.)	Province	District	Facility type	Facility no.	Isolate short name	Matrix	*spa* type
1.	Southern	Choma	Farm	44	**C39**	pooled milk	t521
2.	Southern	Choma	Farm	44	**C93**	pooled milk	t521
1.	Southern	Choma	Farm	60	**C46**	pooled milk	t521
2.	Southern	Choma	Farm	60	**C100**	pooled milk	t521
1.	Southern	Choma	Farm	63	**C48**	pooled milk	t521
2.	Southern	Choma	Farm	63	**C103**	pooled milk	t521
1.	Southern	Choma	Farm	86	**C66**	pooled milk	t521
2.	Southern	Choma	Farm	86	**C110**	pooled milk	t521
1.	Southern	Choma	Farm	96	**C69**	pooled milk	t521
2.	Southern	Choma	Farm	96	**C116**	pooled milk	t521
1.	Lusaka	Chilanga	Farm	4	**L1**	pooled milk	t189
2.	Lusaka	Chilanga	Farm	4	**L114**	pooled milk	t189
1.	Lusaka	Chilanga	Farm	11	**L72**	bucket swab	t189
2.	Lusaka	Chilanga	Farm	11	**L117**	pooled milk	t189
1.	Lusaka	Chilanga	Farm	21	**L75**	bucket swab	t189
2.	Lusaka	Chilanga	Farm	21	**L121**	pooled milk	t189
1.	Lusaka	Chilanga	Farm	29	**L49**	mastitis milk	t189
2.	Lusaka	Chilanga	Farm	29	**L126**	pooled milk	t189
1.	Lusaka	Chilanga	Farm	39	**L19**	pooled milk	t189
2.	Lusaka	Chilanga	Farm	39	**L130**	pooled milk	t189
1.	Lusaka	Chongwe	Farm	56	**L27**	pooled milk	t189
2.	Lusaka	Chongwe	Farm	56	**L138**	pooled milk	t189
1.	Lusaka	Chongwe	Farm	75	**L96**	bucket swab	t189
2.	Lusaka	Chongwe	Farm	75	**L147**	pooled milk	t189
1.	Lusaka	Chilanga	Farm	1	**L70**	bucket swab	t237
2.	Lusaka	Chilanga	Farm	1	**L112**	pooled milk	t237
1.	Lusaka	Chilanga	Farm	9	**L2**	pooled milk	t267
2.	Lusaka	Chilanga	Farm	9	**L116**	pooled milk	t267
1.	Lusaka	Chilanga	Farm	27	**L77**	bucket swab	t267
2.	Lusaka	Chilanga	Farm	27	**L125**	pooled milk	t267
1.	Lusaka	Chilanga	Farm	44	**L87**	bucket swab	t267
2.	Lusaka	Chilanga	Farm	44	**L133**	pooled milk	t267
1.	Lusaka	Chilanga	Farm	40	**L83**	bucket swab	t355
2.	Lusaka	Chilanga	Farm	40	**L131**	pooled milk	t355
1.	Lusaka	Chongwe	Farm	50	**L65**	hands swab	t521
2.	Lusaka	Chongwe	Farm	50	**L134**	pooled milk	t521

Appendix H: Reprint of published material

Bruno S.J. Phiri, Mushekwa Sakumona, Bernard M. Hang'ombe, Alexandra Fetsch, Sara Schaarschmidt. 2021. The traditional dairy value chain in Zambia and potential risk factors to microbiological food safety. Food Control. 124, 107885, ISSN 0956-7135, https://doi.org/10.1016/j.foodcont.2021.107885.