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OCCURRENCE AND ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM RAW MILK AND TRADITIONALLY PROCESSED DAIRY PRODUCTS IN AND AROUND HAWASSA TOWN, ETHIOPIA

MSc THESIS

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DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH

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BISHOFTU, ETHIOPIA

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A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfilment of the requirements for the degree of Master of Sciences in Veterinary Public Health

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The thesis entitled "Occurrence and antimicrobial susceptibility profile of *Staphylococcus aureus* from raw cow milk and traditionally processed dairy products in and around Hawassa town, Sidama, Ethiopia" was prepared by Eskedar Wodaje under the supervision of the MVSc research advisors. As the advisors, we have carefully read and assessed the thesis, and we recommend that it be approved as fulfilling the thesis requirement for the Master of Science in Veterinary Public Health degree.

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As members of the examining board for MVSc open defense, we have carefully read and reviewed **Eskedar Wodaje Ayalew's** thesis, titled **"Occurrence and antimicrobial susceptibility profile of *Staphylococcus aureus* from raw cow milk and traditionally processed dairy products in and around Hawassa town, Ethiopia."** Based on our careful examination of the thesis, we accepted that it be approved as satisfying the requirement for the thesis of a Master of Science in Veterinary Public Health degree.

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I, Eskedar Wodaje, hereby state that I am the writer of this thesis and have properly credited all the sources of information used. The Addis Ababa University College of Veterinary Medicine and Agriculture has accepted this thesis as partial fulfilment of the requirements for an MSc degree. This thesis is now housed in the university or college library and will be accessible to borrowers according to the library's policies.

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ABBREVATIONS

BTS -QC	Bacterial Test Standard Quality Control
CDC	Center For Disease Prevention and Control
CHCA	Cyano Hydroxycinnamic Acid
CNS	Coagulase Negative Staphylococcus
CPS	Coagulase Positive Staphylococcus
ESI	Electrospray Ionization
FBD	Food Borne Disease
LDI	Laser Desorption Ionization
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time of Flight
MRSA	Methicillin-Resistant *Staphylococcus aureus*
PCR	Polymerase Chain Reactions
PMF	Peptide Mass Fingerprint
SCV	small colony variations
SEA	Staphylococcus Enterotoxin A
SEB	Staphylococcus Enterotoxin B
SEC	Staphylococcus Enterotoxin C
SED	Staphylococcus Enterotoxin D
SEE	Staphylococcus Enterotoxine E
SELDI	Surface-Enhanced Laser Desorption Ionization
SFP	Staphylococcus Food Poisoning
WHO	World Health Organization

ABSTRACTS

Staphylococcal foodborne poisoning is a well-known global health concern. This study aimed to investigate the occurrence of *Staphylococcus aureus* and its antimicrobial susceptibility profiles in raw cow milk and traditionally processed dairy products in Hawassa town, Sidama Region, Ethiopia. A cross-sectional study conducted from December 2023 to May 2024. Totally 386 samples were collected and cultured using standard microbiological techniques and further identification and confirmation of *S. aureus* was done using MALDI-TOF method and conventional PCR was used to detect the resistance gene. *S. aureus* was isolated and identified in 54 (13.99%) of the samples, with the highest prevalence in raw milk (20.7%), followed by bulk tank milk (5.0%) and yoghurt (2.4%). Number of *S. aureus* was not detected in cheese samples. Antimicrobial susceptibility testing revealed high resistance to Tetracycline (46.3%), Penicillin G (85.2%), Ampicillin (92.6%), and Amoxicillin (100%). Notably, 87.4% of the *S. aureus* isolates exhibited multiple drug resistance, posing a significant public health concern. PCR-based detection showed that all 25(46.3%) *S. aureus* isolates were positive for the *nuc* gene, but none possessed the *mecA* or *blaZ* genes. A questioner survey found that 40(40.0%) of the population consumed raw milk, 61(61.0%) had no food safety knowledge, and 77(77.0%) were unaware of Staphylococci foodborne illness. These findings demonstrate the need for improved food safety and hygiene practices, as well as the development of new antimicrobial containment strategies to address the high prevalence of MDR *S. aureus* in milk and milk products. Further investigation is warranted to understand the mechanisms of *S. aureus* resistance to methicillin resistance and β-lactam antibiotics.

Keywords: *Antimicrobial resistance, Milkers hand, S. aureus, Dairy products, MALDI-TOF*

1. INTRODUCTION

Milk is the most nutritious and well-balanced food. Although milk is naturally nutrient-rich, it also provides a perfect growing environment for numerous microorganisms, including harmful bacteria (Leser, 2013). In healthy mammary glands, milk is generally free of germs. However, milk can become rapidly contaminated by spoilage bacteria and pathogens after it is produced from the udder. The main method by which pathogenic bacteria enter the milk supply is by contaminating milk before it is secreted from the udder. Feed, water, dairy farm environments, udder and teat surfaces, equipment, raw milk tanks, and workers are some of these sources of contamination (Owusu-Kwarteng *et al*., 2020).

In Ethiopia, like most other African countries, unpasteurized milk is sold through informal, small-scale routes that dominate the dairy market and commerce. The traditional products, processing methods, and retail techniques that are prevalent in the informal market are typically associated with it, as is the limited availability of infrastructure such as refrigeration, water, electricity, and sanitary facilities, in addition to the minimal or lack of food safety regulations in place (Roesel and Grace, 2014; Blackmore *et al*., 2020).

Animal mucous membranes and skin naturally contain *S. aureus* (Rajkovic *et al.,* 2019). *S. aureus* seriously damages the global dairy industry by causing mastitis in dairy cows and causing large financial losses. *S. aureus* presents a serious risk to the public's health since it can be shed into milk from udders that have mastitis. *S. aureus* is the most prominent human pathogenic staphylococcal species that can cause a wide range of illnesses, from minor skin blemishes to severe disorders (Tong *et al*., 2015; Haag *et al*., 2019).

Haag *et al*. (2019) report that enterotoxins can cause food poisoning. *S. aureus* is a significant opportunistic pathogen of raw milk. Milk and milk products are major sources of enterotoxin-producing *S. aureus*. Staphylococcal food poisoning (SFP) is one of the most common causes of gastroenteritis worldwide. It is caused by consuming enterotoxins that have already been formed by *S. aureus* in food. A small number of staphylococcal enterotoxins (SEs) can be harmful (Rajkovic *et al*., 2019).

Antibiotic therapy is a crucial tool for treating infections in both people and animals. However, overuse and abuse of antibiotics have resulted in the emergence of resistant strains, which has decreased the drug's efficacy in treating diseases in both people and animals (Economou and Gousia., 2015).

S. aureus has quickly developed resistance to several medicines. Treatment of human and animal *S. aureus* infections is severely hampered by this resistance. Additionally, *S. aureus* generates enzymes that neutralize antibiotics, leading to a variety of drug resistance mechanisms (Egorov *et al*., 2018).

The diversity, evolution, and dissemination of bacterial resistance to antibiotic pressure are significantly influenced by enzymes implicated in antibiotic resistance. Bacteria that produce antibiotics resistance require ways to produce degradative enzymes to offset the lethal effects of chemicals. However, the extensive use of antibiotics in people, animals, and the environment has created a selection pressure that has led to the development of resistant microbial strains (Larsson and Flach, 2022).

Antibiotics are overused and misused in impoverished nations, such as Ethiopia, because they are easily obtained over the counter, patients cannot get proper antimicrobial therapy, supply chains are unregulated, anyone can acquire antibiotics without a prescription, and inexperienced individuals are used to treating animals (Manyi-Loh *et al*., 2018; Kimera *et al*., 2020). The food chain can allow antibiotic-resistant microorganisms linked to animals to spread to people (Guo *et al.,* 2021).

Although food-borne diseases of public health importance have been identified in raw milk by multiple studies conducted in different parts of Ethiopia however, people continue to drink it because it is preferred, readily available, inexpensive, and they believe it has greater nutritional worth (Ayele *et al*., 2017; Amenu *et al.,* 2019; Keba *et al*., 2020).

Due to *S. aureus* remarkable capacity to become resistant to antibiotics used in therapies, treatment choices for both humans and animals are limited. Ethiopia has been shown to have a widespread disregard for responsible drug use and a regulatory environment that promotes the development of drug-resistant infections (Kifle and Tadesse 2014). Although Antibiotic therapy is an essential stage to treat infections in both humans and animals, nowadays they

are becoming less effective against pathogens including *S. aureus*. Numerous studies have been conducted on the prevalence of *S. aureus* in Ethiopian dairy value chains, but there is scarcity of research done on molecular characterization, resistance gene detection, and enterotoxin gene detection in the study area (Hawassa Town).

Therefore, the general objective of this research was:

- To determine the prevalence of *S. aureus* and associated risk factors in raw milk, traditionally processed dairy products, their antimicrobial resistance profile using microbiological and molecular methods in and around Hawassa town, Sidama region of Ethiopia.

The specific objectives of this research were:

- To isolate and identify *S. aureus* from raw milk, traditionally processed dairy product, milk containers, and milkers' hand using standard microbiological, biochemical and MALDI-TOF method
- To determine the antimicrobial susceptibility level of the isolate using disc diffusion test
- To detect the presence of resistance genes (*nuc, mecA and blaZ* resistance genes) using PCR techniques
- To assess the knowledge and practice of milk value chain actors on the risk factors for *S. aureus* contamination and its public health significance in the study area

2. BACKGROUND

2.1. General Features of Staphylococci

A genus of gram-positive, spherical bacteria called Staphylococcus is frequently responsible for respiratory illnesses, food poisoning, mastitis, and skin and surgical infections. When staphylococci were initially discovered in pus from a surgical abscess in a knee joint in 1880, Sir Alexander Ogston a Scottish surgeon noted that "the masses looked like bunches of grapes." In 1884, German physician Friedrich Julius Rosenbach distinguished between *S. aureus*, which comes from the Latin aurum, which means gold, and *S. albus,* which comes from the Latin for white, based on the colour of their colonies. Due to *S. albus's* widespread presence on human skin, it was eventually called *S. epidermidis* (Sejvar, 2013).

All parts of the environment air, dust, sewage, water, natural surfaces, people, and animals are habitats for Staphylococci. Despite being mostly located on the skin, skin glands, and mucous membranes of mammals and birds, they can occasionally lead to illness. Based on their capacity to manufacture coagulase, staphylococci have been recognised as more than 50 species and 28 subspecies to date. According to Podkowik *et al.* (2013), this classification separates strains of coagulase-positive staphylococcus (CPS) from those that do not (coagulase-negative staphylococcus, or CNS). Most staphylococci that do not produce hemolysis are not dangerous. They are classified as food-grade because it is widely known that a number of CNS species are required for the fermentation of meat and dairy products (Abebe *et al*., 2016).

Over the world, it has been demonstrated that pathogenic strains are usually coagulase positive and cause illness in their hosts. About 20% to 30% of individuals are long-term carriers of *S. aureus*, a common pathogen that colonises their upper respiratory, gastrointestinal, and urogenital systems. Animals that provide healthy food act as reservoirs. Animal skin, specifically the skin of the teat and teat canal, becomes colonised by *S. aureus* (Gulzar and Zehra, 2018). Animal skin, especially the skin of the teat and teat canal, becomes colonised by *S. aureus* bacteria. The pathogenicity of *S. aureus* is higher than that of *S. epidermidis* and *S. saprophyticus*, the other common members of the genus. *S. aureus* can cause a wide range of diseases. Although they can enter the body through the genitourinary tract, the respiratory system, or the skin, the portal of entry varies (Gulzar and Zehra, 2018).

2.2. *Staphylococcus aureus*

According to Radigue and Vandenesch (2014), *S. aureus* is a Gram-positive, cocci-shaped bacterium that frequently forms clusters resembling "grape-like" bunches. The name "aureus" (golden or yellow) refers to the fact that these organisms can grow in up to 10% salt on medium. These species can grow at temperatures ranging from 18 to 40 degrees Celsius, both aerobically and anaerobically (facultatively). Radigue and Vandenesch (2014) also state that *S. aureus* is positive for several common biochemical tests, such as coagulase-positive (which distinguishes *S. aureus* from other Staphylococcus species), novobiocin-sensitive (which distinguishes *S. aureus* from *S. saprophyticus*), mannitol fermentation-positive (which distinguishes it from *S. epidermidis*), and catalase-positive (which detects all pathogenic Staphylococcus species).

Most healthy people's skin and mucous membranes, especially the nasal area, are habitat to *S. aureus*, as common human flora, and environmental pathogen. Even though *S. aureus* seldom causes infections on healthy skin, if it enters the circulation or internal tissues, it can lead to several potentially fatal disorders. Cattle suffering from simple abscesses and mastitis to the more dangerous toxic shock syndrome can be afflicted by *S. aureus* (Larsson and Flach, 2020).

Farzana *et al*. (2004) state that many bacteria, including *S. aureus*, can grow well in milk. A milking operation's ability to prevent bacterial contamination depends on many factors, including environmental sanitation, personnel, milking utensils, and the milking technique. Additionally, it can be the consequence of microorganisms getting into the udder via the opening of teat canal. Kashif *et al*. (2019) further explain that the growth of enterotoxigenic *S. aureus* strains in food, especially milk, can result in the production of thermostable enterotoxins, which when consumed, can cause gastrointestinal symptoms like nausea, vomiting, and diarrhoea.

According to Boucher and Corey (2008) and Rasigade and Vandenesch (2014), Staphylococcal infections are common in hospital-acquired and community settings, and treatment management is still challenging due to the emergence of multi-drug-resistant strains like MRSA (Methicillin-resistant *Staphylococcus aureus*). The *mecA* gene is present on the bacterial chromosome of MRSA strains. This gene gives resistance to several drugs

and is a part of the larger Staphylococcal chromosomal cassette *mec* (SCCmec) region. The protein known as penicillin-binding protein 2a, or PBP-2a, is produced by the *mec* gene. PBP-2a, sometimes referred to as penicillin-binding protein (PBP), is an enzyme that is crucial to the formation of peptidoglycan in the bacterial cell wall (Boucher and Corey, 2008). Compared to other PBPs, PBP-2A has a reduced affinity to bind to beta-lactams and other penicillin-derived antibiotics, which means that even in the presence of several antibiotics, PBP-2A continues to catalyse the manufacture of the bacterial cell wall. Because of this, MRSA strains that produce PBP-2A are resistant to a wide range of antibiotics, and *S. aureus* strains that produce it can flourish in the presence of numerous drugs. methicillin, nafcillin, oxacillin, and cephalosporins are frequently resistant to MRSA strains (Rasigade and Vandenesch, 2014).

2.3. Modes of transmission

S. aureus is a bacterium that can easily spread between different species, including from people to animals and vice versa. This is due to its ability to colonize a wide variety of hosts, including all mammals like rats and lagomorphs (Gulzar and Zehra, 2018). Zoonotic infections caused by Staphylococcus bacteria can be transmitted to humans through animal bites, scratches, handling of infected animal tissue or bones, and contact with skin lesions or carrier animals. *S. aureus* can also transfer from healthy food animals to contaminate a range of food items, leading to food poisoning (Gulzar and Zehra, 2018). Both direct contact with infected individuals and exposure to contaminated surfaces and materials can facilitate the spread of *S. aureus* between people). Animal-to-animal transmission of the pathogen can occur through contaminated milking equipment and from hands. Environmental surfaces and objects frequently shared by household members, such as towels, remote controls, and bedding, have been found to be significant reservoirs for *S. aureus*, potentially leading to recurrent infections within households (Mork *et al*., 2018; Hogan *et al*., 2020).

According to Taponen and Pyorala. (2009), due to its virulence factors, including extracellular toxins and enzymes, *S. aureus* can cause serious animal diseases like mastitis, arthritis, and urinary tract infections in cows. Mastitis can range from mild to severe, with subclinical infections often persisting. The alpha toxin produced by *S. aureus* can cause tissue necrosis and lead to increase somatic cell counts in milk, degrading its quality.

2.4. The Prevalence and Significance of *Staphylococcus aureus* in the Dairy Sector

S. aureus may enter the milk supply chain from production to processing and storage through the milking animal, animal handlers, milking environment, utensils, industry pipes, packing environment, and storage environment. The global supply chain, customer safety, demand, and overall quality are all significantly impacted when *S. aureus* is present in the milk supply chain. The public's health is greatly impacted by animals and food derived from them since food derived from animals might contain one or more staphylococcal enterotoxins that are already established and harmful to humans (Pal *et al*., 2020).

Many factors, such as the direct using of raw milk, insufficient pasteurization, contamination after pasteurization, and the emergence of resistance, can lead to *S. aureus* contamination of processed milk products. Milk provides a rich supply of nutrients that promote the growth of *S. aureus* and the production of enterotoxins. It's common to find *S. aureus* in raw milk, processed milk, and milk products, including multi-drug resistance strains (Zeinhom and Abed, 2020). *S. aureus* can spread readily from raw milk to processed milk products, as evidenced by the numerous reports of contamination in milk products all over the world. The dairy supply chain is in danger of contamination from cows who have subclinical mastitis because they can act as secondary *S. aureus* reservoirs. Decreased milk production, spoilage, reduced nutritional content, unstable flavor, shorter shelf life, and a lower yield of milk products are all consequences of *S. aureus*-caused clinical and subclinical mastitis in cows (Pal *et al*., 2020).

2.5. Role of *Staphylococcus aureus* Toxic Proteins in Mastitis

The main physical and immunological barrier against bacterial infection is the skin. After encountering microorganisms, some epidermal cells, like keratinocytes, express recognition receptors. The skin also produces antimicrobial peptides, which are the first step in the early cutaneous immune response (Ageitos *et al*., 2017). The skin also contains macrophages, mast and plasma cells, natural killer cells, T and B cells, Langerhans and dendritic cells, and other immune cells. Commensal bacteria like *Propionibacterium acnes* and *Staphylococcus epidermidis* that are found in the skin operate as an extra line of defence by stopping the proliferation of pathogens (Reddy *et al*., 2017).

However, *S. aureus* pathogenicity involves the bacterium generating virulence factors that help the organism evade host defences and enter the mammary tissues of cows. They comprise structural elements (e.g., hemolysins, collagen, fibrinogen, elastin binding proteins, penicillin binding protein, teichoic acid, protein α, β-lactamase, proteases, capsules, and slimes), enzymes (coagulase, staphylokinase, DNase, phosphatase, lipase, phospholipase, hyaluronidase), and toxins. Moreover, *S. aureus* has special mechanisms that help it adhere to and invade mammary epithelial cells, such as the capacity to form biofilms. Enterotoxins produced by *S. aureus* include exfoliative toxins, staphylococcal enterotoxins (SEs), and toxic shock syndrome toxin 1 (TSST-1). Exfoliative toxins (ETs), which are serine proteases, prefer desmoglein 1 (Dsg1), which facilitates *S. aureus* colonization of mammalian skin (Imanishi *et al*., 2019).

Hemolysins, which include β, α, and δ toxins, target cell membranes resulting in ischemia, necrosis, destructive lysosomes, and damage to platelets. As a beta-barrel pore-forming toxin, hydroxylase (α hemolysin) damages the cell membrane, resulting in permanent osmotic alterations and apoptosis. A wide range of cells, including lymphocytes, red blood cells, platelets, and endothelial cells, can have their membranes harmed by Hla. A transmembrane channel is formed when Hla attaches to the target cell through its cellular receptor, the transmembrane protein ADAM10. This results in the formation of a heptameter of β-barrels that push up the lipid bilayer. Because of this, cells become more permeable, which causes cell death and stimulates the production of inflammation (Oliveira *et al*., 2018).

A lysogenic bacteriophage encodes beta hemolysin (Hlb), which, by itself, is not capable of killing most cell types, but exposes susceptible cells to additional lytic proteins, including leukocidins and Hla (Xiaohong and Yanjun, 2011). This toxin also referred to as sphingomyelinase, damages keratinocytes and exhibits strong hemolytic activity against sheep erythrocytes, which aids in the bacterium's colonization of mammalian skin. The amount of sphingomyelin in the cells may be the cause of the differences in the susceptibility of erythrocytes from various species to Hlb. Moreover, leukocidins are two-component poisons that form pores and target immune cells (Oliveira *et al*., 2018).

Table 1: Principal *S. aureus* exotoxins involved in food poisoning

Toxin	Principal toxin in food poisoning	*Gene*	Activity
Toxic shock syndrome toxin 1 (TSST-1)	TSST-1	*tst*	Superantigen
Staphylococcal enterotoxins (SE)	SEA, SEB, SEC1, SEC2, SEC3 and SEE. SEG to SER and SEU	*sea to see, seg to ser* and *seu*	Superantigen
SE-like toxins	SEG, SHE, AEI, SER, SES, SEIY and SET	*Seg,she,sei,ser,ses,seiy* and *set*	Superantigen, without emetic properties or have not been tested yet
Leukocidins	Panto-valentine leucocidin (PVL), LukPQ, LukMF,LukAB and LukED	*LukPV,lukPQ,lukM,lukA, and lukB* genes and *lukED*	Pore-forming toxins
Hemolysins	α hemolysin (Hla) and β hemolysin (Hlb)	*hla* and *hlb*	Pore-forming toxins
Exfoliative toxins (ETs)	ETA to ETE	*eta to ete*	Serine proteases that specifically cleave *Dsg1*

Source: Abril *et al*. (2020)

Furthermore, *S. aureus* can produce small colony variations (SCV), which can lead to recurrent infections and are resistant to aminoglycoside medications. Because host defence mechanisms have less of an effect on SCV, they are better suited to survive in mammalian cells. However, mutations usually cause a phenotypic to revert to normal, meaning that their effect is transient (Vaughn *et al*., 2020). It has been demonstrated that Staphylococcal isolates from intramammary infections in cow's exhibit a variety of virulence traits, and naturally occurring strains of *S. aureus* show a notable degree of genetic diversity. It seems that the bacteria have developed special virulence mechanisms to target these three types of ruminants. Comparing the genotypes of *S. aureus* isolated from mastitis-stricken bovine, ovine, or caprine animals indicates that distinct *S. aureus* clonal types exist for each animal species (Hoekstra *et al.,* 2018; Vaughn *et al*., 2020).

2.6. *Staphylococcus aureus* Exotoxins in Food

Various foods, such as raw milk and dairy products, are good substrates for the growth of *S. aureus*. This suggests that there are multiple pathways by which the pathogen can enter dairy products meant for human consumption: through dairy industry workers, the environment and equipment used for milking, or directly from the animals themselves since the pathogen can be found in soiled udders and surrounding skin. One important additional potential source of milk contamination is when dairy animals, such as goats, cattle, or sheep, develop mastitis caused by *S. aureus* (Wu *et al*., 2016). When a person consumes food contaminated with staphylococcal enterotoxins (SEs), food poisoning (SFP) outbreaks can be caused by *S. aureus*. Symptoms of SFP poisonings typically include vomiting, diarrhoea, nausea, and cramping in the abdomen that last for two to six hours. Resilient adults only need a few micrograms of SEs to produce SFP, whereas youngsters only need 100 ng (Wu *et al*., 2016).

When staphylococcus enterotoxin poisoning occurs, the body mounts an inflammatory response that damages the jejunum and ileum severely and modifies the production of T cell metabolites, macrophages, monocytes, mastocytes, and cytokines (Wu *et al*., 2016). These are transcribed by plasmid, chromosomal, or phage genes. They are rich in residues of lysine, aspartic acid, glutamic acid, and tyrosine. Many of them have a cystine loop, which is essential for proper conformation and most likely causes emetic activity (Tuffs *et al*., 2018).

Currently, the antigenicity of over twenty-three staphylococcal enterotoxins has been determined. Food poisoning-causing *S. aureus* produces several heat-stable enterotoxins, such as enterotoxin A, as well as a number of cell membranes that target toxins (alpha, beta, gamma, and delta) and other extracellular proteins, including hemolysins and leukocidins (Kashif *et al*., 2019). The well-characterized staphylococcal enterotoxins with the names SEA, SEB, SEC, SED, and SEE have lengths of 220 –240 amino acids and molecular weights of 25–30 kDa. Staphylococcal enterotoxins, also known as Classical staphylococcal toxins, or SE-A to SE-E, are known for their remarkable stability and resistance to numerous proteolytic enzymes, including pepsin and trypsin. These toxins account for more than 90% of food poisoning cases that arise from *S. aureus* epidemics. Furthermore, they can tolerate temperatures of 100°C for at least 30 minutes, and potentially longer, because to their excellent heat tolerance (Wang *et al*., 2018). SE food poisoning is typically associated with improper handling or storage of prepared food, which encourages the growth of bacteria and

the production of toxins. Nonetheless, the generation of enterotoxins can also be influenced by other variables such as pH, water activity, and temperature. Foods heavy in protein and carbohydrates, like meat products, eggs, and dairy products, are the main sources of these enterotoxins (Denayer *et al*., 2017).

Pasteurisation and cooking kill heat-labile staphylococci cells, whereas thermostable SEs typically retain their biological activity. Staphylococci bacteria are less heat stable than SEs, hence when a food product is tested, SEs may yield good results whereas staphylococci cultures may yield negative results. This means that even in cases where no viable bacteria can be identified from the purportedly contaminated food, diseases may still appear. The number of enterotoxins generated depends on several factors, including temperature, duration, and the makeup of the meal. Competition from other microbes appears to limit the growth of staphylococci, which in turn impacts the generation of enterotoxins (Soejima *et al.,* 2007).

The antigenic types of the 14 different SE types of family, which have sequence and structural properties and are most found in SFP, have been identified. They are classified as SE-A, B, C, D, and E (Kerouanton *et al*., 2007). Enterotoxin F (SEF) does not exist because the toxic shock syndrome toxin was initially identified as such by mistake. Overall, SE-A recovers from food poisoning outbreaks more often than any other, followed by SE-D, while SE-E has the fewest outbreaks. Mild exposure to staphylococcal enterotoxin B may cause fever, coughing, headaches, vomiting, and mild nausea (Walderhaug, 2007).

The SE family now includes SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SER, SES, SET, SElU, SElV, and SElW. Given that several of these newly discovered enterotoxins have structural similarities to traditional enterotoxins, consuming enough of these may result in foodborne disease (Rajkovic *et al*., 2019).

2.7. Public Health Impact of *Staphylococcus aureus*

Food-borne illness is a major global health hazard that is only becoming worse. Both the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC) state that many people get food-borne illnesses every year (WHO, 2015). Worldwide, diarrheal infections claimed the lives of an estimated 2 million people in 2005; more than

70% of these illnesses are foodborne. It is predicted that up to 30% of people in some developed countries get a foodborne illness each year (Jahan, 2012). In global public health initiatives for foodborne diseases (FBD), Staphylococcal food poisoning (SFP) is a major concern. The discovery of *Staphylococcus aureus* in milk and other dairy products presents significant hazards to public health as it is one of the major foodborne pathogens that can cause a wide range of ailments in people (Bintsis, 2017). Staphylococcal bacteria alone are responsible for hospitalization rates of up to 14% (Kerouanton *et al*., 2007). While Staphylococcus enterotoxin does not pose a substantial risk to health, large doses of the substance can be lethal. The mortality rate varies from 0.03% in the general population to 4.4% in more vulnerable groups, including the elderly, children, and immunocompromised individuals (Kerouanton *et al*., 2007).

Since FBD surveillance systems are absent in developing nations, assessing the problem's true impact is challenging. Nevertheless, the study from the European Food Safety Authority revealed that *S. aureus*, after foodborne viruses, Salmonella, and Campylobacte, was the fourth most common cause of foodborne outbreaks that were documented in 2008 (Leuschner *et al*., 2010). Several foods have been connected to outbreaks of staphylococcal food poisoning, including salads, cream-filled bread goods, eggs, dairy, and poultry, as well as beef and meat products (Rajkovic *et al*., 2019).

Antibiotic resistance has become a zoonotic concern for public health because of the bacteria's ongoing potential to seriously endanger human health (Pal., 2020). *S. aureus* isolates that are resistant to antimicrobials, particularly methicillin-resistant *S. aureus* (MRSA), have a significant detrimental impact on public health when it comes to healthcare costs and hospital stays (Zhen *et al*., 2020). Subclinical mastitis, a more pernicious type of the disease, can spread widely throughout dairy herds, lowering milk yield and quality, and potentially resulting in systemic illnesses that force the culling of afflicted animals (Ayele *et al*., 2017).

2.8. Application of Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry in Microorganisms Identification

These days, laser desorption/ionization (LDI), electrospray ionization (ESI), surface-enhanced laser desorption/ionization (SELDI), and matrix-assisted laser

desorption/ionization (MALDI) are the most widely utilized ionization techniques for studying chemical structures in biological systems. With the introduction of soft ionization in the late 1980s, protein analysis rapidly advanced and transformed mass spectrometry. Complete bacterial cells may be distinguished utilizing MALDI in combination with a time of flight (TOF) analyzer, as demonstrated by the innovative application of mass spectrometry in microbiology in the late 1990s. Due to these successes, MALDI-TOF MS system techniques have advanced and are now potentially useful tools for characterizing bacteria, fungi, viruses, and even worms microbiologically (Torres-Sangiao *et al*., 2021).

For the quick, accurate, and economical characterization and identification of microorganisms, matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a useful technique. In addition to being ideal for precise microbial identification at the genus and species levels, it also has the potential to be used for strain typing and identification. It employs distinct mass spectral fingerprints, which are the distinct signatures of every microorganism (Croxatto *et al*., 2012). Based on the cellular proteome, a representation of the organism's metabolic byproducts and gene products, the analytical method known as MALDI-TOF mass spectrometry examines a plethora of proteins, primarily ribosomal ones, found in microorganisms weighing between 2,000 and 20,000 Daltons. These proteins are ionised into charged molecules by the addition or removal of one or more protons, allowing for the determination of the mass-to-charge (m/z) ratio (Vrioni *et al*., 2018).

The sample to be examined is mixed with an energy-absorbing material called a "matrix." The trapped sample crystallizes as well once the matrix dries. Subsequently, the material is ionised by a laser beam, yielding single protonated ions. Following their acceleration at a specific potential and separation from one another, the time it takes for each ion to transit the length of the flight tube also known as the time of flight, or TOF is used to calculate the m/z ratio of these ions (Vrioni *et al.*, 2018). The "Peptide Mass Fingerprint" (PMF) is a distinct mass spectrum created using the TOF data. Next, this PMF, which features peaks specific to genera and species that set them apart from other types of microorganisms, is contrasted with a database. A family, genus, and species level identification of the unknown organism can be done by comparing the PMF of the unknown microbial isolates to known microbial isolates that have been stored in the database (Nomura, 2015; Vrioni *et al*., 2018).

Numerous microorganisms, such as bacteria, fungi, and viruses, have been discovered using MALDI-TOF MS. MALDI-TOF offers potential applications in a wide range of fields, including biodefense, environmental monitoring, medical diagnostics, and food quality management, due to its rapid microbe characterization capabilities. When identifying microorganisms, MALDI-TOF MS offers a high-throughput, reasonably priced substitute for conventional laboratory biochemical and molecular identification technologies (Croxatto *et al.,* 2012).

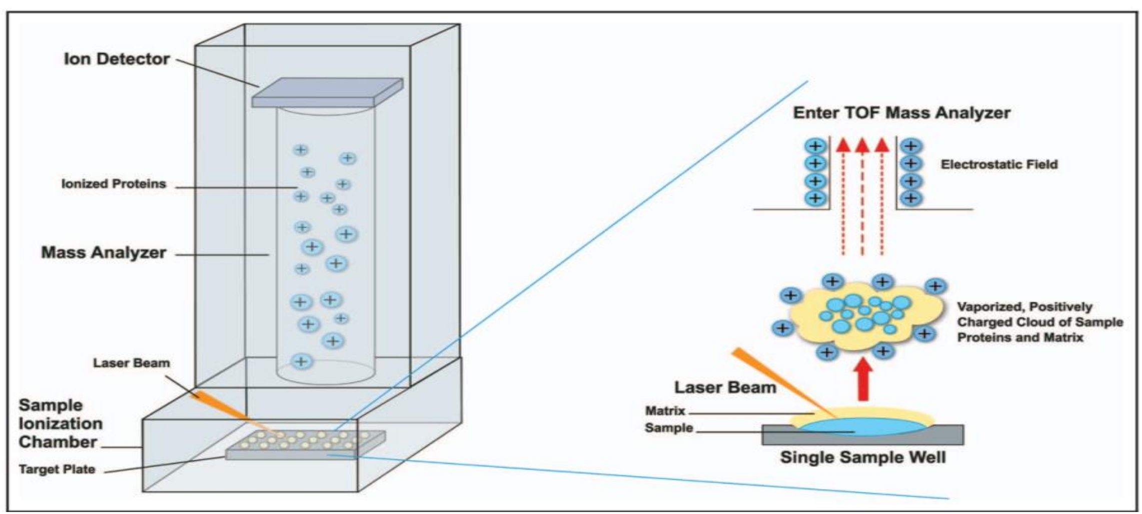


Figure 1: MALDI-TOF mass spectrometer
Source: Patel (2015).

2.9. Prevention and Control Measures of *Staphylococcus aureus*

Because of their extensive dissemination, staphylococci cannot be totally removed from the environment. Reducing or eliminating the number of germs present in food during the stages of harvest, processing, packaging, and storage before consumption has long been thought to be a successful preventative measure. Adherence to specified contact precautions and appropriate infection control principles is necessary to prevent *S. aureus* infections (Calfee *et al*., 2014). One of the most economical ways to prevent *S. aureus* infections both inside and outside of the healthcare setting is to practice good hand hygiene. Preventing *S. aureus* infections has been demonstrated to be possible with care bundles focused on enhancing adherence to quality-of-care measures. Antimicrobial prophylaxis is also advised in some situations to prevent surgical infections caused by *S. aureus* (Bratzler *et al*., 2013).

Depending on the circumstances, staphylococci can be killed or injured by lethal or sublethal doses of heat, cold, drying, radiation, or chemicals. While it is possible for these organisms to be destroyed, sublethal injury can also happen, providing the organism with a chance to recover and spread if the right conditions are met. However, it is crucial to teach and instruct everyone involved in food handling on proper hygiene practices. Routine medical and laboratory testing for individuals who work with food is useless in stopping the transmission of foodborne illnesses (Argaw and Addis, 2015).

Optimising hygiene practices is key to preventing *S. aureus* infections. These practices include covering open wounds, washing your hands frequently, taking regular baths, putting up a barrier between bare skin and shared surfaces (like gym or athletic equipment), and not sharing towels or personal hygiene products like deodorant, razors, or cosmetics. Patients may also be advised to avoid bath or shower loofas, launder towels and washcloths after each use, use lotions in pump or pour bottles to prevent contamination, and keep their fingernails neatly and short (Kaplan *et al*., 2014).

Understanding ways to prevent various modes of transmission is crucial to prevent staphylococcal infections and poisoning. These control programmes include, but are not limited to, the establishment of personal hygiene habits among healthcare workers and food handlers, the cleaning of equipment, surfaces, and clothing, the careful use of antibiotics, proper food preparation and storage, and screening initiatives. Foods must be stored at -10°C or 45°C to stop the growth of organisms and the production of toxins. To ensure quick cooling, foods should also be chilled in small quantities or in shallow layers (Alreshidi *et al*., 2015).

2.10. Treatment and challenge of drug resistance of *Staphylococcus aureus*

The type of infection and the presence of drug-resistant strains are the two main variables influencing the treatment of *S. aureus* infections. When antimicrobial therapy is required, the type of sickness and other factors influence the length of time and type of treatment that should be administered (Tong *et al.*, 2015). Penicillin is still the preferred antibiotic for sensitive isolates (MSSA, or methicillin-sensitive *S. aureus* strains), while vancomycin is used for MRSA isolates. Combining antibiotic medication with other forms of treatment is

sometimes required. For example, toxins-induced illness often requires therapy with fluid replacement, while catheter-related or prosthetic valve infections usually require the removal of foreign objects. Because several strains of MRSA are resistant to a broad variety of medications, MRSA infections are becoming serious illnesses in both hospital and community settings (Boucher and Corey, 2008).

The treatment of *S. aureus* infections is similarly complicated by the bacteria's ability to build biofilms, in addition to the traditional mechanisms of resistance, including antibiotic-modifying enzymes, altered cell-binding sites, and efflux pumps. One of the main therapeutic challenges in treating these infections is the difficulty in eliminating biofilms because they can resist much higher concentrations of antibiotics than planktonic *S. aureus* and can evade multiple host clearance mechanisms (Watkins *et al*., 2019). Even with innovative therapies like bacteriophages or bacteriophages antibiotic combos, this observation held true. Compared to planktonic growth, *S. aureus* susceptibility to tetracycline, benzylpenicillin, and vancomycin decreased by 2–10 times in the biofilm environment (Kebriaei *et al*., 2022).

Planktonic bacteria are generally thought to be resistant to antibiotic treatment because of one of two mechanisms: either they can multiply in the presence of antibiotic concentrations through resistance mechanisms, or they can exhibit tolerance, which is characterised as nongrowing but persistent existence in the presence of antibiotics (Conlon *et al*., 2016; Waters *et al*., 2016). Yet, biofilm-associated bacteria cannot completely be treated under the concepts of tolerance or resistance. Indeed, a different definition of "recalcitrance" is put out to explain how biofilm bacteria manage to withstand antibiotic treatment (Waters *et al*., 2016). In addition to being non-heritable, this biofilm recalcitrance phenomena can also be reversed or go away when the biofilm dissolves and the bacteria go back to their planktonic stage. According to Lebeaux *et al*. (2014), the recalcitrance phenomenon is characterised by several characteristics, including antibiotic-restricted penetration, resistance genetic material transfer, the presence of persisting cells, and phenotypic variability.

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The research was carried out in Hawassa City and its surrounding areas in the Sidama Region of Ethiopia. Hawassa is the capital city of the Sidama Region, which has a population of 157,879 people. Hawassa is located 275 km south of the country's capital, Addis Ababa. The city is situated at an elevation of 1,750 meters above sea level, with geographic coordinates of 7°03'1.35" N latitude and 38°29'43.81" E longitude. The region has an average annual temperature of 22°C and a mean relative humidity of 51.8%. It receives between 800-1,000 mm of rainfall annually, with 67% of this rainfall occurring during the long rainy season from June to September. The dominant vegetation in the area is a dry savanna and shrub-like landscape. The total livestock population in the Sidama region, which includes Hawassa, is estimated to be: 2,413,482 cattle, 308,903 goats, 467,858 sheep, 34,709 horses, 16,376 donkeys, 1,824,841 poultry, and 44,364 beehives (CSA, 2021).

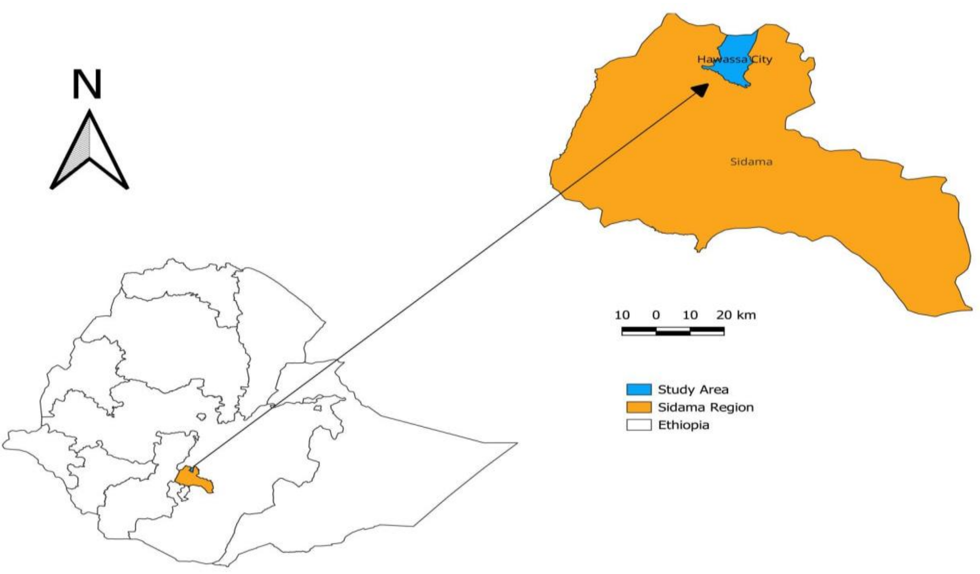


Figure 2: Map of the study area

3.2. Study Setting

The study setting focused on the milk value chain in and around Hawassa town, including intensive and semi-intensive dairy farms within Hawassa and the surrounding areas, which represent the primary production stage; milk collection facilities that receive and aggregate milk from the dairy farms before distribution; and retail stores and outlets that sell both packaged/processed milk products as well as traditionally processed dairy products like yogurt and Ethiopian cottage cheese, representing the final distribution and consumption points of the milk value chain. Additionally, the study included the collection of environmental samples, such as swabs from the hands of milkers at the dairy farms and swabs from milk containers used at various points along the milk value chain, which help us to assess potential sources of microbial contamination throughout the supply chain in the Hawassa town.

3.3. Study populations

The study population for this research comprised lactating dairy cows in and around Hawassa town. The cows included both exotic breeds as well as crossbred cows, which were hybrids of Holstein and zebu (indigenous) breeds. Cows that were not actively lactating during the research sampling period were excluded from the data collection. The information from the cow owners on various parameters, including the animals' ages, breed, lactation stage, parity (number of calves), and the socioeconomic status of the owners were gathered. This data provided important context about the characteristics of the dairy cows that were sampled as part of the study. By focusing on the lactating dairy cow population, both exotic and crossbred, we were able to assess the microbial quality and safety of milk produced across the different cattle types present in the Hawassa milk value chain.

3.4. Study Design and Sample Size Determination

The study utilized a cross-sectional design and was conducted from December 2023 to May 2024 in and around Hawassa town. The sample size was calculated based on the techniques suggested by Thrusfield *et al*. (2018) and previous studies on *S. aureus* prevalence in the study area. According to Mekuria *et al*. (2013) and Megersa (2022), the prevalence of *S.*

aureus was 15.5% in raw milk and 5% in dairy products like cottage cheese and yogurt respectively. The sample size was calculated as:

$$n = \frac{1.962\ p\ exp\ (1-p\ exp)}{d2}$$

where: - n=required sample size

P exp = expected prevalence

d = desired absolute precision level at 95% confidence level

1.96 = constant from normal distribution at a given confidence level

The estimated sample size for the study was 75 farms, with random selection involving 10 lactating dairy cows from large farms, 5 from medium farms, and 3 from small farms. Additionally, 84 samples of dairy products (42 ergo/yoghurt and 42 ayib or cottage cheese) and 242 samples of raw cow milk were collected. To increase the study power, 20 bulk tank milk samples from collection centers, 20 swabs from milkers' hands, and 20 swabs from milking equipment were also included, resulting in a total of 386 samples considered for the study.

3.5. Inclusion and Exclusion Criteria

The inclusion criteria for participation in the study included farms with one or more crossbreed lactating cows, as well as farms with a minimum of five or more lactating cows. Additionally, dairy farm owners who were willing to supply milk and milkers who were willing to participate in the study and provide necessary information via questionnaire were included. Conversely, exclusion criteria comprised non-lactating cows and Zebu lactating cows not raised under intensive or semi-intensive management systems. Furthermore, farmers who were absent during the study or declined to participate, farm owners with hearing impairments unable to provide required information, and those unable to complete questionnaire interviews were excluded from the study.

3.6. Sampling Strategy and Methodology

Based on a prior study conducted by Demissie *et al.* (2022), the farms were divided into three categories: small (5–20 cows), medium (21–40 cows), and large herds (>40 cows). In

all, 819 dairy farms were in the research region, according to information gathered from the Hawassa City Agriculture Office. Proportional sampling was used to calculate how many cows from each category (farm) should be chosen. In the study population, the estimated percentage of small, medium, and big farms was 93%, 6%, and 1%, respectively. Using a systematic random sample technique, representative dairy farms were chosen from the city based on the list of dairy farm owners. A list of households registered with the Hawassa Farmers were used as the sampling frame for choosing the dairy farms.

Each farm received a certain number of cows in proportion to its size. The lactation stages of cows are divided into three categories: early, which lasts from 0 to 3 months, mid, which lasts from 4 to 6 months, and late, which lasts from 7 months to gestation. Young cows, or those under five years old, and adults, or those beyond five, were the two age groups into which the cows were divided. Furthermore, according to Dabele *et al*. (2021), the cows were split into three parity categories: few (1-2 calving), medium (3-5 calving), and many (more than 5 calving). Yoghurt and cottage cheese (n = 84) were collected from dairy product vendors in the study region, while bulk tank milk and raw cow milk were obtained directly from milk collecting centres and dairy farms, respectively. Stores selling conventionally processed yoghurt and cottage cheese were identified along the way during visits to dairy farms; additional retailers were taken into consideration until the necessary sample size was obtained.

3.7. Sample Collection and Transportation

Before sampling, the udder and teats of the cows were washed and dried using clean towels. The teat canal was cleaned by discarding the first few squirts of milk, and then composite milk samples (40 ml) were collected from each cow using sterilized falcon tubes labeled accordingly. Equipment surfaces and milkers' hands were sampled using sterile cotton swabs moistened with Stuart transport media. The swabs were then placed in labeled test tubes containing Stuart medium. Bulk tank milk samples were collected from the top of the bulk milk from Milk Collection Centers (MCCs). Then all samples, including milk products (cottage cheese and yogurt), were transported to the Animal Health Institute's Microbiology Laboratory (AHI), Sebeta, using an icebox containing ice packs and on arrival, the samples were immediately stored at 4°C until culturing for bacterial isolation was done.

3.8. Primary Isolation of *Staphylococcus aureus* and Biochemical Tests

The first step in the enrichment process was to mix 1 millilitre of milk sample with 9 millilitres of peptone water (TM Media, India) and incubate for 24 hours at 37 hours. A loopful of bacterial growth was taken from peptone water, streaked over mannitol salt agar (HiMedia, India), and incubated for 24 hours at 37 °C. The plates were then examined to check for the growth of bacteria and the fermentation of mannitol. A bacterium showing clusters resembling grapes and golden-yellow colonies was streaked over nutrient agar (HiMedia, India) and cultured at 37 °C for 24 hours. Finally, the automated device known as Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonik, Germeny) was used to confirm the representative (pure) colonies, which were typical grapes like arrangement and golden-yellow colour from nutrient agar.

3.9. MALDI-TOF MS Based Identification of *Staphylococcus aureus*

The method for species identification was carried out using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik) according to the company's instructions (Bizzini *et al*., 2010). In short, representative colonies were placed in 300 µl of deionized water, vortexed, and precipitated with 900 µl of ethanol (96% vol/vol) after all putative isolates were sub-cultured on nutrient agar (HIMEDIA, INDIA) and incubated at 37 °C for 24 hours overnight. Following a 2-minute centrifugation at 15,000 rpm, the pellet was reconstituted using a solution of 25 µl formic acid (70% vol/vol) and 25 µl acetonitrile. After two minutes of centrifugation at 15,000 rpm, 1µl of the supernatant was spotted on an MSP 96 target plate that had been polished with Steel BC. The plate was then left to air dry at room temperature. For every designated BTS QC spot, 1 µl of BTS was applied, and it was left to air dry. After covering the entire area with a droplet of 1µl of matrix solution (CHCA), the region was once more fully dried at room temperature. Lastly, analysis was carried out using biotyper software after inserting the MALDI target into the MALDI-TOF mass spectrometer.

3.10. Antimicrobial Susceptibility Test Using Disc Diffusion Method

After identifying bacterial isolates with MALDI-TOF (Bruker Daltonik), susceptibility of antimicrobial testing was done using the disc diffusion method (Kirby Bauer technique) (Khalili *et al.*, 2012). Ten (10) antimicrobial discs were prioritized including penicillin G (10µg), Ciprofloxacin (5µg) Erythromycin (15µg), Trimethoprim-Sulphamethoxazole (25µg), Ampicillin(10µg), Tetracycline(30µg), Gentamycin (10µg), Clindamycin (2µg) cefoxitin (30µg), and Amoxicillin 2 µg based on their availability and distribution to nearby veterinary clinics and pharmacies.

Representative colonies of isolates were suspended in sterile 0.85% NaCl solution and adjusted to a turbidity of 0.5 McFarland units using a densitometer. A sterile cotton swab was dipped into the suspension and spread on Muller Hinton agar. Antimicrobial discs were applied, and plates were incubated at 37°C for 18 hours. Zone diameters were measured and interpreted as sensitive, intermediate, or resistant according to Clinical and Laboratory Standards Institute guidelines (CLSI) (2022) (Table 2). *S. aureus* isolates resistant to three and above antimicrobial classes were considered multidrug-resistant.

Table 2: The antimicrobial disc used to measure the zone of inhibition of *S. aureus* susceptibility, together with its interpretations

Types antimicrobial discs	Symbol	Susceptible	Intermediate	Resistance
Ciprofloxacin	CIP (5 µg)	≥21	16-20	≤15
Penicillin G	P (10 µg)	≥29	-	≤28
Trimethoprim-Sulfamethoxazole	SXT (25 µg)	≥16	11- 15	≤10
Cefoxitin	FOX (30µg)	≥22		≤21
Gentamycin	CN (10 µg)	≥15	13-14	≤12
Erythromycin	E (15 µg)	≥23	14-22	≤13
Tetracycline	TE (30 µg)	≥19	15-18	≤14
Clindamycin	DN (2 µg)	≥21	15-20	≤14
Ampicillin	AMP (10µg)	≥29	-	≤28
Amoxicillin	AX (2 µg)	≥29	-	≤28

3.11 Detection of Resistance Gene by conventional PCR

3.11.1. Bacterial DNA Extraction

Using the Qiagen DNease extraction kit (Thermo Scientific, Germany) and lysis buffer, DNA was extracted from overnight broth cultures of *S. aureus* isolates that had been incubated at 37°C for 24 hours as per the manufacturer's instructions.

3.11.2. Gene Amplification Using PCR

The *mecA*, *nuc*, and *blaZ* genes of isolates of *S. aureus* were tested by PCR amplification. 0.5 µl of Taq polymerase enzyme, 12.7 µl of RNase-free water, 5 µl of PCR buffer, 0.8 µl of dNTP, 2 µl of forward and reverse primers, and 2 µl of each DNA component made up the 25 µl master kit combination used for the procedure. As the negative control, sterile water was used their MRSA, thermostable, and penicillinase enzymes were the targets of the primers *mecA*, *nuc* (Haque *et al*., 2018), and *blaZ* (Takayama *et al*., 2018). The procedure for amplifying the genes was started by adding the mixture to the PCR tubes, the genes were amplified by lightly labelling the tubes. The PCR tubes and their components were processed using a Flex thermal cycler (Biometra Gmbh, Germany) (annex 5). The heat cycler was configured to repeat 40 cycles: initial denaturation at 94°C for 5 minutes, final denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes (Table 3).

Table 3: Specific Primers that were utilised to find resistance genes

Forward and reverse primers	Gene length	References
F: 5’ TACAACTGTAATATCGGAGGG 3’	*blaZ*	Takayama *et al.*
R: 5’ ATTACACTCTTGGCGGTTTC 3’	(846bp)	(2018)
F:5’GTAGAAATGACTGAACGTCCGATGA 3’	*mecA*	Haque *et al.*
R: 5’CCAATTCCACATTGTTTCGGTCTAA 3’	(163 bp)	*(*2018)
F: 5’ GCGATTGATGGTGATACGGTT 3’	*nuc*	Haque *et al.*
R: 5’ AGCCAAGCCTTGACGAACTAAAGC 3’	(279bp)	*(*2018)

F (forward primer), R (reverse primer)

3.11.3. Gel electrophoresis and visualization of amplicons

Electrophoresis was used to separate the PCR products that were stained with ethidium bromide on a 1.5% (w/v) agarose gel. After mixing the PCR result with the loading dye (bromophenol blue), it was added to the agarose gel slots. A maximum of 600 bp of DNA markers (Qiagen, Germany) with 100 bp interval DNA fragments were used to measure the length of the amplicons. Then, using 1X TBE buffer, electrophoresis was conducted for an hour at 110V. After electrophoresis, the separated PCR products were exposed to ultraviolet light for photography and recorded using a gel documentation system (Gel DocTM XR+, BioRAD; Germany) (Annex 5).

3.12. Questionnaire Survey

Interviews and first-hand observations were used to get data about animals during sample collection from farms. This included details about breed, age, parity, lactation stage, herd size, breed hygiene, use of disinfectants, usage of towels, udder cleaning procedures, and management system. A semi-structured questionnaire was developed using information from guidelines and literatures to evaluate potential risk factors as well as study participants' knowledge, attitudes, and practices regarding food safety training, handling, and consumption of raw milk in the study area. Interviews were conducted with 100 participants in the milk value chain, including 42 retailers, 11 milk collectors, and 47 farmers. The central limit theorem was used to determine this sample size ($n = 0.25/SE^2$). Where SE stands for standard error, which, according to Arsham (2002), is 0.05 with a 95% confidence range.

3.13. Data Management and Analysis

STATA statistical software version 14.1 was used for analysis after collected data was filed, cleaned, and coded using Microsoft Excel sheets. The prevalence of *S. aureus,* questioner survey and the information on antibiotic susceptibility were compiled using descriptive statistics. To evaluate the relationship between the frequency of *S. aureus* and putative risk factors in raw cow milk, univariate and multivariable logistic regression analyses were carried out. The multivariable model included non-collinear variables selected from the

univariable analysis with a P value of less than 0.25. Ultimately, at all analytic levels, the results were deemed significant at $P < 0.05$.

3.14. Ethical Consideration

Before commencing the study, a request explaining the goal of the investigation was presented to the animal research ethics and review committee of the Addis Ababa University College of Veterinary Medicine and Agriculture. After obtaining an acceptance certificate from the committee (Annex 6), bearing reference **No. VM/ERC/02/17/16/2024**, the research work was conducted.

4. RESULTS

4.1. Prevalence of *Staphylococcus aureus*

The present study found that the overall prevalence of *S. aureus* was 13.99% from 386 different sample types. In raw milk samples, 242 were tested, and 50 were found to be positive, resulting in a prevalence of 20.7%. For bulk tank milk, hand swabs, and bucket swabs, 20 samples of each were tested, with 1 positive sample in each category, corresponding to 5.0% prevalence. Cheese samples showed no positive results out of 42 tested, indicating 0.0% prevalence. In yogurt, 42 samples were tested, with 1 positive, equating to 2.4% prevalence. These findings suggest raw milk had the highest rate of *S. aureus* contamination, while cheese appeared to have no detectable presence of the bacteria. The other sample types, including bulk tank milk, milkers' hand swabs, bucket swabs, and yogurt, exhibited lower prevalence levels 5% or less (Table 4).

Table 4: *S. aureus* prevalence in raw cow milk and milk products in the research area

Sample	No. of sample	No. positive	Prevalence (%)	95% CI
Raw milk	242	50	20.7	0.157 - 0.264
Bulk tank milk	20	1	5.0	0.001 - 0 .249
Hand swabs	20	1	5.0	0.001 - 0 .249
Bucket swabs	20	1	5.0	0.001 - 0 .249
Cheese	42	0	0.0	0.001 - 0.126
Yoghurt	42	1	2.4	0.0006 - 0.1257
Overall	386	54	13.99	0.1069 - 0.1786

CI (Confidence interval at 95%)

4.2. Risk Factors Associated with *Staphylococcus aureus* Occurrence in Raw Milk at Farm Level

The study found that 50 out of the 242 raw milk samples collected (20.7%) were positive for *S. aureus.* A univariable logistic regression analysis revealed that several farm management

factors were statistically significantly correlated (p<0.05) with the prevalence of *S. aureus* at the farm level. These factors included cow age, cow lactation stage, cow housing floor type, water source used for equipment wash, whether udder and teats were washed before milking, and the presence of mastitis. Specifically, higher prevalence of *S. aureus* was found in dairy cows with mastitis (70.8%), on farms where udder and teat washing were not practiced prior to milking (43%), in farms using ground water sources (39%), in farms with muddy soil flooring (38%), and in cows older than five years (35%). However, the relationship between farm size and *S. aureus* prevalence was not statistically significant (p>0.05).

The findings from the multivariable logistic regression analysis indicated that the prevalence of *S. aureus* at the farm level was statistically significantly (p<0.05) associated with cow age, lactation stage, presence of mastitis, and the water source used for washing equipment (table 5).

Table 5: Univariate and Multivariable logistic regression analysis of factors associated with the occurrence of *S. aureus* in raw milk.

Factors	Categories	No. tested	No. positive %	Crude OR [95% CI]	P value	Adjusted OR [95% CI]	P value
	Small scale	207	41(20)	1.0			
Farm size	Medium scale	25	6(24)	1.28 (0.480 - 3.405)	0.623		
	Large scale	10	3(30)	1.74 (0.43 - 7.00)	0.439		
	Intensive	213	40(19)	1.0			
Farm management	Semi-intensive	29	10(34)	2.28 (0.983 - 5.269)	0.055	1.23(0.219 - 6.883)	0.817
Breed	Jersey	16	2(12)	1.0			
	HFX	226	48(21)	1.89 (0.415 - 8.592)	0.411		
Age of Cow (year)	<-5	144	16(11)	1.0			
	>5	98	34(35)	4.25 (2.184 - 8.269)	0.000	3.98(1.226 - 12.919)	0.022
Parity status	>5 calves	66	12(18)	1.0			
	1-2 calves	88	19(22)	1.24(0.554 - 2.773)	0.602		
	3-5 calves	88	19(22)	1.24(0.554 - 2.773)	0.602		
Lactation stage	early	101	14(14)	1.0			
(month)	Mid	68	13(19)	1.47 (0.6424 - 3.358)	0.362	3.032(0.614- 14.971)	0.173
	Late	73	23(32)	2.86 (1.350 - 6.051)	0.006	8.978(1.89 - 42.596)	0.006
Housing floor used	Concrete	210	38(18)	1.0			
	Muddy soil	32	12(38)	2.72(1.224 - 6.028)	0.014	1.77(0.35 - 8.781)	0.483
Barn cleaning (per	Three times	20	3(15)	1.0			
day)	Twice	189	38(20)	1.426 (0.397- 5.118)	0.586		
	Once	33	9(27)	2.125 (0.500 - 9.030)	0.307		
Farm hygiene	Good	113	18(16)	1.0			
	Moderate	78	19(24)	1.699 (0.826 - 3.498)	0.150	1.61(0.426- 6.068)	0.483
	Poor	51	13(25)	1.81 (0.806 - 4.045)	0.151	2.84(.534 - 15.118)	0.221
Farm disinfection	No	193	36(19)	1.0			
	Yes	49	14(29)	1.75 (0.851- 3.576)	0.129	0.64(0.137 - 2.989)	0.569
Milking equipment	Warm water with detergent	39	7(18)	1.0			
wash practice.	Cold water with detergent	174	36(21)	1.193 (0.487- 2.922)	0.700		

	Warm water only	29	7(24)	1.45 (0.447- 4.733)	0.534		
Water used for equipment wash	Tap	209	37(18)	1.0			
	Ground	33	13(39)	3.02 (1.380 - 6.614)	0.006	11.15(2.174 - 57.14)	0.004
Hand washing before milking	Yes	230	46(20)	1.0			
	No	12	4(33)	2.0 (0.577- 6.932)	0.274		
Udder and teat washing before milking	Yes	228	44(19)	1.0			
	No	14	6(43)	3.14 (1.035- 9.502)	0.043	4.22(0.365 - 48.87)	0.249
Milking equipment used	Wide necked aluminium	53	10(19)	1.0			
	Plastic jar	172	35(20)	1.09 (0.503- 2.400)	0.814		
	Narrow necked aluminium	17	5(29)	1.79(0.513 - 6.252)	0.360		
	All are normal	201	40(20)	1.0			
Teat status	One is blind	20	4(20)	1.00 (0.318 - 3.175)	0.992		
	Two is blind	11	3(27)	1.51 (0.383 - 5.948)	0.556		
	Three is blind	10	3(30)	1.73(0.427- 6.968)	0.444		
Milking techniques	Manual	221	44(20)	1.0			
	Machine	21	6(28.6)	1.61 (0.590 - 4.386)	0.352		
Towel use	Individual	94	17(18.1)	1.0			
	Common	148	33(22.3)	1.29 (0.677- 2.496)	0.431		
Mastitis	Negative	177	4(2.3)	1.0			
	Positive	65	46(70.8)	104.7(33.95- 322.9)	0.000	248.9(45.37-1366.1)	0.000
Milker's gender	Female	59	8 (13.6)	1.0			
	Male	183	42 (22.9)	1.89(0.835 - 4.3163)	0.126	1.21(0.199 - 7.31)	0.838
Milkers' educational status	Secondary	29	3 (10.3)	1.0			
	College & above	29	4(13.8)	1.39 (0.282- 6.83)	0.688	0.33(0.021 - 5.237)	0.434
	Cannot read and write	53	11(20.7)	2.2 (0.578 - 8.906)	0.240	2.20(0.172 - 28.196)	0.544
	Primary	131	32(24.4)	2.80 (0.795 - 9.874)	0.109	0.86(0.089- 8.28)	0.893

Interviews were conducted with 100 participants in the value chain of milk, including farm owners, farm attendants/milkers, milk collecting facilities, and sellers of conventionally processed dairy products. The survey findings revealed significant gaps in food safety awareness and training among the respondents. Only 39% had received any form of food safety training, while the majority (61%) had not. Regarding raw milk consumption, a substantial proportion (40%) of respondents reported consuming raw milk, posing a potential health risk.

Awareness of specific food safety issues was alarmingly low. Only 23 (23.0%) of the respondents were aware of staphylococci food poisoning, while the majority (77%) lacked knowledge of this significant food safety concern. In contrast, 79 (79.0%) of the respondents reported having general knowledge about milk-borne diseases. The educational background of the respondents was mixed, with over half (57%) having only primary-level education, 24% being unable to read and write, 8% having secondary education, and 11% having college or higher education. The sample was also heavily skewed towards male respondents, with 83% being male and only 17% female. The predominant type of milk equipment used was plastic (72%), followed by narrow-necked aluminium (19%) and wide-necked aluminium (9%). Overall, the findings highlight the need for targeted food safety interventions and educational campaigns to address the significant gaps in training, awareness, and practices observed in the study population. The educational background and gender imbalance should be considered when designing and implementing these initiatives to ensure their effectiveness (table 6).

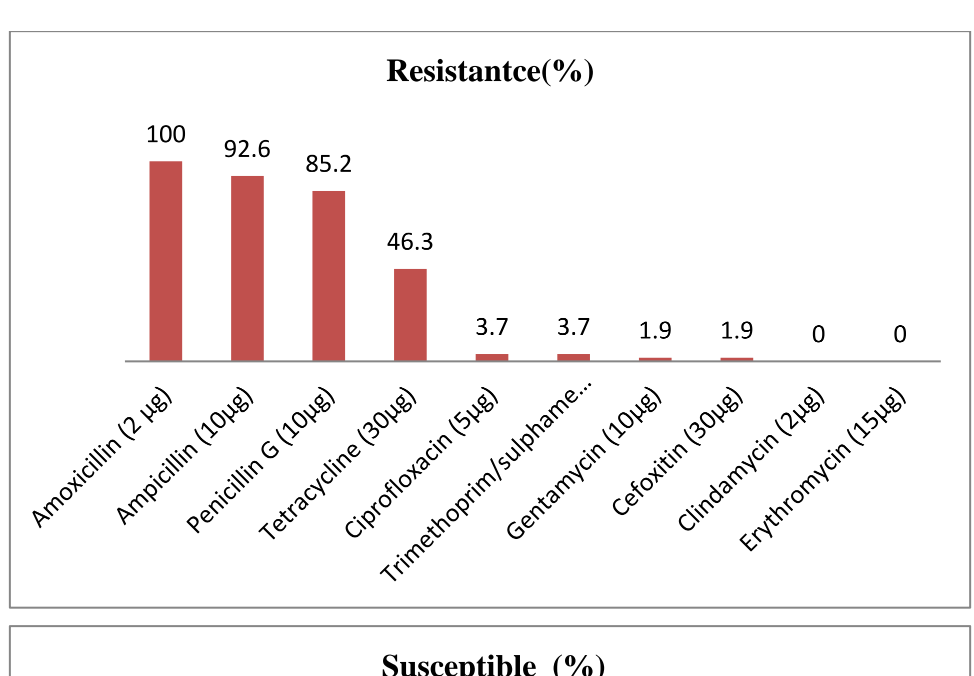
Table 6 : Descriptive statistics of questioner survey on knowledge and practice of milk value chain actors in the research area

Variable	Categories	Respondents No	Percentage (%)
Food safety training	Yes	39	39
	No	61	61
Raw milk consumption habit	Yes	40	40
	No	60	60
Awareness of Staphylococcus food poisoning	Yes	23	23
	No	77	77
Knowledge on milk borne disease	Yes	79	79
	No	21	21
Educational status	Secondary	8	8
	Primary	57	57
	Can't read and write	24	24
	College and above	11	11
Gender	Male	83	83
	Female	17	17
Milk equipment used	Wide necked Aluminium	9	9
	Narrow necked Aluminium	19	19
	Plastic	72	72

4.3. Antimicrobial Susceptibility Profile of the *Staphylococcus aureus* Isolated

The antimicrobial resistance profile revealed concerning levels of resistance among the isolates. Amoxicillin displayed the highest resistance, with 100% of the isolates being resistant to this antimicrobial. Similarly, Ampicillin exhibited a very high resistance rate, with 92.6% of the isolates classified as resistant. Penicillin G also showed substantial resistance, with 85.2% of the isolates being resistant. On the other hand, the data also

highlights the antimicrobials that displayed high susceptibility among the isolates. Cefoxitin demonstrated the highest susceptibility, with 98.1% of the isolates classified as susceptible. Clindamycin and Trimethoprim/sulphamethoxazole also exhibited favourable susceptibility profiles, with 98.1% and 94.3% of the isolates, respectively, being susceptible to these antimicrobials. Ciprofloxacin and Gentamycin also had high susceptibility rates, with 96.3% and 90.7% of the isolates being susceptible, respectively. These findings suggest the potential for the effective use of these antimicrobials in the treatment of infections within the milk value chain, provided that appropriate antimicrobial stewardship practices are in place (Figure 3).



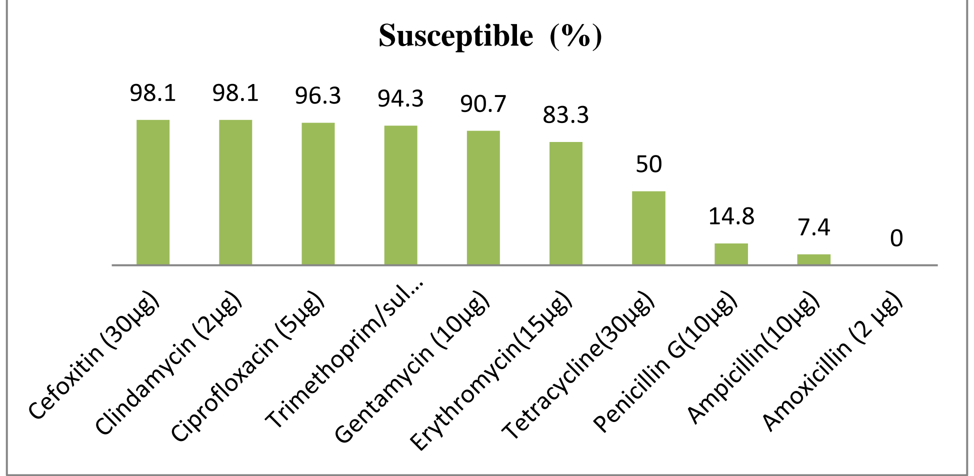


Figure 3 : Antimicrobial Susceptibility Profile of the *S. aureus*

The data also reveals the presence of intermediate susceptibility for some antimicrobials. Erythromycin had the highest percentage of isolates (16.67%) classified as intermediate, indicating a potential risk of resistance development if the usage of this antimicrobial is not carefully monitored. Gentamycin and Tetracycline also displayed a small proportion of isolates (7.4% and 3.7%, respectively) with intermediate susceptibility. The existence of these intermediate profiles highlights the need for continuous monitoring and the implementation of strategies to prevent the further development of antimicrobial resistance within the milk value chain (Table 7).

Table 7: Percentage of antimicrobial susceptibility profile of *S. aureus* isolates

Antimicrobial Disk (µg)	**Susceptible Number (%)**	**Intermediate Number (%)**	**Resistant Number (%)**
Tetracycline(30µg)	27 (50)	2(3.7)	25(46.3)
Cefoxitin (30µg)	53(98.1)		1(1.9)
Erythromycin(15µg)	45(83.33)	9(16.67)	0(0)
Ampicillin(10µg)	4(7.4)	-	50(92.6)
Gentamycin (10µg)	49(90.7)	4(7.4)	1(1.9)
Trimethoprim/sulphamethoxazole (25µg)	51(94.3)	1(1.9)	2(3.7)
Ciprofloxacin (5µg)	52(96.3)	-	2(3.7)
Clindamycin (2µg)	53(98.1)	1(1.9)	0(0)
Penicillin G(10µg)	8(14.8)	--	46(85.2)
Amoxicillin (2 µg)	0(0)	--	54(100)

4.3.1. Multidrug Resistance

The data shows that the *S. aureus* isolates from milk displayed concerning levels of multidrug resistance, with a significant proportion (35.2%) exhibiting resistance to three antibiotics - Ampicillin (AMP), Penicillin (P), and Amoxicillin (AX), an even larger group (48.2%) being resistant to four different antibiotics, including the three mentioned above and additional resistance to Tetracycline (TE), Gentamycin (CN), Ciprofloxacin (CIP), or Trimethoprim/Sulfamethoxazole (SXT), and a small number of isolates (4%) displaying

resistance to five different antibiotics, including combinations of TE, AMP, AX, CIP, SXT, and FOX (Cefoxitin); these findings indicate a worrying trend of multidrug resistance development among the *S. aureus* isolates from milk, posing a significant public health concern within the milk value chain that will require a comprehensive approach to address, including enhanced antimicrobial stewardship, improved infection control practices, and potentially the exploration of alternative therapeutic strategies to ensure the continued safety and effectiveness of dairy products (Table 8).

Table 8: The isolates of *S. aureus* in the study area exhibit a multidrug resistance pattern

No. of antibiotics	Resistance pattern	Number of isolates	Percentage (%)
Three	AMP, P, AX	19	35.2
Four	AMP, P, AX, TE	23	48.2
	AMP, P, AX, CN	1	
	AMP, P, AX, CIP	1	
	AMP, P, AX, SXT	1	
Five	TE, AMP, AX, CIP, SXT	1	4
		1	
	TE, AMP, AX, FOX, P		

P(penicillin), AMP (ampicillin), FOX(Cefoxitin), TE (tetracycline), CN(Gentamycin), AX(Amoxicillin), SXT(Trimethoprim/sulphamethoxazole), CIP (Ciprofloxacin)

4.4. Antibiotic and Resistance Gene Detections

The study examined 25 isolates of *S. aureus* that developed antibiotic resistance. The presence of three specific drug resistance genes in these isolates: *blaZ, mecA, and thermonuclease* (*nuc*) were assessed. These three genes are known to confer resistance to certain antibiotics and their detection can help understand the mechanisms underlying the observed antibiotic resistance in these *S. aureus* samples. The analysis of 25 *S. aureus* isolates that had developed antibiotic resistance revealed the presence of the 279 bp long *nuc gene* (encoding the *thermonuclease* enzyme) in all the isolates, as confirmed by traditional PCR (figure 4). However, none of the isolates were found to possess the 163 bp long *mecA*

gene (associated with methicillin resistance) or the 846 bp long *blaZ* gene (associated with β-lactam resistance).

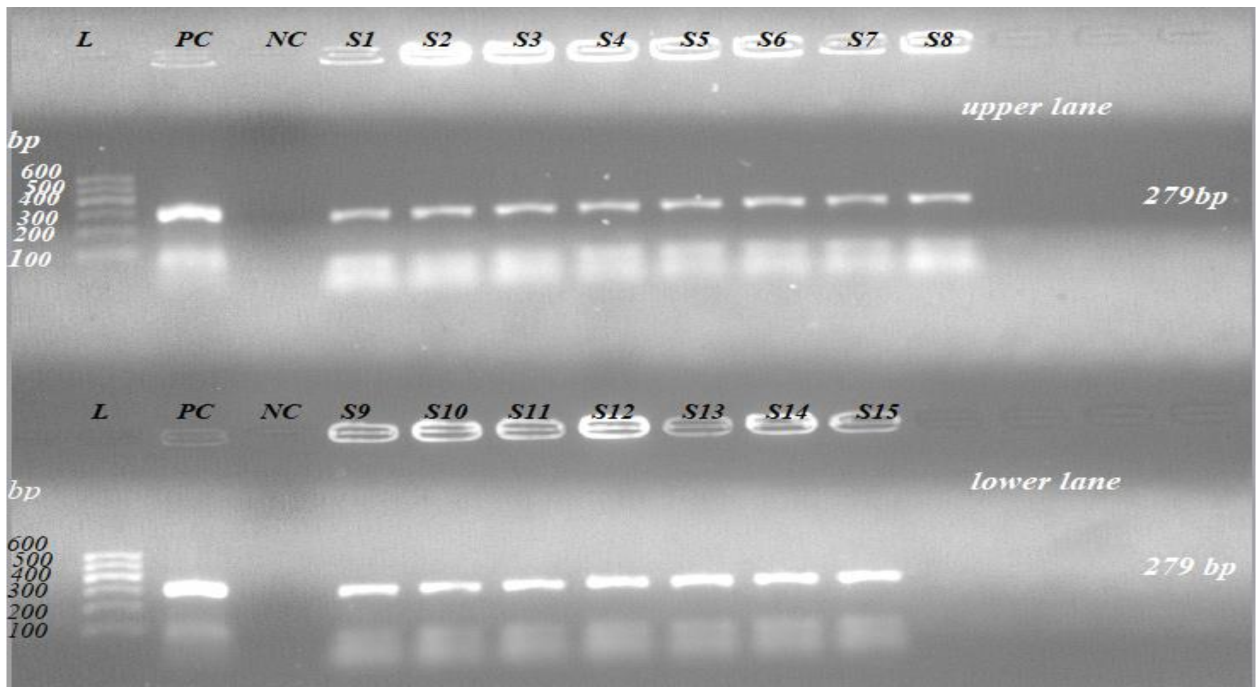


Figure 4: PCR product photograph using a UV illuminator following electrophoresis on a 1.5% agarose gel.

PC = Positive control, L= ladder, S = samples, length of ***nuc gene = 279****, NC = negative control, and bp = base pairs.*

5. DISCUSSIONS

The current study found an overall *S. aureus* prevalence of 13.99% in milk and dairy products, which is consistent with previous reports from Ethiopia, Italy, and Nigeria. Studies from Holeta, Asella, Ambo and Bako, Ethiopia have reported *S. aureus* prevalence ranging from 10.69% to 15.64% in milk and dairy products (Ayano *et al*., 2013; Abunna *et al*., 2016; Gebremedhin *et al*., 2022; Borena *et al*., 2023). Similarly, studies from Italy and Nigeria have found prevalence rates of 12.9% and 12.6%, respectively (Basanisi *et al., 2*017; Umaru *et al*., 2014).

However, the current finding was lower than some other studies, which reported prevalence as high as 19.6%, 20.8%, 40.6% and 24.9% in Holeta, Addis Ababa, Hawassa, and Wolayita Sodo Southern Ethiopia (Beyene *et al*., 2017; Ayele *et al.,* 2017; Daka *et al.*, 2012; Biniam *et al*., 2017). Conversely, the current prevalence was higher than the 10.7% reported by Mekibib *et al*. (2010) in Holeta Town.

The variability in *S. aureus* prevalence across studies may be attributed to differences in study location, sampling protocols, farm management practices, and hygienic standards. Gebremedhin *et al*. (2022) noted that screening methods like the California Mastitis Test can increase detection of *S. aureus* in milk samples.

The current study found *S. aureus* contamination of 20.7% of raw cow milk. This is consistent with previous reports of 21% positivity in raw cow milk from the central highlands of Ethiopia (Tigabu *et al*., 2015) and 23.45 - 25% positivity in the Hawassa, Addis Ababa and Sebeta town (Daka *et al*., 2012; Lemma *et al*., 2021; Ayele *et al*., 2017). However, the prevalence was slightly higher than the 15.29% -17.3% reported in Ambo and Bako, Addis Ababa, Bishoftu, the West Shewa Zone (Borena *et al*., 2023; Mekuria *et al*., 2013; Feyissa *et al.,* 2023; Gizaw, and Duguma., 2014), but lower than the 50% prevalence previously reported in Addis Ababa (Beyene *et al.,* 2017).

Variations in the prevalence of *S. aureus* contamination can be attributed to different sampling methods, hygienic milking practices, handling techniques, and the use of shared towels to dry udders. Observations during the farm-level sample collection process also revealed unsanitary milking and raw milk processing practices. Since *S. aureus* is typically

found on the udders or teat surfaces of infected cows, this is the primary means of transfer between uninfected and infected udder quarters, often occurring during milking (Abebe *et al.*, 2016).

Poor hand-washing practices before milking can increase the risk of intramammary *S. aureus* infection, and people working on dairy farms are more likely to contract the infection (Azevedo *et al.,* 2016). In this study, raw milk had a higher *S. aureus* contamination rate compared to yogurt and cottage cheese. This could be due to environmental contamination, contamination from cows with subclinical mastitis, or improper hygiene during or after milking, such as not cleaning hands before handling milk storage equipment (Quigley *et al.*, 2013).

The current findings showed a 2.4% prevalence of *S. aureus* in conventionally processed dairy products (yoghurt and cheese), which was consistent with Megersa (2018) report of a 5% prevalence in the Ethiopian towns of Ambo and Guder. However, other research on conventionally processed dairy products indicated a comparatively higher occurrence of 11.3% (Lemma *et al*., 2021), 31.15% (Borena *et al*., 2023), and 14.3% (Argaw and Addis, 2015). Traditional yoghurt, which is made by letting the milk to ferment at room temperature without pasteurisation and adding of bacteria to initiate the fermentation process. However, traditional cheese is made by heating milk from which butter has been separated after churning (Berhe *et al*., 2017). Therefore, the reason *S. aureus* is present in yoghurt may be because the bacteria can withstand the lactic acid created by competent bacteria. To safeguard consumers from milk-borne hazards, the milk chain must improve the hygiene of food handlers and equipment and implement cold chain facilities (Tarekgne *et al*., 2015). Dhanashekar *et al*. (2012) also noted that dairy products can be heated or fermented in traditional or modern ways to reduce staphylococcal infection.

In this investigation, 70.8% of dairy cows with mastitis were found to *have S. aureus*. The likelihood of finding *S. aureus* in milk from mastitis-affected cows was 105 times higher than in milk from mastitis-free cows. *S. aureus* is one of the primary causes of mastitis in cattle and can be found on the outside of the udder and teats, as well as in the teat canal (Svennesen *et al*., 2019).

Cow age was identified as a risk factor for the occurrence of *S. aureus*, consistent with previous findings (Zenebe *et al.*, 2014; Kerro and Tareke, 2003). The prevalence of *S. aureus* was also significantly correlated with the type of bedding used or the state of the farm, with mud floors having a higher prevalence of *S. aureus* than concrete floor types (Abera *et al.,* 2013; Biffa *et al.,* 2005). This may be due to the spread of infections in dirty, muddy communal barns, as *S. aureus* is an environmentally hardy organism that can tolerate a wide range of temperature and moisture conditions and easily adhere to the roughened epithelium at teat orifices (Kibebew, 2017).

The prevalence of *S. aureus* was found to be substantially lower in early-lactation cows compared to mid- and late-lactation cows in the current study. While no notable difference was observed between the early and mid-lactation stages, this result aligns with previous research of (Argaw, 2016; Fursova *et al.,* 2020; Baraki *et al*., 2021; Sarba and Tola, 2017; Belayneh *et al*., 2013). In contrast, a much higher prevalence was reported in the middle stage of lactation by Mureithi and Njuguna (2016) in Kenya. Furthermore, Gebremedhin *et al.* (2022) and Abebe *et al*. (2016) noted a higher *S. aureus* prevalence in the early stages of lactation. Variations in the age, parity, and breed of the sampled animals across studies may account for the differences in the effects of lactation phases, and further investigation is required to determine the true cause of this variation.

The current study found that the prevalence of *S. aureus* was 19% in intensive management systems and 34% in semi-intensive management systems. This is consistent with the findings of Borena *et al*. (2023), who reported a higher prevalence in semi-intensive management (28.57%) than in intensive management (23.53%) in Ambo and Bako towns, Oromia, Ethiopia. Similarly, Abebe *et al*. (2016) observed a higher incidence of *S. aureus* in semi-intensive management (63.2%) compared to intensive management (36.8%) in Hawassa, Southern Ethiopia. This may be due to the increased stress and tick infestation experienced by animals when grazing in the field under semi-intensive management, which can expose the teats to pathogenic microbes like *S. aureus*.

Antimicrobial susceptibility testing conducted in this investigation revealed that *S. aureus* exhibited resistance to certain widely used antimicrobials. However, the susceptibility of the *S. aureus* isolates to ciprofloxacin (96.3%), trimethoprim/sulphamethoxazole (94.3%), clindamycin (98.1%), gentamycin (90.7%), erythromycin (83.33%), and cefoxitin (98.1%)

is presented in this study. Nonetheless, it was discovered that the isolates exhibited a high level of resistance to tetracycline (46.3%), ampicillin (92.6%), penicillin G (85.2%), and amoxicillin (100%). Similar results were obtained by Marami *et al*. (2022) from smallholder dairy farms in central Oromia, where the isolates exhibited a strong resistance pattern to ampicillin and amoxycillin, 100% and 90%, respectively. The *S. aureus* isolates showed 95% and 98.48% resistance to ampicillin and amoxycillin by Gebremedhin *et al.* (2022) and Borena *et al*. (2023). In contrast to the present results, *S. aureus* isolates were shown to be resistant to amoxycillin by Abera *et al.* (2010) (36.1%) and Ayele *et al.* (2017) (60.9%). It is not unusual that people have developed resistance to ampicillin and amoxicillin as these medications have long been the most widely used antimicrobials in Ethiopian veterinary and human medicine (Befikadu *et al*., 2017).

The study by Gebremedhin *et al*. (2022), Marami *et al. (*2022), and Lemma *et al*. (2021) reported similar levels of tetracycline resistance in *S. aureus* isolates at 32.5%, 40%, and 46.2%, respectively. In contrast, Ayele *et al*. (2017), Borena *et al*. (2023), Jamali *et al*. (2015), and Feyissa *et al*. (2023) reported higher tetracycline resistance at 68.3%, 83.33%, 56.1%, and 100%, respectively. The 46.3% tetracycline resistance found in the current study was lower than the previous reports. Past research in other area by El-Jakee *et al.* (2008) and Daka *et al*. (2012) also revealed alarming levels of *S. aureus* resistance to tetracycline, ampicillin, and amoxicillin, suggesting this may have developed due to prolonged and careless use of these antimicrobials.

The penicillin G resistance in the current *S. aureus* isolates was 85.2%, which was lower than the previously reported range of 94.4%-100% from various sources, including Borena *et al*. (2023), Feyissa *et al.* (2023), Ayele *et al*. (2017), Marami *et al*. (2022), and Abera *et al*. (2013). Antimicrobial resistance has partially arisen due to the frequent use of antibiotics in dairy animals. The production of beta-lactamase, an enzyme that renders penicillin and closely related drugs ineffective, contributes to *S. aureus* resistance (Daka *et al*., 2012). The frequency of antimicrobial used might be attributed to variation in resistance results in the study area.

The 2% cefoxitin-resistant *S. aureus* isolates found in raw milk in this study are consistent with the findings of Feyissa *et al*. (2023), but lower than the results reported by Borena *et al*. (2023), Beyene *et al*. (2017), and Lemma *et al.* (2021). The cause of the occurrence of

antimicrobial-resistant *S. aureus* isolates could be attributed to the arbitrary use of antimicrobials, self-medication, administration of sub-therapeutic doses to livestock as a preventative measure, and the limited updating of long-used drug groups (Haftay *et al.*, 2018).

In the current investigation, 87.4% (47/54) of the *S. aureus* isolates from milk and milk products exhibited multiple drug resistance. In contrast, studies by Borena *et al*. (2023), Gebremedhin *et al*. (2022), and Regasa *et al*. (2019) reported higher (62.5%), and lower (34.94%, 2.4%) percentages of multidrug-resistant *S. aureus* isolates from milk and milk products.

The high prevalence of multiple drug resistant (MDR) *S. aureus* isolates (87.4%) in milk and milk products is a significant public health concern. MDR pathogens can compromise the ability to effectively treat and control infectious diseases, leading to increased morbidity, mortality, and the potential for the emergence of untreatable infections (Ventola., 2015). The rapid spread of MDR infections within healthcare settings and the community is a particular risk, as MDR pathogens are more easily transmitted due to their resistance to common antimicrobial agents used for prevention and control (Bassetti *et al*., 2017). Additionally, the management of MDR infections can be more costly, placing an economic burden on the healthcare system due to the need for more expensive or alternative antimicrobial agents and prolonged hospital stays (Prestinaci *et al*., 2015). The high prevalence of MDR *S. aureus* in milk and milk products highlights the urgent need for improved food safety and hygiene practices, as well as the development of new antimicrobial strategies to address this issue and mitigate the public health implications (Wieczorek and Osek, 2013).

Factors contributing to the emergence of antibiotic resistance include not only the overuse of antibiotics but also the inappropriate use (inappropriate choices, inadequate dosing, and poor adherence to treatment guidelines). Under the selective pressure of antibiotics, susceptible bacteria are killed or inhibited, while bacteria that are naturally (or intrinsically) resistant or that have acquired antibiotic-resistant traits have a greater chance to survive and multiply (Prestinaci *et al*., 2015).The evolution of antibiotic resistance poses a risk to public health due to the potential difficulty in treating foodborne outbreaks and the fact that multidrug-resistant *S. aureus* is a reservoir for communicable resistant genes in the food supply (Haftay *et al.,* 2018).

The current investigation found that the *S. aureus* isolates were 85.2% resistant to Penicillin G, 100% resistant to Amoxicillin, and 92.6% resistant to Ampicillin. However, the results of the traditional PCR analysis revealed that all 25 isolates that underwent DNA amplification were positive for the *nuc* gene, indicating they were thermostable (González-Domínguez *et al*., 2020). Interestingly, the *mecA* and *blaZ* genes were not detected in these isolates.

Similar to this finding, a Nigerian investigation (Olayinka *et al*., 2009) found that 139 *S. aureus* isolates exhibiting phenotypic resistance to oxacillin (methicillin) were all negative for the *mecA* gene using classical PCR detection. According to a prior Sudanese investigation, 9.8% of MRSA strains recovered from various clinical samples lacked the *mecA* gene (Elhassan *et al*., 2015). It has long been believed that finding the *mecA* or *mecC* genes is a key confirming technique for MRSA (Stegger *et al*., 2012). Nevertheless, the lack of *mecA* genes in this and previous investigations raises the prospect that the MRSA phenotype is caused by different mechanisms. Similar results from earlier research have been reported, pointing to potential alternative genetic processes (Elhassan *et al*., 2015). According to González-Domínguez *et al*. (2020), the *blaZ* gene encodes the β-lactamase enzyme, which is responsible for hydrolyzing beta-lactam antibiotics such as Ampicillin, Penicillin, and Amoxicillin.

A limitation of this research is that molecular characterization and enterotoxin gene detection were not carried out. Additionally, only 25 out of the 54 isolates were subjected to DNA amplification for resistance gene identification, due to a lack of resources in terms of money and primers. Therefore, for future studies of this nature, it would be beneficial to incorporate molecular characterization, resistance gene detection, and enterotoxin gene detection to provide a more comprehensive understanding of the *S. aureus* isolates.

6. CONCLUSIONS AND RECOMMENDATIONS

The results of this study demonstrate that *S. aureus* is commonly present in raw milk, milking equipment, milkers' hands, and milk products in and around the Hawassa, town. The high rate of *S. aureus* isolation indicates that the public health is at significant risk due to the widespread consumption of raw milk and milk products in the area, as the survey findings showed that raw milk consumption is commonplace, there is a lack of food safety training, and the milking practices in the research area are unhygienic. The antibiotic susceptibility testing revealed that the majority of the *S. aureus* isolates have acquired multidrug resistance and are highly resistant to commonly used antibiotics in the local veterinary service. Importantly, the isolates were found to be heat-stable (thermonuclease positive) based on the PCR analysis, suggesting their potential to produce food poisoning toxins.
Based on the conclusions of this study, the following recommendations are proposed:

- Develop and implement comprehensive containment plans for the prevention and management of *S. aureus* infections to reduce the prevalence of the disease.
- Introduce and provide antimicrobials with more potent therapeutic responses to address the multidrug-resistant nature of the isolates.
- Ensure that antimicrobial usage is prescribed by qualified professionals and that antimicrobial resistance is routinely monitored and assessed.
- Provide food safety training to consumers and those handling milk products on the importance of good hygiene practices and the potential zoonotic diseases associated with *S. aureus*.
- Conduct further research to investigate the discrepancy between phenotypic and genotypic detection of resistance genes, in order to better understand the mechanisms by which *S. aureus* develops resistance, especially to β-lactam antibiotics.

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8. ANNEXES

Annex 1: Questionnaire survey.

NO.	Questionnaires for dairy farmers	Responses
	1. General characteristics of individuals	
1.1	Age	-----------
1.2	Sex	Female [] Male [ ]
1.3	Level of Education	Informal Education [] Illiterate [ ]Primary Education [ ] Secondary Education [ ] degree [Other (Specify)………………
1.4	What is your position at the farm?	attendant [] milker [ ] Veterinarian [ ] Other (specify)_____________
1.5	Which kind of management system is in use	extensive [] Semi- intensive [] Intensive
1.6	Which kind of bedding was used?	Clean floor [] Muddy soil floor [] Concrete [] Other specify__
1.7	type of animal Breed	HF Cross [] Local [] Jersey [] other ______
1.8	Animal Age	>5 years [] <5 years []
1.9	Herd size	21-40 [] 5-20 [ ] > 40[ ]
1.10	cow's parity status	2-3 calving [] 1-2calving [] >5 calving [ ]
1.11	The cow's lactation stage	3-6month [] 0-3month [ ] ≥7month [ ]
1.12	How many of the teats are working?	one [] All [ ] two [ ] three [ ]
1.13	Has disinfection been used on the farm?	No [] Yes [ ]
1.14	When milking a cow, do you wash your hands?	Yes [ ] No[ ]
1.15	Have you used hand dying cloth after washing	Yes [] No [ ]
1.16	Before milking a cow, do you wash its teat and udder?	Yes [] No[ ]
1.17	What kind of water source was used, if yes for Q1.16?	Tap water [] ground water[ ] river water [ ]
1.18	Practice used for Teat and udder washing	Cold water and detergent [] Cold water [] Warm water and detergent [] Warm water []

1.19	milking procedure used	machine [] hand [ ]
1.20	How often is the barn cleaned?	daily [] More than two per a week [] Once per week [ ]
1.21	Which kind of milking utensils used?	Wide necked-plastic () Narrow Wide necked-aluminium [] necked plastic containers [ ] Other(s) specify [ ]
1.22	The Source of water used for cleaning milk equipment's	Wells (pond) [ ] Pipelines (tap) [ ] River [] (other)___
1.23	Which kind of procedure is applied for cleaning milk equipment?	Warm water and detergent [] Cold water and detergent [ ] Warm water only [ ] Cold water only [ ]
1.24	Do you conduct tests on milk quality at the farm level?	YES [] NO [ ]
1.25	If yes for Q1.24, what kind of test is used?	clot on boiling test [] organoleptic test [] lactometer test [] Alcohol test [] Others specify
1.26	Do your dry teat using a towel?	YES [] NO [ ]
1.27	How are drying cloths (towels) used?	Individual cloth [] Common cloth []
1.28	Have you taken any training on food safety?	YES [] NO [ ]
1.29	Do you have information about diseases that are transmitted through milk?	YES [] NO []
1.30	Do you consume raw milk	YES [] NO []
1.31	Do you have a refrigerator where you can keep the leftover milk from your daily sales?	YES [ ] NO [ ]
1.32	Do you have knowledge regarding food poisoning caused by staphylococci?	YES [] NO []
	Questionnaire for product vendors	
2.	Age	-----------
2.1	Sex	Female [] Male []
2.2	Level of education	Informal Education [] Illiterate [] Primary Education [ ] Secondary Education [ ] degree [Other (Specify)………………

2.3	Have you taken any training on food safety?	YES [] NO []
2.4	Do you have information about diseases that are transmitted by milk?	YES [] NO []
2.5	Do you consume raw milk	YES [] NO [ ]
2.6	Do you have knowledge regarding food poisoning caused by staphylococci?	YES [] NO []
	Questionnaire for Milk collectors	
3	Age	-----------
3.1	Sex	Male [] Female [ ]
3.2	Level of education	Informal Education [] Illiterate [] Primary Education [ ] Secondary Education [ ] degree [Other (Specify)………………
3.3	Equipment used for storing milk in bulk	Wide necked-plastic(cadisco) Wide necked-aluminium [ ] Narrow necked plastic containers [ ] Other(s) specify [ ]
3.4	The source of water utilized for milk equipment cleaning.	Wells (pond) [ ] Pipelines (tap) [ ] tap water River [ ] (other)___
3.5	What kind of procedure is employed to clean milk processing equipment?	Warm water and detergent [ ] Cold water and detergent [ ] Cold water only [ ] Warm water only [ ]
3.6	Do you check milk quality test?	YES [ ] NO [ ]
3.7	If yes for Q.36 what kind of test used	organoleptic test [ ] clot on boiling test [ ] lactometer test [ ] Alcohol test [ ] Others specify __________
3.8	How many hours on average does fresh milk take to finish?	3 hrs after collection 2) 6 hrs after collection 3) 9 hrs after collection 4) 12 hrs after collection 5) Other(s) specify
3.9	When do you receive milk or milk products from the supplier(s)?	1) Morning hours 2) Afternoon hours 3) Evening hours 4) Other (s) specify______
3.10	Do you have a refrigerator where you can keep the leftover milk from your daily sales?	YES [] NO [ ]
3.11	Do you smoke milk equipment using the traditional method?	YES [] NO [ ]

3.12	Do you have information about diseases that are transmitted through milk?	YES [] NO [ ]
3.13	Do you consume raw milk	YES [] NO []
3.14	Have you taken any food safety related training	YES [] NO []
3.15	Do you have knowledge regarding food poisoning caused by staphylococci?	YES [] NO []

Annex 2: Research Informed Consent Form

Title: Prevalence, isolation, identification, and antimicrobial susceptibility profile of *Staphylococcus aureus* from raw cow milk and traditionally processed dairy products in and around Hawassa town, Ethiopia.

Investigator: Dr. Eskedar Wodaje (MSc student at AAU College of veterinary Medicine and agriculture, Bishoftu, Ethiopia

A few general guidelines regarding this research that you should be aware of.

You are asked to take part in a research study. You are under no obligation to participate in this study, and you are free to decline participation at any time. The objective of research studies is to acquire additional knowledge regarding a particular topic or situation. Participating in a study does not guarantee that you will gain anything personally. This permission form contains comprehensive information about the study in which you are being asked to participate. The study that you are being asked to join in is described in this consent form.

The goal of the research: Numerous food-borne infections can affect both human and animal health. Ethiopia is afflicted by numerous illnesses. It is crucial that future prevention and treatment programmes be able to concentrate on these problems. The KAPs of the community regarding the handling and consumption of milk in the study area are being assessed, along with the isolation, identification, and antibiotic resistance profile of *Staphylococcus aureus* from raw cow milk and conventionally processed dairy products. You will be asked to answer questions from a survey I will be handing out verbally if you agree to participate in this study. The questionnaire should take you about ten minutes to complete.

This work has two benefits: it identifies the sources of *Staphylococcus aureus* contamination in raw milk and milk products and proposes a mechanism of transmission from animals to humans. This research will provide valuable information for future control efforts against bacterial zoonotic illnesses in developing countries such as Ethiopia. Study-related data shall remain confidential to the maximum degree allowed by law. Nothing that could be used to identify you, either in written or spoken reporting, will mention the study. There will be no need for you to write your name on any study materials, so nobody will be able to associate your answers with you. Participating in this study carries no financial benefits. If you would like to contact me with any questions about the study, my phone number is +251912490484 and my email address is wodaje.e82@gmail.com.

Assent to Take Part: I was given the option to read or hear the information above, and I was aware of it before I agreed to participate. Someone brought me this paperwork. Hereby, I give my permission to take part in this study, knowing that I can leave at any time without incurring any fees or losing any benefits to which I might otherwise be eligible.

Name of participant___________ Date___________ Signature_______Phone №_______

Annex 3: Standard protocol for *S. aureus* identification with MALDI_TOF

1. An Eppendorf tube was filled with 300 µl of water that was suitable for HPLC analysis.
2. After mixing HPLC-grade water with isolated pure colonies from the cultivated plate, a full suspension was made.
3. The suspensions were mixed by centrifugation at 15000 rpm for two minutes after adding 900 µL of pure ethanol.
4. With a pipette, the supernatant was removed.
5. To ensure there was no leftover ethanol, the tubes were centrifuged for two minutes at 15,000 rpm.
6. For five minutes, the tubes were allowed to air dry at ambient temperature.
7. Following the addition of 25 µL of 70% formic acid, the pellets were reconstituted.
8. Centrifugation was performed for two minutes at 15000 rpm after adding 25 µL of 100% acetonitrile.
9. A single, clean MALDI-target plate was coated with 1µl supernatant, which was then allowed to air dry for fifteen minutes.

10. At the designated BTS QC point, 1 µL of BTS was applied, and the mixture was allowed to dry completely.
11. Every sample position and BTS QC position had 1µL of CHCA matrix solution applied over them.
12. The areas were allowed to air dry at ambient temperature.
13. A unique bar code was scanned, and then the target plate was placed into the mass spectrometer.

Annex 4: Methods for DNA extraction.

1. In the Eppendorf tube containing TSB, 1.75 ml of MALDI-TOF-confirmed bacteria were preserved and incubated for 24 hours at 37 degrees Celsius.
2.The tubes were centrifuged for five minutes at 20,000 rpm.
3. After discarding the supernatant, 180µl of lysis buffer (ATL) was added to each tube.
4. The tubes were vortexed for 30 seconds and then incubated at 37 degrees Celsius.
5. 200 µl of lysis buffer (AL) and 25 µl of proteinase K were added, after 2-minute vertexing period.
6. For half an hour, the tubes were incubated at 56 oC.
7. After adding 200 µl of 100% ethanol, the tubes were vortexed for a minute.
8. A micropipette was used to transfer all the contents to the labelled DNase main spin column (Qiagen DNease extraction kit) (Thermo Scientific, Germany), and the tubes have been spun for one minute at 10,000 rpm.
9. After being taken out of the collection tubes, the column was put into fresh tubes.
10. After adding 500µl of wash buffer one (AW1), the mixture was then spun for one minute at 10,000 rpm.
11. Following the extraction of the column from the collecting tube, 500 µl of wash buffer two (AW2) was added, and the mixture was centrifuged for three minutes at 20,000 rpm.
12. After that, the extracted DNA was put onto a 1.5 ml tube column, filled with 200 µl of elution buffer (AE), and allowed to dry at room temperature for a minute.
13. The DNA extracts were then kept at -20 oC until another extraction could be done after being centrifuged for one minute at 10,000 rpm.

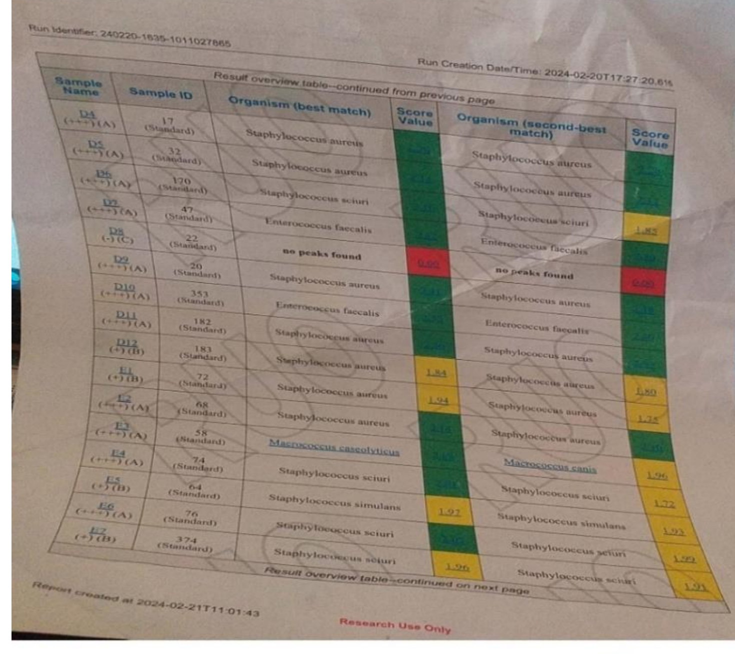
Annex 5: Laboratory work results and instrument used photos

Media preparation

Bacterial Inoculation

Colony characteristics of S. aureus on mannitol salt agar media with negative control

MALD-TOF MS



Run Identifier: 240220-1635-1011027865

Run Creation Date/Time: 2024-02-20T17:27:20.615

Result overview table--continued from previous page

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
D4 (+++)(A)	17 (Standard)	Staphylococcus aureus	[illegible]	Staphylococcus aureus	[illegible]
D5 (+++)(A)	32 (Standard)	Staphylococcus aureus	[illegible]	Staphylococcus aureus	[illegible]
D6 (+++)(A)	170 (Standard)	Staphylococcus sciuri	[illegible]	Staphylococcus sciuri	1.85
D7 (+++)(A)	47 (Standard)	Enterococcus faecalis	[illegible]	Enterococcus faecalis	[illegible]
D8 (-)(C)	22 (Standard)	no peaks found	0.00	no peaks found	0.00
D9 (+++)(A)	20 (Standard)	Staphylococcus aureus	[illegible]	Staphylococcus aureus	[illegible]
D10 (+++)(A)	353 (Standard)	Enterococcus faecalis	[illegible]	Enterococcus faecalis	[illegible]
D11 (+++)(A)	182 (Standard)	Staphylococcus aureus	[illegible]	Staphylococcus aureus	[illegible]
D12 (+)(B)	183 (Standard)	Staphylococcus aureus	1.84	Staphylococcus aureus	1.80
E1 (+)(B)	72 (Standard)	Staphylococcus aureus	1.94	Staphylococcus aureus	1.75
E2 (+++)(A)	68 (Standard)	Staphylococcus aureus	[illegible]	Staphylococcus aureus	[illegible]
E3 (+++)(A)	58 (Standard)	Macrococcus caseolyticus	[illegible]	Macrococcus canis	1.96
E4 (+++)(A)	74 (Standard)	Staphylococcus sciuri	[illegible]	Staphylococcus sciuri	1.72
E5 (+)(B)	64 (Standard)	Staphylococcus simulans	1.97	Staphylococcus simulans	1.93
E6 (+++)(A)	76 (Standard)	Staphylococcus sciuri	[illegible]	Staphylococcus sciuri	1.99
E7 (+)(B)	374 (Standard)	Staphylococcus sciuri	1.96	Staphylococcus sciuri	1.91

Result overview table--continued on next page

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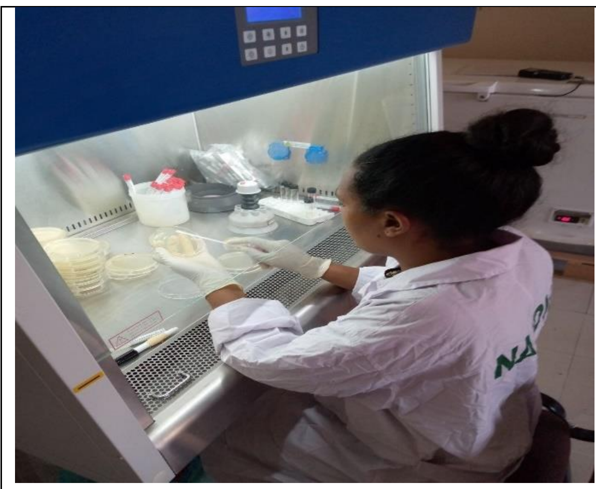
Research Use Only

CamScanner

MALDI- TOF out put results



Inoculation *S. aureus* isolates on Muller agar

Measuring the inhibitory zone using calliper

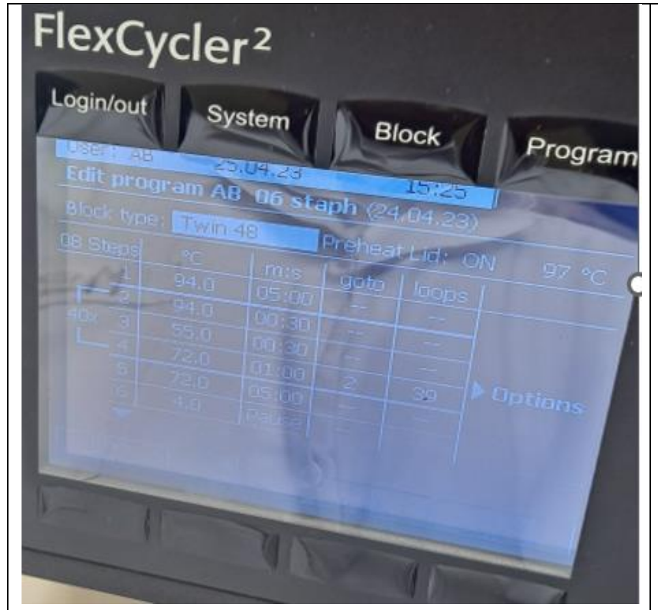
McFarland Densitometer

Antimicrobial susceptibility test results

Agarose gel slot

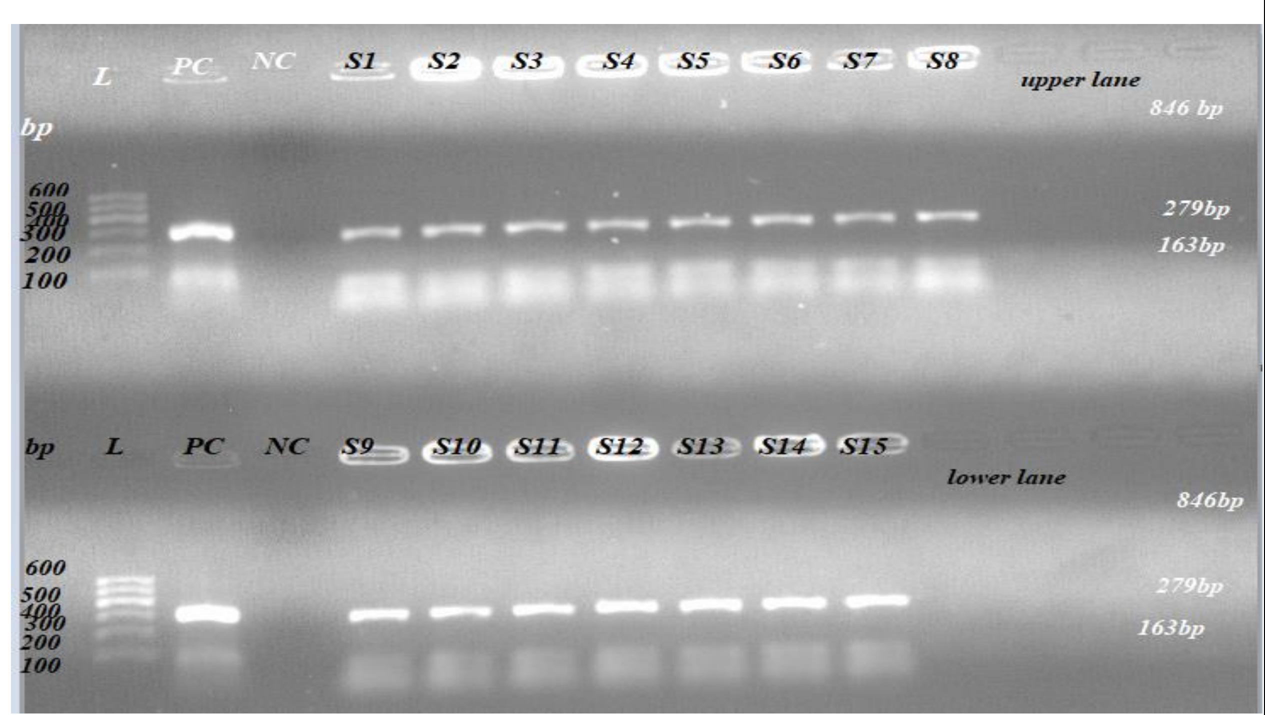
Electrophoresis

Agarose gel after electrophoresis



Thermal cycler

Gel documentation Machine



Conventional PCR amplification of the resistance gene imaging with a UV-illuminator following 1.5% agarose gel electrophoresis.

Annex 6: Ethical clearance.

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/02/17/16/2024

Name of Applicant: **Eskedar Wodaje (DVM, MSc student)**

Address: Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Prevalence, isolation, identification and antimicrobial susceptibility profile of S. aureus from raw cow milk and traditionally processed dairy products in and around Hawassa town, Sidama-Ethiopia*

Date of application:	**December, 2023**
Nature of the project:	**Field investigation**
Target animal species:	Dairy **cattle**
Number of animals involved:	**242**
Study area:	**Hawassa- Ethiopia**

Minutes No. and date of review: **VM/ERC/02/16/024, 26/03/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Eskedar Wodaje.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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