ASSESSMENT OF BOVINE MASTITIS IN LACTATING COWS, AND CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* FROM THE COWS AND DAIRY PRODUCTS IN WEST SHOWA ZONE, ETHIOPIA

PhD Dissertation

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PhD Program in Applied Microbiology

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Addis Ababa, Ethiopia

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A Dissertation submitted to the Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences of Addis Ababa University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Applied Microbiology

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STATEMENT OF THE AUTHOR

I first, declare that this dissertation is my bonafide work and that all sources of materials used for this dissertation have been duly acknowledged. This dissertation has been submitted to the requirements for PhD Degree at Addis Ababa University, College of Natural and Computational Science and is deposited at the University's Library to be made available to borrowers under rules of the library. I solemnly declare that this dissertation is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this dissertation are allowable without special permission provided that accurate acknowledgment of source is made. Requests for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major Department or the Dean of the School of Graduate Studies when in his judgment the proposed use of the material is in the interest of scholarship. In all other instances, however, permission must be obtained from the author.

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BIOGRAPHCAL SKETCH

I, the author of the dissertation was born on June 6/1977 in a village named Meti of Saphera Meti peasant association in the then Limu District of Wollega Province, and now bounded to Gida Ayana district of East Wollegga Zone, West of Oromia region. I was schooled my elementary level at Labu Boko Elementary School (1986-1990), junior secondary school at Gida Ayana Junior Seconary School (1991-1992), and the secondary school at Gida Ayana Secondary School (1993-1996). In Ethiopian School Leaving Certificate Exam, (ESLCE) I scored a point for diploma and joined Addis Ababa University faculty of veterinary medicine and graduated in Animal Health Assistance in 1998. After successful completion of my diploma in the aforementioned profession, I was then employed by Oromia Agricultural Bureau in Abe Dongoro District of Horo Guduru Wollega Zone in 1999. I have served in Animal Health Assistant, team leader, deputy head of the Agricultural Office, and head of Animal Health and Production Agency in Abe Dongoro District. I, in turn, pursued the summer program of Addis Ababa University College of Veterinary Medicine and Agriculture, and attained my BSc. in Veterinary Laboratory Technology in 2010. I graduated from the college in July 2010 and was employed by Ambo University department of Veterinary Laboratory Technology at Graduate Assistant level in 2011. As a graduate assistant, I had been teaching General Veterinary Microbiology, Diagnostic veterinary bacteriology, and Diagnostic Veterinary Virology and Mycology. From 2013-2014, I pursued MSc. in Veterinary Microbiology in Addis Ababa University College of Veterinary Medicine and Agriculture in regular program. After completion of MSc, I have been teaching courses related with veterinary microbiology including, Virology, Bacteriology, Mycology, Immunology and Molecular Biology in Ambo University Guder Mamo Mezemir Campus department of Veterinary Laboratory Technology. I have published 8 articles in reputable journals. After 4 years of experience I joined Addis Ababa University, College of Natural and Computational Science in September 2018 to pursue a PhD study in Applied Microbiology (Anex 8).

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LIST OF ABBREVIATIONS

AMP	Antimicrobial Peptide
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
BHI	Brain Heart Infusion
Cbp	Collagen-binding protein
CHIPS	Chemotaxis Inhibitory Protein of *S. aureus*
CI	Confidence Interval
CL	Confidence Level
Clfs	Clumping factors
CMT	California Mastitis Test
CV	Core Variable
CWA	Cell Wall Associated
Eap	Extracellular adherence protein
ECM	Extracellular Matrix
EDT	Extended Direct Transfer
ETB	Ethiopian Birr
FBP	fibrinogen-binding protein
FLIPr	Formyl peptide receptor-like 1 inhibitory protein
FnBP	Fibronectinbinding Proteins
GDP	Gross Domestic Product
LAB	Lactic Acid Bacteria
MALDI-ToF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MGE	Mobile genetic elements

MIC	Minimum Inhibitory Concentration
MLS	Macrolide Lincosamide Streptogramin
MRSA	Methicillin Resistance *Staphylococcus aureus*
MSCC	Microscopic Somatic Cell Count
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecule
NAHDIC	National Animal Health Diagnostic and Investigation Center
O-F	Oxidation-Fermentation
PA	Peasant Associations
PBP	Penicillin Binding protein
PCR	Polymerase Chain Reaction
PSM	Phenol-soluble Modulins
SAK	Staphylokinase
SCC	Somatic Cell Count
SCIN	Staphylococcal Complement Inhibitor
SCM	Subclinical Mastitis
SE	Staphylococcal Enterotoxin
SEl	SE-like
SERAM	Secreted Expanded Repertoire Adhesive Molecules
SFMT	Surf field Mastitis Test
SLST	sodium Lauryl Sulphate Test
SpA	Staphylococcal protein A
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TSST	Toxic Shock Syndrome Toxin

USD	USA Dollar
WHO	World Health Organization
WST	Whiteside Test

ABSTRACT

Mastitis, to which *Staphylococcus aureus* is one of the main causative agents, is the costly mammary disease which adversely affects dairy industry worldwide in general and in developing countries in particular. *S. aureus* is a Gram-positive coccus resides on the skin and mucus linings, and associated with various diseases in animal and human. The virulence factors of *S. aureus* associated with disease causing potential and/or survive in the harsh melena include different enzymes and toxin productions, antibiotic resistance, and survival in dry conditions and relatively high concentration of salt. A cross-sectional study with objectives of determining the prevalence of mastitis in lactating cows, and assessing the prevalence and virulence factors of *S. aureus* isolated from the lactating cows, and bulk milk, and milk products (yogurt and cheese) was, conducted from May 2020 to March 2021 in West Showa Zone of Oromia, central Ethiopia. Stratified random sampling method was applied to select the districts. The PAs, the farm (herds), and individual sampling units were selected randomly. Mastitis was diagnosed physically by observations and palpation for clinical mastitis, and by California Mastitis Test (CMT) method for subclinical mastitis. Teat milk from the lactating cows, bulk milk, yogurt, and cheese samples were collected for bacterial isolation. Farmers and milk product retailers were interviewed using structured questionnaire to assess the hygienic milk collection and storage practices in the study area. The biological samples were aseptically collected, labeled, and were transported under cold chain to the Zoonosis and Food Safety Research Laboratory of Ambo University for bacterial isolation. *S. aureus* was selectively isolated on a manitol salt agar at 37°C for 24 hours, and presumptively identified by growth and rapid manitol fermentation within 24 hours, Gram staining and observation under microscope, and catalase and coagulase tests. Finally, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-ToF) test was used to confirm the species. The enzyme production ability of the isolates was determined using heated plasma agar for staphylokinase, butterfat-supplemented brain-heart infusion agar for lipase, and skimmed milk agar for protease. The enterotoxin genes, resistant genes (*blaZ*, and *mecA*), and thermo nuclease gene (*nuc)* were assessed using a specific primer-based conventional polymerase chain reaction (PCR). In addition, the antibiotic susceptibility pattern of the isolates was determined using the disc diffusion method on Muller Hinton agar. The data obtained from the field assessment, laboratory experiment, and questionnaire surveys were analyzed using SPSS version 20 and R-statistical software. . The data were summarized using frequencies' and percentage, and presented in graphs and tables. The risk-factors associated with the prevalence of mastitis and *S. aureus* , and virulence factors of *S. aureus*, were analysed and interpreted by using chi-square test, logistic regression, t-test, and ANOVA at 95% confidence interval (C.I.) with $p<0.05$ significance levels. Accordingly, 258 lactating cows were diagnosed for mastitis and 97 (37.6%) of them were mastitis-positive. The prevalence of mastaitis was found significantly increased with the advancement of lactation stage of the cows, in those milked with unwashed hands and unwashed udders before milking, and in cows infested by ticks when compared with early lactation stage, those milked with washed hands and udder before milking, and tick free cows respectively ($p<0.05$). Subclinical mastitis was significantly more prevalent than clinical mastitis ($p<0.05$). The result of the study also disclosed that 94 (15.36%) of 612 samples were positive for *S. aureus*. The isolation rate of *S. aureus* was significantly higher in mastitis-positive cows (35.05%) than in mastitis-negative cows (5.59%) ($p<0.05$). The prevalence of *S. aureus* was also significantly higher in cows reared in farms with large number of lactating cows and tick-infested cows than in cows of farms with small number of lactating cows and in tick free cows, respectively ($p<0.05$). In addition, 354 bulk milk and milk product samples were collected and

the bacterium was significantly more frequent in bulk milk (20.3%) and cheese (14.9%) than in yogurt (4.8%). Moreover, 71 (77.2%), 51 (55.4%), and 62 (67.4%) of 92 *S.aureus* isolates were positive for protease, staphylokinase and lipase respectively. The PCR result revealed that the *S.aureus* isolates harbored *sea*, *seb*, and *tsst-1* genes at rate of 12/24 (50%), 3/24 (12.5%), and 4/24 (16.6%) respectively. The *S. aureus* isolates were resistant to penicillin(100%), tetracycline (100%), oxy-tetracycline (100%), amoxicillin (100%), ampicillin (83.3%), and to norfloxacin (75%). Of the 92 isolates tested 12(13%) were found resistant to oxacillin (1 μg) and were considered MRSA. The PCR test of the resistant genes (*mecA* and *blaZ*) revealed that 10/44 (22.73%) were *mecA* positive; but none of them harbored the *blaZ* gene. In addition, all (100%) of the 34 isolates tested for the thermonuclease (*nuc)* gene were positive to the gene. The questionnaire survey also indicated that all of the 140 respondents milked the animals manually. More than 50% of them have not washed their hands before milking; have not used the udder drying clothes; and/or used it for more than one cows; used plastic utensils for collecting and storing milk and milk products; and have not smoked the utensils. Therefore, it is concluded that mastitis was prevalent in the study area, and *S. aureus* might be the major cause of the disease. In addition, the milk and fermented milk products might not be safe for human consumption. Moreover, the unhygienic milking practices and tick infestation were one of the factors playing role in the prevalence of mastitis in the study area. The milk collection and storage practices might exacerbate the cross contamination of the dairy products by pathogens from the miller's hand, utensils and/or the environment so that the source of the *S. aureus* could be the animals, the handlers or the utensils. The presence of the enterotoxin genes in the bacterium is indicative for the food intoxications caused by the toxins. Furthermore, different enzymes producing ability of the isolates, summed up with resistance to commonly used antibiotics revealed that the *S. aureus* isolates are highly virulent to cause serious and incurable infections. The availability of the resistant genes (*mecA*) particularly in the isolates from milk and milk products can be evident for the possible transfer of the gene to other bacterium if co-infection occurs or to the enterobactriacae found in the GIT. Therefore, other mastitis causing agents and their antibiogram characteristics and virulence factors, and the types of MRSA distributed in the area should be studied. In addition, in order to prevent and control the disease and cross contamination of the dairy product, the animal and dairy product handlers should be trained about the hygienic milking practices and food handling methods.

Key Words: Lacattin Cows, Mastitis, Milk and Milk Products, *S. aureus*, Virulence Factors.

1. INTRODUCTION

1.1. Background

More than 80% of the livelihood of Ethiopia's population relies on various agricultural activities. This fact maintains the agricultural sector as the major mainstay of the economy of the country (Asresie & Zemedu, 2015; Bekuma & Galmessa, 2018; Endalew & Ayalew, 2016). The sector contributes 33% to the total gross domestic product (GDP), 87% to national exports, and 72.7% to total employment (Addfiisu & Gadisa, 2022).

Agriculture in Ethiopia is structurally categorized into three sub-sectors: crop production, forestry, and livestock production. Among the categories, crop and animal productions are the two dominant subsectors accounting for 60% and 20% of agricultural and gross domestic GDP respectively (Solómon *et al*., 2021). Livestock production alone contributes more than 16% of the total national GDP (Gi̊rma & Tamir, 2022).

Livestock production is an essential component of agriculture which has been proposed to play a significant role in the accomplishment of the sustainable development goals of the United Nations, particularly in developing countries (FAO, 2018; Gebreyohanes *et al*., 2021). This agricultural subsector plays an important role in Ethiopia's economy. It is a source of income and employment possibilities, ensures food security, and provides draught services. Furthermore, the animal production resources are considered as an asset and have social, cultural, and environmental significances (Duguma, 2022; Endalew & Ayalew, 2016; FAO, 2018; Getabalew *et al*., 2020). Without considering the draught power, and manure contributions, the subsector contributes 15–17% to national GDP, 35–49% to agricultural GDP, 15% to total export, 30% to agricultural employment, 80% to rural support, and 37-87% to household income (Asresie & Zemedu, 2015; Kidane et al., 2019; Leta & Mesele, 2014).

In Ethiopia, there are about 65 million cattle, 40 million sheep, 51 million goats, 2 million horses, 9 million donkeys, 0.4 million mules, 8 million camels (Duguma, 2022), and 57 million poultry (Urgesa & Urgesa, 2023). About 80% of the total cattle are kept in cereal-producing regions, where there is more competition on farmland between crops and animals (Endalew &

Ayalew, 2016; Mêkuria *et al*., 2018), and Zebu cattle is the dominant breed (Asresie & Zemedu, 2015; Duguma, 2022).

Among the livestock productions, dairy is the oldest livestock subsector in Ethiopia and remain a vital pillar for the rural economy (Duguma, 2022; Gebreyohanes *et al*., 2021). It reduces poverty, guarantees food security, and creates jobs for farmers (FAO, 2018; Gebreyohanes *et al*., 2021). Dairy has recently gained popularity as a kind of investment, notably in and around the cities and the center of the country (Minten *et al*., 2020).

Milk for human consumption in the country is sourced from cattle, camels, goats, and rarely sheep (Duguma, 2022; Kedija *et al*., 2008; Tegegne *et al*., 2013). Cow milk is the most popular type of milk consumed in all agro-ecological areas and is the milk type from which milk products such as cheese, yogurt, and butter are mostly made (Abêbe *et al*., 2020; Amenu *et al*., 2017; Kedija *et al*., 2008).

However, various challenges have been reported to negatively affect the dairy product in Ethiopia. These include microbial contamination of milk and milk products, animal diseases, insufficient veterinary services, lack of feed, lack of adaptable breeds of animals with high milk yields, insufficient official markets, and lack of knowledge (Gebreyohanes *et al*., 2021; Yassin *et al*., 2022; Zerssa *et al*., 2021). Among these constraints, microbial contamination of milk and milk products, and the mammary disease called mastitis play substantial roles in adversely affecting the health of the consumers. They can be the sources of pathogenic microorganisms which may cause foodborne poisoning, and infections (Deddefo *et al*., 2023; Dhanashekar *et al*., 2012). The food poisonings are resulted from the fact that some of the pathogenic microorganisms, such as *Staphylococcus aureus*, *Escherichia coli*, and *Shigella* spp. can be induced to release enterotoxins in the fermented milk products, and the toxins may result in life-threatening foodborne illnesses (Ortega *et al*., 2010; Pinchuk *et al*., 2010).

The Gram-positive cocci bacteria, *Staphylococcus aureus* (*S. aureus*) is one of the most important microorganisms that can infect both humans and animals and/or produce enterotoxins in foods. Since it lives on the superficial linings of the body (skin and mucus membranes) of animals and humans as a microflora, it might cause various diseases as an opportunistic pathogen (Kadariya *et al*., 2014; Lozano *et al*., 2016). It causes diseases ranging from moderate local

wound infections to very serious life-threatening disorders, particularly in immunocompromised individuals (Dropulic & Lederman, 2016; Tong *et al.*, 2015). *S. aureus* may also generate biofilm, which may be connected to medical devices, and infect skin, soft tissues, wounds, endocarditis, osteomyelitis, bacteremia, and other chronic infections (Kwiecinskia *et al.*, 2019). In general, *S. aureus* infections are commonly recognized as local infections that can occasionally spread to other organs of the body, resulting in severe symptoms such as necrotizing fasciitis and pneumonia, sepsis, toxic shock, and finally death in up to 50% of cases (Adalbert *et al.*, 2021; Kwiêcinski & Horswill, 2020; Thorlacius-Ussing *et al.*, 2019).

In animals, *S. aureus* causes mastitis in cattle, small ruminants, and rabbits (Campos *et al.*, 2022; Kotzamanidis *et al.*, 2021). It can also result in joint infections, osteomyelitis, dermatitis, arthritis, and septicemia in poultry (El-ghany, 2021; Jin *et al.*, 2021; Mcnamee *et al.*, 2000). Moreover, it is not uncommon to isolate the bacterium from pustular and exudative dermatitis, subcutaneous abscesses, conjunctivitis, purulent rhinitis, and pododermatitis in rabbits (Corpa & Hermans, 2009; Viana *et al.*, 2010) , septicemia, and exudative epidermitis in pigs. Furthermore, *S. aureus* has also been determined to be associated with dermatitis and cellulitis in horses and other animal species (Sheehan & Sheehan, 2018; Tessema, 2017).

Mastitis is one the serious diseases that affect the dairy sector. Depending on whether or not the symptoms manifest themselves, the disease is categorized as clinical and subclinical (Goulart & Mellata, 2022; Kitila *et al.*, 2021; Tezera & Ali, 2021). In clinical mastitis changes in milk's color and consistency, and classic indicators of inflammation, such as heat, redness, pain, and swelling are prominent (Ashraf & Imran, 2018; David & Daum, 2020). However, in subclinical mastitis, only reduction of milk yield is apparent (Cobirka *et al*., 2020; Khasanah *et al.*, 2021), which can also masquerade as a feed shortage, estrus, and other chronic illnesses. Therefore, it is diagnosed by recognizing the increment of milk cellular count and the California Mastitis Test (CMT). The basic premises of the diagnostic methods of subclinical mastitis depend on the unusual incline of nucleated cells number in the milk (Birhanu *et al.*, 2017).

The pathogenesis of infective *S. aureus* is mostly attributed to its many virulence factors, including pathogenic antigens and surface proteins (Engidaw *et al*., 2020; Kolar *et al*., 2013), enzyme production, membrane-damaging toxins, toxic shock syndrome toxins (TSSTs), and

proteins that penetrate or escape the immune system (Ahmad-Mansour *et al*., 2022; Pollitt *et al*., 2018; Sheehan & Sheehan, 2018). As a cause of foodborne poisoning, it secretes a variety of staphylococcal enterotoxins (SEs), and SE-like toxins (SEls) (Sheehan & Sheehan, 2018). Moreover, *S. aureus* is known in developing resistance swiftly to newly formulated antibacterial agents. As the result, nowadays, it tolerates several classes of antibiotics used as drug of choices for treating infections caused by the pathogen. It has been repeatedly reported that *S. aureus* is becoming resistant or intermediate resistant to most β–lactam drugs such as methicillin and vsncomycin (Dadashi *et al*., 2020; Yakubu *et al*., 2020).

Evidences reported that the distribution of the pathogen and the virulence factors can vary with geographical difference (agro ecology) and/or with types of samples (Wilson *et al*., 2016). In addition, since *S. aureus* is intimately associated with animals' body, and the food toxications caused by the bacterium are most frequent in foods of animal origins in general and in dairy products in particular, isolation of the pathogen from the dairy cows and dairy products has epidemiologic importance. Furthermore, determination of the virulence factors of the isolates may enforce the evidences of risks facing the community and the animals in the locals.

1.2. Statements of the problem

Bovine mastitis is one of the most costly diseases in dairy industry. The economic importance of mastitis is associated with reductions in milk yield, disposal of milk, increased treatment costs, loss of body condition and/or death of the animals due to the disease, and early culling of cows. It also increases the work load, which indirectly affects the income of the farms. As of the 2016 reports, the losses due to mastitis in dairy sector worldwide was estimated to be 19.7- 32 billion EUR (Nale & Mcewan, 2023). In Ethiopia, the annual economic loss due to bovine mastitis is estimated to be 150 million US$ (Bekuma & Galmessa, 2018).

In addition to its economic importance, bovine mastitis might be one of the sources of microbial contamination in foods, and the microbes, in turn, can result in foodborne diseases. Bacteria, fungi, protozoa, and viruses can contaminate milk from mastitic animals. Some of these microorganisms, particularly bacteria and fungi, are able to directly infect consumers and/or produce toxins in foods, which result in food intoxications. Bacteria such as *S. aureus* and *E. coli* are repeatedly implicated as causes of foodborne infections and poisonings.

From its nature as microflora on the body of the animals, *S. aureus* has potential to cause opportunistic diseases including mastitis. In addition, its ability to produce toxins to cause foodborne intoxications, assigns it as the most important pathogen in animal and human. In addition to its pathogenic potential, the widely distributed nature of *S. aureus* can also seek the attention of the scientific world. As repeatedly stated by different researchers, residence on the surface of the animal's skin and mucosal linings as a microflora, relative tolerance to a variety of environmental conditions, and swift developing antibacterial resistance to newly formulated drugs are considered as important features of *S. aureus* to be ubiquitous.

Traditional milk collection and processing methods which are widely practiced in Ethiopia may encourage bacterial contamination of milk and milk products. These practices vary among the practitioners because of various factors including awareness, cultural differences, education, advancement of technologies, availability of infrastructures, and economic importance of the dairy farm for the family. In addition, in most locals of the country, little is known about bovine mastitis serving as sources of contamination in dairy products. Moreover, the virulence factors such as enterotoxins, staphylokinase, lipase and proteases productions, and antibiotic resistance of *S. aureus* isolates from lactating cows, milk and milk products in West Showa zone have not been studied intensively. Traditional milk collection and handling practices such as smoking, types of utensils used for milk collection and storage, using udder clothes, and hand washing before and with intervals of each milking have also not been widely assessed in some districts of West Showa Zone.

1.3. Objectives of the study

1.3.1. General objectives

The general objective of the study is, therefore:

- To determine the prevalence of mastitis in lactating cows and to isolate and identify *S. aureus* from lactating cows and whole milk and milk products (yoghurt and cheese), and to determine the prevalence, antibiotic resistance, and virulence factors, including enterotoxins, lipase, and staphylokinase production, of the isolates in West Shewa Zone

1.3.2. Specific objectives

- To determine the prevalence of mastitis in lactating cows in West Showa Zone.
- To determine the risk factors associated with mastitis.
- To assess the prevalence of *S. aureus* in milk, bulk milk, yoghurt and cheese in the project area
- To determine the antibiotic susceptibility characteristics of the *S. aureus* isolates in the study area.
- To assess the drug resistance and enterotoxin genes in the *S. aureus* isolates.
- To assess the enzyme production characteristics of the *S. aures* isolates in the study area.

1.4. Research questions

The study was conducted to answer the following questions.

1. What is the overall prevalence of mastitis in lactating cows in the study area?
2. Does the prevalence of mastitis vary with agro ecology?
3. Is *S. aureus* isolated from both mastitis positive and mastitis negative with equal rate?
4. Is *S. aureus* isolated from bulk milk and fermented milk products with equal rate?
5. Are staphylokinase, lipase and protease produced by all *S. aureus*?
6. What is the proportion of the methicillin resistant *S. aureus* (MRSA) in the *S.aureus* isolates?
7. What is the prevalence of penicillinase (*blaZ*), thermonuleus (*nuc*), methicillin resistant *(mecA)* and staphylococcal enterotoxin genes (sea, seb and tsst-1) genes in the *S. aureus* isolates?
8. What are the animal, environmental and hygienic risk factors associated with mastitis in bovine?
9. What are the milking, milk storage and hygienic practices likely to occur as risk factors associated with bacterial contamination of milk and milk products in the study area?
10. To which antibiotics are the *S. aureus* isolates resistant?
11. Do the means of multidrug resistance indices vary between MRSA and MSSA strains?

1.5. Conceptual Framework

For the simplicity of understanding, this study used the following framework as road map (Fig.1).

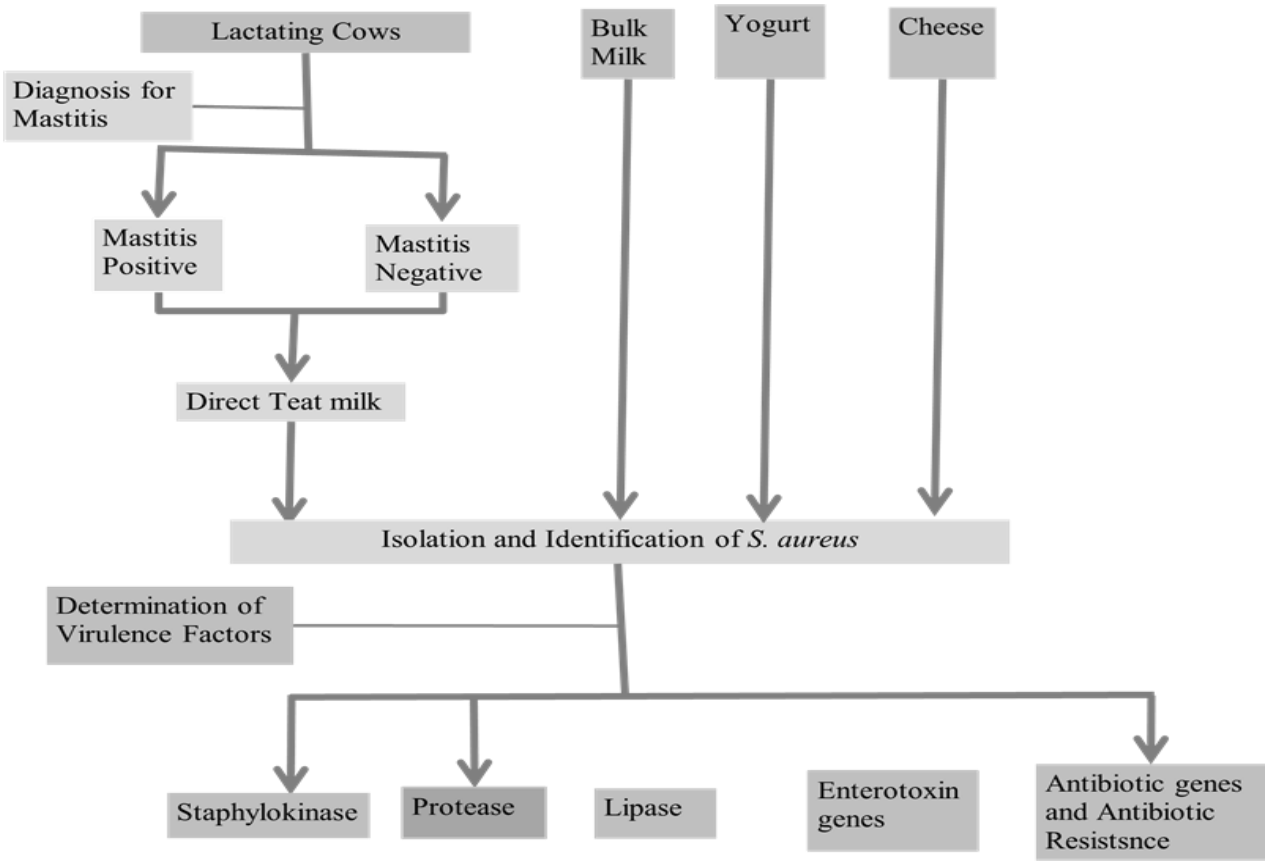

Figure 1: Conceptual Framework of the study

1.6. Significance of the study

This study focused on determining the prevalence rate of mastitis in lactating cows and evaluating the prevalence and virulence characteristics of *S. aureus* isolated from lactating cows, bulk milk, and fermented milk products. The samples were collected directly from the animals or were taken from bulk milk and milk products (yogurt and cheese). The significance of the study is, therefore, mainly to set the baseline for further scientific studies on the issues in the country in general and in the study area in particular. This research has provided significant insights on the distribution of clinical and subclinical mastitis in cows, as well as the antibiogram and enterotoxin-producing traits of *S. aureus* isolates from milk and milk products. Since the studies

of virulence factors of *S. aureus* isolates from dairy products particularly enzyme and enterotoxin have been rare in Ethiopia, the study lays important base line for further studies.

This information is significant for veterinarians and related professionals in the prescription of the types of antibiotics for the treatment of animal diseases, particularly mastitis. Policymakers can also be benefited from the results of the study to design methods for the prevention and control of mastitis and drug resistance, both in animal and human medicines. In addition, it is very important to provide awareness to society about the safety practices needed when collecting, storing, and consuming milk and milk products.

1.7. Limitation of the study

Even though the result and evidences provided by the study are robust, the study had many limitations. First, the study was conducted only in four districts with limited number of kebeles of West Showa zone. Second, the bulk milk and milk product samples were collected only from the towns and local markets so that majority of the products have been expected left unstudied. Third, the sample size expected to be collected had not been attained because of the CoVD-19 and security issues. In addition, all the virulence factors were not assessed and even not all the isolates of the *S. aureus* have been characterized for their virulence factors. Furthermore, the potential of some of the virulence fuctors particularly the enterotoxins in contaminated foods is directly associated with concentration of the secretions which in turn directly associated with toxigenic bacterial population in the food staffs. This indicates that in addition to looking for the toxigenic bacteria, determination of bacterial load in the food samples is very important to anticipate the food poisoning probability in the foods. However, in the current study, because of the lack of Potassium Tellurite which is the supplement ingredient for Baird-Parker agar, *S.aureus* enumeration has not been carried out. Therefore, these should be taken into consideration and cited wisely when used for further studies.

2. REVIEW OF LITERATURE

2.1. General properties of Milk

Milk is a secretion of the mammary gland of most female mammals produced to nourish their offspring (Chamber, 2002; Hassen *et al*., 2022). It is considered as a perfect food containing nutrients needed for growth and health (Angelidis, 2015; Hussien *et al*., 2021; Melesse & Beyene, 2009; Zakary *et al*., 2011). Although there might be slight variation in the concentration and types of the contents based on the species and breeds of the animals, the feeds of the animals, and seasons can vary the content of the milk. Milk generally contains carbohydrates, proteins, minerals, and vitamins (Antunes *et al*., 2023; Girmá *et al*., 2012; Hassen *et al*., 2022). Cow whole milk contains approximately 87%, 3%, 4% and 5% water, protein, fat, and lactose respectively (Chamber, 2002).

Milk proteins are generally divided into two as caseins, and whey proteins (serum proteins) (Antunes *et al*., 2023; Duguma, 2022; Duguma & Janssens, 2021; García-Burgos *et al*., 2020) with proportion of 80% and 20% respectively (Alhussien & Dang, 2018). Caseins are a group of proteins which precipitate at a pH of about 4.6 or less and a temperature of 20°C, but whey proteins remain soluble in whey (Broyard & Gaucheron, 2015; Freire *et al*., 2022; Guyomarc'h *et al*., 2014; Séverin & Wen-shui, 2006; Yè & Harte, 2013).

Milk fat content is much more variable than proteins and lactose in the milk of the same animal. The milking frequency has also a positive effect on fat content, and the morning milk contains more concentrated fat than the evening (Quist *et al*., 2008) . It has been also determined that the crude protein increases with the total fat of a milk. The major lipid component of cow's milk is a triglyceride, which makes up about 98% of milk fat by weight. The other lipids in the milk include diacylglycerides (0.25-0.48%), monoacylglycerides (0.02-0.04%), phospholipids(0.6-1.0%), cholesterol (0.2-0.4%), glycolipids (0.006%) and free fatty acids (0.1-0.4%) (Djordjevic *et al*., 2019; Khan *et al*., 2019; Liu *et al*., 2020; Magan *et al*., 2020; Mansson, 2008).

Among the carbohydrate in the cow's milk, lactose which account to about 5% concentration is the major carbohydrate in milk (Gambelli, 2017; Portnoy & Barbano, 2021). It is relatively less soluble in water than many other sugars, and plays role in colligative properties of milk such as

osmotic pressure, freezing point depression, and boiling points which are important properties in detection of adulteration of the milk (Alhussien & Dang, 2018; Mehta, 2014).

Milk is also a better source of minerals such as calcium, iodine (Górska-Warsewicz *et al*., 2019; Hassen *et al*., 2022; Mihret *et al*., 2017), and vitamins including riboflavin, vitamin A, vitamins C, vitamin B1, vitamin B12, folates, and other vitamins (Antunes *et al*., 2023; Chamber, 2002; Mihret *et al*., 2017; Nazari *et al*., 2014; Rall *et al*., 2008). The minerals are either in their salt or elemental forms. Chlorides, phosphates, citrates, sulfates, and bicarbonates of sodium, potassium, calcium, and magnesium are among the prominent salts found in milk. Some of the salts including the chlorides, sulfates, and compounds of sodium and potassium are soluble in the milk and remain dissolved in the whey after coagulation in the fermented milk. However, calcium and phosphate salts are found in milk partially dissolved and partly insoluble (colloidal) forms, in close associations with the casein micelles. Minerals found in elemental form in milk include zinc, iron, and manganese, and they are found in trace amounts. Some of the minor constituents of milk including enzymes (lactoperoxidase and acid phosphatase) and somatic cells contribute to the inhibition of the growth of microorganisms in milk. Antibodies and disinfectants might also be found in milk as accidental contaminants (Chamber, 2002).

In addition to the aforementioned indigenous and exogenous factors, other potential influences to change the composition of cow milk include mastitis, extreme weather conditions, stress, and exhaustion (Andualem & Geremew, 2014; Argaw, 2016; Bihon *et al*., 2019; Chamber, 2002; Ejeta *et al*., 2022; Kasa *et al*., 2020; Mehamud *et al*., 2017). As Alhussien and Dang (2018) reviewed, the increment of somatic cell in a milk, which is due to mastitis infection, has effect on the total milk yield and concentration of protein and lactose but not on the fat of the milk. As the result, there has been increased in protein but decrease in lactose observed in milk from mastitis infected animals when compared with non-infected ones. When the milk proteins are compared, however, there is increase in whey protein but decrease in casein in milk from mastitis positive cows. The pH of milk from healthy animals ranges from 6.4 to 6.8 with isotonicity equal to blood plasma. However, in mastitis cases, the lactose and casein concentration reduced that sodium chloride and sodium bicarbonate from plasma ooze to the alveoli to maintain isotonicity so that the pH of the milk increases to become alkaline pH.

2.2. Dairy industry in Ethiopia

Dairy products are excellent sources of nutrients (Górska-Warsewicz *et al*., 2019) and are consumed by people all over the world (Koushalya *et al*., 2022). The most prevalent type of milk consumed worldwide is cow's milk (Antunes *et al*., 2023; Calahorrano-Moreno *et al*., 2022; Visioli & Strata, 2014). In Ethiopia, cattle account for 80% of the country's milk production (Abegaz, 2022; Tegegne *et al*., 2013; TRAIDE Ethiopia, 2021), followed by goats (8%), camels (7%), and sheep (5%) (TRAIDE Ethiopia, 2021). Moreover, cow milk is the sort of milk used to make dairy products like cheese, yogurt, and butter. Camel, goat, and sheep milk are limited in the lowlands of the country (Kedija *et al*., 2008), and rarely traditionally processed into yogurt. According to the report by Farrell (2021), of the total milk produced in Ethiopia, 70% was used for human consumption of which 57% was consumed by rural residents, and the rest 43% was processed into dairy products and delivered to the market.

Around 15% of Ethiopia's overall cattle population are dairy cows, the majority of which are local zebu breeds (Ahmêd *et al*., 2003; Duguma, 2022; Gebre *et al*., 2022). There are more than 10 million dairy cows in the country, with an average milk yield of 1.54 liters per cow per day throughout a lactation period of six months (Getabalew *et al*., 2019). The native breeds are favored because of their disease resistance, environmental flexibility, and capacity to survive low feed availability and subpar management (Alilo, 2019; A. Assefa & Hailu, 2018; Bora *et al*., 2023). As the result, dairy industry in the country is generally characterized by low productivity, an extensive grazing system, and uncontrolled breeding (Ashagrie *et al*., 2023; Gebreyohanes *et al*., 2021).

The four basic categories of dairy cow production systems in Ethiopia are rural smallholder (mixed crop-livestock) production, pastoral and agro pastoral production, urban and peri-urban smallholder dairy production, and commercialized dairy production systems (Kedija *et al*., 2008; Tadese & Yilma, 2018; TRAIDE Ethiopia, 2021). The smallholder production systems cover majority of the production systems and is practiced in rural and semi-urban areas The system uses predominantly indigenous breeds managed under extensive management systems (Dugguma *et al*., 2015; Hailemariam *et al*., 2022; Kena *et al*., 2022; Kitila *et al*., 2021; Korir *et al*., 2023). The extensive management system is characterized by open grazing and watering, and

non-roofed sheltering with no drainage facilities (Meskela & Gashaw, 2017; Temple & Manteca, 2020). In very few areas, however, the smallholder dairy farms may keep a small number (≤10 animals) of cross-bred cows in zero-grazing systems which have been considered to improve the productivity of the animals (Hadush, 2021). In addition, since recent years, because of the expansion of crop farming and reforestation, particularly in the highland part of the country, the fixed grazing method, which limits the long movement of the cattle for grazing, is also being adapted (Tilahun & Schmidt, 2012). This method may encourage the farmers to use crop residue and some concentrates for animal feeds. The commercialized dairy farms, which use improved breeds and semi-intensive or intensive management systems, are still in the initial stages. Indeed, they are expanding in the form of investment in and around the capital city, regional cities, and zonal towns of the country (Ahmêd *et al*., 2003).

Furthermore, dairy farms might also be classified according to herd size, and production level as smallholder farms (<10 animals), medium farms (10 to 50 animals), and large farms with more than 50 animals (Borena *et al*., 2022; Kebebew & Jorga, 2016; Megersa *et al*., 2011; Tschopp *et al*., 2021). Research has determined that herd size affects the management system of the dairy farms (Beggs *et al*., 2019; FAO, 2018; McCarthy & Mallon, 2016; Tilahun & Schmidt, 2012). As the result, it has been found to be one of the risk factors that affect dairy production in general and the prevalence of some diseases, including mastitis in particular (Alemayehu & Simon, 2016; Badua *et al*., 2020; Bandara *et al*., 2011; Borena *et al*., 2022; Duguma & Janssens, 2021; Fesseha *et al*., 2021; Korir *et al*., 2023; Naing *et al*., 2019).

2.3. Milk and fermented milk products in Ethiopia

2.3.1. Milk

It is estimated that about 4.96 billion liters of cow milk (97% from local breeds and 3% from crossbreed or exotic cow) is produced annually in Ethiopia. The rural smallholder farmers, where dairy infrastructures are not well developed, supply more than 90% of the milk production. Households consume approximately about 50% of the milk collected, process 45% to milk products which have longer shelf life, and sell only 5% as raw milk (CSA, 2021).

The whole milk is processed into other products such as butter or Ethiopian cottage cheese (*ayb*) (Gemeda *et al*., 2018). The perishability nature of milk (Pal *et al*., 2017), the lack of refrigerators to keep the milk for long time, and the lack of transportation influence the owners to sell the milk to neighbors or process into butter and cheese locally. When farmers who operate in remote areas are compared to the farmers in areas readily access to market to sell raw milk, the latters are more encouraged to intensify milk production than the formers (Bachewe *et al*., 2018; Gebreyohanes *et al*., 2021; Kena *et al*., 2022). Except in a few areas where dairy producers are organized into milk marketing cooperatives, milk is sold predominantly on a contractual basis through informal/unorganized channels (Ahmêd *et al*., 2003; Deddefo *et al*., 2023; Duguma, 2022; FAO, 2017; Gebreyohanes *et al*., 2021; Gobezie & Aysheshim, 2020; TRAIDE Ethiopia, 2021).

In most rural areas, where the market for fresh whole milk is rare, milk is processed into dairy products mainly to butter and cheese (Abêbe *et al*., 2020; Duguma, 2022; Getabalew *et al*., 2019) following the flow of the processes summarized in **Figure** 2. Milk processing was performed mainly as a value-adding method to provide higher incomes for small-scale producers as well as to reach the markets. Traditional milk processing begins with fermenting milk to make yogurt (Ergo) without using defined starter cultures (Andualem & Geremew, 2014). The main reasons for fermenting milk are the small volume of milk produced per day, the intension to improve the better shelf life of the milk, and preferences of the consumers (Mùrphy *et al*., 2021; Poulsen *et al*., 2022). The major products of fermented milk produced by smallholder farmers by traditional methods include yogurt (*ergo*), traditional butter (*kibe*), cottage cheese (*ayub*), sour defatted milk (*area*), and whey (*aguat*) (Abêbe *et al*., 2020; Ashenafi, 2006; Mattiello *et al*., 2018; Yilma *et al*., 2011). Among these products, yogurt, cottage cheese and butter are the most frequently presented to local and national wide markets (Yilma *et al*., 2011).

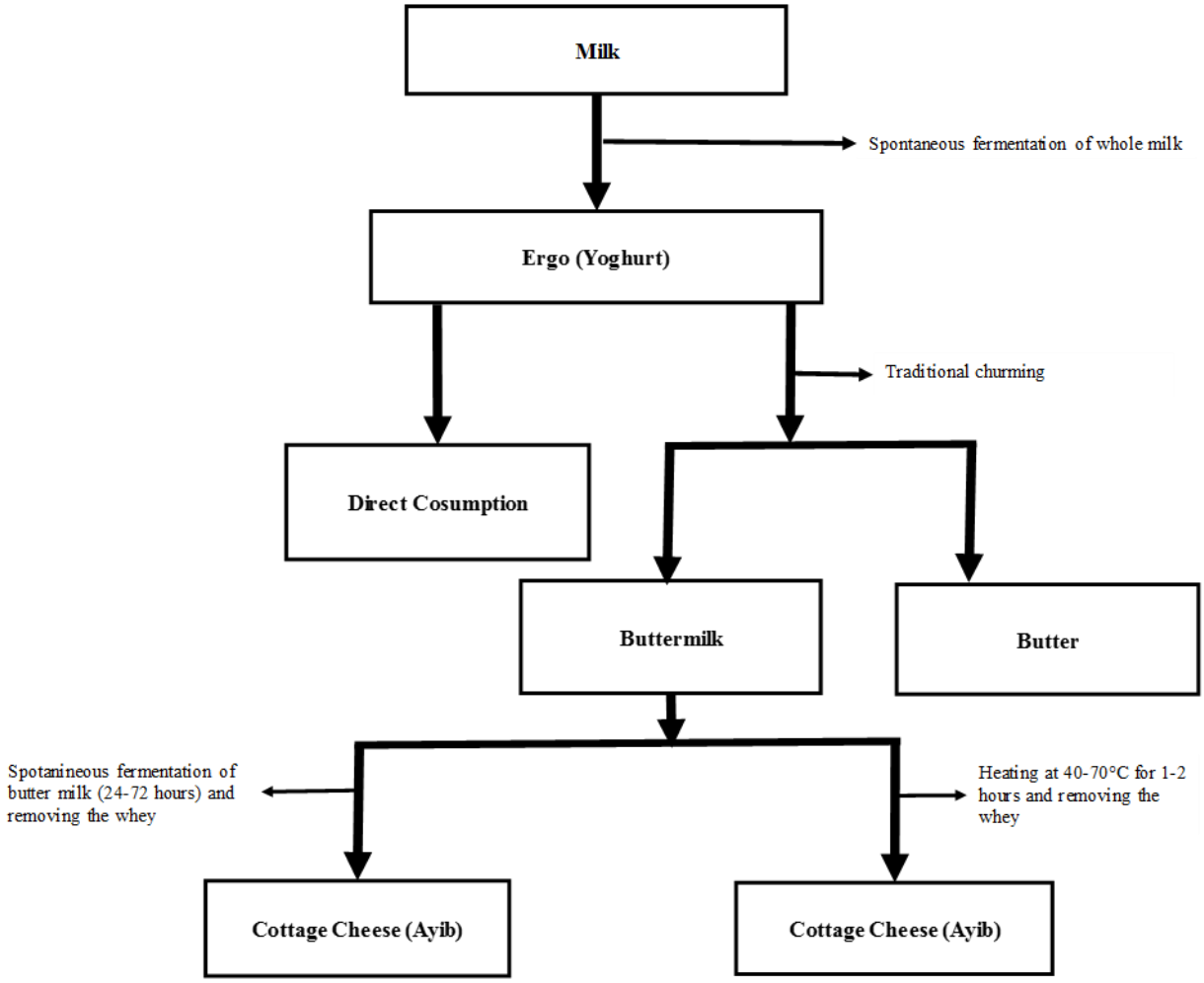

Figure 2: Flow Chart of Common Traditional Milk Processing in Ethiopia

2.3.2. Yogurt

The term "*Ergo"* in *Amharic* or "*Itittu"* in *Afan Oromo* is meaning coagulated milk (fermented whole milk). Ethiopian traditional yogurt (e*rgo*) is a thick, smooth and of uniform appearance naturally fermented milk product, and usually has a white milkish color when prepared carefully (Worku *et al*., 2015) . It is semi-solid and has a pleasant odor and taste. Its consistency and flavor considerably vary among households due to the spices used, and types of smoking materials used to smoke the utensils (Asefa & Abreha, 2018).

Ergo is the product of spontaneous fermentation of whole milk carried out at ambient temperature. The precipitation of the casein is usually the sign of completion of the fermentation. Therefore, it cannot be defined in terms of its microbiological and biochemical properties, and does not have a definite temperature and duration of incubation periods (Ashenafi, 2006). The duration of its incubation varies from place to place depending on the prevailing environmental conditions (Asafa *et al*., 2008; Bêrhe *et al*., 2017) . Since it is produced by a natural fermentation processes, various species of microorganisms, which are either natural microbial flora present in the milk and/or introduced from the surrounding, are involved and contribute to its basic final characteristics (Hotessa & Robe, 2020; Maleke *et al*., 2021; Yilma *et al*., 2011). As the result, the quality and safety of the product might partially depend on the composition of the species of the microorganisms involved in the fermentation processes. In most of the rural and semi-urban areas of the country, *Ergo* can serve as a primary sour milk product from which other products such as butter and cheese might be produced.

The pH value of Ergo ranges from 4.3 to 4.6. However, there were reports with less pH value of *Ergo* in Ethiopia. Yilma *et al.* (2007) and Worku *et al.* (2015) reported that the average pH values of *Ergo* are to be 4.02 and 4.07 respectively. This low pH value grants the relatively long shelf life of the product, which can be stored for 15-20 days at 16 to 18ºC. The acidic condition of Ergo inhibits the growth of spoiling and pathogenic microorganisms in the milk (Bêrhe *et al*., 2017; Mossie, 2019; Tesfaye *et al*., 2011; Worku *et al*., 2015; Yilma *et al*., 2011). However, evidences showed that the low pH may favor the growth of yeast and molds which can spoil the product in some conditions (Matin *et al*., 2018). Even some pathogens such as *S. aureus*, *Salmonella* spp. and *Listeria* spp., can survive in the acidic conditions even though their growth can be hindered (Ashenafi, 2006; Erdoğrul *et al*., 2006). The spices (Geremew *et al*., 2015) and smoking materials (Amênu, 2013; Ashenafi, 2006; Bêrhe *et al*., 2017; Getabalew *et al*., 2020; Japaro, 2021) have also an effect on the survival and multiplication of some microbes in the product.

Even though Ethiopia has an ancient tradition of yogurt consumption, and *ergo* is the major fermented dairy product in all parts of the country (Worku *et al*., 2015), it is consumed only in special revel conditions or by individuals as a sign of respect in rural areas (Andualem & Geremew, 2014). This is because, in rural areas, butter which is the product of *ergo* is commonly

sold with more considerable price than raw milk at the local market. As the result, the farmers prefer to process *Ergo* to butter and cheese than regularly consuming it. Sometimes, it is also fed to sick people, children, and pregnant and lactating women as a good nutritional supplement (Asresie *et al*., 2018; Daba *et al*., 2021).

In rural areas of the country, farmers prefer the raw fresh milk and fermented milk consumption to the boiled ones (Asresie *et al*., 2018; Daba *et al*., 2021). This might be because of that they have thought boiling milk reduces its flavor and taste. In rural areas, the consumption of milk and milk products is heavily influenced by livestock ownership; but in urban areas, the principal determinant of milk and milk product consumption levels is income (FAO, 2017; Getabalew *et al*., 2019; Yilma *et al*., 2011).

Almost none of the traditional yogurts prepared at the smallholder level are supplied to the market. They are used either for household consumption or processed further to other milk products. However, there are commercial *Ergo*s processed and packed available in the domestic markets. Shola yogurt, Mama flavored and non-flavored yogurt, Family yogurt, Holland Dairy yogurt, Berta yogurt, Genesis yogurt, Etete yogurt, Harmey yogurt, and other unbranded yogurts are among the most frequently sold in tailor shops. Besides to these, ergo is most commonly prepared from raw milk in towns and cities, and sold in restaurants. However, as there is no quality control, and the products are directly prepared from raw milk bought from local dairy farms without pasteurization, the safety of the latter is doubtful.

2.3.3. Cheese

Cottage cheese is a soft curd-type product obtained by draining coagulated skimmed or partially skimmed milk. The principle of its processing is based on the coagulation of the protein in the milk to be a mass curd and then draining the liquid part called whey (Alemayehu & Simon, 2016; Ashenafi, 2006; Duguma, 2022; Getabalew *et al*., 2020; Japaro, 2021; Mattiello *et al*., 2018; Mossie, 2019; Tegegne *et al*., 2013). The microbiological and biochemical processes of fermented milk products involve the acidification of the milk by metabolites of the lactobacilli and coagulation by the plant or animal rennet. The acids dissolve calcium phosphate and the aggregation of the milk proteins enables the formation of networks to coagulate the milk by the rennet that removes the casein macropeptide from k-casein, and paracasein micelles to firm the

cheese at which level the whey is easily drained (Coelho *et al*., 2022; Harper *et al*., 2022; Nyamakwere *et al*., 2021).

Cheese-making methods may differ based on the adapted technologies. Cheese is made in almost every country of the world and there exist more than 2000 varieties (Hagi *et al*., 2022; Zheng *et al*., 2021). Therefore, traditional cheese-making methods are vigorously diversified except that they use fermented milk as a common raw material for the product. However, in modernized factory farms, most of the cheese producers use specific lactic acid bacteria (LAB) as starter cultures, bacterial or plant renin, and apply heat to stop the further multiplication of LABs, to increase curd contraction and whey expulsion(Coelho *et al*., 2022; Hosken *et al*., 2023).

In Ethiopia, local cheese ("*Ayib*" in Amharic or "*Badu*" in Afan Oromo) is made by churning the buttermilk (at the household level) or skim milk (in farm or cooperative milk product producers) without using specific starter culture nor external rennet (Andualem & Geremew, 2014; Mossie, 2019). Briefly, sour milk (fermented milk) is primarily defatted either by skimming or by churning the milk to separate the butter. The buttermilk is then heated at low temperatures (approximately 50-60°C) until a soft curd-type cheese distinctly forms and floats over the whey(Asresie *et al*., 2018; Japaro, 2021; Mattiello *et al*., 2018). In some parts of the country particularly in the western part, however, the buttermilk is left at room temperature for 1-2 days to coagulate to be cheese. In the latter method, the amount of whey removed is so small that the consistency of the cheese is softer than the former cheese. The whey is drained through a strainer or cheesecloth and the mass is collected as cheese (Bêrhe *et al*., 2017; Geremew *et al*., 2015). Sometimes salt is added to cheese to preserve it if it is expected stored for long period, or it is stored in the refrigerator if available (Dandessa, 2019) .

Very few studies have been conducted on the physicochemical characteristics of Ethiopian cheese ("*Ayib*"). However, it has been considered as it is acidic (pH 3.7) similar to *ergo* (Andualem & Geremew, 2014; Ashenafi, 2006). *Ayib* has been reported containing about 1.8% fat, 15% protein, 80% moisture, and 1% ash. The ash proportion indicates its metal composition (Ashenafi, 2006; Bêrhe *et al*., 2017; Dandessa, 2019).

2.4. Constraints of dairy products in Ethiopia

Despite the huge involvement of dairy products in the local and national economy in Ethiopia, little attention has been given to the sector. The dairy production encounters several constraints among which the microbial contamination of the products (Gebreselassie, 2019; Gobezie & Aysheshim, 2020; Mossie, 2019), animal diseases (Fesseha *et al*., 2021; Kedija *et al*., 2008; Mihret *et al*., 2017; Tadesse, 2016; Yilma *et al*., 2011), insufficient veterinary services (Argaw, 2016; FAO, 2017; Getabalew *et al*., 2019; Ma'alin *et al*., 2022; Mihret *et al*., 2017), lack of feed quantitatively and quantitatively, lack of adaptable high milk yield animal breeds, lack of formal markets, and lack of knowledge are the prominent ones (Alemayehu & Simon, 2016; Gebreselassie, 2019; Gebreyohanes *et al*., 2021; Getabalew *et al*., 2019; Ma'alin *et al*., 2022).

Since the majority of the milk and milk products are produced in traditional methods at the farmer level, the contamination of the products is uncommon. Particularly, the microbial contaminations of the products can cause serious foodborne illnesses on the consumers. The pathogens cause disease in two or combined ways. Some of the pathogens infect the consumer resulting in gastrointestinal infections and/or septicemia (Bhunia, 2008; Borena et al., 2022; Gizaw et al., 2020; Imre et al., 2020; Mølba et al., 2016; Oliver et al., 2005; Saka & Terzi Gulel, 2018). However, some microorganisms might produce toxin secretions in the milk and milk products to cause food poisoning when ingested with the foods (Aydin *et al*., 2011; Bintsis, 2017; Borena *et al*., 2022; Gizaw *et al*., 2020; Imre *et al*., 2020; Rahimi, 2013; Sudershan *et al*., 2014). Most of the contaminations occur as the result of a lack of knowledge and facilities to prevent the probability of their occurrences (Abêbe *et al*., 2020).

Animal diseases summed up with insufficient veterinary services, and low milk yield producing local breeds in Ethiopia have a great negative impact on the quality and quantity of dairy products. All diseases affecting milk-producing animals in general and the disease of the teat (mastitis) in particular adversely reduce the production and productivity of milk. Mastitis is scientifically defined as the inflammation of the mammary gland (the organ to produce milk) and is caused by a variety of causative agents which could be non-infectious or infectious (Belay *et al*., 2022; Elbably & Asmaa, 2013; Marama *et al*., 2016; Wolfenson *et al*., 2015). The non-infectious include tumor, trauma, and mechanical occlusion of the teat canal by tissue debris and

coagulation of colostrum (Angelidis, 2015; Sêyoum *et al.*, 2017; T. Zhang *et al.*, 2022). Infectious mastitis is caused by pathogens such as bacteria, fungi, and viruses (Komarov *et al*., 2021).

2.5. Contaminations in milk and milk products

2.5.1. Non-biological contaminations

Naturally, milk is a sterile fluid when secreted into the alveoli of the udder of healthy animals. However, it might be polluted with chemicals in the animal's feeds or if the animal has been treated with inorganic drugs (Melini *et al*., 2017; Mosu *et al*., 2013). The chemicals include mycotoxins (Carvajal, 2008; De *et al*., 2014; El-Sayed *et al*., 2022; Fernandes, 2009; Zebib *et al*., 2022), poisonous plants (Diaz, 2011; Puschner & Varga, 2012; Riet-Correa *et al*., 2017), insecticides (Calahorrano-Moreno *et al*., 2022; Chamber, 2002; Puschner & Varga, 2012; Tufa *et al*., 2018), and treatment drugs (Abebew *et al*., 2014; Egyedy & Ametaj, 2022; Mosu *et al*., 2013; Tufa *et al*., 2018) as well as various heavy metal residues that enter the system of the animal from the external and are secreted with the milk as a byproduct. Some of these chemical or biochemical contaminants may cause serious health problems when consumed at high doses and/or expose the consumers to antibiotic-resistant pathogens (Abdolshahi & Yancheshmeh, 2020). Therefore, it is recommended that the minimal or no usage of veterinary drugs in food animals and environmental protection from chemical residue will minimize the chemical residues in milk and milk products (Chicoine *et al*., 2020).

2.5.2. Microbial contaminations

Milk might also be commonly contaminated by microorganisms from the animal it has been obtained or from the environment (Abebe *et al*., 2016; Calahorrano-Moreno *et al*., 2022; Dittmann *et al*., 2017; Hassen *et al*., 2022; Hógan *et al*., 2020; Jahan *et al*., 2015; Sadiku *et al*., 2020; Yambise *et al*., 2020). The microorganisms of animal origin might be either the microflora found in the orifices of the teats or on the superficial skin of the teat (Akkaya *et al*., 2014; Calahorrano-Moreno *et al*., 2022; Chamber, 2002; Deresse, 2014), or pathogenic infectious microbes in the animals body. In the case of animals with infected teats, the pathogenic microorganisms can shed in the milk during milking (Owusu-kwarteng *et al*., 2020; Sheehan &

Sheehan, 2018). The environmental contaminations might occur from the environment or due to less hygienic conditions of the handler's body or/and the containers of the milk (Guessan *et al*., 2015; Gwandu *et al*., 2018; Sadiku *et al*., 2020). The microbial contaminants might be reduced by subjecting the milk to heat (Godfroid *et al*., 2010) or through fermentation to decrease the pH of the products, and subsequently following the good hygienic procedure in the preparations of milk products (Lemma *et al*., 2021; Melini *et al*., 2017; Owusu-kwarteng *et al*., 2020; Tesfaye *et al*., 2011; Thaker *et al*., 2013).

Bacteria, fungi, viruses, and protozoans have been identified from milk (Cisak *et al*., 2017; FAO & WHO, 2023; Grossi-Soyster *et al*., 2018; Owusu-kwarteng *et al*., 2020; Saad & Hussein, 2018). The pathogenic microorganisms might infect the consumer and/or produce toxins that can cause foodborne intoxication (Berhe *et al*., 2020; Bintsis, 2017; Engidaw *et al*., 2020; Gutema & Engidawork, 2018; Mølba *et al*., 2016; Sudershan *et al*., 2014). Apart from causing foodborne diseases, microbial contamination of milk and milk products can also reduce the acceptability of the products by altering the color, taste, smell, or texture. For example, the mixed growth of *Pseudomonas* spps and *Streptococcus lactis* results in the blue coloration of milk (Chiesa *et al*., 2014; Gwandu *et al*., 2018; Maske *et al*., 2021; Quintieri *et al*., 2021). The growth of encapsulated *Bacillus* species hydrolyzes starch and protein in milk and as a consequence of which the product become repines (Yang *et al*., 2021)(Berthold-Pluta *et al*., 2019). However, there are bacteria especially the species of lactobacilli of which acidic metabolic products have the potential to inhibit the multiplication of pathogenic microorganisms and they can selectively be added to the milk as starter culture in fermented milk products in the dairy industry (Admassu, 2019; Coelho *et al*., 2022; Fernández *et al*., 2015).

As the fact that milk and milk products contain relatively high amount of proteins and carbohydrates which support bacterial growth, they are perished swiftly when contaminated with bacterial (Berhe *et al*., 2020; Tasse *et al*., 2022). Bacterial contamination of milk and milk products is a widespread issue in the dairy industry in the globe in general (Qiangchuan Hou *et al.*, 2015), and, in developing countries including Ethiopia in particular (Abêbe *et al*., 2020; Adugna *et al*., 2018; Dagnew *et al*., 2012; Sadiku *et al*., 2020).

Bacteria can contaminate milk and milk products during milking or after milking. The sources of the bacteria are skin and teat canal, or miller's hands, the vessels and equipment used for milking and storage, air, and the milking surroundings (Deddefo *et al*., 2023; Garedew, Mengesha, *et al*., 2015; Seid Geletu *et al*., 2022; Zhong *et al*., 2020). The bacterial contamination of milk in Ethiopia has been found largely associated with poor animal health services, poor hygiene, lack of milk storage and cooling facilities, and insufficient quality checks at collection centers (Amentie *et al*., 2016; Berhe *et al*., 2020). Therefore, the safety of milk needs to be kept by having healthy cows, keeping the sanitation of the milking environment, and utensils for milking and storage of the milk. Milk with minimum contamination and low bacterial numbers is essential for the manufacture of dairy products such as butter, cheese, and fermented milk (Andualem & Geremew, 2014).

Pathogenic bacteria such as *Brucella abortus*, *Escherichia coli* 0157: H7, *Mycobacterium bovis*, *Campylobacter jejuni*, *Salmonella* spp., *Clostridium* spp., and *Staphylococcus aureus* are responsible for nearly 90% of infections associated with dairy products (Cancino-padilla *et al*., 2017; Gwandu *et al*., 2018). Fungi are also incriminated in producing mycotoxin that causes food poisoning which may lead to death. Especially *Aspergillus flavus* and *A. parasiticus* produce aflatoxin which is among the natural contaminants of milk and milk products and lead to jaundice, edema, gastrointestinal hemorrhage, liver cancer, and ultimately death (Bhunia, 2008; Zebib *et al*., 2022).

Of the bacterial pathogens, *Staphylococcus aureus* is the most common cause of milk and milk product-associated staphylococcal disease outbreaks (Borena *et al*., 2023; Fernandes, 2009). This is because, not only milk and milk products but also generally foods of animal origin with high protein contents such as meat, meat products, salads and bakery products favor the growth of bacteria, and they are frequently incriminated in *S. aureus* outbreaks (Alkhafaji *et al*., 2019).

Staphylococcal food poisoning caused by *S. aureus* is one of the most common types of foodborne illness worldwide and is mostly characterized by severe vomiting and diarrhea. The symptoms result from the fact that *S. aureus* produces a large number of toxins and enzymes, of which the enterotoxins are most important in inducing gastroenteritis (Aycicek *et al*., 2005; Borena *et al*., 2023).

In addition, since the pathogen commonly resides as microflora on superficial skin and in the mucosa of nasal, buccal, urogenital, and teat canals of animals and humans, it is identified frequently in association with infections in various organs of the hosts (Dego *et al*., 2020; Haag *et al*., 2019; Lozano *et al*., 2016). It may cause infectious diseases ranging from mild skin infections to life-threatening sepsis in both animals and humans. The pathogenic strains of the bacterium are known in causing skin and soft tissue infections, wound infections, endocarditis, osteomyelitis, bacteremia, and chronic infections by forming biofilms in association with medical devices (Balasubramanian *et al*., 2017; Howden *et al*., 2023). Occasionally, the skin infections can spread to other organs of the body with more severe symptoms, including necrotizing fasciitis, and necrotizing pneumonia (tissue destruction), followed by sepsis and toxic shock, and then death ranging in 15-40% of the cases (Kimmig *et al*., 2021; Tong *et al*., 2015).

2.6. Bovine mastitis

2.6.1. Etiology of mastitis

Mastitis is defined as the mammary gland inflammation which can result from pathogenic microorganisms, trauma, and/or chemical irritants (Shiferaw & Telila, 2016; Varela-Ortiz *et al*., 2018). Regardless the etiology, since the disease is the inflammation of the parenchymal part of the gland, it is characterized by various physical and chemical alteration in milk and tissues of the mammary gland. As the result the color and consistence of the milk might be changed; immediate clots or uncoagulation of the milk might be observed; there might be swelling, heat and pain (clinical mastitis) of the teat area (Beyenê & Tolosa, 2017).

Microorganisms such as bacteria, viruses, fungi and their toxins are reported to cause mastitis in dairy animals (Argaw, 2016; Mbindyo *et al*., 2020). Bacteria are the most common causative agents of mastitis (Bihon *et al*., 2019). According to the sources, the agents and sometimes the disease are generally categorized as contagious and environmental. The contagious agents reside in the teats and udder and the organs serve as reservoir for the agents, whereas the environmental agents are obtained from the surrounding environment due to poor hygiene of housing and milking conditions (Asmare & Kassa, 2017; Wedad *et al*., 2020). The contagious causative agents spread among the animals during milking, and tend to cause chronic and subclinical

mastitis. These include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp. and *Corynebacterium bovis*. *S. aureus* and *Streptococcus agalactiae* are the most common types of the of the contagious pathogens causing mastitis (Dego *et al*., 2020; Tezera & Ali, 2021).

However, the environmental agents infect the mammary from the environment in which the animals are living. These include the coliform bacteria such as *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., and non-coliform Gram-negative bacteria such as *Serratia spp., Pseudomonas spp., Proteus spp., Gram-positive bacteria such as Enterococcus faecium and E. faecalis, some Bacillus spp. and streptococci other than Str. agalactiae*. In most of the cases, the environmental pathogens cause clinical mastitis of short durations (Abebe *et al*., 2016; Angelidis, 2015; Belay *et al*., 2022; Bikila & Soressa, 2022; Tolosa *et al*., 2013).

2.6.2. Classification of mastitis

2.6.2.1. Classification based on the clinical signs

In clinical mastitis, the reduction of milk yield accompanied by the cardinal symptoms of inflammation including heat, redness, pain, and swelling are obviously observed. Sometimes, the only change in color and consistency of milk might be observed particularly at the early stage of this mastitis type, and it will progress to the development of the inflammation (Kurjogi & Kaliwal, 2014; Muturi, 2020; Zeryehun *et al*., 2013). The color or consistency change in milk might include blood tinged, presence of clots in the milk, and watery appearance (Beyenê & Tolosa, 2017; Biffa *et al*., 2005; Dego & Tareke, 2003; Kebebew & Jorga, 2016). Clinical mastitis is an acute with short duration, and detected by physical observation (visual and palpation) of the udder for the presence of clinical signs. This type of the disease can easily be identified by non-professional individuals (Kurjogi & Kaliwal, 2014).

Clinical mastitis is commonly caused by environmental pathogens particularly *E. coli* (Wolfenson *et al*., 2015). However, other bacteria including *S. aureus, Str. Agalactiae, Klebsiella spp.* and *Enterobacter* spp. have been incriminated (Abebe *et al*., 2016; Belay *et al*., 2022; Bikila & Soressa, 2022; Cobirka *et al*., 2020). As the clinical type of mastitis is characterized by bacteriological, chemical and physical change of the milk (Kebebew & Jorga, 2016), it adversely affects the milk yield and quality in dairy industry (Muturi, 2020).

In contrast to the clinical mastitis, no observable clinical signs except reduction in milk production characterize the sub-clinical mastitis (SCM). It can remain undetected as a disease for a long period (Cobirka *et al*., 2020) and can be divisive with problems associated with stress factors (Almaw *et al*., 2012; Belachew, 2016; Egyedy & Ametaj, 2022; Mungube *et al*., 2004; Sanotharan *et al*., 2016; Shiferaw & Telila, 2016; Wolfenson *et al*., 2015; Zeryehun *et al*., 2013). Factors which affect the wellbeing of cows and decrease milk production to be confused with subclinical mastitis include advanced in lactation stage, estrus cycle (Delbecchi & Lacasse, 2006; Lopez *et al*., 2004), age of the cow, nutrient deficiency, change in environmental temperature (Liiu *et al*., 2019; Toghdory *et al*., 2022), chronic diseases, and transportation (Hong *et al*., 2019). Hence, SCM has been considered the more devastating disease for a long time, and highly prevalent than clinical mastitis. It has been estimated that SCM alone can cause more than 90% of total milk losses in dairy farms in Ethiopia (Birhanu *et al*., 2017).

Subclinical mastitis is commonly caused by the contagious bacteria which reside in teat canal and on the skin of the udder of the animals (Dego *et al*., 2020; Tezera & Ali, 2021). This type of the disease is caused particularly by gram positive bacteria such as *S. aureus* and *Str. agalactiae* (Argaw, 2016). These pathogens mostly cause subclinical mastitis probably because of the immune development of the animal against the pathogen, and hence maintain persistent infection (Belay *et al*., 2022).

The difficulty of the sub-clinical mastitis detection is due to the absence of the apparent clinical signs observed but reduction in milk production. Various diagnostic methods have been developed for of this type of mastitis. These include California Mastitis Test (CMT), Whiteside test (WST), Surf field mastitis test (SFMT), sodium lauryl sulphate test (SLST), and Microscopic Somatic Cell Count (MSCC) (Mpatswenumugabo *et al*., 2017; Mureithi & Njuguna, 2016).

The principle of CMT and SCC is that during mastitis the white blood cells (inflammatory cells) migrate to the mammary gland as a response to the infection. This situation increases the somatic cells in the milk secretion. The CMT reagent lyses the cells and the DNA of the cell dissolves in the reagent to give a viscous (jelly formation) appearance to the mixture of the reagent and the tested milk. Therefore, CMT is the qualitative method to measure the increment of the somatic cell in the milk; whereas the SCC involves the direct count of the cells in the milk sample

(Farschtschi *et al*., 2022). Both are preferable because they are ease to use, give rapid and satisfactory result in screening SCM (Mureithi & Njuguna, 2016). CMT is the most sensitive, inexpensive and reliable cow side test to detect the disease (Asmare & Kassa, 2017; Mpatswenumugabo *et al*., 2017; Sanotharan *et al*., 2016).

2.6.2.2. Classification based on sources causative agents

Based on the sources of the causative agents mastitis can be classified as contagious and environmental (Bihon *et al*., 2019; Kitila *et al*., 2021). The importance of these categories of mastitis may vary with the management practices of a specific area (Ndahetuye *et al*., 2019). Contagious mastitis is the most important type, and caused by pathogens for which the mammary of the lactating cows serves as the reservoir (Belay *et al*., 2022; Bihon *et al*., 2019; Ejeta *et al*., 2022). Since the causative agents of contagious microbes are from the animal itself, this types of the disease is the most prevalent type (Kasa *et al*., 2020). These pathogens spread from animal to animal or from teat to teat mainly during milking due to poor hygienic practices (Mpatswenumugabo *et al*., 2017; Sant'Anna & Paranhos da Costa, 2011). Milking practices such as using common udder clothes and common milkers, milking of the mastitis cows before the health ones, and lack of milking cows in age order can be vector for the contagious types of the agents (Aki *et al*., 2017; Bikila & Soressa, 2022). In addition, the spread of the pathogens among the quarter is during the milking process or teat preparation by contaminated hands, udder clothes, cleaners, milking hands and milking machine (Egyedy & Ametaj, 2022; Ejeta *et al*., 2022).

On the other hand, environmental mastitis is the form of mastitis caused by pathogens contracted from the environment which include the soil, contaminated water, manure, and bedding (Argaw, 2016; Egyedy & Ametaj, 2022). Most of the causative agents of the environmental mastitis reside in the environment of the animals. However, because of their nature of resistance in environmental conditions, some bacteria such as *S. aureus* and *Str. agalactiae* can cause both contagious and environmental mastitis (Cobirka *et al*., 2020). Majority of this type of the disease is clinical mastitis and last for short time. Environmental mastitis is associated with poor farm hygiene, poor slope of the stable settings, poor sanitation of milking materials, lack of using

individual towels, not using dry-cow therapy[1] (Belay *et al*., 2022). The environmental mastitis is the most prevalent and economically important in modern dairy farms where mastitis is controlled through mastitis prevention program (Klaas & Zadoks, 2018). Therefore, this type of the disease is controlled by taking action to reduce the number of bacteria to which the teat is exposed, enhancing the immune resistance of the animal, pre milking teat dipping with a germicidal, cleaning and drying animals house, and antibiotic treatment of clinical and sub clinical mastitis (Argaw, 2016).

2.6.3. Bovine mastitis prevalence in Ethiopia

The prevalence and incidence of bovine mastitis may vary with type of the disease, breed, age, and parturition of the animal, and management systems of the farm. Bovine mastitis in Ethiopia has been reported by many researchers. According to Argaw (2016) the overall prevalence of bovine mastitis in Ethiopia ranges from 23% to 85%. More recently, the systematic review and meta-analysis conducted by Girma and Tamir (2022) revealed that the overall pooled prevalence of mastitis in dairy cows in Ethiopia is to be 43.60%, of which 12.59% are clinical and 32.21% are subclinical cases. The analysis also reported that the pooled prevalence of the disease increases with year of study that the highest prevalence was recorded in studies conducted between 2017 and 2022 (46.83%) followed by in studies conducted between 2005 and 2016 (39.97%). Mihret *et al.* (2017) also reviewed that the prevalence of bovine mastitis in Ethiopia is as less to be 3.8% for clinical and 2.1% for blocked teat cases and as high to be 42.1% for sub clinical.

2.6.4. Economic and public health importance of mastitis

Mastitis is called the costly disease which affects the dairy industry worldwide in various ways. The losses in dairy farms is due to mastitis arise from reduction of milk yield, pharmaceutical and veterinary serves cost, labour, defect in milk quality, investment cost for mastitis management, mastitis test cost, culling of the premature dairy animal, and occasionally death (Belay *et al*., 2022; Khannal & Pandit, 2013; Mpatswenumugabo *et al*., 2017; Rainard *et al*., 2017). Studies have revealed that both clinical and sub-clinical mastitis affect the reproductive

[1] Dry-cow therapy is the antibiotic treatment given, specially with the long-acting antibiotics, to control mastitis during dry phase of the dairy animals (Crispie *et al*., 2004; Niemi *et al*., 2021).

performance of the affected animals. Mastitis has been considered delaying the postpartum ovarian function, change the ovulation, fertilization, pregnant maintenance, and implantation. It also increases the calving to conception interval, and calving to first service interval, and increase the number of services per conception (Argaw, 2016).

It has been estimated that the expense cost of clinical and subclinical mastitis per case is to be $ 287 and $102 respectively (Rainard *et al*., 2017). In USA the economic losses due to mastitis is estimated to about $185.00 to 265.00 per cow per year (Argaw, 2016). Studies conducted by Getaneh *et al.* (2017) and (Mekonnen *et al*., 2019) estimated the average total failure costs of mastitis in Ethiopia was to be 4765 Ethiopian Birr (ETB) (1 ETB = 0.0449 USD) per farm per year and 54% of the cost was due to sub-clinical mastitis. The same study also revealed that the total failure cost due to mastitis in lactating cows per cow per farm per year was to be 1961 ETB which varied with range of 0 to 35 084 ETB between farms. In addition, Beyene and Tolosa (2017) found that the total financial losses caused by mastitis in Horo Guduru Wollega Zone (West Ethiopia) was estimated to be 2,949.8 USD (59,719.08ETB) according to the USA Dolar exchange rate of that time. Another study conducted by Mungube *et al.* (2004) in urban and peri-urban of Addis Ababa of Ethiopia reported that the economic loss due to mastitis was estimated to be $ 58 per cow per lactation. The studies indicated that mastitis causes enormous economical losses worldwide in general and in Ethiopia in particular.

The public importance of mastitis is linked with the contamination of milk and milk products with potential zoonotic microorganisms which might enter into to the milk from the mastitis dairy animals. Some of these microbes can cause serious public health problems though food poisoning and/or infection. The illnesses include Staphylococcal food poisoning, diphtheria, brucellosis, caseous, leptospirosis, listeriosis, melioidosis, scarlet fever, Q-Fever, toxoplasmosis and tuberculosis Hameed 2006, (Tillahun & Aylate, 2015). Raw milk has been found spreading diseases caused by *Listeria* spp., *Campylobacter* spp., *Yersinia* spp, *Salmonella* spp, *Staphylococci* spp, and *E. coli* (Hameed *et al*., 2007). These pathogens enter into the food chain specially when they are found in milk from subclinical mastitis where no obvious changes observed in the milk color or consistence (Hameed *et al*., 2007).

2.6.5. Control and prevention of mastitis

Most of the mastitis-related losses in Ethiopia are due to the fact that dairy farms are less implementing the mastitis prevention practices (Abebe *et al*., 2016). Many researches have recommended various mastitis control and prevention methods to combat the economic and public impacts of the disease. The methods may vary with the types of the causative agents (environmental or contagious), and other risk factors. Generally, Hygienic practices; pre-treatment testing, dry-cow therapy, and culling of persistent mastitis in dairy cows are recommended to control and prevent mastitis. The hygienic practices include washing and drying teats before milking, early maintenances of milking machines, post milking teat disinfection, and milking of non-infected cows before infected ones (Biffa *et al*., 2005; Cheeng & Han, 2020; Workineh *et al*., 2002). Decreasing the number of cows in a house and hygienic bedding may decrease the chance of the animal to contract the pathogen from the environment (Dabele *et al*., 2021; Klaas & Zadoks, 2018). The application of post-milking disinfection is the best method to prevent the occurrence of contagious mastitis (Schnitt & Tenhagen, 2020)

Antibiotics have been used for control and prevention of bacterial infections of the mammary glands (Stevens *et al*., 2016). However, antibiotic resistance have been incriminated to tackle the efficiency of the treatments (Wedad *et al*., 2020). The existing infection can be eliminated through treatment during lactation or at dry off (Kibebew, 2017). Furthermore, vaccines against front line pathogens have also been developed. Mastitis vaccines have mainly focused on *E. coli*, *S. uberis* and *S. aureus*. The efficacy of the vaccines is evaluated based on the criterion including the prevention or reduction of severity in clinical mastitis, reduction of milk loss, and reduction in mortality, and particularly for *S. aureus* vaccine, enhancing the chance of cure and hindering transmission (Klaas & Zadoks, 2018; Kurlenda & Grinholc, 2012).

2.7. *Staphylococcus aureus*

2.7.1. Discovery of S. aureus

In 1880, Alexander Ogston discovered a bacterial group known as ‘Staphylococcus’ as the major cause of pus. The name was coined from the Greek word “Staphyle” meaning “a bunch of grapes” and kokkos meaning a grain or a berry. The name was provided from the appearance of

the bacteria that the member of the group revealed grape-like clusters when first isolated from pus in the of abscess from human. At the time, they were also determined to produce toxins responsible for food poisoning (Bhunia, 2008). Four years later of Ogston's discovery, Rosenbach, a German surgeon, isolated two species of staphylococci, and named them based on the color of their colonies: 1. yellow pigmentation named as *Staphylococcus aureus*, from the Latin *aurum* for gold, and 2. white named *Staphylococcus albus* (now called *epidermidis*), from the Latin *albus* for white. Currently, about 40 species and 17 subspecies of Staphylococci have been recognized (Maktabi *et al*., 2021).

The most important species in the genus Staphylococcus is *S. aureus*. It is Gram-positive cocci (1 μm in diameter) and appears as grape-like clusters under the microscope. It is non-motile and most of the time produces golden yellow colonies on a nutrient agar plate. As far as the cell component of the bacterium is concerned, *S. aureus* is unique in that its cell wall contains protein A. The protein is covalently linked to the peptidoglycan and is characterized by its ability to bind the *Fc* component of the immunoglobulin in plasma causing auto-agglutination. Protein A is absent in the cell wall of most of the other species of staphylococci (Quinn *et al*., 2004).

2.7.2. Laboratory characteristics of S. aureus

The genus *Staphylococcus* may sometimes be confused with other Gram positive cocci such as *Micrococcus* and *Streptococcus*. Micrococcus and Staphylococcus resemble each other under microscope in some conditions forming clusters and both are catalase (an enzyme that catalizes H_2O_2 to water and Oxygen) positive. However Staphylococcus is differentiated from Micrococcus by using oxidation-fermentation (O-F) test in that Micrococcus is oxidative that ferments glucose in the presence of oxygen whereas Staphylococcus is fermentative which ferment glucose both aerobically and anaerobically. The main difference between Streptococcus and Staphylococcus is that the latter is catalase positive while former does not produce catalase so that it cannot catalize H_2O_2 into water and oxygen (Quinn *et al*., 2004).

Enrichment isolation on selective or nonselective media and direct plating are the most commonly used approaches for detecting and enumerating *S. aureus*. *S. aureus* can grow in an extensive range of temperatures, pH values, sodium chloride concentrations and water activity. Taking these conditions in consideration, the principal diagnostic features of *S. aureus* on

contemporary media include ability to grow in the presence of 7.5 or 10% NaCl, 0.01–0.05% lithium chloride, and 0.12–1.26% glycine, or 40 ng/mL polymyxin. It reduces potassium tellurite to produce black colonies aerobically and anaerobically and is also able to hydrolyze egg yolk. In addition, the colonial form, appearance, size, and pigmentations are some of the presumptive characteristics of the species. The aforementioned features summed up with its microscopic appearance and cellular arrangements, coagulase and thermo nuclease activity, acid production in a solid medium, and growth at 42–43°C on selective agar will lead ones to conclusive diagnosis. Media used in the detection and enumeration of *S. aureus* may employ one or more of these diagnostic features (Bennet & Monday, 2003).

S. aureus is a Gram-positive coccus appearing microscopically as grape-like clusters. They are nonmotile and produce golden yellow colonies. The cell wall of *S. aureus* contains protein A, which is covalently linked to the peptidoglycan and particularly is characterized by its ability to bind to Fc component of the immunoglobulin in plasma causing autoagglutination. Most of the other species of staphylococci lack protein A in their cell wall (Quinn *et al*., 2004).

The growth temperature of *S. aureus* ranges from 7 to 47.8°C, with an optimum temperature of 35°C. The pH range for growth of *S. aureus* is between 4 and 10, with the optimum between pH 7.0 and 7.5. However, the minimum pH for its growth is dependent on the degree to which all other parameters are at optimal conditions. With regard to water activity (Aw), the staphylococci are unique in being able to grow at as low as 0.83 Aw which is ideally too low for the growth of many competing organisms (Bennet & Monday, 2003; Montanari *et al*., 2014).

S. aureus grow on many non-selective general purpose media. Their colonies on trypticase soy agar or blood agar are opaque, 1 to 3 mm in diameter, and yellow, orange, or white in color. They are salt-tolerant that they grow well on media containing 10% sodium chloride. They ferment mannitol aerobically to produce acid. They also produce alpha toxin that causes a wide zone of clear (beta-type) hemolysis on blood agar. This alpha toxin can cause local necrosis and death if inoculated in rabbit (Benson, 2002).

The other cultural and biochemical characteristics of *S. aureus* include that it is coagulase positive which coagulates rabbit plasma relatively quicker than *S. intermedius* and *S. hyicus* subsp. *hyicus* which produce delayed coagulation, facultative anaerobic, and grows abundantly

under aerobic conditions. Under aerobic conditions, it produces acetoin as the end product of glucose metabolism. It causes coagulation of rabbit plasma, produces thermo nuclease, and is sensitive to lysostaphin. *S. aureus* is relatively resistant to drying and heat (Quinn *et al*., 2004).

2.7. 3. Virulence factors of S. aureus

S. aureus has some remarkable features that enable it to survive in various hosts. First, there is a large genetic diversity amongst *S. aureus* isolates. Accordingly, in addition to a highly conserved backbone of core genes, there are more than 700 core variable (CV) genes in strains of *S. aureus* (Dancer, 2008; Stefania *et al*., 2012). Second, it possesses a wide variety of virulence factors and quorum sensing mechanisms, which enable it to survive the immune defense mechanisms of its host and to cause several types of diseases(Dancer, 2008) . Moreover, *S. aureus* can acquire new genes, (e.g. antimicrobial resistance genes) mainly through mobile genetic elements. These genetic elements help the bacterium to adapt to new environmental conditions or selective pressures (Stefania *et al*., 2012) so that some of the strains are resistant to highly effective antibiotics such as methicillin (methicillin-resistant (MRSA)) and vancomycin (vancomycin-resistant). Such characteristics of the pathogen make the infections caused by the strains fatal because of the lack of alternative antibiotics (Torrence & Isaacson, 2003).

More than 50 virulence factors with a wide range of biological activities have been determined in *S. aureus*. Some of the frequently identified include lipase, protease, coagulase, haemolysins (α,β,γ and δ), deoxyribonuclease (DNase), leukocidin, exfolative toxins (A and B), toxic shock syndrome toxin 1 (TSST-1), a family of emetic pyrogenic superantigens (Sags) and enterotoxins (SEs). These are responsible for a variety of toxin-mediated and suppurative diseases in their host. On the basis of their localization, however, the factors are generally categorized into two main classes as cell-surface-associated (surface proteins) and secreted proteins (exotoxins) (Green & Mecsas, 2016; Tam & Torres, 2019; Tarekgne, 2016) (Table.1).

The staphylococcal toxins are mediated by specific genes carried either in chromosome and/or in plasmids of the strains. However, the presence of the enterotoxin genes might not indicate that the genes are surely expressed. Paulin *et al.* (2012) reviewed that 12% to 77% of *S. aureus* have been determined expressing their enterotoxin genes. Fooladi *et al.* (2010) determined that genotypically 31.1% of the isolates were enterotoxin gene positive. However, only half of the

enterotoxin gene positive isolates were found expressed the gene phenotypically. Likewise, Santana *et al.*, (2010) and Rahimi (2013) reported that only 9.81% of 14 and 43.75% of 16 enterotoxin gene positive respectively were found producing enterotoxin in growth media. Other researchers have also observed that not all enterotoxin genes are expressed. Klotz *et al.* (2003) reported 40 of 44 enterotoxin gene positive produced the toxin in the media. Jørgensen *et al.* (2005) found none of the 75 enterotoxin gene positive *S. aureus* produced the enterotoxin.

Most of the enterotoxin genes are carried on mobile genetic elements such as pathogenicity islands , plasmids, or bacteriophages that they are commonly transferred among the strains by horizontal genetic transfer (Pinchuk *et al*., 2010). The expression of the enterotoxin genes in *S. aureus* can be enhanced by the lipid contents of the medium in which the strain is grown (Medveďová *et al*., 2009). In addition, the enterotoxin gene expression is influenced by the genetic elements which the gene is carried on. The genes can be present on different mobile genetic elements and so that their expression processes need complex genetic regulations (Cretenet *et al*., 2011; Cunha *et al*., 2007). For example, the expression of some SE genes (seb, sec, sed) is associated with quorum sensing via the *agr* (accessory gene regulator) system that significantly high concentration of the cells should be present for enterotoxin production to occur (Ortega *et al*., 2010). Furthermore, the concentrations of the toxins vary with types of the toxins and the strains of the species of bacterium (Paulin *et al*., 2012). The expression of some enterotoxin specially that of *sea* is partially linked with SEA-encoding prophage in contrast to the non-prophage enterotoxin uch as seb, sec and sed (Schelin *et al*., 2011).

In addition, the growth conditions such as temperature, pH, and the medium contents, including salt concentration, water activity and nutrients, have been reported affecting the production of the staphylococcal enterotoxins (Al-nabulsi *et al*., 2020; Sandel & McKillip, 2004). The temperature ranges for staphylococcal enterotoxin production are between 10 and 45°C with optimum of 35 to 40°C (Paulin *et al*., 2012). Evidences revealed that the production of SEA increase with temperature in range of 15 to 32°C (Schelin *et al*., 2011). Concerning pH value, the toxins are produced in between 4 and 9.6. If high concentration of bacterial cell available (10^8CFU/ml) in Brain-Heart-Infusion broth media, the enterotoxogenic strains of *S. aureus*, without adding salt, the bacterium can produce the toxin at PH range of 4 to 9.8. However the enterotoxin has not been produced when NaCl was added at concentration of 12%. For example,

enterotoxins in cheese have not been reported so far; although isolates are occasionally tested for both the production of enterotoxin and possession of enterotoxin genes. This might be attributed to the low pH value of the product (Paulin *et al*., 2012). Low water activity (wa) has also been seen affecting the production of some enterotoxin (SEB) than others (SEA) (Hennekinne *et al*., 2012). Furthermore, the addition of carbon sources such as glucose has been determined limiting of the enterotoxins; whereas the addition of peptides increase their production (Paulin *et al*., 2012).

The virulence factors have been found generally involve in adhesion, invasion and immune evasion (Heffernan *et al*., 2015) and their production can be limited to the growth phase of the bacterium. Accordingly, in general terms, proteins facilitating adhesion and invasion of *S. aureus* are manufactured during the exponential phase of the bacterial growth, while enzymes and exotoxins, including the SEs, are synthesized in the post-exponential phase of growth (Tarekgne, 2016).

S. aureus expresses several adhesion factors that mediate interactions with host cells and extracellular matrix (ECM), and allow efficient colonization. These adhesins can be classified into two groups: surface components which specify adhesive matrix molecules (MSCRAMMs), and secreted expanded repertoire adhesive molecules (SERAMs). The major components of MSCRAMMs include fibronectin binding proteins (FnBPs), clumping factors (Clfs), staphylococcal protein A (SpA) and collagen-binding protein (Cbp). SERAMs are secreted adhesins, including the fibrinogen-binding protein (FBP) A, the coagulase (Coa), the extracellular fibrinogen-binding protein (Efb), the ECM-binding protein and the extracellular adherence protein (Eap). SERAMs interact with a broad array of host ligands, thereby mediating bacteria adhesion. However they may also interfere with host defense mechanisms (El-baz *et al*., 2016).

Invasion is the ability *S. aureus* to express proteolytic enzymes that facilitate the distribution of the bacterium in the body of the host. It is facilitated by the host cell fibronectin receptor, (integrin α5β1). Various enzymes and toxins including coagulase, hyaluronidase, leukocidins, DNAse Proteases, nucleases, phospholipase C, metalloproteases (elastase) and haemolysins have been incriminated as invasion factors (Kurlenda & Grinholc, 2012). The main function of these

proteins may be to damage local host tissue and convert it into nutrients required for bacterial growth (Bien *et al*., 2011). *S. aureus* produces several different lipases or lipid hydrolases; an enzyme which help in infecting skin and subcutaneous tissues. Lipases break fats down into their fatty acid and glycerol components (Cadieux *et al*., 2014). Coagulase together with MSCRAMMs, activates prothrombin to which in turn converts fibrinogen to fibrin results in the formation of microthrombin (Cheng *et al*., 2010; McAdow *et al*., 2012). Hyaluronidases hydrolyse hyaluronic acids, the acidic mucopolysaccharides that hold together certain cells of the body, particularly cells in the connective tissue. It is, therefore, used to break down the connective tissue which is used as a barrier in the body (Thomas *et al*., 2019).

S. aureus avoid the success of the innate and adaptive immune components by various mechanisms. It evades innate immune components through a hide and seeks strategy including interfering with Toll-like receptor (TLR) ecognition, restraining complement deposition or activation, and the chemotaxis of neutrophils. In addition, several members of cell wall associated (CWA) proteins such as ClfA, Cna, SdrE and protein A, also interfere with innate and adaptive immune responses. Moreover, *S. aureus* produces several secreted proteins with lytic properties on neutrophils. For example Phenol-soluble Modulins (PSMs) are small, amphipatic, α-helical peptides having been found effectively lyse human neutrophils and the integrity of the plasma membrane of neutrophils (Surewaard *et al*., 2013). This indicates that the primary role of PSMs is to destroy leukocytes, and thus facilitate *S. aureus* evasion of host immune defense systems. Other protein used to evade host immune defense mechanism is Protein A (SpA). Protein A (SpA) is a conserved, multifunctional surface protein of *S. aureus* whose N-terminus contains five tandemly linked triple-helical bundle domains which are important for binding to IgG and other ligands, such as tumor necrosis factor (TNF) receptor 1 (TNFR1) and von Willebrand factor (VWF). In addition it has a number of immunosuppressive traits and is one of *S. aureus*' most important mechanisms of immune evasion. Furthermore, SpA binds to the Fcγ portion of IgG, resulting in the bacteria becoming coated in IgG bound in the incorrect orientation, leading to decreased recognition by neutrophils and consequently evasion of phagocytosis (Falugi *et al*., 2013; Kim *et al*., 2012). SpA is also a B cell super antigen that binds to the VH3+ immunoglobulin on the surface of B cell receptors, causing clonal expansion, which results in apoptosis (Fox *et al*., 2021; Shi *et al*., 2021).

However, except for the toxin-mediated diseases such as toxic shock syndrome (TSS), other staphylococcal infections are produced by multiple virulence factors in a single disease condition. Various factors participate in a stepwise manner in various disease conditions. In each step, one or more virulence factors may be involved (Tarekgne, 2016).

Table 1: Virulence factors and their respective functions in *S. aureus* pathogenicity

S.No	Virulence factors	Putative Function
1	Cell Surface Factors	
1.1	Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)	
	Staphylococcal protein A (SpA)	Bind to IgG, interfering with opsinization and phagocytosis
	Fibronectin-binding proteins (FnbpA and FnbpB)	Attachment to fibronectin and plasma clot
	Collagen-binding protein	Adherence to collagenous tissues and cartilage
	Clumping factor proteins (ClfA and ClfB)	Mediate clumping and adherence to fibrinogen in the presence of fibronectin
1.2	Capsular polysaccharides	Reduce phagocytosis by neutrophils; enhance bacterial colonization and persistence on mucosal surfaces
1.3	Staphyloxanthin	Resistance to neutrophil reactive oxidant-based phagocytosis
2	Secreted Factors	
2.1	*Superantigens*	
	Staphylococcal enterotoxins (SEA, B, C, D, E, G and Q)	Massive activation of T cells and antibody presenting cells
	Toxic shock syndrome toxin-1 (TSST-1)	Massive activation of T cells and antibody presenting cells
2.2	*Cytolytic toxins*	
	Cytolysins	
	α-hemolysin	Induce lysis on a wide spectrum of cells, mainly platelets and monocytes
	β-hemolysin	Hydrolysis of sphingomyelin of the plasmatic membrane of monocytes, neutrophils and lymphocytes; make cells susceptible to other lytic agents
	γ-hemolysin Leukocidin family	Induce lysis on erythrocytes and leukocytes

Cont.

S.No	Virulence factors	Putative Function
	Leukocidins E/D and M/F-PV	Induce lysis on leukocytes
	Panton-Valentine leukocidin (PVL)	Induce lysis on leukocytes
2.3	Various exoenzymes	
	Lipases	Inactivate fatty acids
	Nucleases	Cleave nucleic acids
	Proteases	
	Serine (e.g. exfoliative toxins ETA and ETB)	Inactivate neutrophil activity; activate T cells (only ETA and ETB)
	Cysteine (e.g. staphopain)	Block neutrophil activation and chemotaxis
	Aureolysin	Inactivate antimicrobial peptides
	Hyaluronidase	Degrade hyaluronic acid
	Staphylokinase (SAK)	Activate plasminogen; inactivate antimicrobial peptides
3	Miscellaneous proteins	
	Staphylococcal complement inhibitor (SCIN)	Inhibit complement activation
	Extracellular fibrinogen binding protein (Efb)	Inhibit complement activation
	Chemotaxis inhibitory protein of *S. aureus* (CHIPS)	Inhibit chemotaxis and activation of neutrophils
	Formyl peptide receptor-like 1 inhibitory protein (FLIPr)	Inhibit chemotaxis of neutrophils
	Extracellular adherence protein (Eap)	Inhibit neutrophil migration

Source: Tarekgne (2016)

2.7.4. Antibiotic resistance in S .aureus

Penicillin is the earliest antibiotic to be discovered in 1928 (Vineetha *et al*., 2015). It is one of the antibacterial drugs in the superfamily of beta lactam antibiotics, of which common core structure is the presence of 4-membered β-lactam ring. The β-lactam antibiotics (commonly called beta lactams) have bactericidal properties by interrupting the synthesis of cell wall. The drugs can cause this by binding to the penicillin binding proteins (PBPs), the enzyme which catalyzes the cross-linking of peptidoglycan (Aragão *et al*., 2019; Bush & Bradford, 2016; Lakhundi & Zhang, 2018). However, *S. aureus* has been repeatedly reported that majority of the

strains develop resistance to most of the β-lactams particularly to penicillin. The main resistance mechanism of *S. aureus* to penicillin is by producing the extracellular enzyme β-lactamase, the enzyme which hydrolyses the lactam ring of the drugs. This Beta lactam hydrolyzing enzyme (beta lactamase) is coded by *blaZ*-gene in the plasmids of the beta lactam resistant strains of the species (Lowy, 2003). Another mechanism by which *S. aureus* resist β-lactam drugs is by producing the mutated type of penicillin binding protein known as penicillin-binding protein 2a (PBP 2a). This type of protein is the alternative enzyme to penicillin binding protein to catalyze the cross link of peptidoglycan during the cell wall synthesis. PBP 2a is not inhibited by β-lactam antibiotics because it has less affinity to β-lactams (Fuda *et al*., 2005). Studies have disclosed that the PBP 2a in MRSA is more similar with PBP 2B of *Bacillus subtilis* (52% similarity) and with PBP 3 of *E. coli* (48% similarity) than with PBP of *S. aureus* (43% similarity) (Sauvage *et al*., 2008).

The ability of a microorganism to withstand the effect of an antibiotic to which it has been susceptible previously is known as antimicrobial resistance (AMR) (Barhe *et al*., 2021; Nelson *et al*., 2019; Prestinaci *et al*., 2015). Such characteristic evolves either through natural selection by random mutation or through acquisition of resistance gene from other bacteria by horizontal or vertical gene transfer. Horizontal fashion of gene transfer involves plasmid exchange among the bacteria living in the same vicinity; whereas vertical gene transfer is the mechanism through which the transfer takes place from mother cell to daughter cells during cell division. The gene may carry several resistance genes which are responsible to withstand many antibiotic; the situation result in evolution of multi-drug-resistant microorganisms (Palmer & Kishony, 2013; Wilson *et al*., 2016). Apart from plasmid transfer, antibiotic resistance may occur due to the movements of transposable elements which jump from place to place in a single DNA. These elements may also carry the codes for several factors and can insert themselves on either side of a pathogenicity island (Bennett, 2008; Partridge *et al*., 2018; Tarekgne, 2016).

AMR poses serious threats to our chemical shield that it has been increasingly leaked. As the result the world is on the brink of losing the miracle curing ability of the antibiotics developed so far. The reduction in efficacy among the antibiotics due to the emergence of resistant bacteria has limited the opportunities to control pathogenic bacteria in public infections particularly when multidrug resistant bacteria, which prohibit most of drug treatment alternatives, are emerged.

More importantly, the phenomenon of antibiotic resistance becomes extremely threat because the rate at which it develops is unmatched with drug discovery and development (Ahmèd *et al*., 2023; Patell & Saiman, 2010; Shinu *et al*., 2022).

The emergence and distribution of the drug resistant bacteria is so intense that each year at least two million illnesses and 23,000 deaths are caused by drug-resistant bacteria in the United States alone (CDC, 2019; Reygaert, 2018). Hence, World Health Organization (WHO) urged that in the absence of timely corrective and protective actions, the world is heading toward a post-antibiotic era, in which many common infections will no longer have a cure and once again, kill unabated (WHO, 2015).

The alarm of AMR has imposed member states to request WHO to develop a global priority pathogens list (global PPL) of antibiotic-resistant bacteria to help in prioritizing the research and development of new and effective antibiotic treatments. Accordingly, the organization listed the pathogenic bacteria into three categories according to the urgency of need for new antibiotics: critical, high and medium priority. The critical group includes all multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and among patients whose care requires devices such as ventilators and blood catheters. They can cause severe and often deadly infections such as bloodstream infections and pneumonia. The high and medium priority categories contain other increasingly drug-resistant bacteria that cause common diseases such as gonorrhea and food poisoning (WHO, 2019).

1. The Priority 1 (Critical) groups carbapenem-resistant Acinetobacter, carbapenemresistant Pseudomona aeruginosa, and carbapenem-resistant and 3 rd generation cephalosporinresistant Enterobacteriaceae (including *Klebsiella* spp., E. coli, *Serratia* spp., and *Proteus* spp).

2. The Priority 2 (High) include vancomycin-resistant Enterococcus faecium, methicillin-resistant and vancomycin intermediate and resistant *Staphylococcus aureus*, clarithromycin-resistant Helicobacter pylori, fluoroquinolone-resistant Campylobacter spp., fluoroquinolone-resistant Salmonella spp., and 3rd generation cephalosporinresistant and fluoroquinolone-resistant Neisseria gonorrhoeae.

3. The Priority 3 (Medium) are penicillin-non-susceptible Streptococcus pneumoniae, ampicillin-resistant Haemophilus influenzae, and fluoroquinolone-resistant Shigella spp.

However, Mycobacteria (including Mycobacterium tuberculosis) was not included into these categories in this prioritization programme as it is already a globally established priority for which innovative new treatments are urgently needed (WHO, 2019)).

S. aureus had been convicted to develop drug resistance as early as the introduction of chemotherapy in 1941, only after 2 years since the introduction of penicillin in clinical therapy and now day's antibiotic resistance is among the pathogenicity factors of the species. Unlike methicillin-resistance which is chromosomic, penicillin resistance in the species is plasmidic, and therefore it is distributed very quickly to several other strains. The extended use and misuse of antibiotics (e.g. in agriculture, stock-farming and in the treatment of human diseases) have been incriminated to result in enhancing and increasing the number of bacteria that are resistant to antimicrobial agents. Now days, *S. aureus* is notorious for its ability to become resistant to many antibiotics (multidrug resistant) and is becoming a global problem (Pesavento *et al*., 2007). *S. aureus* resist antibiotics in three main ways two of which are due to resistance gene acquisition.

1. The resistance genes may be carried by mobile genetic elements (MGE) such as bacteriophages, plasmids, naked DNA or transposons which transfer through horizontal gene transfer. There have been reported that MGE can be transferred in the same lineage at high frequency by generalized transduction; although conjugation and transformation can also occur at much lower frequency (Lindsay, 2013). Some transposons /naked DNA contains more complex genetic material integrons that contain a site for integrating different antibiotic resistance genes and gene cassettes in tandem for expression from a single prompter(Sabbagh *et al*., 2021; Ye *et al*., 2020).

2. Resistance could also occur through the chromosomal mutation. This type of antibiotic resistance is characterized by step-wise progression from low level to high level (Gostev *et al*., 2023; Vestergaard *et al*., 2019).

3. The bacteria may also resist to certain antibiotics because of innate ability of the species to resist activity of a particular antimicrobial agent through inherited structural or functional characteristics. Such characteristic is known as intrinsic type of resistance (Cox & Wright, 2013; Guo *et al*., 2020).

Various resistance genes responsible to different antibiotic resistance have been identified in *S. aureus* of which methicillin resistance genes (*mecA* and *mecC*) are clinically the most important since a single genetic element confers resistance to the most commonly prescribed class of antimicrobials- the β-lactam antibiotics, which include penicillin, cephalosporin and carbapenems (Grundmann *et al*., 2006). In addition to Mec genes encode for resistance to β-lactam antiotics, many antibiotic resistance genes play role in *S. aureus* resistance. These include macrolide resistance encoded by the erm gene, aphA3 and sat genes for kanamycin and streptomycin resistance and accA-aphD and tet genes for gentamicin, tobramycin and tetracycline resistance (Pekana & Green, 2018).

In laboratory, antibiotic resistance of a bacterium is tested in vitro by either phenotypic characterization (antimicrobial susceptibility testing) or molecular methods. Antimicrobial susceptibility testings (ASTs) are conducted to choose the best therapeutic drugs of alternatives. The two methods of in vitro antibiotic susceptibility tests used for *S. aureus* are genotypic (molecular method) and phenotypic methods. There are, in turn, three approaches used to conduct phenotypic antimicrobial susceptibility testings. These are broth dilution, agar dilution and agar diffusion (disk diffusion) tests (Karatuna, 2012; Ortez, 2005; Rankin, 2005).

Broth dilution method is the technique preformed in incremental concentrations of an antimicrobial agent in a standardized liquid medium against a standardized suspension of bacteria. The minimum amount of antibiotic that inhibits the visible growth of an isolate or minimum inhibitory concentration (MIC) which is critical for antimicrobial resistance monitoring programmes will be determined by the test. Bacterial isolate is subjected to various dilutions of antibiotics. The highest dilution of antibiotic that has inhibited the growth of bacteria is considered as MIC. There are two dilution MIC tests named as macro- and microbroth dilution MIC test. In macrobroth dilution MIC tests A serial two-fold dilution of antibiotic are made in test tubes from 0 to maximum concentration that is achieved in vivo without toxic effect on

patient. The inoculum density of bacterial isolate to be tested is standardized with 0.5 McFarland turbidity standards. The suspension should have a final inoculum of 5 x 105 cfu/ml. 1ml of bacterial suspension is added to rows of antibiotic solution and incubated at 37°C overnight. The lowest concentration of antibiotic that completely inhibits visual growth of bacteria (no turbidity) is recorded as MIC. In the case of microbroth dilution MIC tests, a polystyrene tray containing 80 wells is filled with small volumes of serial two-fold dilutions of different antibiotics. The inoculum suspension and standardization is done according to McFarland standard. The bacterial inoculum is then inoculated into the wells and is incubated at 37°C overnight and MIC is determined as in macro-broth dilution test. Broth dilution method can be performed either in tubes containing a minimum volume of 2 ml (macrodilution) or in small volumes (50–100 microliter) using microtitration plates (microdilution). Despite the method is reproducible; it is relatively expensive and requires specialized equipment and training (Ortez, 2005; Torrence & Isaacson, 2003).

Agar dilution is similar to broth dilution, except that the antimicrobial agent is incorporated into an agar medium, and the bacterial inoculum is applied to the surface of that medium, usually by using a multipin replicating apparatus. The MIC is determined by unaided visual inspection of the agar surface. Using agar dilution methods has several advantages. The medium is very flexible, allowing one to extend the antibiotic concentration range as far as necessary. It allows the testing of multiple bacteria (32–36 isolates) simultaneously on the same set of agar plates (Torrence & Isaacson, 2003).

In disk diffusion method, a known amount of drug is incorporated into a paper disk, which is placed on the agar surface. After incubation, the diameter of the zone of inhibition is measured and used to predict drug susceptibility. The technique is carried out in such a way that the standardized bacterial isolate is spread on an agar plate and then paper disc containing specific concentration of antibiotics are placed and incubated at 37°C over night. The susceptible isolate to the antibiotic does not grow around the disk thus forming a zone of inhibition. Strains resistant to an antibiotic grow up to the margin of disk. The diameter of zone of inhibition will be measured and result read in comparison to the predetermined results as sensitive, intermediate or resistant. As long as standardized methodologies are employed and zone interpretive standards are in place, reliable results can be obtained with disk diffusion tests. Disk diffusion is, therefore,

technically straightforward to perform, reproducible, and does not require expensive equipment. The main advantages of the disk diffusion method are the low cost and the ease in modifying test formats when needed (Kayser *et al*., 2005; Torrence & Isaacson, 2003).

Most of the antibiotic resistance of *S. aureus* is due to the presence of resistance genes in Staphylococcal cassette chromosome *mec* (SCCmec). The most common identified resistance genes in strains of *S. aureus* are *mecA* and *mecC* genes for β-lactam resistance, *erm* gene for macrolide resistance, aphA3 and sat genes for kanamycin and streptomycin resistance, and accA-*aphD* and *tet* genes for gentamicin, tobramycin and tetracycline resistance(Pekana & Green, 2018). *MecA* gene is the most characterized gene and has been identified encoding penicillin binding protein (PBP2a) and different version of PBP2a respectively. PBP2as are the proteins which have low affinity to methicillin and other beta-lactam antibiotics (Eshetie *et al*., 2016). There is scarcity of information on the molecular characterization of *S. aureus* in most developing countries especially in the Ethiopia. Better understanding of *S. aureus* antibiotic susceptibility profiles and molecular characterization of antibiotic resistance genes are, therefore, of supreme importance for initiating effective control measures and reducing staphylococcal infections in human and animals. Molecular testing for the resistant genes or their products can, therefore, be performed to determine the resistant phenotypes. Molecular tests and characterizations for the resistance genes including a cycling probe reaction and polymerase chain reaction (PCR) are available worldwide. Moreover, PBP2a can also be detected by a commercially available latex agglutination test (Cavalieri, 2005).

2.7 5 Public health and veterinary importance of S. aureus

2.7.5.1. *S. aureus* infections in human

S. aureus inhabits the skin, upper respiratory tracts, and urogenital tracts in humans (Bhunia, 2008). Approximately half of the human population is expected to be an asymptomatic carrier of which about 40% being a persistent and 60% an intermittent carriers (Chambers & DeLeo, 2009; Wertheim *et al*., 2005). Higher loads of one *S. aureus* clone have been detected in the cases of persistent carriages; whereas, in intermittent carriages, there are lower loads of various *S. aureus* clones. The nasal carriage of *S. aureus* has been identified as a common risk factor for subsequent infections caused by the bacterium (Wertheim *et al*., 2005).

S. aureus is one of the most important bacterial opportunistic pathogens which cause infections in various organs. It may cause various human infections ranging from mild infections (for example furuncles and abscesses) to more severe and more invasive infections such as pneumonia, septicemia, endocarditis, and septic arthritis. Some of these infections can even be life-threatening in immunodeficient individuals. In addition, the toxins of *S. aureus* are capable of causing diseases such as food poisoning, toxic shock syndrome, or staphylococcal scalded skin syndrome. It has been estimated that about 40% of the mortality from nosocomial infections worldwide is caused by *S. aureus* (Bhunia, 2008; Cosgrove *et al*., 2003; Durai *et al*., 2010; Wertheim *et al*., 2005).

2.7.5.2. *S. aureus* infections in animals

In animals, pathogenic Staphylococcus species known to cause infections include *S. aureus*, *Staphylococcus hyicus* and the *Staphylococcus intermedius*. *S. aureus* causes mastitis in cattle and small ruminants, joint infections, osteomyelitis, dermatitis, arthritis and septicemia in poultry, mastitis, pustular and exudative dermatitis, subcutaneous abscesses, conjunctivitis, purulent rhinitis and pododermatitis in rabbits, septicemia and exudative epidermitis in pigs, dermatitis and cellulitis in horses and other animal species. *S. hyicus* attributes to exudative epidermitis, sporadic joint infections or cystitis in pigs and exudative skin infections in cattle, horses and poultry. The *S. intermedius* species group which consists of *Staphylococcus pseudointermedius, S. intermedius* and *Staphylococcus delphini* and is responsible for septicemia in ducks and pigeons and dermatitis in mink and horses. *S. pseudointermedius* is the predominant species causing skin infections in dogs and *S. delphini* causes skin diseases in horses (Bhunia, 2008; Quinn *et al*., 2004).

2.7.6. Control and prevention of S. aureus infections

β-lactam antibiotics such as Penicillin G, Methicillin, Ampicillin, Carbenicillin, Azlocillin, Cephalothin, Cephaloridine, Cefoxitin, Cefotaxime, Ceftazidime, Cefepime, Aztreonam and Imipenem, have long been used for treatments of *S. aureus* infections both inhuman and animal. Of the aforementioned drugs, methicillin has been found the most effective drug of all β-lactam antibiotics. However the pathogen has been determined that it is prone to develop resistance

easily and fast to the β-lactams. Most of the strains of *S. aureus* which developed resistance to Methicillin, are resistant to all other β-lactams (Alghamdi *et al*., 2023; Glazer & Nikaido, 2007).

Currently the macrolide–lincosamide–streptogramin (MLS) group of antibiotics especially erythromycin and its derivatives, Vancomycin, have been used extensively for the treatment of MRSA infections as drug of choice. Furthermore, because of antibiotic-resistance problem, several alternatives to antibiotics including bacteriocins and bacteriophages are recently becoming in the spotlight for the treatments of drug-esistant *S. aureus* infections. Bacteriocins, are small ribosomally synthesized antimicrobial peptide (AMP) produced by bacteria and are lethal to other bacteria as a mechanism of defense. The peptides insert into the membrane of the target bacteria, causing pores on the membrane and consequently lysis is the general mode of action (Snyder & Worobo, 2013). Bacteriophages, viruses that infect bacterial cells, may be used as therapeutic agent to treat infections caused by multi-drug resistant bacteria (Patel *et al*., 2015) and to alleviate the risk of transmitting pathogenic bacteria via food commodities (Premarathne *et al*., 2017).

Effective infection control practices include strengthening surveillance, screening of high-risk population (Islam *et al*., 2011) routine disinfection of surfaces (Oie *et al*., 2007), and wearing protective devices such as gloves, masks, and protective clothes when patients are handled. In case of animals, strict implementation of hygienic measures might be difficult on livestock farms. However, it is recommended that as hand hygiene is an essential part of any infection control program, hand-washing between each patient handling could logically decrease the chance of spread of the microbes. It should also be known that *S. aureus* is susceptible to various disinfectants including sodium hypochlorite, alcohols, benzalkonium chloride, iodophors, phenolics, chlorhexidine, glutaraldehyde, formaldehyde, and a combination of iodine and alcohol. This organism can also be destroyed by heat. However, the exotoxins responsible for food poisoning are relatively heat stable, and can persist after live *S. aureus* has been eliminated (Cancino-padilla *et al*., 2017). Furthermore, the production chain is one of the ways to transmit animal associated antibiotic resistant pathogens, improving sanitary control measures between herds and during transport is among the ways to reduce the dissemination of MRSA from farm to farm (Catry *et al*., 2010).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in 4 purposively selected districts namely Wolmera, Ambo, Toke Kutaye, and Bako Tibe of the 19 districts of the West Showa zone of Oromia region using stratified random sampling method. The total population of the zone is estimated to be 2,058,700, of whom 1,028,500 are male and 1,030,200 are female. There are about 2.3 million cattle, 1.07 million sheep, 265,000 goats, 541,000 equines, and 1.3 million chickens reared in the zone. (CSA, 2021) (**Fig. 3**). The intensive and semi-intensive dairy farms are more limited to the centers (towns) of the districts in the West Showa zone.

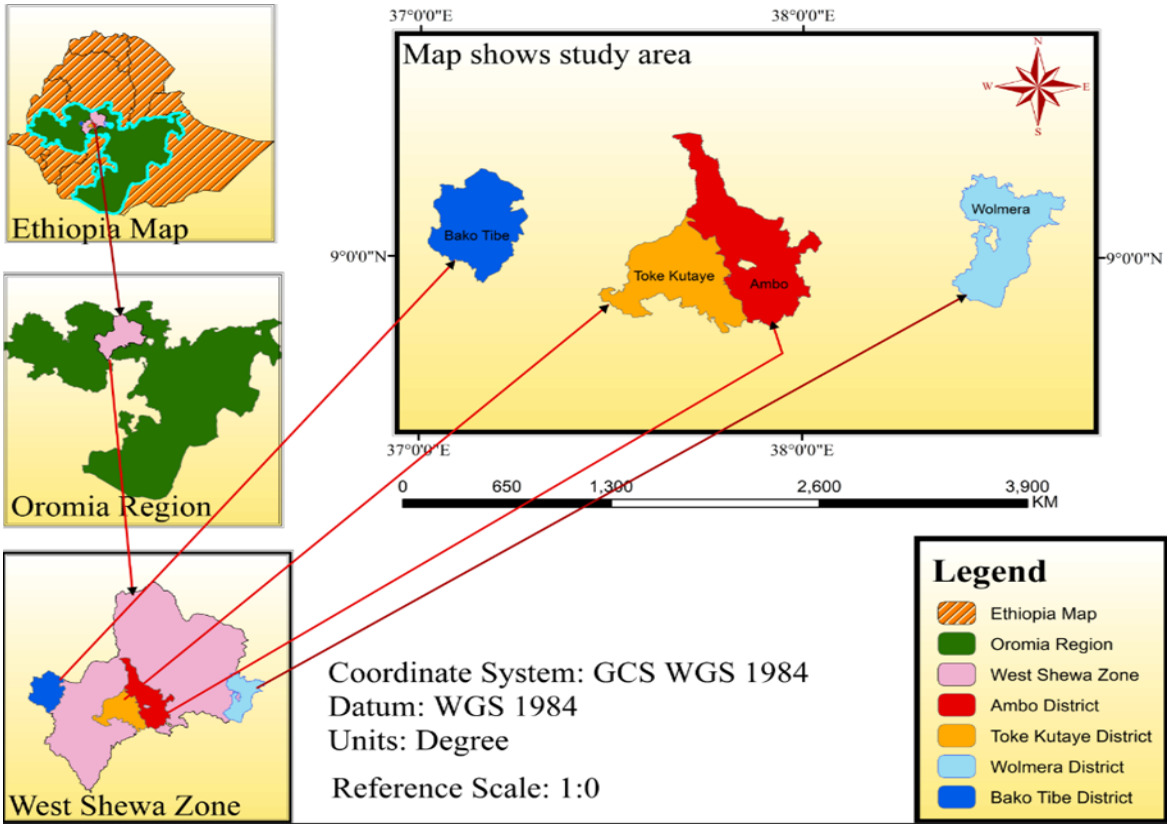

Figure 3: Map of the study area

Wolmera (center Holeta) is situated 34 kilometers west of Addis Ababa at 8°50'-9°15'N latitude and 38°25'-38°45'E longitude. The altitude of the district ranges from 2060 to 3380 meters above sea level (masl). The annual average temperature of the area is 15.76ºC, ranges from 9.13ºC to 19.66ºC, and receives about 1,100 mm of rainfall with a relative humidity of about 60%. The

cattle population of Wolmera district is estimated to be 190, 000 (S. Hailu, 2021). In Holeta town, there were 20 regestered and many smallholder dairy farms and the herd size of the farm ranged from 5 to 300 heads. The number of lactating cow ranged between 2 to 140 per farm (Borena et al., 2022)..

Ambo (center Ambo), is situated 114 kilometers west of Addis Ababa and the town serves as administrative center for the West Shewa Zone. Its coordinates are 8° 47'-9° 21'N latitude and 370 32' - 380 3' E longitude. The average annual temperature of Ambo district is 22°C, with ranges between 15°C and 29°C. The district is elevated between 1800 and 3200 masl and gets about 900 mm of rainfall annually. There were 165,000 cattle expected to be reared in Ambo district (Bayisa & Duguma, 2022). There were 8 regestered and many smallholder dary farms whose herd sizes were ranged between 3 and 65 with number of lactating cows of 2 to 32 per farm (Borena et al., 2022).

Toke Kutaye (center Gudar) is a district of the west Shewa zone, found 12 kilometers from Ambo and 123 kilometers from Addis Ababa in the western direction. The district is located at 37° 46' 27.6' E longitude and 08° 59' 01.1' N latitude. The altitude of the district ranges about 1600 to 3194 masl. The temperature of the district fluctuates from 16°C to 29°C, with an average of 22.5°C. The district receives an average annual rainfall of 900 mm. The cattle population of Toke Kutaye district was estimated to be 175, 000 animals (Dabele et al., 2021). There was one registered located at Birbirsa kebele near Guder town and many smallholder dairy farms in Guder town (Geddafa et al., 2022).

Bako Tibe (center Bako) district is situated about 260 km west of Addis Ababa. The district is situated at 9° 06' N latitude and 37° 09' E longitude in its geographical location. Its elevation ranges between 1570 and 2600 masl, and annual average temperature is 29°C, which ranges from 28°C to 31°C. The estimated annual rainfall of the district is 887 mm. The cattle population of Bako Tibe district was estimated to be 137,00 (Kebede et al., 2016). There are five registered dairy farms in Bako town. The farms consist of 7 to 315 animals per farm and the number of lactating cows ranged between 2 and 152 per farm (Borena *et al*., 2022).

3.2. Study design

A cross-sectional study was designed to determine the prevalence rate of mastitis in lactating cows, the isolation rate, and virulence factors of *S. aureus* from lactating cows, bulk milk, yogurt, and cheese in four randomly selected districts of the Wets Showa Zone in Oromia regional states, Ethiopia. The study design was set to include different agro-ecologic areas for comparison. At the time of sample collection,the zone structurally comprised 19 Districts of which 7 (Gindaberet, Wolmara, Dire Incini, Chaliya, Ilfata, Jaldu and Jibat) were found in high land ("Bada"), 8 (Abuna Gindaberet, Meta Robi, Adea Barga, Ejere, Dandi, Ambo, Tokkee Kutaye, and Midakegn) were in mid highland ("Bada Dare") and the rest 4 (Nono,Dano, Bako Tibe, and Ilu Galan) were found in low land agro-ecologic areas. From these agro-ecologic categories of the districts, four districts were selected purposevly based on the craterion including accessibility to vehcle, proximity to the laboratory and relatively secured. Accordingly, Wolmera district was selected as the representative for the highland; Ambo and Toke Kutaye were for mid highland; and Bako Tibe was for low land areas (Duguma & Janssens, 2021). The research was conducted from May 2020 to March 2021.

3.3. Study population

The sources of data for this study included lactating cows, dairy products (bulk milk, yogurt and cheese), and farm owners and/or farm managers and milk and milk product tailers (at market levels and restaurants). Data of investigating mastitis were obtained by physical diagnosis (observation, pulpation) and using Calfornia mastitis test. The data generated from isolation and characterization of *S. aureus* was obtained by conducting laboratory experiments on collected samples of dairy products and recording results. The assessment of risk factors associated with the prevalence of mastitis, and bacterial contamination of milk and milk products were collected from the farms, milk and milk product tailers using a structured questionnaire.

The number of lactated cows on the farms was classified as small (1–5 heads), medium (6–10 heads), and large (>10 heads). The lactation stages of the animals were categorized in such a way that 0–3 months of "early," 3-6 months of "mid," and >6 months of "late" lactation. Age-wise, the cows were categorized as young (≤4 years), medium (4-6 years), and adult (> 7 years). The

cows were also grouped according to their parity levels as few (1-2 calving), medium (3-5 calving), and many (≥6 calving) (Dabele *et al*., 2021).

The bulk milk, yogurt, and cheese were collected from the administrative-center towns of the selected districts, namely Holeta, Ambo, Guder, and Bako. Milk and yogurt samples were collected at the household level, whereas cheese samples were collected at the local market level, except for Bako town, where the samples were collected only from the households.

These towns were selected based on the fact that raw milk and traditionally processed milk products are sold in local markets. Particularly, cheese has been prepared by the local farmers and supplied for sale informally to the local market. Even though yogurt is not prepared and supplied to local markets, restaurants, or hotels, the raw milk supplied by the farmers was fermented and commonly sold in restaurants without the application of further decontamination techniques. Furthermore, the towns vary agro-ecologically from one another (Duguma & Janssens, 2021).

3.4. Sample size determination

In the study of the prevalence of mastitis in lactating cows and *S. aureus* in lactating cows, bulk milk and milk products, the sample sizes were calculated based on the recommended formula given by Thrusfield (2007).

$$n = \frac{Z^2 p(1-P)}{d^2}$$

For prevalence of mastitis, n- is sample size, Z- is the standard normal distribution at 95% confidence interval = 1.96, p-is the expected prevalence from a previous study = 70.6%=0.706 and d-is the absolute desired precision at 95% confidence which is 0.05. The previous studies conducted in the study area have reported a 70.6% prevalence of mastitis in cows (Emeru *et al*., 2019). However, since there have not been reports on the prevalence of *S. aureus* in bulk milk and milk products, the expected prevalence of the bacterium was taken to be 50%. As a result, at a 95% level of confidence (CL) and a 5% desired level of precision, the minimum sample size for the study of prevalence of mastitis was conducted as follows. The sample size for the prevalence of mastitis in lactating cows was calculated as $n = \frac{1.96^2 * 0.71(1-0.71)}{0.05^2}$ =318.

For the prevalence of *S. aureus* in milk and milk products, the sample size was calculated as $n=\frac{1.96^2(0.5-0.5)^2}{0.05^2}=384$

Therefore, a total of 318 cows were expected to be tested for mastitis and sampled for bacterial isolation. According to the cattle population of the districts, it was planned to examine 90 cows in Wolmera, 80 cows in Ambo, 83 cows in Toke Kutaye, and 65 cows in Bako Tibe. However, only 258 animals were checked for mastitis. In regard to bulk milk and milk products (yogurt and cheese), 354 accepteable samples were processed for bacterial isolation and identification in the laboratory. Eventhough, 414 bulk milk and milk product samples were collected, 60 samples were rejected for different reasons among which leakage of the sample was the major onces. However, since there was no statistically published information about the number of lactating cows in the zone in general and in Walmera, Ambo, Toke Kutaye and Bako Tibe in particular, it was difficult to distribute the sample size to the ditricts according to the number of the lactating cows in the districts.

For the questionnaire survey, however, since the owners of the sampled cows and dairy products were expected to be interviewed, the research did not use any formula for the calculation of the sample size. Rather the researcher subjectively interviewed 35 respondents per distrct. Therefore, a total of 140 respondents were interviewed to assess the milk collection and storage practices of the study area.

3.5. Sampling methods

The districts were randomly selected from agro-ecologically stratified districts. The peasant associations (PAs) of the selected districts and the farmers who had lactating cows at the time of sampling were also randomly selected using a simple random sampling method. However, since the advanced (semi-intensive and intensive) dairy farms were few in number in selected PAs, all of them were purposively selected for the study.

The clinical-mastitis-positive cows were sampled purposively. However, the lactating cows, which have not shown noticeable signs of clinical mastitis, were selected randomly to check subclinical mastitis using the California mastitis test (CMT).

The bulk milk samples at the household level were randomly selected. Similarly, the cheese samples at the household and market levels were selected randomly. Cheeses, which were sampled from market were collected on the market day when the owners presented the product to the market. In the case of yogurt samples, some were collected with bulk milk samples if available. The remaining yogurt samples were collected from the restaurants that deliver yogurt to customers after buying bulk milk from the locals in the selected towns.

3.6. Sample collection

A physical diagnosis of clinical mastitis was made based on the clinical signs of mastitis at the herd level. The clinical mastitis was diagnosed by determining the change in color and consistency of milk and the cardinal signs of udder inflammation, including redness, pain, swelling, and heat in the area. The diagnosis of clinical mastitis was performed by physical observation and/or palpation.

The clinical-mastitis-negative lactating cows were tested for subclinical mastitis by using the California mastitis test (CMT) following the procedure for CMT stated by Quinn *et al.* (2004). To put it briefly, a milk sample from each udder quadrant was squirted into each cup of the CMT paddle, and a similar amount of 3 percent CMT reagent was added to the tested milk sample and gently agitated by moving the puddle. The kind of coagulation and viscosity of the mixture (milk and CMT reagent), which indicate the presence of the infection, were recorded as positive for subclinical mastitis. The milk samples were collected from all functional quarters of a cow in a single test tube as the pooled sample for bacterial identification.

Milk sample collection and transportation were according to the guide line stated by Quinn *et al.* (2004). Milk samples from lactating cows were collected in such a way that bacterial contamination from the milker’s hands, the environment of the barn, and the skin of the udder and teats was avoided by following strict aseptic procedures. Before sampling, teat ends were quickly washed and sterilized with 70% ethanol, and the foremilk (initial jets) was expelled. The sample collector's hands were protected with gloves and were then disinfected with 70% ethyl alcohol. A milk sample of 3 to 5 mL was drawn into a sterile universal bottle. The bottle was then labeled with the animal ID and the date of collection

About 25 mL milk sample was collected from the bulk milk container after agitation to homogenize the content. The sample was taken from the top of the bulk tank using a sanitized dipper and added into a sterile screw-caped milk collection vial. In the case of yogurt samples, the owners were asked to add about 25 mL to sterile screw-caped wide-mouthed sample collecting containers directly from the container. In the case of cheese sample collection, approximately 25 g of cheese samples were taken with sterile spoons and separately added to screw-capped wide-mouthed tubes. The containers were labeled with sample ID, date of collection and sample types (Lemma *et al*., 2021; Oliveira *et al*., 2020)

The samples were then transported under cold chain in ice box to the Zoonosis and Food Safety Research Laboratory of Ambo University. At the arrival of the laboratory, the samples were either processed as soon as possible or stored at +4 °C for a maximum of 12 hours until processed.

3.7. Bacterial isolation and identification

3.7.1. Isolattion and primary identification methods of S. aureus

Brain Heart Infusion (BHI) broth (Accumix, Vena, India) was used to enrich the samples. For samples collected directly from lactating cows, the milk sample was mixed with about 5 mL of sterile BHI broth (Thaker et al., 2013). In the cases of bulk milk, and yoghurt, 100 mL of BHI broth was added directly to 25 mL of milk or yogurt samples. The cheese samples were enriched in such a way that the sample (about 25g) was first suspended in 100 mL sterile saline water (0.86% m/v NaCl). About 5 mL of the suspension of the cheese sample was mixed with 5 mL of the BHI broth (Lemma *et al*., 2021).

The enriched samples were incubated at 37°C for 24 h (4% CO_2). A loop full of the culture was streaked on a Mannitol Salt Agar (MSA) (Accumix, Vena, India) plate and incubated at 37°C for 24 hours (4% CO_2). The growth of the bacterium on MSA was checked, and repeated subculturings would be performed in the cases of mixed and undispersed colonies. *S. aureus* was suspected if the bacteria could resist the high salt content (7.5%) of MSA to grow and ferment mannitol rapidly within 24 hours. The mannitol fermentation was determined by observing the yellowish-colored colony on MSA. (Sharp & Searcy, 2006). The typical presumptive colonies

were transferred to BHI agar plates and incubated for 24 hours at 37 °C (4% CO_2), and the purity of the colony was visually checked for uniformity in color and form. The isolates on the BHI agar plates were then tested by Gram staining for cell morphology and arrangements (Quinn *et al*., 2004).

The presumed isolates were further tested using primary biochemical tests, including catalase and coagulase tests. The catalase test was performed by mixing a loop full (using a plastic loop) of the overnight cultured bacteria on a nutrient agar plate with two drops of 3% H_2O_2 (Sheba Pharmaceuticals PLC). The immediate production of babble was considered catalase-positive. The tube coagulase test method was used to test the coagulase enzyme production of the isolates. It was carried out in such a way that 0.5 mL of a 24-hour culture in BHI broth was mixed with an equal amount of rabbit plasma (obtained from the National Veterinary Institute [NVI], Bishoftu, Ethiopia). The mixture was incubated at 37°C and checked for the presence of clots at every 30 minutes' interval for 4 h. If no clotting was observed within 4 h, the sample would wait for 24 h. The presence of the clot was considered coagulase-positive, and the isolates with no clot within 24 hours were recorded as coagulase-negative (Quinn *et al*., 2004),

3.7.2. S. aureus identification using MALDI-TOF

The coagulase-positive isolates were further confirmed as *S. aureus* by the Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) biotyper mass spectrometry Identification Standard method (Singhal *et al*., 2015) at the National Animal Health Diagnostic and Investigation Center (NAHDIC), Sabata, Ethiopia. The extended direct transfer (EDT) method was used (Chên *et al*., 2018). Briefly, a single colony of the 24-hour BHI agar culture was picked up by wooden application sticks and smeared as a thin film directly onto a spot on a MALDI target plate. The smeared material was overlaid with 70% formic acid and allowed to dry at room temperature. The material was then overlaid with Matrix (α-cyano-4-hydroxycinnamic acid) and allowed to dry at room temperature. *E. coli* ATCC 8739 was used as a quality control for the reading of the machine. The plate was then inserted into the MLD-ToF machine and run, and the result was displayed on the computer connected to the machine.

3.8. **Virulence Factors of *S. aureus***

3.8.1. Antibiotic resistance test

3.8.1.1. Disc diffusion tests

The Kirby-Bauer disk diffusion antibiotic sensitivity test (Khalili *et al*., 2012) was carried out in the National Animal Health Diagnostic and Investigation Center (NAHDIC) laboratory. The test was performed to check the susceptibility of the isolates to 10 antibiotics, including ciprofloxacin [5 μg], penicillin G [10 units], trimethoprim/sulfamethoxazole [1.25/23.75 μg], cefoxitin [30 μg], oxytetracycline [30 μg], gentamycin [10 μg], erythromycin [15 μg], tetracyclin [30 μg], clindamycin [2 μg] and novobiocin [5 μg]. The antibiotics were selected based on their availability, and their usage in veterinary and public health in the study area.

The disc diffusion method was conducted according to CLSI, (2020). Briefly, the *S. aureus* isolates were first cultured on BHI agar plates, at 37 °C for 24 hrs and were checked for purity based on the colony characterization followed by Gram stain method. Two to three colonies of the fresh (24 hrs) culture were mixed with 5 mL of sterile normal saline (0.85% NaCl). The concentration of the bacterium was measured at 0.5 McFarland by using McFarland Densitometer (Grant bio DEN-1, biosan Medical-Biological Research and Technology, Germany). The swab was dipped into the suspension of the test organism and was streaked evenly on a Muller-Hinton agar plate of 90 mm diameter. The antibiotic discs were then placed on the surface of the plate using the disk dispenser. The discs were gently pressed by sterile thumb forceps taking care not to touch the surface of the plate. The plates were incubated at 37 °C. The diameter of the inhibition zones of the antibiotics was measured in centimeters using a caliper after 18 hrs of incubation at 37 °C. The width of the inhibition zone was then compared with the standards published by the Clinical and Laboratory Standard Institute (CLSI, 2020).

3.8.1.2. Mannitol salt agar-cloxacillin test for screening MRSA strain

The *S. aureus* isolates were screened for methicillin resistance by using a selective medium, which was cloxacillin-supplemented mannitol salt agar. The technique was carried out according to the method obtained from Mir *et al.*,(1998), with slight modifications. Briefly, the mannitol salt agar was supplemented with cloxacillin (**Annex 6**) at a rate of 6 μg/mL (GMP Medicine,

China). The cloxacillin suspension was prepared in such a way that 500 mg of cloxacillin, which was obtained from a local drug store, was dissolved in 10 mL of sterile distilled water to obtain a stock solution of 50 mg/mL (50 µg/µL).

The disk diffusion antibiotic sensitivity test using cefoxitin [30 µg] on Muller Hinton agar, and the *mecA* gene test using PCR were carried out, as per CLSI, (2020) on the MRSA suspected isolates to confirm the isolates MRSA. In addition, the antibiotic susceptibility of the MRSA isolates were, in turn, tested for their resistance pattern against ciprofloxacin [5µg], penicillin G [10 units], Trimethoprim/Sulfamethoxazole [1.25/23.75µg], Oxytetracycline [30µg], Gentamycin [10 µg], Erythromycin [15 µg], Tetracyclin [30 µg], Clindamycin [2 µg] and Novobiocin [5µg]. The disc diffusion test method on Muler Hinton agar was used for the susceptibility test (Khalili *et al*., 2012).

3.8.2. Antibiotic resistance and enterotoxin genes

3.8.2.1. Bacterial DNA extraction

DNA extraction was performed using the Qiagen DNeasy extraction kit for bacterial culture following the manufacturer's protocol (Thermo Scientific, Germany). Two loops full of 24-hrs-grown *S. aureus* isolates on BHI agar were suspended in 600 µL of sterile phosphate buffered saline (PBS) buffer. The suspension was centrifuged at 20,000 x g for 5 min. The supernatant was discarded, and the pellet was resuspended in 180 µL of enzymatic lysis buffer. After vigorous vortexing for 10–20 sec, the mixture was incubated at 37 °C for 30 min in the water bath. The suspension was then mixed with 25 µL of proteinase K and 200 µL of AL buffer, briefly vortexed, and was incubated at 56 °C for 30 min in the water bath. Finally, 200 µL of 100% ethanol was added and vortexed.

The entire contents of the extracted sample were transferred to the spin column tube and centrifuged at 10,000 x g for 1 minute at room temperature. The content of the column was transferred to a new collection tube, suspended in 500 µL AW1 buffer, and centrifuged at 10,000 x g for 1 minute at room temperature. The column was placed in another collection tube, and 500 µL of AW2 buffer was then added and centrifuged at 20,000 x g for 3 min at room temperature. The collection tube was carefully removed from the centrifuge, the supernatant was discarded,

and the column was transferred to a 1.5-mL tube. Two hundred µL of AE buffer was added, and stood at room temperature for 1 minute. Finally, the column was centrifuged at 10,000 x g for 1 minute. The pure DNA was then collected, quantified using Nanodrop 1000 spectrophotometry instrument (Thermo Scientific, Loughborough, UK), and stored at -20 ºC until used for further analysis (Meshref *et al*., 2019).

3.8.2.2. Gene amplification using PCR

Six genes (*mecA, nuc, blaZ, sea, seb,* and *tsst-1*) of *Staphylococcus aureus* isolates were subjected to PCR for amplification. Oligonucleotide primers *mecA, nuc* (Hoque *et al*., 2018), and *blaZ* (Okiki *et al*., 2020) were used to target oxacillin-resistant, thermoneuclease, and penicillinase genes, respectively; Whereas, the primers *sea, seb,* and *tst1* were to target the staphylococcal enterotoxins A, B, and toxic shock syndrome toxin 1, respectively (Cunha *et al*., 2007) (Table 2)**.**

Table 2: Specific PCR primers for the respective genes and product length

S.No	Primer name	5'-3'	Product Length	References
1	*mecA*-F	GTAGAAATGACTGAACGTCCGATGA	163 bp	(Hoque *et al*., 2018)
	mecA-R	CCAATTCCACATTGTTTCGGTCTAA		
2	*nuc*-F	GCGATTGATGGTGATACGGTT	279 bp	
	nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
3	*blaZ* -F	TACAACTGTAATATCGGAGGG	846 bp	(Okiki *et al*., 2020)
	blaZ-R	CATTACACTCTTGGCGGTTTC		
4	*sea*-F	GGTTATCAATGTGCGGGTGG	120 bp	(Cunha *et al*., 2007)
	sea-R	CGGCACTTTTTTCTCTTCGG		
5	*seb*-F	GTATGGTGGTGTAACTGAGC	478 bp	
	seb-R	CCAAATAGTGACGAGTTAGG		
6	*tsst1*- F	ACCCCTGTTCCCTTATCATC	350 bp	
	tsst1- R	TTTTCAGTATTTGTAACGCC		

The primers for *mecA, nuc,* and *blaZ* were purchased from Eurofins Genomics India Pvt. Ltd.. The procedure was used for amplification of *mecA, nuc*, and *blaZ*. For each gene it used a 23 µL mixture master kit whose contents were 2 µL (50 pmol) of each primer, 1.5 µL of 2.5 mM $MgCl_2$, 5 µL of PCR buffer, 0.5 µL of dNTP, 0.2 µL of Taq polymerase enzyme, 11.8 µL of RNase-free water, and 2 µL of extracted DNA. Sterile water was used as the negative control.

The oligonucleotide primers for enterotoxin gene amplification were obtained from Integrated DNA Technologies (USA). The PCR was performed following a slight modified procedure used by Cunha *et al*. (2007). The reaction mix was prepared separately for each type of toxin gene under test. Accordingly, it used a 21-µL mixture master kit whose contents were 2 µL (20 pmol) of each primer, 1.5 µL of 2.5 mM $MgCl_2$, 5 µL of PCR buffer, 0.5 µL of dNTP (2.5U), 0.2 µL of Taq polymerase enzyme (2.5U), 11.8 µL of RNase-free water, and 2 µL of extracted DNA (~200 ng).

The amplification of the genes was carried out so that the PCR tubes containing the mixture were tapped gently and the PCR tubes with all the components were transferred to a thermal cycler (Flex cycler, Biometra Gmbh, Germany). The thermocycler was programmed as an initial denaturation at 95 °C for 5 min. After the initial denaturation, the sequential steps of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min were repeated 37 cycles with final extension at 72°C for 10 min (Salisbury *et al*., 1997).

3.8.2.3. Gel electrophoresis and visualization of the amplicons

Electrophoresing the PCR products on a 1.5% (w/v) agarose gel containing 0.5 mg/mL ethidium bromide (2 µL) was used to separate and visualize the amplicons. The ethidium bromide intercalates into the DNA to initiate the visualization of the DNA. The electrophoresis was performed by mixing an 8 µL amplification product of each sample with 2 µL of loading dye and applying it to the slots of agarose gel. The DNA marker 100 bp (Qiagen; Germany) was used to estimate the fragment length of the DNA fragments. The electrophoresis (Apelex Midigel XL, France) was then performed in 1X TBE buffer at 120 volts for one hour. The separated PCR products in electrophoresis were visualized with UV illumination (Gel DocTM XR+, BioRAD; Germany) and imaged with a gel documentation system (Adam *et al*., 2016).

3.8.3. Detection of staphylokinase (SAK) production

Heated plasma assay

The production of SaK was carried out in Satoh's medium, which contained 1% nutrient broth, 0.3% yeast extract, 0.5% sodium chloride, and 1% glycerol with a pH of 7.4 adjusted with 1 N NaOH. The seed culture was inoculated in the medium and was maintained at 37 °C for 24 h in a shaker water bath (200 rpm). After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered (Annex 4). The staphylokinase-producing potential of the isolates was determined by a heated plasma assay (Devi *et al*., 2015).

The bovine plasma was prepared by collecting blood from apparently health animal with EDTA anticoagulant. The blood was centrifuged at 2000 rpm for 10 minutes. The supernatant was collected in sterile screw capped test tubes. The plasma was stored at +4 °C until used (Srinivasan *et al*., 2013)

In the heated plasma assay method, the nutrient agar was melted on bunson burner, outoclaved at 121°C for 15 mins and was allowed to cool to 56 °C in the water bath. The bovine plasma was heated at 56 °C for 20 mins and then added to the melted nutrient agar at concentration of 30% v/v (Devi *et al*., 2015). The mixture was dispensed on the Petri dish to make agar plate.

Production of the enzymes was determined by observing the clear zone around the colony on recommended agar plates and/or the enzyme extract of bacteria in broth. In the later method, wells of about 3 mm diameter each were formed by using cork borer to cut the surface of the agar plate. Then 25 µL of the supernatant was added into the well and was incubated at 37 °C for 24 hours. Finally, the zone of hydrolysis was measured and recorded **(Annex 2)**. A clear zone around the colonies and around the crude extract of the enzyme after applying in well formed on the surface of heated plasma agar was considered positive (Srinivasan *et al*., 2013).

3.8.4. Casine hydrolysis assay for protease

Casine hydrolysis activity of protease enzyme was determined using skim milk agar according to the method stated by Devi (2015). The skim milk agar was supplemented with 1% bovine serum

and was dispensed on the Petri dish. The isolates were streaked on the plate and incubated at 37°C for 24 hrs. The zone of the hydrolysis was subjectively checked by observation (Annex 3).

3.8.5. Lipase production

The lipase production ability of the isolates was determined according to the previous research with slight modifications (El-baz *et al*., 2016). Briefly, the brain heart infusion agar was supplemented with melted local butter at rate of 1% (w/v). The medium was then sterilized in autoclave at 121 °C and 1 bar pressure for 15 minutes. The agar plate was made by pouring about 15 mL of the medium, and allowed to dry.

A single colony of each isolate of *S. aureus* was streaked on the agar plateand the plate was incubated at 37°C for 24 hrs, and checked for the clear zone of hydrolysis around the colony. Based on the presence of the zone of lipid hydrolysis, the result was then recorded qualitatively as positive or negative. The lipid hydrolysis by lipase enzyme was considered positive if it manufested clear zone of hydrolysis arround the colonies inoculated on nutrent agar supplimented by 1% butter (w/v) (Annex 5).

3.9. Questionnaire survey

A structured pretested questionnaire was used to determine the associated risk factors with bovine mastitis, and contamination of milk and milk products in the study areas. The questionnaire was pretested in other Ejere district of West Showa zone. Various farm level and animal level factors thought affecting the prevalence of mastitis in the dairy herds was assessed by interview and personal observations. Major risk factors that were considered in the current study include the districts/agro-ecology, breeds, age group, lactation stage, parity, body condition, types of farms, and management system. In addition the hygienic practices during milking such as washing hands before milking, washing udder before milking and the use of fabrics for drying the udder were also assessed.

The risk factors associated with contamination of milk and milk products, and the risk of distribution of the foodborne diseases were also assessed by the questionnaire survey. Accordingly, the practices including the hygienic practices practiced during and after milking such as cleaning, washing and smoking utinsils were asked (**Annex 1**).

3.10. Data management and analysis

Data obtained from bacteriological isolation and identification and questionnaire data were entered into an Excel Microsoft Spreadsheet and were coded. The mastitis prevalence data were transferred to SPSS version 20 and the prevalence and riskfactors of *S.aureus* were exported to R statistical software for analysis. Descriptive statistics such as frequency, percentage, and means were used to display them in tables and graphs. The risk factors associated with bovine mastitis and isolation rates of *S. aureus* were analyzed based on the significance level of the $\chi 2$ test and logistic regression. A one-way ANOVA and t-test were used to analyze the variation of the multi-antibacterial resistance index of the isolates among the study districts, sample types, and mastitis positivity of the animals (Wanja *et al*., 2020).

3.11. Ethical considerations

All the data collected from the owners was based on their consent, and the information was kept secret and confidential. The names, pictures, and addresses of the individual participants have not been displayed in the dissertation and in the publications related to the dissertation.

4. RESULTS

4.1. Bovine mastitis in lactating cows

4.1.1. Prevalence of mastitis in lactating cows

In the current study, a total of 258 lactating cows were diagnosed for mastitis. Among these, 97 (37.6%) were found to be mastitis positive, of which 38 (39.2%) and 59(60.8%) had clinical and subclinical mastitis, respectively.

The distribution of mastitis according to environmental and animal related factors is presented in **Table 3**. The highest prevalence rate of mastitis was observed in mid highland (40.62%), in the cows managed under extensive farm management (41.21%), in herds with many lactating cows (55.56%), in animals whose milkers do not wash their hands before milking (83%) and in animals with tick infestation (84%). Taking the animal related factors as determinant of the prevalence of the mastitis in the studied cows, the highest occurance rate of the disease was determined in cows with ≤4 years old (42.86%), late lactating stage (41.79%), many parity level (60%), and good body condition (50%).

Table 3: Prevalence of bovine mastitis in West Showa Zone in respect to factors

Characteristics	Category	Diagnosed	Mastitis Positive (%)	Mastitis Type	
				Clinical	Subclinical
Sampling Area	Wolmera	81	28(34.57)	12(42.86)	16(57.14)
	Ambo	77	33(42.86)	17(51.52)	16(48.48)
	Toke Kutaye	51	19(37.25)	5(26.32)	14(73.68)
	Bako Tibe	49	17(34.69)	4(23.53)	13(76.47)
Agro-ecology	Highland	81	28(34.57)	12(42.86)	16(57.14)
	Midland	128	52(40.62)	22(42.31)	30(57.69)
	Lowland	49	17(34.69)	4(23.53)	13(76.47)
Breeds	Local	115	42(36.52)	16(38.1)	26(61.9)
	Cross	143	55(38.46)	22(40.0)	33(60.0)
Farm Types	Intensive	27	4(14.81)	1(25.00)	3(75.00)
	Semi intensive	49	18(36.73)	5(27.78)	13(72.22)
	Extensive	182	75(41.21)	32(42.67)	43(57.33)
Age Group of the Animal	Adult	122	43(35.25)	15(34.88)	28(65.12)
	Intermediate	87	33(37.93)	18(54.55)	15(45.45)
	Young	49	21(42.86)	5(23.81)	16(76.19)
Lactation Stage	Early	96	28(29.17)	16(57.14)	12(42.86)
	Medium	95	41(43.16)	13(31.71)	28(68.29)
	Late	67	28(41.79)	9(32.14)	19(67.86)
Parity Level	Few	86	43(50.00)	17(39.53)	26(60.47)
	Moderate	167	51(30.54)	20(39.22)	31(60.78)
	Many	5	3(60.00)	1(33.33)	2(66.67)
Animal's body condition	Good	34	17(50.00)	7(41.18)	10(58.82)
	Moderate	177	57(32.20)	20(35.09)	37(64.91)
	Poor	47	23(48.94)	11(47.83)	12(52.17)
Number of lactating cows in the Herd	Small	224	80(35.71)	33(41.25)	47(58.75)
	Medium	16	7(43.75)	1(14.29)	6(85.71)
	Many	18	10(55.56)	4(40.00)	6(60.00)
Washing milker's hand before milking	Yes	154	18(11.69)	10(55.56)	8(44.44)
	No	104	79(75.96)	28(35.44)	51(64.56)
Udder washing before milking	Yes	158	14(8.86)	8(57.14)	6(42.86)
	No	100	83(83.00)	30(36.14)	53(63.86)
Current tick infestation of the teat	Yes	50	42(84.00)	33(78.57)	9(21.43)
	No	208	55(26.44)	5(9.09)	50(90.91)

4.1.2. Analysis of factors associated with bovine mastitis

UnivariableAs indicated in the **Table 4** blow, among the animal related factors the lactation stages and parity level of the animals showed statistically significant differences among the categories. Significant associations between the disease and risk factors such as washing of milkers' hands and udder of the animals before milking, and tick infestation were also revealed. Accordingly, mastitis was more prevalent with the advancement of the lactation stage such that as the lactation time increases in one month the cow was 1.26 times more likely to be mastitis positive than at zero level of lactation stage ($p<0.05$). In respect to the parity number, even though the animals with few parity level were more affected (OR=2.27) by the disease than the moderate levels, the advancement in the parity number did not show significant difference among the animals ($p>0.05$). The milking hygiene such as washing of milker's hand and udder of the cow before milking were also inversely associated with the prevalence of the disease in the study animals. Cows milked with milkers who did not washed their hands before milking, had about 30 times more likely to have mastitis when compared with those milked by the milkers who washed their hands before milking. Similarly, the cows whose udder were washed before milking were less affected than those milked without washing their hands (OR=69, 95% CI: 30.83- 154.18). Furthermore, cows with tick infestation on their udder were 14 times more likely to be affected by mastitis than tick free ones. However, significant difference in the distribution of the disease was not found among the categories of agro-ecology, breeds, age of the animals, and herd size ($p>0.05$).

Table 4: Logistic regression analysis of bovine mastitis against the risk factors

Characteristics	Category	Mastitis Positive (%)	Univariable COR (95% CI)	Univariable p-value	Multivariable AOR (95% CI)	p-value
Districts	Wolmera	28(34.57)	1.0			
	Ambo	33(42.86)	1.42(0.75-2.71)	0.285		
	Toke Kutaye	19(37.25)	1.12(0.54-2.33)	0.754		
	Bako Tibe	17(34.69)	1.01(0.47-2.11)	0.988		
Agro-ecology	Highland	28(34.57)	1.0			
	Midland	52(40.62)	1.30(0.73-2.32)	0.381		
	Lowland	17(34.69)	1.01(0.47-2.11)	0.988		
Breeds	Local	42(36.52)	1.0			
	Cross	55(38.46)	1.09(0.65-1.81)	0.749		
Farm Types	Intensive	4(14.81)	1.0		1.0	
	Semi intensive	18(36.73)	3.34(1.07-12.75)	0.051	2.06(0.27-18.87)	0.501
	Extensive	75(41.21)	4.03(1.48-14.17)	0.013	6.83(1.08-58.75)	0.057
Age Group of the Animal	Adult	43(35.25)	1.0			
	Intermediate	33(37.93)	1.12(0.63-1.99)	0.691		
	Young	21(42.86)	1.38(0.70-2.71)	0.353		
Lactation Stage	Early	28(29.17)	1.0		1.0	
	Medium	41(43.16)	1.84(1.02-3.38)	0.045	5.09(1.50-19.37)	0.012
	Late	28(41.79)	1.74(0.91-3.37)	0.096	6.12(1.57-27.05)	0.012
Parity Level	Medium	51(30.54)	1.0		1.0	
	Few	43(50.00)	2.27(1.33-3.90)	0.003	2.40(0.82-7.32)	0.111
	Many	3(60.00)	3.41 (0.55-26.49)	0.186	0.04(0.00-1.90)	0.139
Animal's body condition	Moderate	57(32.20)	1.0		1.0	
	Good	17(50.00)	2.11(1.00 -4.45)	0.049	0.65 (0.13-3.19)	0.595
	Poor	23(48.94)	2.02 (1.05-3.89)	0.035	0.98(0.25-3.68)	0.976
Number of lactating cows in the Herd	Small	80(35.71)	1.0			
	Medium	7(43.75)	1.40(0.48-3.90)	0.520		
	Large	10(55.56)	2.25(0.85-6.11)	0.101		
Milker's hand washing before milking	Yes	18(11.69)	1.0		1.0	
	No	79(75.96)	23.88(12.55-47.75)	0.000	16.10(5.65-51.55)	0.000
Udder washing before milking	Yes	14(8.86)	1.0		1.0	
	No	83(83.00)	50.22(24.35-111.32)	0.000	36.87(12.28-134.1)	0.000
Current tick infestation of the teat	No	55(26.44)	1.0		1.0	
	Yes	42(84.00)	14.60(6.77-35.34)	0.000	40.42(9.93-195.15)	0.000

AOR=Adjusted Odds Ratio, COR= crude Odds Ratio

The risk factors, which were found significantly associated with the prevalence of mastitis in Univariable logistic regression analysis, were farther analysed by multivariable logistic regression for their predictor strength. Of the factors significantly varied in the Univariable

logistic regression, lactating stage, washing of milker's hand and udder of the animal before milking, and tick infestation at 95% confidence, and farm type at 90% confidence were significantly associated with bovine mastitis. The result revealed that animals with above mid lactating stage, and managed under extensive farm type were significantly attacked by the disease than those with early lactating stage ($p<0.05$) and managed in intensive farm type ($p<0.1$), respectively. In addition, the disease was significantly prevalent in cows milked without washing milker's hand and udder of the animal when compared with the animals milked by milker's hand and udder of the animals were unwashed before milking. Furthermore, tick infested animals contract mastitis more frequently than tick free cows ($p<0.05$).

The model of fitness determined by the Fisher's exact test revealed that subclinical mastitis was significantly higher ($p<0.05$) in the lactating cows than clinical mastitis. As revealed in **Table 5**, the odd ratio of the clinical mastitis to subclinical did not significantly differ among the districts, animal-related factors such as breeds and body condition, and hygienic practices including washing of milker's hands and udder before milking. The ratio also does not vary with number of lactated cows, and previous tick infestation ($p>0.05$). However, the subclinical mastitis was significantly higher in animals with a mid-lactating stage, medium parity level, and negative for *S. aureus* when compared with early lactating stage, few levels of parity, and positive for *S. aureus* respectively. However, clinical mastitis was found significantly more prevalent in cows with current tick infestation on their teats than those free of the parasite. The multivariable logistic regression analysis of the variables significantly associated with the variation of the types of mastitis showed that current tick infestation of the teat was the strongest predictor for the prevalence of the clinical mastitis($p<0.05$).

Table 5: Logistic regression analysis of the comparison between clinical and sub-clinical mastitis in respect to the risk factor

Characteristics	Category	Types of Mastitis		Univariable		Multivariable	
		Clinical (%)	Subclinical (%)	COR(95% CI)	*p-value*	AOR(95% CI)	*p-value*
Districts	Ambo	17(51.52)	16(48.48)	1.0			
	Wolmara	12(42.86)	16(57.14)	1.42(0.52-3.96)	0.500		
	Toke Kutaye	5(26.32)	14(73.68)	2.97(0.91-10.98)	0.082		
	Bako Tibe	4(23.53)	13(76.47)	3.45(0.99-14.33)	0.064		
Agro-ecology	Highland	12(42.86)	16(57.14)	1			
	Midland	22(42.31)	30(57.69)	1.02(0.40-2.59)	0.195		
	Lowland	4(23.53)	13(76.47)	2.44(0.67-10.39)	0.962		
Breeds	Cross	22(40.0)	33(60.0)	1.0			
	Local	16(38.1)	26(61.9)	1.08(0.48-2.49)	0.849		
Farm Types	Extensive	32(42.67)	43(57.33)	1.0			
	Semi intensive	5(27.78)	13(72.22)	1.93(0.66-6.53)	0.252		
	Intensive	1(25.00)	3(75.00)	2.23(0.27-46.28)	0.495		
Age Group	Intermediate	18(54.55)	15(45.45)	1.0		1.0	
	Adult	15(34.88)	28(65.12)	2.24(0.89-5.77)	0.089	2.18(0.53-9.80)	0.282
	Young	5(23.81)	16(76.19)	3.84(1.19-14.05)	0.030	7.01 (1.32-45.76)	0.029
Lactation Stage	Early	16(57.14)	12(42.86)	1.0		1.0	
	Medium	13(31.71)	28(68.29)	2.87(1.07-7.97)	0.038	0.96 (0.19-4.34)	0.956
	Late	9(32.14)	19(67.86)	2.81(0.96-8.66)	0.063	2.03(0.41-10.39)	0.383
Parity Level	Few	17(39.53)	26(60.47)	1.0			
	Moderate	20(39.22)	31(60.78)	1.01(0.44-2.33)	0.975		
	Many	1(33.33)	2(66.67)	1.31(0.12-29.44)	0.832		
Body condition	Poor	11(47.83)	12(52.17)	1.0			
	Good	7(41.18)	10(58.82)	1.31(0.37-4.77)	0.676		
	Moderate	20(35.09)	37(64.91)	1.70(0.63-4.57)	0.292		
Lactating cows in the Herd	Few	33(41.25)	47(58.75)	1.0			
	Medium	1(14.29)	6(85.71)	4.21(0.68-81.54)	0.193		
	Many	4(40.00)	6(60.00)	1.05(0.28-4.39)	0.940		
Hand washing before milking	Yes	10(55.56)	8(44.44)	1.0			
	No	28(35.44)	51(64.56)	2.28 (0.81-6.61)	0.120		
Udder washing before milking	Yes	8(57.14)	6(42.86)	1.0			
	No	30(36.14)	53(63.86)	2.36(0.75-7.78)	0.140		
Tick infestation	Yes	33(78.57)	9(21.43)	1.0		1.0	
	No	5(9.09)	50(90.91)	36.67(12.21-132.41)	0.000	67.9(15.5-444.2)	0.001
S. aureus	Positive	19(55.88)	15(44.12)	1.0		1.0	
	Negative	19(30.16)	44(69.84)	2.93 (1.25-7.08)	0.015	0.55 (0.10-2.31)	0.445

AOR=Adjusted Odds Ratio, COR= crude Odds Ratio

Among the milking practices washing of the hands of the milkers and udder of the cows before milking were determined inversely associated with the percentage of mastitic cows in the herd. As illustrated in **Table 6**, the percentage of mastitic cow significantly increased in association with being milked by milkers who have not washed their hands and udder before milking. In addition, the percentage of the mastitis infected cows was significantly less in the herd milked with washing hands of the milkers in interval of milking each animal than in the herd where it was not practiced ($p<0.05$). However, which were worn only during milking, and using pieces of fabrics for drying the wet udder were not found associated with the percentage of the disease in the herd.

Table 6: Association of summarized mean of the mastitis prevalence with the risk factors

Factors	Responses	Means of Mastitis percentage	t	df	*p-value*
Hand washing before milking	Yes	14.92%	6.390	138	0.001
	No	54.74%			
Washing the udder before milking	Yes	31.21%	2.933	138	0.004
	No	52.68%			
Washing hands with interval of milking each cow	Yes	18.05%	2.438	138	0.016
	No	41.47%			
Using cloth/fabrics to dry the udder	Yes	40.72%	-0.303	138	0.762
	No	37.55%			
Using the towel for more than a cow	Yes	36.67%	1.079	16	0.297
	No	61.00%			

However, there was great variability considered in the distribution of the prevalence of the disease in herds where hand washing before milking was practiced. That is although hand washing has been practiced, there were cases where high percentage of mastitis was determined. The percentage of the mastitic cases in the herd lied in the ranges of the confidence interval (CI) when the udder washing before milking was taken as explanatory variable (**Fig, 4A and B**).

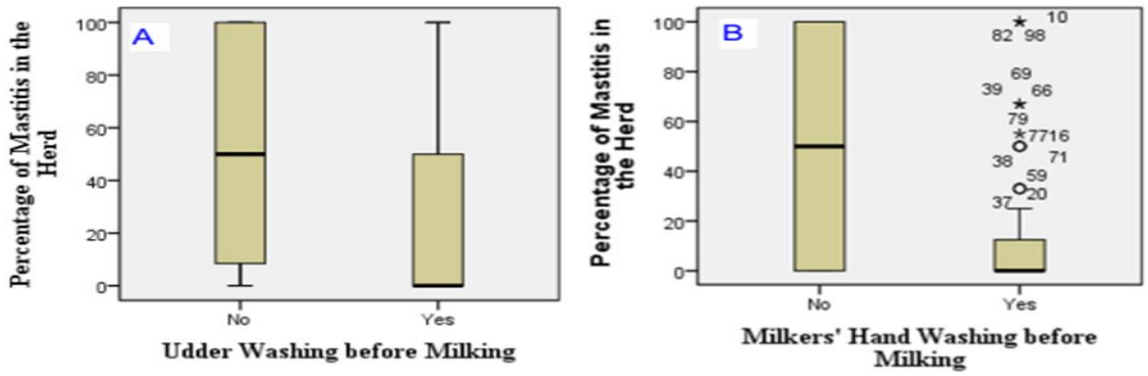

Figure 4: Comparison of variability of average mastitis prevalence between (A) udder washing and (B) Milkers' hand washing

4.2. Isolation of *S. aureus* from lactating cows

S. aureus was isolated from 43 (16.6%) of the 258 lactating cows (**Table 7**). The Univariable logistic regression analysis at 95% CI revealed that the rate varied with the sampling area, parity level and body condition of the animals, milker's hands and udder washing before milking, the number of lactating cow in the herd, and the presence of mastitis in the animal. However, in the multivariable logistic regression analysis only the number of lactating cows in the herd and the current tick infestation of the teat were significantly associated with the isolation rate of *S. aureus*. Accordingly, when the number of lactating cows was increased, the probability of the cows to have *S. aureus* significantly was increased. The result of the analysis revealed that the *S. aureus* positivity of the cows in the large and medium herd size were about 13 and 10 times more likely than those from small herd size, respectively. Similarly, the animals with tick infestation on their teats were found significantly more infected (OR= 27.69, 95% CI= 9.71-93.01) with *S. aureus* than those free of ticks ($p<0.05$).

Table 7: Prevalence of *S. aureus* in lactating cow and associated risk factors

Characteristics	Category	Samples	*S. aureus* Positive Samples (%)	Univariable		Multivariable	
				COR(95% CI)	*p-value*	AOR(95% CI)	*p-value*
Districts	Toke Kutaye	51	4(7.84)	1.0		1.0	
	Wolmara	77	18(22.22)	3.36(1.16-12.20)	0.037	3.85(0.92-19.96)	0.081
	Ambo	81	16(20.78)	3.08 (1.05-11.30)	0.057	3.20(0.66-18.40)	0.164
	Bako Tibe	49	5(10.20)	1.34(0.33 -5.70)	0.681	0.92(0.14-6.10)	0.931
Agro-ecology	Lowland	49	5(10.20)	1.0			
	Midland	128	20(15.62)	1.63(0.61-5.14)	0.358		
	Highland	81	18(22.22)	2.51(0.92-8.07)	0.089		
Breeds	Cross	115	21 (14.69)	1.0			
	Local	143	22 (19.13)	1.37(0.71-2.66)	0.342		
Farm Types	Intensive	27	1(3.70)	1.0		1.0	
	Semi intensive	49	36(12.24)	3.63(0.58-70.5)	0.245	0.89(0.08-23.35)	0.930
	Extensive	182	36(19.78)	6.41(1.29-116.31)	0.073	1.77(0.22-40.61)	0.642
Age Group of the Animal	Adult	87	17 (13.93)	1.0			
	Intermediate	122	16 (18.39)	1.39(0.66-2.95)	0.385		
	Young	49	10 (20.41)	1.58(0.65-3.71)	0.297		
Lactation Stage	Late	67	8 (11.94)	1.0			
	Medium	95	17 (17.89)	1.61(0.67-4.17)	0.304		
	Early	96	18 (18.75)	1.70 (0.71 -4.39)	0.246		
Parity Level	Moderate	167	24 (14.37)	1.0			
	Few	86	18(20.93)	1.58(0.79-3.09)	0.186		
	Many	5	1(20.00)	1.49(0.07-10.61)	0.727		
Animal's body condition	Moderate	47	22 (12.43)	1.0		1.0	
	Poor	34	11 (23.40)	2.15(0.93-4.77)	0.063	1.20(0.33-4.24)	0.778
	Good	177	10 (29.41)	2.94(1.20- 6.86)	0.014	2.67(0.69-10.41)	0.152
Number of lactating cows in the Herd	Few	224	33 (14.73)	1.0		1.0	
	Medium	16	4 (25.00)	1.93(0.52- 5.93)	0.279	9.99(1.70-57.56)	0.009
	Large	18	6 (33.33)	2.89(0.95- 8.02)	0.047	12.58(2.33-68.54)	0.003
Washing milker's hand before milking	Yes	154	16 (10.39)	1.0		1.0	
	No	104	27 (25.96)	3.02(1.55-6.07)	0.001	0.46(0.11-1.69)	0.262
Udder washing before milking	Yes	158	12 (7.59)	1.0		1.0	
	No	100	31 (31.00)	5.47 (2.71-11.68)	0.000	2.12(0.50-9.04)	0.304
Current tick infestation of the teat	No	208	11 (5.29)	1.0		1.0	
	Yes	50	32 (64.00)	31.84(14.22-76.68)	0.000	27.69(9.71-93.01)	0.000
Mastitis	Negative	161	9 (5.59)	1.0		1.0	
	Positive	97	34 (35.05)	9.11(4.29-21.24)	0.000	2.34(0.53-10.86)	0.263

AOR=Adjusted Odds Ratio, CI=confidence interval, COR= crude Odds Ratio

4.3. *S. aureus* in milk and milk products (yogurt and cheese)

4.3.1. Prevalence of S. aureus in milk and milk products (yogurt and cheese)

A total of 354 milk and milk product (cheese and yogurt) samples were collected for isolation and identification of *S. aureus*. The bacteriological tests revealed that 51 (14.4%) of the samples harbored *S. aureus*. The prevalence according to sample type was as displayed in **Table 8**. Significantly higher prevalence rate of the bacterium was determined in whole milk (20.3%) followed by in cheese (14.9%) than in yogurt samples (4.8%) ($p<0.05$).

Table 8: Univariable logistic regression analysis of *S. aureus* prevalence in bulk milk and milk products

Types of Sample	Tested	Positive (%)	Pearson $\chi2$		Bin. Log.	
			$\chi2$	p-value	COR (95% CI)	p-value
Bulk Milk	123	25 (20.3)	9.706	0.008	5.04 (1.683-15.080)	0.004
Cheese	148	22 (14.9)			3.45 (1.146-10.379)	0.028
Yogurt	83	4 (4.8)			Reference	
Total	354	51(14.4)				

COR= Crude Odds Ratio (95% CI given in parenthesis); Bin Log= binary logistic regression.

4.3.2. Assessment of risk factors associated with prevalence of S. aureus in milk and milk products

The types of the sample, sampling area (towns), and sources of the samples were recorded during sampling. The Chi-square analysis of the isolation rates of *S. aureus* in respect to the factors expected to be associated with the isolation rate was as depicted in **Table 9**. Regardless of the sample types, the rate did not differ with the sampling area. However, the isolation rate significantly varied with source of the samples. Accordingly, the bacterium was not found in milk samples collected from market or restaurants. Nevertheless, it was isolated with higher rate in milk sample collected from large dairy farms followed by small holders. In comparison of the isolation rate in milk samples obtained from large dairy farms and smallholders, it was about 4 times more frequent in dairy farm samples than smallholders'($p<0.05$).

In the cases of cheese and yogurt samples, *S. aureus* was not isolated from the samples whose sources were smallholder and large dairy farms. However, it was isolated in the samples of markets or restaurants with rates of 22.2% and 13.3% in cheese and yogurt respectively.

Table 9: Prevalence of *S. aureus* in milk and milk products in respect to districts

Sample Type	Factors	Samples	Positive (%)	$\chi 2$	p-value
	Towns				
Milk	Holata	23	7 (30.4)	3.058	0.409
	Ambo	23	6 (26.1)		
	Gudar	54	8 (14.8)		
	Bako	23	4 (17.4)		
Cheese	Holata	51	13(25.5)	7.643	0.057
	Ambo	44	5 (11.4)		
	Gudar	45	4 (8.9)		
	Bako	8	0(0.0)		
Yogurt	Holata	14	0 (0.0)	2.830	0.502
	Ambo	31	3 (9.7)		
	Gudar	31	1 (3.2)		
	Bako	7	0 (0.0)		
	Source of Samples				
Milk	Small Holder	102	18 (17.6)	8.409	0.024
	Market	6	0 (0.0)		
	Dairy Farm	15	7 (46.7)		
Cheese	Small Holder	44	0 (0.0)	12.790	0.003
	Market	99	22 (22.2)		
	Dairy Farm	5	0 (0.0)		
Yogurt	Small Holder	49	0 (0.0)	8.265	0.022
	Market	30	4(13.3)		
	Dairy Farm	4	0 (0.0)		

4.4. Factors associated with contamination of milk and milk products

4.4.1. Demographic characteristics of the respondents

Equal number of respondents (35 individuals per district) from each of the four districts was selected. As the result a total of 140 respondents were asked to complete the structured questionnaire. The respondents were either the owner or the manager of the dairy farm. The demographic characteristics of the respondents are presented in**Table 10**.

The majority of the respondents were female (88.6%) and aged between 20 and 40 years (67.9%). Regarding the education level of the respondents, 85.7% learned to the maximum of Ethiopian elementary school level (grade 1-6) and only 6.4% learnt to college dipiloma and

above. In addition most of the respondents have additional sources of income other than the dairy farms, only 17% responded that they relied on the farm only as source of income.

Table 10: Demographic characteristics of the respondents

Characteristics	Category	Frequency	Percentage
Sex	Male	16	11.4
	Female	124	88.6
Age	20-40 Years	95	67.9
	40-60 years	36	25.7
	≥60 years	9	6.4
Education	Illiterate or informal education	37	26.4
	Elementary	83	59.3
	Secondary School	11	7.9
	College Diploma or above	9	6.4
Address	Wolmera	35	25.0
	Ambo	35	25.0
	Toke Kutaye	35	25.0
	Bako Tibe	35	25.0
Major source of income	Dairy farm	24	17.1
	Other	116	82.9

As summarized in Table 11, sex and education level of the respondents were found to determine the prevalence of *S. aureus* in the dairy products. The result of the study revealed that the products handled by female were found significantly contaminated than those handled by males. In addition, the dairy products handled by indviduals whose levels of education were to the maximum of elementary were more contaminated by the bacteria than those handled by individuals with education level of secondary school and above.

Table 11: The association between demographic Characteristics of the respondents and *S. aureus* contamination of milk and milk products

Demographic Characteristics of the respondents	Categories	*S. aureus* Positive (%)	Pearson $\chi 2$	df	p-value
Sex	Male	6(37.5)	4.752	1	0.041
	Female	19 (15.3)			
Age in years	20-40	15(15.8)	0.861	2	0.651
	40-60	8(22.2)			
	≥60	2 (22.2)			
Education	Illiterate and Informal education	12(32.4)	8.260	3	0.038
	Elementary	9(10.8)			
	Secondary level school	2 (18.2)			
	College Diploma and above	2 (22.2)			
Major means of income	Dairy Farm	1(4.2)	3.701	1	0.076
	Others	24(20.7)			

4.4.2. Milking practices

As illustrated in **Table 12**, none of the farms in the study area were using milking machine during the study. The nonparametric analysis of $\chi 2$ test revealed that significantly higher number of the respondents has been washing the udder of the cows before milking. However, majority of them had no separate clothes worn during milking time, and had not used fabrics to dry the udder ($p<0.05$). However, most of them washed the drying fabrics twice daily, and did not use for more than one cow per milking time ($p<0.05$).

Table 12: Respondents responses to the milking practice questions

Factors	Response	N (%)	χ2	p-value
Milking Methods	Hand	140(100)	-	-
	Machine	0(0.00)		
Milker wash hands before milking	Yes	59(42.1)	3.457	0.063
	No	81(57.9)		
Milker wash udder before milking	Yes	96(68.6)	19.314	0.001
	No	44(31.4)		
Milker wash hands with interval of milking each cow	Yes	21(35.6)	4.898	0.027
	No	38(64.4)		
Using cloth/fabrics to dry the udder	Yes	18(12.9)	77.257	0.001
	No	122(87.1)		
Using the towel for more than a cow	Yes	3(16.7)	8.000	0.005
	No	15(83.3)		

4.4.3. Hygienic practices of milk collection and storage

With similar to the milking practices, the milk and milk product storage practices-related-risk factors were analyzed for significant differences among the participants. Accordingly, the respondents who had been using plastic containers for milk collection and storage were significantly higher than those that used metal, wooden/grass made or clay ($p<0.05$).

As good practices, however, most of them had been washing the milking containers twice a day using soap and hot water. In addition, in the cases where the non-plastic containers were used, they had been smoking the milking and storage containers on least daily basis, and 3-4 days per week, respectively (**Table 13**).

Table 13: Hygienic practices implemented in milk collection and storage in the study area

Factors	Response	No (%)	χ2	p-value
Type of milking materials	Plastic	115(82.1)	150.186	0.001
	Metal	14(10)		
	Wooden or calabash	11(7.9)		
Types of container for milk storage	Plastic	64(45.7)	32.629	0.001
	Metal	23(16.4)		
	Wooden or Calabash	29(20.7)		
	Clay	24(17.1)		
Wash the milk equipment	Yes	140(100)	-	-
	No	0(0.00)		
Frequency of washing milking equipment	Twice per day	129(92.1)	99.457	0.001
	Daily	11(7.9)		
Smoke the milking equipment	Yes	25 (17.9)	57.857	0.001
	No	115(82.1)		
Frequency of smoking the milking equipment	Daily	8(32)	4.600	0.204
	Two days/ week	9(36)		
	3-6 days/week	6(24)		
	Weekly	2(8)		
Smoke storage container	Yes	70(50)	0.000	1.000
	No	70(50)		
Interval of smoking milk storage	Two days/week	32(45.7)	26.771	0.001
	3-4 days /week	35(50)		
	Weekly	3(4.3)		
Using hot water to clean milk equipment	Yes	131(93.6)	106.314	0.001
	No	9(6.4)		
Frequency of using soap to wash milk equipment	Not at all	6(4.3)	62.800	0.001
	Some times	52(37.1)		
	Always	82(58.6)		
Using refrigerator in case of dalliance for consumption or sell	Yes	65(46.4)	0.714	0.398
	No	75(53.6)		

4.4.4. Food intoxication information and associated risk factors

The study also attempted to determine the association between the occurance of the symptoms of food intoxication, and the storage materials used for milk products, hygeinic proctices of the handlers of the dairy products. Accordingly, the origin of the materials, the washing and smoking practices with their frequency, the use of hot water and soap to clean the utensils, and storage of the food staffs in refrigerator were analysed using Univariable logistic regression. However since there was only one factor which showed significant differences among its categories in Univariable logistic regression, the multivariable logistic regression analy was not performed for food intoxication. The food intoxication, which is manifested by clinical signs including vomiting, diarrhea, and stomach pain, was taken as dependent variable for the analysis.

As indicated on **Table 14**, the output of the analysis revealed that not all the factors except using hot water to wash the equipment were found to be associated with food intoxication revealed after milk and milk product consumption. The food intoxication clinical signs were found significantly less prevalent in families washing the equipment with hot water than who did not (*p<0.05*).

Table 14: Food intoxication experience and associated risk factors in the study area

Factors	**Response**	**Food Intoxication Clinical Signs Observed**		**COR (95%CI)**	***p*-value**
		No (%)	**Yes (%)**		
Type of milking materials	Plastic	78 (67.8)	37(32.2)	1.186(0.35-4.03)	0.785
	Metal	10 (71.4)	4(28.6)	Ref.	
	Wooden/ Calabash	7 (63.6)	4(36.4)	1.429(0.264-7.737)	0.679
Types of container for milk storage	Plastic	42(65.6)	22(34.4)	1.484(0.512-4.301)	0.467
	Metal	17 (73.9)	6(26.1)	Ref.	
	Wooden/Calabash	20(69)	9(31)	1.275(0.377-4.313	0.696
	Clay	16(66.7)	8(33.3)	1.417(0.402-4.99)2	0.588
Frequency of washing milking equipment	Twice per day	89(69)	40(31)	Ref.	
	Daily	6(54.5)	5 (45.5)	1.854 (0.534-6.433)	0.331
Smoking the milking equipment	Yes	17(68)	8(32)	Ref.	
	No	78(67.8)	37(32.2)	1.008(0.399-2.547)	0.987
Frequency of smoking the milking equipment	Daily	5(62.5)	3(37.5)	4.200(0.332-53.123)	0.268
	Two days/ week	5(55.6)	4(44.4)	5.600 (0.472-66.447)	0.172
	>3 days/week	7 (87.5)	1(12.5)	Ref.	
Smoking storage container	Yes	49(70)	21(30)	Ref.	
	No	46(65.7)	24(34.3)	1.217(0.598-2.478)	0.587
Frequency of smoking milk storage	Two days/week	24(75)	8(25)	Ref.	
	≥3 days /week	25(65.8)	13(34.2)	1.560(0.549-4.430)	0.404
Using hot water to wash milk equipment	Yes	92(70.2)	39(29.8)	Ref.	
	No	3(33.3)	6 (66.7)	4.718(1.123-19.826)	0.034
Frequency of using soap to wash milk equipment	Not at all	3(50)	3(50)	2.467(0.447-13.625)	0.300
	Some times	37 (71.2)	15(28.8)	Ref.	
	Always	55(67.1)	27(32.9)	1.211(0.568-2.580)	0.620
Use refrigerator for storage of milk products	Yes	43(66.2)	22(33.8)	Ref.	
	No	52(69.3)	23(30.7)	(0.568-2.354)	0.688

COR= Crude Odds ratio

4.4.5. Milk and milk product consumption and marketing habits

In the current study, the result of the milk and milk product consumption, and marketing habits of the localities was as depicted in **Table 15.** More than 80% of the respondents and all of them or the family members have been respectively selling or consuming at least one of the dairy products (milk, yogurt or cheese). Specially, significantly higher numbers of them had been selling milk and cheese. However, very few (3.6%) of them had been engaging in selling yogurt. It was determined that 32% of the respondents had encountered the food intoxication clinical signs on themselves and/or family members after consumption of dairy products.

Table 15: Milk and milk products consumption and marketing habit of the residents

Factors	**Response**	**No (%)**	**$\chi 2$**	***p*-value**
Milk selling	Yes	85(60.7)	6.429	0.011
	No	55(39.3)		
Cheese selling	Yes	94(67.1)	16.457	0.000
	No	46(32.9)		
Yogurt selling	Yes	5(3.6)	120.714	0.000
	No	135(96.4)		
Selling dairy products to processing plant	Yes	0(0.00)	4.114	0.043
	No	140(100)		
Selling dairy products to local consumers	Yes	82(58.6)	6.429	0.011
	No	58(41.4)		
Selling dairy products to restaurants	Yes	85(60.7)	16.457	0.001
	No	55(39.3)		
Selling dairy products to local market	Yes	94(67.1)	16.457	0.001
	No	46(32.9)		
Raw milk consumption	Yes	73(52.1)	0.257	0.612
	No	67(47.9)		
Yogurt and or cheese consumption	Yes	140(100)	-	-
	No	0(0.00)		
Having observed food poisoning symptoms following consuming milk and/or milk products	Yes	45(32.1)	17.857	0.001
	No	95(67.9)		

4.4.6. Milking practices in respect to the study area

Some of the milking practices were found varied with the districts. Significantly higher number (65.7%) of the respondents in Ambo had been washing their hands before milking ($p<0.05$). In addition majority of the Ambo (97.1%), Toke Kutaye (80%) and Bako Tibe (62.9%) people had been washing the udder of the cows before milking when compared with Wolmeras' ($p<0.05$) .

Of the individuals using fabrics for drying udder, more than 60% of the Toke Kutayes, Bako Tibes, and Wolmeras had been washing the fabrics twice daily.

As illustrated in **Table 16**, in general good safety milking activities had been practiced in Ambo than the other districts. Particularly, in washing hands and udder before milking, the respondents from Ambo district had better practiced than others.

Table 16: The hygienic practices of milking in the residents according to their district

Factors	Response	Districts				$\chi 2$	p-value
		Wolmera No (%)	Ambo No (%)	Toke Kutaye No (%)	Bako Tibe No (%)		
Milker wash hands before milking	Yes	17(48.6)	23(65.7)	11(31.4)	8(22.9)	15.56	0.001
	No	18(51.4)	12(34.3)	24(68.6)	27(77.1)		
Milker wash udder before milking	Yes	12(34.3)	34(97.1)	28(80)	22(62.9)	35.00	0.001
	No	23(65.7)	1(2.9)	7(20)	13(37.1)		
Milker wash hands with interval of milking each cow	Yes	5(14.3)	12(34.3)	3(8.6)	1(2.9)	15.41	0.002
	No	30(85.7)	23(65.7)	32(91.4)	34(97.1)		
Using cloth/fabrics to dry the udder	Yes	12(34.3)	2(5.7)	2(5.7)	2(5.7)	19.13	0.001
	No	23(65.7)	33(94.3)	33(94.3)	33(94.3)		
Using the towel for more than a cow	Yes	11(91.7)	0(0.0)	2(100)	2(100)	11.40	0.010
	No	1(8.3)	2(100)	0(0.0)	0(0.0)		
Frequency of washing the towel	Milking interval	1(8.3)	1(50)	0(0.0)	0(0.0)	4.71	0.858
	Twice daily	9(75)	1(50)	2(100)	2(100)		
	Daily	1(8.3)	0(0.0)	0(0.0)	0(0.0)		
	More than two days	1(8.3)	0(0.0)	0(0.0)	0(0.0)		

As displayed on **Table 17,** the odds ratio of washing hands before milking was about 6 times in Ambo and 3 times in Wolmera than in Bako Tibe district; whereas, udder washing was practiced 65, 7 and 3 times more in Ambo, Toke Kutaye and Bako Tibe districts respectively when compared with Wolemera ($p<0.05$) .

Table 17: binary logistic regression of milker's hands and cow's udder washing before milking with respect to the districts

Districts	Wash hands before milking			Wash udder before milking		
	Yes (%)	**COR (95% CI)**	***p*-value**	**Yes (%)**	**COR (95% CI)**	***p*-value**
Wolmera (N= 35)	17(48.6)	3.187 (1.137-8.932)	0.027	12 (34.3)	Ref.	
Ambo (N= 35)	23(65.7)	6.469 (2.256-18.548)	0.001	34 (97.1)	65.167 (7.920-536.172)	0.001
Tokke Kutaye (N= 35)	11(31.4)	1.547 (.534-4.482)	0.422	28 (80)	7.667 (2.595- 22.646)	0.001
Bako Tibe (N= 35)	8(22.9)	Ref.		22 (62.9)	3.244 (1.219- 8.629)	0.018

COR=Crude Odds Ratio, Ref= Reference category, N= total number of the respondents

4.4.7. Hygienic practices of milking and milk storage equipment with respect to the districts

Although using plastics as primary milk collection, and storage containers was highest in all the districts, the proportion was found different among the districts. Plastic containers were used frequently in Ambo followed by in Toke Kutaye and Wolmara districts than in Bako Tibe districts (**Table 18**).

Table 18: Milking and Milk Storage habit of the respondents according to their districts

Factors	Response	Districts				χ^2	p-value
		Wolmera No (%)	Ambo No (%)	Toke Kutaye No (%)	Bako Tibe No (%)		
Type of milking materials	Plastic	30(85.7)	34(97.1)	30(85.7)	21(60)	19.25	0.003
	Metal	4(11.4)	0(0.0)	2(5.7)	8(22.9)		
	Wooden or Calabash	1(2.9)	1(2.9)	3(8.6)	6(17.1)		
Types of container for milk Storage	Plastic	20(57.1)	23(65.7)	14(40)	7(20)	35.22	0.000
	Metal	8(22.9)	0(0.0)	11(31.4)	4(11.4)		
	Wooden or Calabash	4 (11.4)	8 (22.9)	4(11.4)	13(37.1)		
	Clay	3(8.6)	4(11.4)	6(17.1)	11(31.4)		
Washing the milk equipment	Yes	35(100)	35(100)	35(100)	35(100)	-	-
	No	0(0.00)	0(0.00)	0(0.00)	0(0.00)		
Frequency of cleaning milking equipment	Twice per day	31(88.6)	35(100)	29(82.9)	34(97.1)	8.98	0.030
	Daily	4 (11.4)	0(0.0)	6(17.1)	1(2.9)		
Smoking the milking equipment	Yes	5(14.3)	1 (2.9)	5(14.3)	14(40)	17.68	0.001
	No	30(85.7)	34(97.1)	30(85.7)	21(60)		
Interval of smoking the milking equipment	Daily	3(60)	0(0.0)	1(20)	4(28.6)	10.68	0.299
	Two days/ week	1(20)	1(100)	4(80)	3(21.4)		
	3-6 days/week	1(20)	0(0.0)	0(0.0)	5(35.7)		
	Weekly	0(0.0)	0(0.0)	0(0.0)	2(14.3)		
Smoking storage container	Yes	13(37.1)	12(34.3)	18(51.4)	27(77.1)	16.11	0.001
	No	22(62.9)	23(65.7)	17(48.6)	8(22.9)		
Interval of smoking milk storage	Two days/week	4(30.8)	5 (41.7)	8(44.4)	15(55.6)	8.62	0.196
	3-4 days /week	8(61.5)	5(41.7)	10(55.6)	12(44.4)		
	Weekly	1(7.7)	2(16.7)	0(0.0)	0(0.0)		
Using hot water to wash milk equipment	Yes	32(91.4)	33 (94.3)	33(94.3)	33(94.3)	0.36	0.949
	No	3(8.6)	2(5.7)	2 (5.7)	2(5.7)		
Frequency of using soap to wash milk equipment	Not at all	1(2.9)	1(2.9)	0(0.0)	4(11.4)	25.92	0.000
	Some times	28(80)	23(65.7)	22(62.9)	9(25.7)		
	Always	6 (17.1)	11(31.4)	13(37.1)	22(62.9)		
Using refrigerator in case dalliance for consumption or sell	Yes	20(57.1)	23(65.7)	16(45.7)	6(17.1)	18.93	0.000
	No	15(42.9)	12(34.3)	19(54.3)	29(82.9)		

The use of plastic container as milk and milk product primary collection and storage material was found differ with the districts. Accordingly, the use of the plastic materials as primary milk collection was found 23 times more frequent in Ambo, 4 times in Toke Kutaye and Wolmara

than in Bako Tibe district. Similarly using similar type of the container in Ambo and Wolmera as milk storage was 8 and 5 times more frequent than in Bako Tibe district. The locals have used hot water for washing the containers more frequently regardless of the districts. However, using soap always for washing the containers was about 8 times more frequent in Bako Tibe than in Wolmera ($p<0.05$).

4.4.8. Risk factors associated with consumption of dairy products in the districts

The analysis of the questionnaire responses displayed in **Table 19** disclosed that there was significant variation in selling of milk and milk products (particularly cheese) to different categories of the clients among the residents of the districts ($p<0.05$). However, selling yogurt was less familiar in the locals regardless of the districts ($p>0.05$).

Table 19 Milk and milk product marketing habits of the residents according to their district

Factors	Response	Districts				χ^2	*p-value*
		Wolmera No (%)	Ambo No (%)	Toke Kutaye No (%)	Bako Tibe No (%)		
Milk Selling	Yes	28(80)	25(71.4)	19(54.3)	13 (37.1)	15.902	0.001
	No	7(20)	10(28.6)	16 (45.7)	22(62.9)		
Cheese selling	Yes	32(91.4)	30 (85.7)	30(85.7)	2(5.7)	80.167	0.000
	No	3 (8.6)	5(14.3)	5 (14.3)	33 (94.3)		
Yogurt selling	Yes	2(5.7)	2(5.7)	1(2.9)	0 (0.0)	2.281	0.516
	No	33(94.3)	33 (94.3)	34(97.1)	35(100)		
Selling dairy products to Local consumers	Yes	26(74.3)	25(71.4)	18(51.4)	13(37.1)	13.305	0.004
	No	9(25.7)	10(28.6)	17 (48.6)	22(62.9)		
Selling dairy products to restaurants	Yes	28(80)	25(71.4)	19(54.3)	13(37.1)	15.902	0.001
	No	7(20)	10(28.6)	16(45.7)	22(62.9)		
Selling dairy products to local Market	Yes	32(91.4)	30(85.7)	30(85.7)	2(5.7)	80.167	0.000
	No	3(8.6)	5 (14.3)	5(14.3)	33(94.3)		
Raw Milk consumption	Yes	15(42.9)	17(48.6)	21(60)	20(57.1)	2.605	0.457
	No	20(57.1)	18 (51.4)	14(40)	15(42.9)		
Having observed food poisoning symptoms following consuming milk and/or milk products	Yes	18(51.4)	8(22.9)	10(28.6)	9(25.7)	8.220	0.042
	No	17(48.6)	27(77.1)	25(71.4)	26(74.3)		

Milk has been more frequently sold in Wolmera (OR=6.8, 95%CI=2.31-19.84), Ambo (OR=4.23, 95%CI=1.55-11.55) compared to Bako Tibe district ($p<0.05$). It was determined that cheese was sold in Wolmera (OR=176, 95%CI= 27.56-1123.92), Ambo (OR=99, 95%CI=17.86-548.87 and Toke Kutaye (OR=99, 95%CI=17.86- 548.87) districts more frequently than it was sold in Bako Tibe ($p<0.05$).

Furthermore, there was significant difference among the districts regarding who bought the dairy products.. The local consumers had bought the milk and/or milk products 5 and 4 times more frequently in Wolmera and Ambo, respectively, than in Bako Tibe. The odds of restaurant and hotels purchasing the dairy products in Wolmera and Ambo were 7 and 4 times more than they did in Bako Tibe. Similarly, the products were sold in local market as 176, 99 and 99 times more frequent in Wolmera, Ambo and Toke Kutaye, respectively, than in Bako Tibe ($p<0.05$).

4.4.9. Effects of education of respondents on milking practices

The result of the study revealed that the milking practices in the study area except using fabrics to dry the udder were not found significantly differ with the educational levels of the owners or the mangers of the farms. In such cases the tendency of using the fabrics increased with the level of their education (**Table 20**). The individuals in secondary, and college and above level were found using the fabrics about 14 and 40 times more than the illiterate individuals $p<0.05$).

Table 20: Hygienic milking and storage practices in respect to education level

Factors	Responses	Education Level				$\chi 2$	*p-value*
		Level[1*]	Level[2*]	Level[3*]	Level[4*]		
Hand Washing before milking	Yes	13(35.1)	40 (48.2)	3(27.3)	3 (33.3)	3.275	0.355
	No	24(64.9)	43(51.8)	8(72.7)	6(66.7)		
Udder washing before milking	Yes	24(64.9)	59(71.1)	5(45.5)	8(88.9)	4.931	0.185
	No	13(35.1)	24 (28.9)	6 (54.5)	1(11.1)		
Using cloth/fabrics to dry the udder	Yes	3(8.1)	2(2.4)	6(54.5)	7(77.8)	59.749	0.000
	No	34(91.9)	81(97.6)	5(45.5)	2(22.2)		
Towel for more than a cow	Yes	3(100)	2(100)	6(100)	4(57.1)	5.657	0.143
	No	0(0.0)	0(0.0)	0 (0.0)	3(42.9)		
Frequency of washing the drying clothes	Milking interval	0(0.0)	0(0.0)	0 (0.0)	2(28.6)		
	Twice daily	2(66.7)	2(100)	5(83.3)	5(71.4)	10.378	0.391
	Daily	1(33.3)	0(0.0)	0(0.0)	0 (0.0)		
	> 2 days	0 (0.0)	0 (0.0)	1(16.7)	0(0.0)		
Type of Milking materials	Plastic	32 (86.5)	68(81.9)	9(81.8)	6(66.7)	4.244	0.650
	Metal	2 (5.4)	8(9.6)	2(18.2)	2 (22.2)		
	Wooden/Grass	3 (8.1)	7 (8.4)	0(0.0)	1(11.1)		
Types of container for Milk Storage	Plastic	16(43.2)	39(47)	4 (36.4)	5(55.6)	4.410	0.882
	Metal	4 (10.8)	16 (19.3)	2 (18.2)	1(11.1)		
	Wooden/Grass	8 (21.6)	17 (20.5)	3(27.3)	1(11.1)		
	Clay	9(24.3)	11(13.3)	2 (18.2)	2(22.2)		
Rate of washing milking equipment	Twice per day	33(89.2)	78(94)	10(90.9)	8(88.9)	0.986	0.864
	Daily	4(10.8)	5(6)	1(9.1)	1(11.1)		
Smoking the milking equipment	Yes	5(13.5)	15(18.1)	2(18.2)	3(33.3)	1.949	0.617
	No	32(86.5)	68(81.9)	9(81.8)	6(66.7)		
Interval of smoking the milking equipment	Daily	1(20)	5 (33.3)	1(50)	1(33.3)	3.391	0.981
	Two days/ week	2(40)	6(40)	0(0.0)	1(33.3)		
	3-6 days/week	1(20)	3(20)	1(50)	1(33.3)		
	Weekly	1(20)	1(6.7)	0(0.0)	0 (0.0)		
Smoking storage container	Yes	16(43.2)	44(53)	5(45.5)	5(55.6)	1.179	0.801
	No	21(56.8)	39(47)	6(54.5)	4(44.4)		
Interval of smoking milk storage	Two days/week	9(42.9)	19(48.7)	2 (33.3)	2(50)	3.943	0.696
	3-4 days /week	12(57.1)	18(46.2)	3(50)	2(50)		
	Weekly	0 (0.0)	2 (5.1)	1(16.7)	0(0.0)		
Using hot water to clean milk equipment	Yes	31(83.8)	81(97.6)	11(100)	8(88.9)	9.205	0.038
	No	6 (16.2)	2 (2.4)	0(0.0)	1(11.1)		
Using soap for washing equipment	Not at all	1 (2.7)	5(6)	0 (0.0)	0 (0.0)	5.475	0.464
	Some times	18(48.6)	25(30.1)	5(45.5)	4(44.4)		
	Always	18(48.6)	53(63.9)	6(54.5)	5(55.6)		
Using refrigerator for storage	Yes	8(21.6)	49(59)	3(27.3)	5(55.6)	16.383	0.001
	No	29(78.4)	34(41.0)	8(72.7)	4(44.4)		

[1*]= illiterate and informal education, [2*]= elementary school, [3*]= ethiopian secondary school, [4*]= college diploma and above

4.5. Virulence factors of the *S. aureus* isolates

Among many virulence factors protease, staphylokinase and lipase production, staphylococcal enterotoxigenecity (SEA, SEB, and TSST-1), thermostability, and antibiotic resistance patterns of *S. aureus* isolates were tested in the current study. Since the labels of 2 of the isolates had been lost, only 92 of the total 94 isolates of the *S. aureus* were included for the assessement of the virulence factors. Accordingly, 92 of them were tested for protease, staphylokinase and lipase production, and 24 for harboring the staphylococcal enterotoxin genes. In the cases of antibiotic resistance 92 of the isolates were screened for the Methecillin resistant (MRSA) using the cloxacillin-supplemented mannitol salt agar (MSA). In addition, 12 to 46 isolates were tested for susceptibility to different antibiotics using antibiotic disc diffusion method. Furthermore, 44 and 34 of the isolates were tested for the presence of *mecA* gene and *blaZ* gene, respectively, and 34 for thermostable gene (*nuc* gene) using PCR method.

4.5.1. Protease production

Ninety-two isolates were tested for protease production on skimmed milk agar, and 71 (77.2%) showed clear zone of protein hydrolysis on the plates (Annex 3). There was no significant difference among the types of the samples, and districts from where the isolates were obtained ($p<0.05$) (**Table 21**).

Table 21: Protease producing isolates in respect to sample type and districts

Factors	**Category**	**Protease**			
		Isolates tested	**Positive (%)**	**Person χ2**	***p*-value**
Origin of the Isolates	Lactating cows	43	36(83.7)	6.526	0.095
	Bulk milk	24	15(62.5)		
	Yogurt	4	2(50)		
	Cheese	21	18(85.7)		
Districts	Wolmera	38	31 (81.6)	2.924	0.442
	Ambo	29	23(79.3)		
	Toke Kutaye	16	12(75)		
	Bako Tibe	9	5(55.6)		

4.5.2. Staphylokinase and lipase production

Staphylokinase and lipase were determined to be generated by 51 (55.4%) and 62(67.4%) of the isolates, respectively. Thirthy-three (34.9%) of the examined isolates were capable of producing

both staphylokinase and lipase. Nevertheless, only one of the two enzymes was generated by 29 (31.5%). That is, 17(33.3%) of the 51 isolates that were positive for staphylokinase were negative for lipase; and among the 62 lipase positive isolates, 28 (45.3%) were staphylokinase negative. However, out of the 92 isolates, 13 (14.1%) were neither lipase- nor staphylokinase-positive **(Fig 5)**.

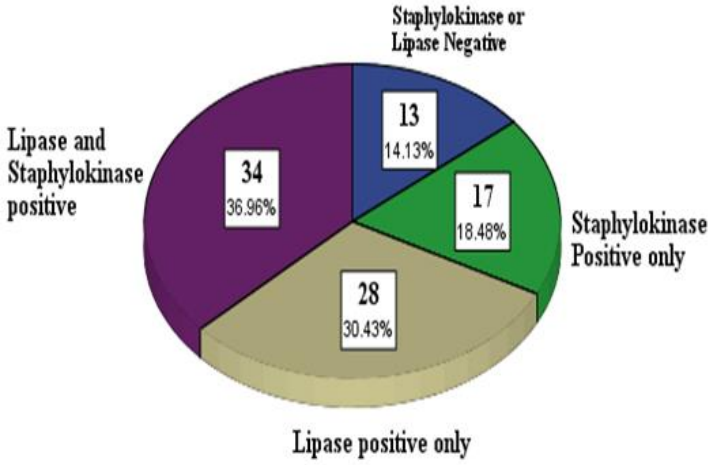

Figure 5: Multi-enzyme production of the isolates

The staphylokinase and lipase positivity of the isolates with respect to sample type was analyzed in **Table 22**. As a result, the isolates tested positive for the enzymes regardless of their origins (milk from a milking cow directly, bulk milk, yogurt, or cheese) ($p>0.05$).

Table 22: Staphylokinase and lipase production with respect to the orgion of the isolates

Factors	Category	Total isolates tested (%)	Staphylokinase positive (%)	Lipase positive (%)	Staphylokinase + Lipase positive (%)
Origin of the isolates	Lactating cows	43	29(67.4)	32 (74.4)	21 (48.8)
	Bulk milk	24	12(50)	12(50)	6(25)
	Yogurt	4	1(25)	3(75)	0(0.0)
	Cheese	21	9(42.9)	15(71.4)	7 (33.3)
$\chi 2$			5.641	4.531	6.541
p-value			0.134	0.207	0.085

4.5.3. Enterotogenicity characteristics of the S. aureus isolates

The enterotoxogenicity of the *S. aureus* was tested by specific-primer-based conventional PCR. Three enterotoxin genes were assessed in the current study. These were *sea*, *seb* and *tsst-1*which code for staphylococcal enterotoxin type A(SEA), staphylococcal enterotoxin type B (SEB), and Toxic shock syndrome toxin-1(TSST-1).

A total of 24 (10 from lactating cow, 6 from bulk milk, 1 from yogurt, and 7 from cheese isolates) *S. aureus* isolates were selected for enterotoxogenic characteristics using the PCR test (**Fig. 6**).

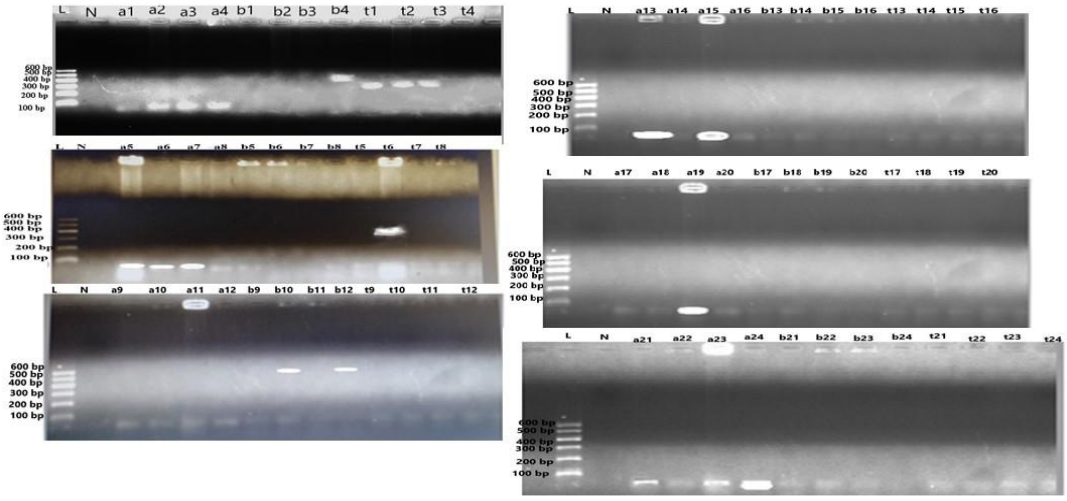

Figure 6: UV-illuminator imaged PCR product of enterotoxin genes after electrophoresis in 1.5% agarose gel.

L = ladder, a 1– a 24 denote samples tested for *sea* gene, b 1- b 24 denoted samples tested for *seb* gene and t 1- t 24 denotes samples tested for *tst-1* gene; N stands for negative control. Description: the length of *sea* = 120 bp, *seb*= 478 bp, and *tst-1*= 350 bp. Note: bp represents base pairs.

Twenty four isolates were tested for the presence of the *sea*, *seb* and *tsst-1* genes and 12(50%), 3(12.5%) and 4(16.6%) of them were respectively positive to the genes (**Fig. 7**). Some of the isolates were positive for two toxic genes simultaneously. Accordingly, 1(4.2%) held *sea* and *seb*, 3(12.5%) bore *sea* and *tsst-1*. However, none of them were found containing *seb* and *tsst-1*. The research also determined that significantly higher number of the isolates were *sea* positive than *seb* and *tsst-1* ($p<0.05$). However, 8 (33.3%), 2(8.3) and 1(4.2) of them held only one of each genes- *sea*, *seb* and *tsst-1*, respectively.

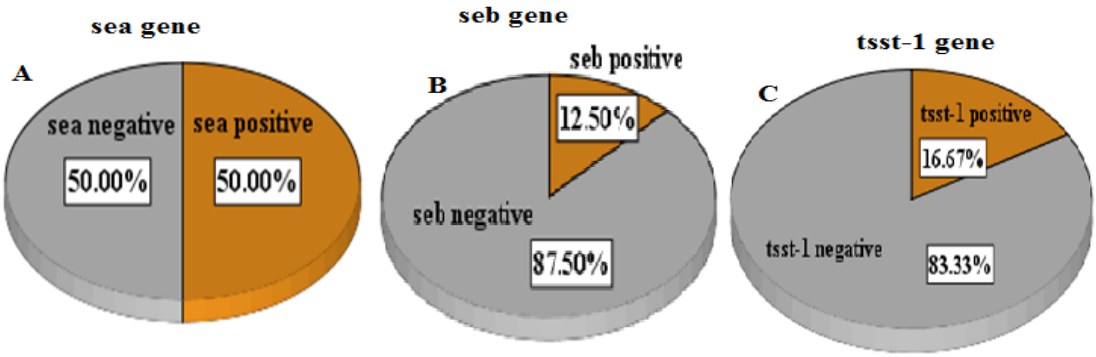

Figure 7: Prevalence of enterotoxin genes in the *S. aureus* isolates

The enterotoxogenicity of the isolates according to the origion of the samples is presented in **Table 23**. The *sea* gene was detected in isolates of all origions. However, *seb* was identified in

isolates from lactating cows. Moreover, *tsst-1* was determined in isolates of lactating cows and cheese samples.

Table 23: *sea, seb* and *tsst-1* genes harboring of the isolates with respect to the origions of the isolates

Factors	**Category**	**Total isolates tested (%)**	***sea* positive (%)**	***seb* positive (%)**	***tsst-1* positive (%)**
Origin of the isolates	Lactating cows	10	5(50)	3(30)	1(10)
	Bulk milk	6	1(16.7)	0(0.0)	0(0.0)
	Yogurt	1	1(100)	0(0.0)	0(0.0)
	Cheese	7	5 (71.4)	0(0.0)	3(42.9)
$\chi 2$			4.952	4.800	5.18
p-value			0.183	0.211	0.276

The study determined that only 1 of the 12 *sea* positive isolates which was isolated from lactating cows was found to harbor *seb* genes simeltanously **(Table 24**). Further more, three isolates (1 from lactating cow and 2 from cheese) positive for *sea* were also positive for *tsst-1* gene.

Table 24: Proportion of *S. aureus* isolates harboring multiple enterotxin genes with respect to origion of the isolates

Factors	**Category**	**Total isolates tested (%)**	***sea +seb* positive (%)**	***sea+tsst-1* (%)**
Origin of the Isolates	Lactating cows	10	1(10)	1(10)
	Bulk milk	6	0(0.0)	0(0.0)
	Yogurt	1	0(0.0)	0(0.0)
	Cheese	7	0(0.0)	2(28.6)
$\chi 2$			1.461	2.710
p-value			1.000	0.503

4.5.4. Antibiogram Activity of S. aureus Isolates

S. aureus were tested for susceptibility to various antibacterial drugs. Generally the antibiogram characteristics of the isolates were as displayed in **Table 25**. Accordingly, 46 isolates were tested against penicillin, Oxy-tetracycline, tetracycline, Trimethoprim/sulfamethoxazole, and gentamycin, 44 against erythromycin, 34 against cefoxitin, clindamycin, and 12 against oxacillin, amoxicillin, norfloxacin, and ampicillin. The isolates were found highly resistant to penicillin

(100%), amoxicillin (100%), Tetracycline (100%), Oxy-tetracycline (100%), ampicillin (83.3%) and Norfloxacin (75%). They were less resistant to gentamycin (8.7%), Trimethoprim/sulfamethoxazole (19.6%), erythromycin (20.5%) and cefoxitin/oxacillin (26.1%).

Table 25: Antibiogram characteristics of *S. aureus*

Antibiotics	Total tested	Susceptible (%)	Intermediate (%)	Resistant (%)
Growth on Cloxacillin-MSA	92	80(87)	0(0.00)	12(13)
Penicillin G	46	0(0.00)	0(0.00)	46(100)
Cefoxitin	34	34(100)	0(0.00)	0(0.00)
Oxacillin	12	0(0.00)	0(0.00)	12(100)
Clindamycin	34	34(100)	0(0.00)	0(0.00)
Amoxicillin	12	0(0.00)	0(0.00)	12(100)
Norfloxacin	12	2(16.7)	1(8.3)	9(75)
Ampicillin	12	0(0.00))	2(16.7)	10(83.3)
Tetracycline	46	0(0.00)	0(0.00)	46(100)
Oxy Tetracycline	46	0(0.00)	0(0.00)	46(100)
Trimethoprim/sulfamethoxazole	46	37(80.4)	0(0.00)	9(19.6)
Gentamycin	46	42(91.3)	0(0.00)	4(8.7)
Erythromycin	44	33(75)	2(4.5)	9(20.5)
Ciprofloxacin	34	34(100)	0(0.00)	0(0.00)
Novobiocine	34	34(100)	0(0.00)	0(0.00)

The isolates were highly resistant to penicillin (100%), tetracycline (100%), and oxy-tetracycline (100%), followed by ampicillin (83.3), and norfloxacin (75%). However the isolates were highly susceptible to clindamycin (100%), ciprofloxacin (100%) followed by gentamycin (91.3%), trimethoprim/sulfamethoxazole (80.4%), and cefoxitin/oxacillin (74%).

More specifically, 34 (24 from milk and milk products, and 10 from lactating cows, 11 form bulk milk, 11 from cheese and 2 from yogurt) of the *S. aureus* isolates, which did not grow on the cloxacillin supplemented manitol salt agar, were selected to determine the antibiotic susceptibility characteristics of the isolates. These isolates were found 100% resistant to penicillin, tetracyclin and oxytetracyclin (Table 26). Since all the members in the genus staphylococcus have been considered susceptible to Novobiocin [5ug], the drug was used as supplementary test for confirmation of the *S. aureus* isolates.

Table 26: Antibiotic susceptibility test result of isolates not grown on cloxacillin supplemented MSA

Antibiotics	Total Tested	Susceptibility		
		Susceptible (%)	Intermediate (%)	Resistant (%)
Ciprofoxacin	34	34(100)	00(0.00)	00(0.00)
Penicillin G	34	00(0.00)	00(0.00)	34(100)
Trimethoprim/sulfamethoxazole	34	34(100)	00(0.00)	00(0.00)
Cefoxitin	34	34(100)	00(0.00)	00(0.00)
Oxytetracycline	34	00(0.00)	00(0.00)	34(100)
Gentamycin	34	34(100)	00(0.00)	00(0.00)
Erythromycin	34	31(91.18)	2(5.88)	1(2.94)
Tetracycline	34	00(0.00)	00(0.00)	34(100)
Clindamycin	34	34(100)	00(0.00)	00(0.00)
Novobiocin	34	34(100)	00(0.00)	00(0.00)

4.5.5. Methicillin resistant S. aureus (MRSA)

Among, 92 *S. aureus* isolates were screened for MRSA on the cloxacillin supplemented MSA, 12(13%) grew on the plate, showed typical characteristics of *S. aureus*, and were found resistant to oxacillin. Ten of the MRSA strains were tested for harboring *mec*A gene and were positive (**Fig. 8**).

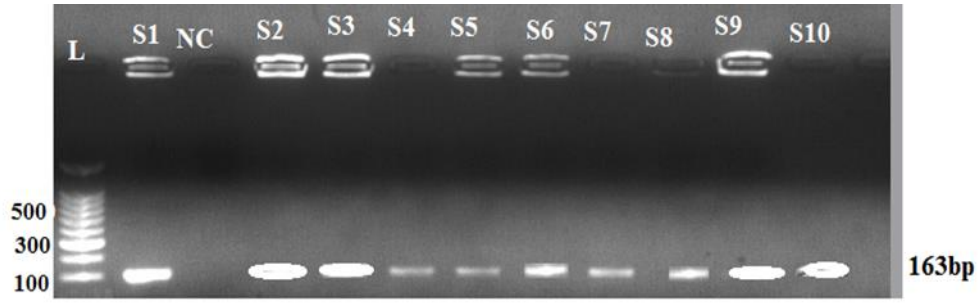

Figure 8**:** UV-illuminator imaged PCR product of mecA gene after electrophoresis in 1.5% agarose gel.

L = ladder, S1-S10 denote samples tested for *mecA* gene; NC stands for negative control. Description: the length of *mecA*= 163 bp. Note: bp represents base pairs.

In the current study MRSA was identified regardless of the origin of the sample, and study area. Furthermore, there was no significant variation in rate of MRSA between isolates from mastitis positive and mastitis negative animals (*p>0.05*) (**Table 27**).

Table 27: MRSA strains in respect to districts and sample types

Factor	Category	MRSA		$\chi 2$	p-value
		Negative (%)	Positive (%)		
Districts	Wolmera	9(64.3)	5 (35.7)	1.715	0.594
	Ambo	12(85.7)	2(14.3)		
	Toke Kutaye	8(72.7)	3 (27.3)		
	Bako Tibe	5 (71.4)	2 (28.6)		
Original samples	Lactating cow	9(60)	6(40.)	3.871	0.302
	Bulk Milk	12 (92.3)	1(7.7)		
	Yogurt	11 (73.3)	4(26.7)		
	Cheese	2(66.7)	1 (33.3)		
Mastitis	Positive	9 (69.2)	4 (30.8)	3.462	0.143
	Negative	0 (0.0)	2(100)		

The MRSA isolates were also checked for their resistance to other antibiotics. As stated in **Table 28**, all were resistant to pencillin, amoxicillin, tetracycline, and oxytetracycline. They were also highly resistant to ampicillin (83.3%), norfloxacin (75%), Trimethoprim/sulfamethoxazole (75%), and Erythromycin (66.7%). However, MRSA were less resistant to gentamycin (33.3%).

Table 28: Antibiotic resistance pattern of MRSA strains to other antibiotics

Antibiotics	Total tested	Susceptible (%)	Intermediates (%)	Resistant (%)
Oxacillin	12	0(0.00)	0(0.00)	12(100)
Penicillin G	12	0(0.00)	0(0.00)	12(100)
Amoxicillin	12	0(0.00)	0(0.00)	12(100)
Norfloxacin	12	2(16.7)	1(8.3)	9(75)
Ampicillin	12	0(0.00))	2(16.7)	10(83.3)
Tetracycline	12	0(0.00)	0(0.00)	12(100)
Oxy Tetracycline	12	0(0.00)	0(0.00)	12(100)
Trimethoprim/sulfamethoxazole	12	3(25)	0(0.00)	9(75)
Gentamycin	12	8(66.67)	0(0.00)	4(33.33)
Erythromycin	10	2(20.00)	0(0.00)	8(80.00)

4.5.6. Molecular detection of Resistance Genes

By using conventional PCR technique, some of the isolates of *S. aureus* were tested for the presence of resistance genes (44 for *mec*A, 34 for *bla*Z genes) and thermonuclease gene (34 for *nuc* gene). The *mec*A gene was determined in 10 (22.73%) of the tested isolates. All of the *mec*A

gene positive isolates were MRSA which were resistant to oxacillin in antibiotic resistance test. In addition, 34(100%) of the tested isolates were found to harbored *nuc* gene. However, none of the isolates tested for *blaZ* gene were found positive for the gene, despite all of them were resistant to penicillin in antibacterial susceptibility test (**Fig. 9& 10)**.

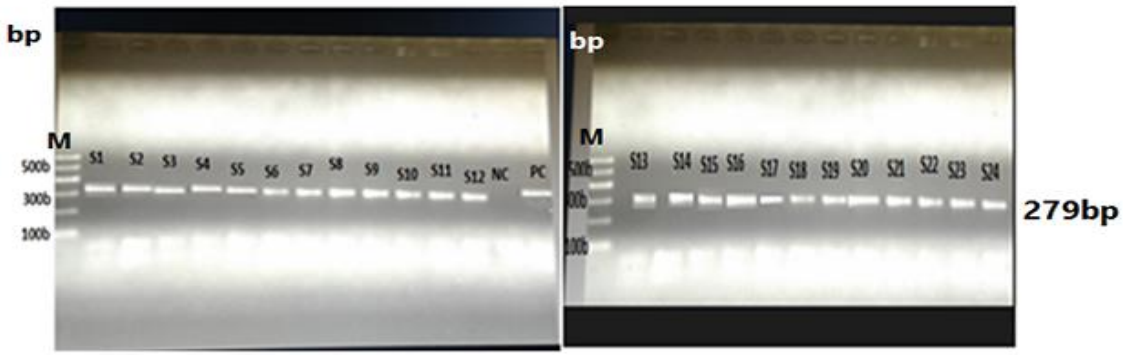

Figure 9*:* PCR product of *mecA, blaZ* and *nuc* genes in *S. aureus isolates* from bulk milk, yogurt and cheese electrophoresed in 1.5% agarose gel

Note: the length of *mecA* is 163bp, *blaZ* is 279 bp and *nuc* is 846 bp. M is the ladder, S1-S24 are the tested samples, NC is the negative control, PC is the positive control.

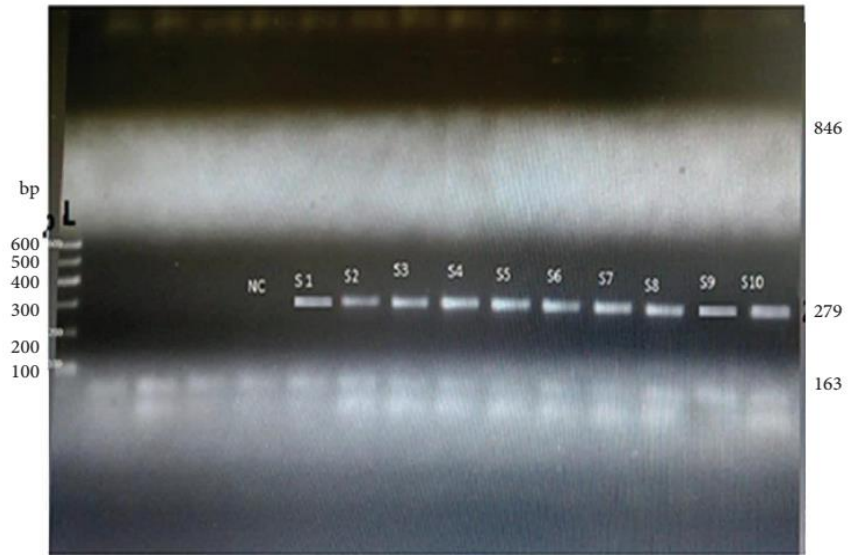

Figure 10: PCR product of *mecA, blaZ* and *nuc* genes in *S. aureus* isolates from lactating cows electrophoresed in 1.5% agarose gel

Note: L = ladder, S1–S10 denote samples, and NC stands for negative control. Description: the length of *mecA* = 163 bp, *nuc* = 279 bp, and *blaZ* = 846 bp. Note: bp represents base pairs.

4.5.7. Multidrug resistance (MDR) characteristics of S. aureus

Generally, the tested isolates were resistant at least to a minimum of 3 and a maximum of 9 of the 10 antibiotics leaving novobiocin as a diagnostic technique. As illustrated in **Table 29**, majority (71.74%) were resistant to 3 antibacterial agents, followed by to 9 antibiotics (4.35%). However, none of the tested isolates were found susceptible to all drugs.

Table 29: Multidrug Resistance pattern of the *S. aureus* isolates

Number of antibiotics	Antibiotics they showed resistance to	Number of Isolates (%)
3	P+OT+TE	33 (71.74)
4	P+OT+TE+E	1(2.17)
7	P+ OX+OT+TE+AMX+NO+E	1(2.17)
7	P+ OX+AMX+AMP+ OT+TE+ SXT	1(2.17)
8	P+ OX+AMX+AMP+TE+OT+ SXT +CN	1(2.17)
8	P+ OX+AMX+NO+AMP+TE+OT+E	1(2.17)
8	P+ OX/OX+AMX+NO+AMP+TE+OT+ SXT	1(2.17)
8	P+ OX+AMX+NO+TE+OT+ SXT +E	1(2.17)
9	P+OX+AMX+AMP+TE+OT+ SXT +CN+E	1(2.17)
9	P+OX+AMX+NOR+AMP+TE+OT+ SXT +E	2(4.35)
9	P+OX+AMX+NOR+AMP+TE+OT+SXT+CN	1(2.17)
9	P+OX+AMX+NOR+AMP+TE+OT+ SXT+E	1(2.17)
9	P+OX+AMX+NOR+AMP+TE+OT+CN+E	1(2.17)
	Total	46 (100)

AMP=Ampicillin, AMX=Amoxicillin, CN=Gentamicin, E=Erythromycin, NOR= Norfloxacin, OT= Oxytetracycline, OX=Oxacillin, P= Penicillin, TE= Tetracycline

4.5.8. MDR index analysis

According to the definition of the MDR index it was calculated as MAR index=a/b, where a represents the number of antibiotics to which an isolate is resistant, and b represents the total number of antibiotics the isolate is tested for susceptibility. In the current study, 46 isolates of *S. aureus* were tested and had >0.2 MDR index values. The indices ranged between 0.33 and 0.9 (**Table 30**) with average MAR index value of 0.47.

Table 30:: MDR indices of the *S. aureus* isolates

Number of antibiotics tested	Number of antibiotics to which the isolates showed resistance	Number of MDR Isolates	MDR Index
9	3	33	0.33
9	4	1	0.44
10	7	1	0.70
9	7	1	0.78
10	8	3	0.80
9	8	1	0.89
10	9	6	0.90
Total		46	

As revealed in **Table 31**, the MDR index of the MRSA was 0.7-0.9 with average of 0.85. The MDR index of MSA (mean=0.85) isolates was significantly higher than the non-MRSA isolates (mean=0.34) ($p<0.05$).

Table 31:MDR indices of MRSA strains

Number of antibiotics tested	Number of antibiotics to which the isolates were resisted	Number of MAR Isolate	MAR Index
10	7	1	0.70
9	7	1	0.78
10	8	3	0.80
9	8	1	0.89
10	9	6	0.90
Total		12	

In this research, the MDR index values of the isolates were not significantly varied with districts, and origin of the sample type but slightly with mastitis positivity (**Table 32**). The value was higher in isolates obtained from mastitis negative than mastitis positive ($p<0.05$).

Table 32: Analysis of the means of MDR indices with respect to districts, sample types and mastitis

Factors	Categories	Mean of MDR Index	95% CI for mean		d.f	F/t	p-value
			Lower	Upper			
Districts	Wolmera	0.52	0.38	0.67	3	0.826	0.487
	Ambo	0.39	0.31	0.48			
	Toke Kutaye	0.49	0.31	0.67			
	Bako Tibe	0.49	0.24	0.75			
Original samples	Lactating cow	0.55	0.39	0.7	3	1.283	0.293
	Bulk Milk	0.38	0.28	0.47			
	Yogurt	0.47	0.35	0.6			
	Cheese	0.48	-0.16	1.12			
Mastitis	Positive	0.49			13	-2.176*	0.049
	Negative	0.89					

* is t-value

Taking the MDR index as measurement of the multi antibacterial resistance pattern of the MRSA isolates, the values varied with the districts from where the isolates were obtained (**Table 33**). Accordingly, the multiple comparison analysis using ANOVA revealed that MDR index of MRSA isolated from Ambo were significantly lower than Wolmera's, Toke Kutaye's and Bako Tibe's ($p<0.05$). However, significant difference was not observed among isolates from Wolmera, Toke Kutaye and Bako Tibe district isolates (**Table 34**).

Table 33: Analysis of the means of MDR indices of MRSA strains in respect to districts, sample types and mastitis

Factors	Categories	Mean of MDR Index of MRSA isolates	95% CI for Mean		d.f	F/t	*p-value*
			Lower	Upper			
Districts	Wolmera	0.84	0.77	0.91	3	6.460	0.016
	Ambo	0.74	0.24	1.23			
	Toke Kutaye	0.90	0.90	0.90			
	Bako Tibe	0.89	0.82	1.0			
Original samples	Lactating cow	0.86	0.780	0.950	3	0.759	0.548
	Bulk Milk	0.90	-	-			
	Yogurt	0.83	0.745	0.905			
	Cheese	0.78	-	-			
Mastitis	Positive	0.85			5	2.205*	0.079
	Negative	0.89					

* is t-value

Table 34: Comparison of MDR indices of MRSA strains among the districts from where the samples were obtained

(I) District	(J) District	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Wolmera	Ambo	0.10	0.024	0.017	0.185
	Toke Kutaye	-0.06	0.095	-0.133	0.013
	Bako Tibe	-0.05	0.172	-0.138	0.029
Ambo	Toke Kutaye	-0.16	0.004	-0.253	-0.07
	Bako Tibe	-0.16	0.007	-0.256	-0.055
Toke Kutaye	Bako Tibe	0.006	0.892	-0.086	0.097

5. DISCUSSION

5.1. Mastitis prevalence in lactating cows

5.1.1. Mastitis prevalence and associated factors

Mastitis has been repeatedly mentioned as the disease of dairy animals, which incurs unnecessary expenditures in the dry industry. It affects the sector in various ways, among which reductions in milk yield and quality, culling of the animals, and treatment expenses have been stated. It might also be sources of toxin-producing microorganisms in foods of milk and milk products (Cobirka *et al*., 2020).

In the current research, a total of 258 cows in the four selected districts of West Showa zone were visited for the presence of mastitis and about 38% of the diagnosed cows were mastitis positive. This finding is in agreement with the findings of Sarba and Tola (2017), and Ejeta *et al* (2022) who reported the rate in Ambo district as 42%. In addition, it was in accordance with the finding of Delelesse (2010) who reported the prevalence rate of bovine mastitis in Holeta as 44%. However, with slightly less than the current report, the overall prevalence rate of the disease have been reported as 30.5% in selected districts of the West Showa Zone (Dabele *et al*., 2021) and as 29% in Shire of Tigray region (Girmay *et al*., 2020). The prevalence rate of mastitis was higher than the findings of Belay *et al.*, (2022) who found 17% prevalence rate of the disease in Gamo zone of Southern Ethiopia.

Neverthless, the prevalence of bovine mastitis in the current study was less than the finding of Mekbib *et al.* (2010) who reported the prevalence of mastitis in cross-breed lactating cows in Holeta district as 71% at animal level. Similarly, Marama *et al.* (2016) and Dereje *et al.* (2018) reported an overall prevalence rate of the disease as 62% in Holeta area and 70% in Holeta Agricultural Research Center, respectively. Similarly, the prevalence rate of mastitis determined in the current study is less than the findings of most studies conducted in different parts of Ethiopia which have reported the rate in the range between 46 and 73% (Abebe *et al*., 2016; Abêra *et al*., 2010; Bekele *et al*., 2019; Emeru *et al*., 2019; Etifu & Tilahun, 2019; Lakêw *et al*., 2019; Lakew *et al*., 2009; Sêyoum *et al*., 2017).

The result of the current study revelaed that from mastitis positive cows, 61% of them were subclinical and 39% were clinical mastitis. Hence, it has been determined that the occurrence of subclinical mastitis was significantly higher than clinical mastitis ($p<0.05$). The prevalence rate of clinical mastitis in this study (14.73% (38/258)) is greater than the rate reported by Abera *et al.* (2010) (10%) in Adama town, Abebe *et al.* (2016) (3.4%) at Hawassa milk shed , and Bekele *et al.* (2019) (10%) in Sebeta town. The current study also found that the distribution of subclinical mastitis in non clinical mastitis cows was 26.81% (59/220). This rate is less than the occurrence rate reported by Abera *et al.* (2010) in Adama, Abebe *et al.* (2016) at Hawassa milk shed, Birhanu *et al.* (2017) in Bishoftu, Baraki *et al.* (2021) in Southern Zone of Tigray, and by Bekele *et al.* (2019) in and around Sebeta town.

The proportion of clinical compared to subclinical mastitis determined in the current study is in agreement with the reports of Shiferaw and Telila (2016) who observed clinical mastitis and subclinical mastitis at rate of 38.80% and 61.19% in and around Wolayta Sodo. Similarly many studies showed that subclinical mastitis is significantly higher than clinical though the ratio might differ (Beyene *et al*., 2017; Mekibib *et al*., 2010; Móges *et al*., 2012; Muturi, 2020). Argaw (2016) also reviewed that the prevalence of clinical and subclinical mastitis in Ethiopia is estimated to be 23 and 85%, respectively. Girma and Tamir (2022) reviewed the prevalence of clinical and subclinical bovine mastitis in Ethiopia and found it to range from 0.9 to 48.1% and 2.5 to 56.8%, respectively.

Bovine mastitis is a polyethiological, complex pathogenesis and multifactorial disease of dairy cows (Zigo *et al*., 2021). That is the prevalence rate of bovine mastitis can vary with various determinants (Dabele *et al*., 2021; Móges *et al*., 2012). Therefore, identification of the risk factors associated with the disease has great importance in epidemiologic studies, and taking measures to prevent and control mastitis accordingly. The factors are generally categorized as intrinsic and extrinsic (Bikila & Soressa, 2022; Tezera & Ali, 2021). Breed, age, lactation stage, and parity level from intrinsic risk factors, whereas agro-ecology, number of milked cows in the herd, milking technique, management system, milking cleanliness (milkers hand and udder washing before milking), and tick infestations from extrinsic risk factors were included in the current study.

Among the intrinsic factors, genetic composition of the animal has been mentioned as the main risk factor in bovine mastitis. When compared to less chosen and lower performing dairy breeds, the selective pressure for enhanced milk output in Holstein Friesian cows have a higher tendency to contract mastitis (Curone *et al*., 2018). However, according to Cheng and Han, (2020), whether they are purebred or crossbred, high-yielding cattle or not, Holstein-Friesian cattle in particular tend to be genetically more susceptible to mastitis than other breeds. It is evidential, therefore, that mastitis is more prevalent in cows of the Holstein Friesian pure breed or cross-breed than in the local pure zebu breeds.

However, the current study did not encounter significant variation of the disease between the cross breed and local breeds ($p>0.05$). With this result, it was in line with the finding of Marama *et al.* (2016). Similarly, Baraki *et al*. (2021) found that the disease was distributed equally in the North Tigray regardless of local and cross breed cows. The distribution of bovine mastitis among the breeds in Ethiopia has not been constant that it is difficult to reach general conclusive summary regarding the prevalence of the disease in respect to the breeds of the animal. Research conducted by Fesseha *et al.* (2021) revealed that the prevalence of bovine mastitis was significantly higher in Jersey pure breed than local zebus, but no difference between the cross breed and local zebus. Furthermore, Moges *et al.* (2012), Meskela and Gashaw (2017) and Ejeta *et al.* (2022) determined that mastitis has been prevalent in pure Holstein-Friesian and Holstein Zebu crossbreed dairy cows than in local Zebu cattle. In addition, studies in different parts of Ethiopia have shown that the disease is highly prevalent in the exotic-local crossbreeds than local cows (Abebe et al., 2016; Alemu et al., 2013; Beyenê & Tolosa, 2017.). There were also reports stating that the high milk yield producer pure exotic breeds were highly infected by mastitis than the local-exotic-crossbreed (Mulshet *et al*., 2017). However, Tilahun and Aylate (2015) found that the prevalence of bovine mastitis was higher in local zebu breed than the crossbreed although less than pure Jersey and Holistein breeds. The variation might be because of the difference in percentage of the exotic gene shared with the cross breed animals. However, the resistance potential of local zebu cattle to the disease has not yet been well understood.

It has been determined that the prevalence of clinical mastitis in the current study varied significantly with the risk factors including the age of the animal, and current tick infestation of the teat. In respect to these factors, the animals in medium age group and contemporarily infested

with tick developed clinical mastitis than the young age and free of tick infestation. In addition, the Univariable logistic regression analysis revealed that the cows with mid lactation stage, few and many parity level and *S. aureus* positives were significantly tended to develop subclinical mastitis when compared with the cows in early lactation stage, medium parity level, and *S. aureus* negative cows. Moges *et al.* (2012) determined that the stage of' lactation and parity number positively associated with the development of subclinical mastitis in bovine.

The age of the animals was also determined as the risk factor for the variation of bovine mastitis in the study area. As mentioned in the methodology part of the study, the age of the cows were categorized as old cows young, intermediate, and adult when their ages were fallen in <4, 4—6, and >6 years old respectively. The study did not find significant prevalence of mastitis among the age groups of the cows *(p>0.05)*. The current finding agrees with the result of research conducted by Balemi *et al.* (2021), on cows, one-humped dromedary camels, and goats in Bule Hora and Dugda Dawa districts of Oromia and by Tillahun and Aylate (2015) on commercial bovine dairy farms of Addis Ababa. However, other studies in Ethiopia have reported the increment of the prevalence of mastitis with age of the cows (Aberá *et al*., 2010; Abêra *et al*., 2010; Birhanu *et al*., 2017; Ejeta *et al*., 2022; Kebebew & Jorga, 2016; Mekibib *et al*., 2010). Kurjogi and Kaliwal (2014) have also reported the increase of bovine mastitis with age of the cow in India. In contrary of these researches, Fesseha *et al.* (2021) reported reduction of mastitis prevalence with age.

The variation of mastitis occurrence in the current study was found increased with the lactation stage of lactating cows. Cows in the late and medium lactation stages were 6 and 5 times more affected by the disease respectively than the cows in early stage of lactation. In accordance with this finding, Biffa *et al.* (2005), Tolosa *et al.* (2013), Tilahun andAylate (2015), Abebe *et al.* (2016), and Shiferaw and Telil (2016) in Ethiopia, Khanal and Pandit (2013) in Nepal, and Kurjogi and Kaliwal *et al.* (2014) in India have also reported the increase of prevalence rate of mastitis in cow with lactation stage. In addition, the result of the current study fairly agrees with the report of Meher *et al.* (2018) of Bangladesh, and Belachew (2016) and Gebisa *et al.* (2019) of Ethiopia. However, there have been studies who reported the highest prevalence of the disease in cows at early stage of lactation (Bihon *et al*., 2019; Bikila & Soressa, 2022; Elbably & Asmaa, 2013; Girmá *et al.*, 2012; Kitila *et al*., 2021; Mulshet *et al.*, 2017; Tezera & Ali, 2021; Zeryehun

et al., 2013). However, Mureithi and Njuguna (2016) and Dabele *et al.*(2021) observed the highest rate of bovine mastitis in mid lactation stage than early and late stages. Nevertheless, Kebebew and Jorga (2016); Asmare and Kassa (2017), Assefa (2021), Baraki *et al.* (2021), Fesseha *et al.* (2021), and Ejeta *et al.* (2022) have not found significant differences of the rate of the disease in respect of lactation stages. The diversity of these reports might be linked with variation in hygienic and farm management practices which differ with the locals, farms and man power of the farms.

Researchers have indicated that the teat canal remain opened for 1-2 hours after milking during which the pathogens enter into the canal to cause mastitis *(*Asmare & Kassa, 2017; Cobirka *et al.,* 2020). The milking frequency of the animal decreases with the lactation stages of the animals (Vijayakumar *et al.,* 2017). As the animal approaches to drying time, during which milking stops at all, the milking frequency decreases. In fact, milking frequency may vary with the availability of feeds and health status of the animal as well (D. Hailu et al., 2016). According to Klaas and Zadoks (2018) the more milking frequency is the more removal of the pathogens from the teat canal and replace by the somatic cells. SCC has long been used as one of the methods for mastitis test. That means, as lactation stage increase, the milking frequency decreases (Vijayakumar *et al.,* 2017), so that the chance of the pathogens to remain in the canal and establish infection increases. Kivaria *et al.* (2007), however, found the reduction of somatic cells count (SSC) with increasing milking frequency.

The parity level and animals' body conditions have shown significant associations with the occurrence rate of bovine mastitis in the Univariable logistic regression. Accordingly, the diseases was prevalent in cows with few (≤2 calving) and many (≥6 calving) parity than the medium, and in cows with poor and good body condition than in medium body condition ($p<0.05$). The mean of parity numbers of mastitis positive and negative cows were 2.93 and 2.81 calving respectively. However, both factors could not remain strong enough to be significant risk factors of the disease in the multivariable logistic regression analysis ($p>0.05$). With this, the current finding agrees with the previous studies who observed mastitis in all cows regardless of their parity level (Aberá *et al.*, 2010; Alebie *et al.*, 2021; Alemu *et al.*, 2013; C. Assefa, 2021; Baraki *et al.*, 2021; Belachew, 2016; Etifu & Tilahun, 2019; Fesseha *et al.*, 2021; Getahun *et al.*, 2008; Mbindyo *et al.*, 2020; Singha *et al.*, 2021; Tezera & Ali, 2021; Tolosa *et al.*, 2013).

However, many researches conducted in Ethiopia (Abebe *et al*., 2016; Argaw, 2016; Asmare & Kassa, 2017; Belay *et al*., 2022; Beyenê & Tolosa, 2017; Biffa *et al*., 2005; Birhanu *et al*., 2017; Ejeta *et al*., 2022; Girmá *et al*., 2012; Kebebew & Jorga, 2016; Lakew *et al*., 2009; Móges *et al*., 2012; Mulshet *et al*., 2017; Shiferaw & Telila, 2016; Tillahun & Aylate, 2015; Zeryehun *et al*., 2013), in Uganda (Abrahmsén *et al*., 2014), in Egypt (Elbably & Asmaa, 2013), in Kenya (Mureithi & Njuguna, 2016), in Bangladesh (Meher *et al*., 2018), and in Rwanda (Ndahetuye *et al*., 2019) determined that the prevalence of the disease increases with parity levels of the animals. Mekibib *et al.* (2010) and, Bihon *et al.* (2019) reported high prevalence of bovine mastitis in cows with medium parity level; whereas, Kitila *et al.* (2021) investigated the reduction of mastitis rate with high parity level.

The Person chi-square test ($\chi 2$ =6.9998, df =2, $p<0.05$) revealed that body condition of the animal was associated with the bovine mastitis in this research. Accordingly, the Univariable logistic regression analysis showed that the cows with good (50%) and poor(49%) body conditions were about 2 times more affected by mastitis than those with moderate body condition ($p<0.05$). However, in the multivariable logistic regression analysis, in which all the variables showing potential association with the disease were entered simultaneously, animal's body condition could not show significant association with the disease ($p>0.05$). Many studies have reported the association between animals body condition and bovine mastitis. Accordingly, Tolosa *et al.* (2013), Birhanu *et al.* (2017), Mulshet *et al* ,. (2017), Hoque *et al.* (2018), and Singha *et al.* (2021) have not observed significant difference of mastitis prevalence among the categories of body condition (body condition score (BCS). However, other studies reported high prevalence rate of the diseases in poor body condition than in good and/or medium ones (Balemi *et al*., 2021; Belay *et al*., 2022; Dereje *et al*., 2018; Ejeta *et al*., 2022; Fesseha *et al*., 2021; Tezera & Ali, 2021).

Despite the prevalence of bovine mastitis has been expected to vary with agro-ecology (Bude & Mengesha, 2018; Fesseha *et al*., 2021; Getahun *et al*., 2008; Wirtu *et al*., 2018), then with the districts in this study, there was no difference in the prevalence rate of mastitis among the study areas ($p>0.05$). However, very few studies conducted in Ethiopia have compared the agro-ecologic fluctuation of bovine mastitis. In accordance with the current study Baraki*et al.* (2021) have not found significant difference of bovine mastitis among the agro-ecology in North Tigray

of Ethiopia. However, Etifu and Tilahun, (2019) determined agro-ecology as the determinant of the variation of bovine mastitis in Rift Valley area of Ethiopia. It has been estimated that the difference in prevalence of mastitis with agro-ecology might be attributed to the dominance of mastitis causing microorganisms in a particular climate (Arga *et al*., 2012).

Furthermore, the occurrence of bovine mastitis was not found varied with the farm types as extensive, semi-intensive and intensive management system in this research. In agreement with the current study, Baraki *et al.* (2021) and Fesseha *et al.* (2021) did not find significant differences of the mastitis among the three types of the management systems in Tigray and Central Oromia respectively. The variation of the prevalence of the disease with the management system of the farm is controversial. Some studies have determined significantly high prevalence rate in extensively managed cattle than in intensive farming system (Belachew, 2016). The others, however, reported high rate of bovine mastitis in animals managed under intensive farm management system than in semi-intensive and/or extensive management system (Abrahmsén *et al*., 2014; Arga *et al*., 2012; Bihon *et al*., 2019; Mbindyo *et al*., 2020; Mpatswenumugabo *et al*., 2017; Tezera & Ali, 2021). There were also reports indicating high prevalence rate of the disease in semi-intensive farms than in intensive and/or extensive management system (Biffa *et al*., 2005; Bikila & Soressa, 2022; Kebebew & Jorga, 2016).

Researchers have indicated that, the high prevalence rate of bovine mastitis in extensive and semi-intensive than in intensive management system might be linked with the fact that the former management systems usually use open grazing system (Hoque *et al*., 2018). The open grazing system, in turn, exposes the animals to various ecto-parasites including ticks, which can cuase mechanical damages to the teats, or they can be vectors for mastitis causing microorganisms (Meskela & Gashaw, 2017). In addition, spiky plants that can mechanically damage the udder or teats of the animal mostly cover grazing fields. Nevertheless, the surpassing of the rate in the intensive management system might be associated with insufficient hygienic measures taken in the farm particularly during milking. In general, the controversial reports of farm type as determinant of bovine mastitis pointed that the farming system alone could not bring significant influence on the rate unless other hygienic practices have been supplemented.

Some studies have related the high prevalence of bovine mastitis in intensive farming system with the welfare of the animals. Arnott *et al.* (2017) evidentially reviewed that in comparison of the prevalence of mastitis within continuously housed and pasture systems, the disease was highly prevalent in the former than in the latter group. According to the review, the confined Holstein cows had a prevalence of 51% v. 31% mastitis, when compared with the pasture-based cows of the same breeds. These studies complained that lack of access to open pasture can expose the animal with an increased risk of compromised udder health. In contrary, however, within pasture-based systems, the risk of so-called 'summer mastitis' is likely to be increased. This type of the disease is an acute clinical mastitis most prevalent in non-lactating cattle feeding at pasture during the summer.

The other extrinsic factors expected to affect the prevalence of bovine mastitis are the hygienic and sanitation practices. Accordingly, for the current study, the hygienic practices expected to influence the occurrence rate of bovine mastitis were assessed by collecting the information from the owners or the manager of the farms using structured questionnaire. These practices include milking methods, hand washing of the milker's hands and udder of the animals before milking, washing hands of the milkers with interval of milking each cow, availability and washing frequency of separate cloth for milking, using cloth/fabrics to dry the udder, using the teat-drying clothes for multiple animals, and frequency of washing the teat-drying fabrics. Among these hygienic practices washing milkers' hand, and udder of the animals before milking, and washing the milkers' hand with interval of milking each cow were the significant associated factors with the prevalence rate of bovine mastitis in the study area. The poor milking hygiene procedures could be the reason for high prevalence of clinical and subclinical mastitis (Miyama *et al*., 2020; Tezera & Ali, 2021).

Tick infestation of inguinal area particularly of teats has been incriminated among potential extrinsic risk factors to exacerbate prevalence of mastitis in cattle. This external parasite result in the development of mastitis due to either injury it causes and/or being vector for pathogenic microorganisms (Gebisa *et al*., 2019; Junaidu *et al*., 2011; Meskela & Gashaw, 2019). In this research, it was found that the bovine mastitis was about 40 times prevalent in lactating cows with teats infested by ticks than in tick free cows. In this regard, the result agreed with the findings of many studies previously conducted in Ethiopia and other parts of the world.

Researches conducted in Oromia region by Tolosa *et al.* (2013) in Jimma, by Gebisa *et al.* (2019) in and around Bedele town and Mettu district, and by Kitila *et al.* (2021) in west Wollega, by Biffa *et al* (2005) in South Nations, Nationalities and People (SNNP) region, by Shiferaw and Telila (2016) in Sidama and by Moges *et al.* (2012) in Welayta zone, in and around Wolayta Sodo, by Belay *et al.* (2022) in Hawassa, and in Gamo Zone, by Bihon *et a*(2019)*l* in Amhara region in and around Gondar), by Abera *et al.* (2010) in Somali region in Jijiga, and by Tezera and Ali (2021) in Benishangul Gumuz region in and around Assosa town) of Ethiopia reported that tick infestation is significantly associated with high prevalence of bovine mastitis. Similarly, Madut *et al.* (2018) in Sudan, and Elbably *et al.* (2013) in Egypt found tick infestation as one of the risk factors of bovine mastitis. However, Ejeta *et al.* (2022), Alemu *et al.* (2013) and Dabele *et al.* (2021) did not find significant difference of mastitis prevalence in cows infested with ticks and those without ticks.

5.1.2. Milking practices and hygienic practices associated with mastitis

For the purpose of the current study, a total of 140 (35 individuals from each district) owners/farm managers were interviewed for the general hygienic practices performed in the farms. According to the responses of the respondents, all of them have been milking manually, 59(42.1%) of them have washed their hands before milking of which 15(35.6%) practiced it with interval of milking each cow. Regarding the udder washing before milking, 96(68.6%) have regularly been practicing it.

The current research revealed that animals which were milked with unwashed hands were about 16 times more likely to contract mastitis than the animals milked with washed hands before milking ($p<0.05$). Shittu *et al.* (2012) determined that cows in herds where hand washing was practiced regularly were less affected by subclinical mastitis than the animals where it had not been practiced in Nigeria. Shem *et al.* (2002) determined that 58.3% of the farmers practiced hand washing before milking in Tanzania.

Hand washing particularly with soap is important practice not only in preventing mastitis in dairy animals, but it is also considered as the method to prevent the contamination of food stuffs by the opportunistic or pathogenic microorganisms (Abbas *et al*., 2016). Yakubu *et al.* (2020) advised the milkers have to wash their hands adequately with detergent and clean water in between

milking each cow in order to avoid cross contamination of milk products from the milkers. The practice is also crucial especially when it is practiced by health care workers as far as the public health issues are concerned. Hand washing with antimicrobial soaps is considered to have potential effect in removing transient bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which are deposited on the skin surface from environmental sources (Chirani *et al*., 2021; Riaz *et al*., 2009) However all the soap should be removed from the hands to avoid the effect of the soap (particularly scent soaps) on the odour of the milk (Linda *et al*., 2010; Muhammad *et al*., 2017).

Since dirty udder has been expected to be one of the farm hygienic risk factors for the high frequency of mastitis in cows, teat or udder washing is one of the hygienic practices to reduce the prevalence of mastitis (Meher *et al*., 2018). In addition, just as milkers' hand washing prevents contamination of milk and milk products, so does udder washing before milking (Ayele *et al*., 2017). Studies have revealed that udder washing has reduced milk contamination with bacteria (Abêbe *et al*., 2020; Bekuma & Galmessa, 2018; Tola *et al*., 2020). Significantly high bacterial count have been determined in milk collected from cows with unwashed udder than in cows milked after washing their udder (Borena *et al*., 2022; Yeserah *et al*., 2019).

In this study the prevalence of mastitis in lactating cows whose udders have not been washed before milking was significantly higher (OR=36.87) than in cows milked after their teats were washed ($p<0.05$). The finding is consistent with Dabele *et al.* (2021) and Mekibib *et al.* (2010) who found that bovine mastitis was significantly higher in animals milked without washing the udder before milking than in the animals milked after washing their udder. Compatibly, Sori *et al.* (2005), Lakew *et al.* (2009) and Abera *et al.* (2013) reported high prevalence of bovine mastitis in less hygienic dairy farms than in good hygienic managed farms. In contrary, Bihon *et al.* (2019) and Fesseha *et al.* (2021) determined higher prevalence of the disease in cows milked with washing their udder. This could be due to following improper procedure of udder washing. The current result was also inconsistent with the finding of Abebe *et al.* (2016) who has not found significant difference in mastitis prevalence between the groups of cows milked after udder washing and unwashed udder. There were also studies reporting the significant reduction of mastitis when the teats of lactating animals were washed and disinfected after milking (Abrahmsén *et al*., 2014; Komarov *et al*., 2021; Linda *et al*., 2010).

5.1.3. Prevalence rate of S. aureus from lactating cows

S. aureus has been identified as one of the primary etiological agents of infectious mastitis in cattle (Franco *et al*., 2008). The bacterium resides on superfcial surfaces of the udder and the teats, as well as in the teat canal (Haveri *et al*., 2008; Svennesen *et al*., 2019). In the current study, *S. aureus* was found in 43 (16.67%) of 258 total milk samples from the lactating cows. The rate was signifcantly higher in mastitis-positive animals than the mastitis negative ones (35% versus 5.6%) ($p<0.05$). Many previous studies have also isolated *S. aureus* at various rates from mastitis-positive cows. This result agreed with Emeru *et al.* (2019) and Girmay *et al.* (2020) reports. However, it is less when compared with the findings of Seyoum *et al.* (2017), Birhanu *et al.* (2017), and Abebe *et al.* (2016), but it exceeded the findings of Bekele *et al.* (2019), Etifu and Tilahun (2019), and Lakew *et al.* (2009).

S. aureus isolation rates were also found to differ between clinical and subclinical mastitis, with the pathogen being significantly more common in clinical mastitis-positive cows (OR= 2.93) than in subclinical mastitis-positive cows if other variables remained constant. In dissimilarity to the current study, Mekibib *et al.* (2010) and Madut *et al.* (2018) determined a higher *S. aureus* isolation rate in subclinical than clinical mastitis.

In the current study, the increase in the number of lactation cows in the herd and tick infestation were linked with the higher isolation rates of *S. aureus*. The number of the lactating cows might affect the hygienic practices such as washing of milker's hands and teats of the animals before milking unless there is an increase in labor resources in the farm. In line with the current fndings, Gebremedhin *et al.* (2022) found a direct correlation between tick infestation and the prevalence rate of *S. aureus* in milk samples, as well as an inverse correlation between teat washing before milking and the isolation rate of *S. aureus*. Vouraki *et al.* (2022) also reviewed that there have been reports of *S. aureus* isolation from *Rhipicephalus* spp. tick. *S. aureus* can spread from infected to uninfected udder quarters during milking if the cow's udder or teats are not cleaned before milking since it lives on the udder or teat surface of cows.

The prevalence of *S. aureus* did not signicantly vary across animal parity levels in the current investigation ($p>0.05$). Birhanu *et al.* (2017), Garedew *et al.* (2015), and Tasse *et al.* (2022), however, reported the increment of the isolation rate of *S. aureus* with the parity number of

cows. Milking practices are also considered as one of the major risk factors in spreading mastitis among the quarters and animals as well as contaminating the milk and milk products if practiced in a poor hygienic ways (Bekuma & Galmessa, 2018; Singh & Ramachandran, 2017). These milking practices, in turn, are influenced by various social and economic determinants. The sex, age, education level, culture and sources of income of the practitioners may determine the application of the practices. In the current study most of the respondents (who were considered as the worker to perform milking) were females, with age range between 20 and 60 years, and elementary and above education level. The aforementioned demographic characteristics of the farmers were good indicator for the expectations that good hygienic milking practices could be implemented in the dairy farms. However, since majority of them had other sources of income other than the dairy farms, attention might not be given to the dairy farm management in general and to milking practices in particular.

Regarding the implementation of the milking practices, in all of the visited dairy farms, milking was practiced manually. In majority of the farms, the milkers have not washed their hands before milking, nor in the interval of milking each cow, and also did not use clothes to dry udder. However, what good practice was that majority of them reported that they wash the udder of the cows before milking. Environmental pathogenic microbes can be removed from the udder when the udder is washed before milking and hence may maintain the health of the teat. The necessity of the hygienic practices depends on the types of the milking practices (hand milking or machine milking). The good practices include clean milker's hands and clothes, being in good health of the milkers, clean milking machine and keeping clean, and being in good condition of the milk storage equipments including milk churns immediately after use (Bekuma & Galmessa, 2018). Milker's hand may spread the pathogens which might originate from the milker, animal and/or environment (Belay *et al*., 2022). Therefore, hand-washing is one of the cheap and effective methods to prevent the distribution of the pathogens among cows (Hogan *et al*., 2016).

A comparative controlled experimental study conducted by Abdalla and Elhagaz (2011) in Sudan, revealed that application of hygienic practices such as milkers' hand washing with soap and water, and rubbing teats and udder with clean wet towels prior to milking showed significant reduction in the total coliform and *S. aureus* count in milk.

5.2. *S. aureus* in milk and milk products and contamination associated risk factors

5.2.1. Prevalence of S. aureus in bulk milk and milk products

In the current study, *S. aureus* was isolated from bulk milk and milk products at rate of 14.4% that it was 25/123(20.3%), 22/148(14.9%), 4/83(4.8%) in bulk milk, cheese and yogurt respectively. The result is in accordance with the result of Gebremedhin *et al.* (2022) who isolated the microbe from 10.7% of the milk and milk products in Holeta Town of Ethiopia, but less than the reports of Ayele *et al.* (2017) and Lemma *et al.* (2021) who isolated the bacterium in 23%, and 20.3% of milk and milk products in Sebata town and Addis Ababa city of Ethiopia respectively. Elsayed *et al.* (2018) with an isolation rate of 46% in Egypt, Pervin *et al.* (2019) with an isolation rate of 69% in Bangladesh, and Saka *et al.* (2018) with an isolation rate of 28% in Turkey also found higher isolation rates of *S. aureus* in milk and milk products than the current result. However, the current result is higher than the findings of Thaker *et al.* (2013) who determined *S. aureus* in 6.25% of milk and milk products in India.

The prevalence of the bacteria was the highest in milk (20.3%) followed by cheese (14.9%) in the current study. The lowest isolation rate was determined in yoghurt (4.8%). The rate was significantly higher in milk and cheese than in yoghurt (OR = 5.04 and 3.45 respectively) ($p<0.05$). However, statistically, the prevalence rate was similar both in milk and in cheese ($p > 0.5$) though figuratively the bacterium was more frequently isolated in the milk than in cheese. In agreement with the current findings, previous studies have also determined significantly highest *S. aureus* isolation rate in raw milk than in fermented dairy products (Al-ashmawy *et al*., 2016; Enquebaher *et al*., 2015; Lemma *et al*., 2021; Sadek & Koriem, 2020; Tarekgne, 2016; Thaker *et al*., 2013). However, Gebremedhin *et al.* (2022)and Zakary *et al.* (2011), determined that raw milk was found less contaminated by *S. aureus* than yoghurt and cheese.

There have been reports indicating that, the organic acids (lactic, acetic, propionic, and butyric acids) produced by lactobacilli in fermented dairy products like cheese and yoghurt are responsible for lower pH values of the products than of raw milk (Amênu, 2013; Girma & Aemiro, 2021; Nyamakwere *et al*., 2021). The acids are expected to inhibit the growth of bacteria in the fermented milk products though some pathogens could survive in the acidic

condition of the products (Girma & Aemiro, 2021; Medveďová *et al*., 2009; Ortiz-Rivera *et al*., 2017).

The isolation rate of *S. aureus* was found to be significantly higher in cheese than in yoghurt. This variation might be associated with the difference in the processes through which the products have been prepared and transported (Andualem & Geremew, 2014). In the study area, cheese has been processed from sour milk in such a way that the milk is heated approximately to 40-70°C for 15-25 min, left stat for 3-5 h to cool, and removing the whey through strainer, and the solid part (called *Badu*) is harvested. The product is transported to market using portable-sized containers, and in turn, transferred to the container of the purchaser.

Fermented milk products such as cheese and yoghurt were expected to affect the survival of *S. aureus* (El-Gawad *et al*., 2014; Felicio *et al*., 2015; Soliman & Ahmed, 2019). However, *S. aureus*, and other pathogenic bacteria such as *E. coli*, *Bacillus cereus*, *Klebsiella* spp., and *Enterobacter* spp., have been repeatedly isolated from traditional Ethiopian cheese (Andualem & Geremew, 2014; Bêrhe *et al*., 2017). Andualem and Geremew (2014) reviewed that the high bacterial load in raw milk which subsequently decreases in cheese due to low PH and heat, can in turn increase in the product due to bacterial entrance from the packaging, flavours, and handlers.

The prevalence rate of *S. aureus* in the samples with respect to other associated risk factors revealed that the rate per sample type did not significantly differ with the towns ($p > 0.05$). However, it was found varied with the sources of the samples (large dairy farm, small householder, and market/restaurant). The pathogen was highly prevalent in milk samples collected from the large dairy farm (46.7%) than from the smallholder (17.6%) and markets (restaurants) (0.0%). In contrast, it was isolated only from cheese and yoghurt obtained from the market at the rate of 22.2% and 14.8% respectively ($p < 0.05$). That is it was not identified in cheese and yoghurt samples collected from large dairy farms and smallholders. In agreement with the current study, Tarekgne *et al.* (2015), Gebremedhin *et al.* (2022) and Tarekgne (2016) found that *S. aureus* has been isolated with a higher rate in milk from large size dairy farms than in the small dairy farms or householders.

5.2.2. Hygeinic risk factors associated with contamination of milk and milk products

The risk factors associated with contamination of milk and milk products include the types of milk collecting and storage materials, rate of washing of the materials, the use of soap and hot water for washing the materials, and smoking and rate of smoking of the materials. Majority of the respondents (82.1%) of this study reported that they were using plastic materials for collection and storage of milk and milk products. In addition, most of the respondents responded that they have been washing the milking materials twice a day (92.14%), and smoking at least two days per week (68%). Higher number of them also reported that they have used hot water (93.57%) and soap always (37.14%) to wash the materials. Utensils used for milk collection, storage, processing and transportation are the main risk factors for safety of the products (Regasa *et al*., 2019). The milk collecting, storage and process practices have been considered the major determinant of post milking milk quality. Among these practices, smoking has been stated as a type of hindering the multiplication of microorganisms and hence used for preserving milk and milk products but for short period of time (Japaro, 2021; Mattiello *et al*., 2018). Various plant species have been used for smoking containers used for collecting and storing milk and milk products in Ethiopia. Among these plants, wooden plants such as Abalo (*Terminalia brownii*) and Cheba (*Acacia* spp) (Asresie *et al*., 2018; Seifu & Tassew, 2014) in Gojam, Ejersa (*Olea africana*), and *Juniperous procera* and *Ocium hardienes* in different parts of Ethiopia (Tadesse, 2016) have been mentioned.

In agreement with the current finding Gebremedhin *et al*. (2022) reported that majority of the farmers in Holeta town used plastic container for collecting and storage of milk and milk products. Similarly Berhe *et al*. (2020) and Ayele *et al*. (2017) respectively reported that majority of the farmers in Tigray and Sebeta used plastic containers for collecting, storing and transporting dairy products. However, Seifu and Tassew (2014) observed that most of the farmers in Bahir Dar Zuria and Mecha Districts used gourd material than other types of utensils for milk collection, storage and churning. Plastic containers have inherent characteristics which render them unsuitable for handling dairy products that they might be the area where the microorganisms can hide themselves and hence could be the sources of the contamination of the dairy broduct by pathogenic microorganisms (Ayele *et al*., 2017; Gebremedhin *et al*., 2022). The

plastic and gourd utensils may not be conducive for heat sterilization even though they are cheap and light for transportation (Ayele *et al*., 2017).

The usage of hot water and detergents for cleaning the milk containers are also important procedure to maintain the safety of the milk and milk products. As mentioned in the results section, majority of the respondents in the current study responded that they have been using hot water and soap for cleaning milking and storage utensils. In agreement with the result, Gebremedhin *et al.* (2022) determined that the farmers in Holeta town frequently used cold water followed by hot water and soap for cleaning the containers. Berhe *et al.* (2020) and Ayele *et al.* (2017) also determined that majority of the farmers used hot water and soap for cleaning utensils used for milk collection, transportation and storage. Similarly, Tadesse (2016) reviewed that many dairy farmers in Ethiopia used cleaning the utensils using hot water followed by smoking.

5.3. Virulence factors of the *S. aureus*

S. aureus produces various extracellular excretions which have been considered as virulence factors in many of the strains. Some of these productions are very important in the survival and hence retrieve in environments where the strains devoid of them are not able to exist. These productions include staphylokinase, lipase, proteases, and alpha-, beta-, delta- hemolysin, (Kumar *et al*., 2018) and enterotoxins (Raineri *et al*., 2022; Yaniarti *et al*., 2017).

5.3.1. Enzyme secretions

In this study, 92 *S. aureus* isolates- (43 from lactating cows, 24 from bulk milk, 4 from yogurt, and 21 from cheese) were tested for the production of protease on skimmed milk agar and of staphylokinase on heated-plasma agar. Accordingly, 71 (77.2%) and 51 (55.4%) of the isolates tested were protease and staphylokinase positive respectively. In this study, it has been determined that 71.83% of the protease positive were found producing staphylokinase. The result also revealed that the staphylokinase was produced at rate of 67.4%, 50%, 25% and 42.9% in the *S. aureus* isolates from the strains isolated from lactating cows, bulk milk, yogurt and cheese respectively. The finding agrees with the result of the investigation made by Adame-Gómez *et al.* (2020). Adame-Gómez *et al.* (2020) reported that 57.8% of *S. aureus* of human foods strain harbored the saK gene which codes for staphylokinase. Similarly Wieckowska-Szakiel *et al.*

(2007) found that more than 62% of the then tested *S. aureus* could contain secreted type of staphylokinase. Zheng *et al.* (2022), and Tam and Torres (2019) reviewed that gene sak, encoding staphylokinase, is conserved in the majority of *S. aureus* strains. Relatively high percentage (60–95%) of human strains of *S. aureus* produce staphylokinase than other strains (Peacock, 2006). Staphylokinase and other virulence factors such as the staphylococcal complement inhibitor proteins, and chemotaxis-inhibitory proteins are mostly carried by prophage gene which is common in human strain so that the proteins are rare in animal strains (Tam & Torres, 2019; Vandenesch *et al*., 2012).

Haghkha (2003) stated that almost all strains of *S. aureus* produce staphylokinase. Staphylokinase, which is also known as staphylococcal fibrinolysin or Mfiller's factor, is a 15.5 kDa protein consisting a 136 amino acids produced by lysogenic strains of *Staphylococcus aureus* and non-aureus at the late exponential growth phase (Adam *et al.*, 2016; Alzahrani & Shawky, 2020; Bokarewa *et al.*, 2006; Hachim *et al.*, 2020; Srinivasan *et al.*, 2013). It is the extracellular protein, detected as a released molecule in supernatants of bacterial cultures and/or attached to the cell surface of bacteria (Bokarewa *et al.*, 2006). It is a fibrin-specific plasminogen activator transforms plasminogen into the active proteolytic and fibrinolytic enzyme plasmin, which can dissolve fibrin clots (Mohanasrinivasan *et al.*, 2014).

The enzyme can be encoded by the bacteriophage (Becker, 2018; Mohanasrinivasan *et al.*, 2014; Schelin *et al.*, 2011) or plasmid pESak (Nguyên & Quyen, 2012) in the *S. aureus*. Staphylokinase's ability to cause plasminogen activation and the consequent cleavage of host-derived fibrin is thought to be the basis for the pathogenicity of the bacterium (Zhêng *et al.*, 2022). It is suspected that its localized fibrinolysis might aid the bacterial spreading (Rohilla, 2010). Furthermore, scientists stated that staphylokinase serves the bacteria to overcome the bacteriostatic effects of antimicrobial peptide (Nguyen & Vogel, 2016). Kwiecinski *et al.*, (2013) found the dual roles of staphylokinase in *S. aureus* skin infections that it indorses the bacteria to

establish the infection while decreasing disease severity. Staphylokinase accelerates healing by draining the infected lesions on the skin surface; at the same time, this drainage might allow bacteria to spread to new sites on the skin. In strengthening of this, Becker (2018) also pointed that with the expression, of the staphylokinase gene (Sak), *S. aureus* is able to activate human plasminogen into plasmin, a fibrinolytic protease, and, thus, to degrade present clumps that results into dissemination of separated cocci. Furthermore, according to Deepa *et al.* (2019), staphylokinase (Sak) increases the proteolytic activity of bacteria, which damages tissue and increases bacterial invasiveness. Staphylokinases and coagulases are cofactors that hijack the coagulation system of the host oppositely. This characteristics of the enzyme categorize it as one of the immune evasion factors; the others include chemotaxis-inhibitory proteins, and staphylococcal complement inhibitor proteins. Staphylokinase is a cofactor, that has no enzymatic activity by itself; but it can activate host zymogens (Tam & Torres, 2019).

From the fact of the fibrinolytic character of staphylokinase (SAK), since resent, staphylokinase has been emerging as a crucial thrombolytic medication for the treatment of individuals with cardiovascular disease that is a promising blood clot dissolving agent (Nguyen and Quyen, 2012, Srinivasan et al., 2014; Chandrappa et al., 2017).

In the current study, the screening of the lipase producing ability of the *S. aureus* isolates revealed that 62(67.4%) of them were lipase positive. Lipase is one of the extracellular active substances produced by strains of *S. aureus* (Tsepo *et al*., 2016). Lipases play important roles during infection (Kumar *et al*., 2021). It has been considerd as a virulence factor of the pathogen by which the bacteria can escape both innate and adaptive immune responses of the host (Divyakolu *et al*., 2019). Bacterial lipase activity on mammalian lipids and phospholipids can promote release of free fatty acids from lipid stores, detoxify antimicrobial lipids, and facilitate membrane dissolution (Chen & Alonzo, 2019). Lipase produced by *S. aureus* has been demonstrated to interfere with the host granulocyte functions and increase the survival of the

bacteria during the host defense by inactivating the bacteriocidal lipids (Chen & Alonzo, 2019; Hu *et al*., 2012). In addition, *S. aureus* lipase (SAL), which belongs to the glycerol ester hydrolases, shows strong cytopathicity to host cells (Tanaka & Kitadokoro, 2018). Apart from this, lipolysis caused by bacterial lipases is of considerable concern to the dairy industry. Lipase can degrade the milk fats essentially triglycerides, to short-chain fatty acids that might be unstable (Alshaibani, 2020). In accordance with the current finding, Alkhafaji *et al.* (2019) reported that the isolates obtained from milk (65.7%), yogurt (75%) and cheese (71.4%) were found positive to lipase.

However, little attention has been given to the importance of the lipase enzyme in dairy products that little recent scientific researches have been conducted on the staphylococcal lipase in dairy products. Few researches performed on the isolates of *S. aureus* obtained from clinical infection of human reveled that considerable number of the isolates were able to produce the enzyme. Accordingly, Orjiakor *et al.* (2020) reported that all of the 26 (100%) *S. aureus* isolates from surgical wound culture were lipase positive. Similarly, El-baz *et al.* (2016) also report that 71.1% of 135 isolates from inpatients were lipase positive.

Regarding the factors determining the the staphylokinase and lipase positivity of the *S. aureus* isolates in the current study, it was determined that the enzymes were produced by the isolates regardless of the sample types from which the isolates were obtained. The staphylokinase positivity of the current finding (55.4%) was greater than the finding of El-baz *et al.* (2016) (41.5%). The result revealed that 37% isolates were found producing both staphylokinase and lipase. The multi-enzyme positivity of the isolates was not found varied with the types of the samples from which the isolates were obtained *($p>0.05$).* This study determined that 76.1%, 50%, 85.7% isolates from milk, yogurt and cheese samples respectively were found producing protease. Alkhafaji *et al*. (2019) reported *S. aureus* isolates from cheese produced lipase and protease. However, the isolates of the same species from meat and meat products found positive for protease but not for lipase. Gündoğan and Devren (2010) also determined that 87.16% of proteolytic but only 7.4% lipolytic *S. aureus* isolated from meat.

5.3.2.Enterotoxogenicity of Staphylococcus aureus isolates

The strains of *S. aureus* produce two types of toxins namely enterotoxins and toxic shock syndrome toxin (TSST -1) (Rohilla, 2010) (Lee *et al*., 2012). These toxins are collectively called pyrogenic toxin super antigens (PTSAgs) that they induce proliferation of T cells regardless of antigenic specificity (Shettigar & Murali, 2020). The supper antigenicity of the toxins is responsible for a variety of pathological conditions including pneumonia, soft tissue infections, toxic shock syndrome, and infective endo-carditis (Elsherif *et al*., 2020).

Staphylococcal enterotoxins are extra-cellular proteins produced by different species of genus *Staphylococcus* including the strains of *S. aureus* (Seo, 2016). They are stable and resistant to proteolytic enzymes including the digestive enzymes as the consequence of which they are remained active in the digestive tract after ingestion (Ortega *et al.*, 2010). They cause gastrointestinal toxicity and have super-antigenic activity. Their enterotoxic activity can result in vomiting and diarrhea when ingested orally and are responsible for staphylococcal food poisoning (Seo, 2016).

The super-antigenic activity of the enterotoxins occurs when they are expressed systemically and is characterized by induction of T-cells to produce cytokines without control that result in toxic shock syndrome (Rohilla, 2010). The toxic shock syndrome is manifested by shock and fever (Seo, 2016; H. Zhang et al., 2016). However, TSST-1 is a supper antigen toxin which is known in causing toxic shock syndrome (Shettigar & Murali, 2020) but have no emetic activity (Ortega *et al*., 2010). The other member of the SAg family is SEI-X and it causes necrotizing pneumonia (Shettigar & Murali, 2020). Since they are supper antigens, they can also trigger the proliferation of T-cells and anti- body presenting cells as the enterotoxin do (El-baz *et al.*, 2016). TSST-1 can also cause allergic and autoimmune diseases (Ortega *et al.*, 2010).

It has been considered that enterotoxin-producing-staphylococci are coagulase and/or thermonuclease positive; so that enterotoxigenic *S. aureus* should be positive for the production of a thermonuclease, or thermostable nuclease (Paulin *et al*., 2012). Although not all enterotoxin genes harbored by strains of *S. aureus* are expressed, scholars have suggested that detection of enterotoxin gene should be taken in consideration in determination of food borne diseases (Gencay *et al*., 2010). The *S. aureus* isolates were tested for harboring the enterotoxin genes.

Accordingly, in the current study the isolates were tested for holding *sea*, *seb* and *tsst-1*which code for staphylococcal enterotoxin type A (SEA), staphylococcal enterotoxin type B(SEB), and Toxic shock syndrome toxin-1(TSST-1) respectively. From a total 24 isolates tested, 12 (50%) held *sea*, 3(12.5%) seb, and 4(16.6%) tsst-1 genes. In the current study, *sea* gene was the most frequently identified genes of the three genes. Enterotoxin type A gene (*sea*) has been considered as the most frequent types of enterotoxin genes in staphylococcus (Adame-Gómez *et al*., 2020). In agreement with the current study, many studies have determined the dominance of *sea* in various *S. aureus* isolates obtained from various samples (Saka & Terzi Gulel, 2018; Seyoum et al., 2016). In a study conducted by Seyoum *et al.*, (2016) (2016) in Selale, Asela, Debre-Zeit, and Addis Ababa of Ethiopia, majority of the isolates (36.7%) held *sea* followed by see (18; 16.5%), seb (17.4%), tsst (16; 14.7%). Adame-Gomez *et al.* (2020) in Mexico also found that 53.1%, 3.1% and 9.3% of *S. aureus* isolates were positive to *sea*, *seb* and *tsst-1* genes respectively. Allaion *et al.* (2022) in Brazil determined that *sea* was the highest gene identified among the classical enterotoxin genes (*sea*, *seb, sec,* and *see*). Rall *et al.* (2008) in Brazil also found that majority (41%) of the isolate of *S. aureus* from raw and pasteurized milk contained *sea*. The former study also revealed seb in 7.7%, sec in 20.5%, sed in 12.8%), and see in 5.1% of the isolates. In addition, Carfora *et al.* (2015) in Italy and Saadat *et al.* (2014) in Iran reported *sea* gene at rate of 12.96% and 31.4% respectively of *S. aureus* isolates from milk and milk products. Chen *et al.* (2018) in Taiwan also identified *sea* genes in 17.26% of the *S. aureus* isolates obtained from food and human patients. Nazari *et al.* (2014) in Iran determined that *sea* found the most frequent enterotoxin in *S. aureus* that it was identified in 30.7% of the isolates followed by *Seb* (26.9%) and Sed (15.37%). Rosengren *et al.* (2010) in Sweden found that *sea* and *sec* are the most frequently determined genes in *S. aureus* isolated from cheese. Filipello *et al.* (2019) in Italy found *sea* to be the most frequent (52.72%) enterotoxin genes identified in 110 isolates of *S. aureus* obtained from traditional dairy products in Italy. The same study also reported seb in 9.1% of the isolates. Sharma *et al.* (2017) in India reported *sea* and *seb* respectively in 10% and 3.3% of *S. aureus* obtained from bovine raw milk.

The result of this study indicated that 13(54.2%) of the total 24 isolates tested, were found positive to at least one of the enterotoxin genes. In agreement with the current study, Seyoum *et al.* (2016) reported that 66.9% of the *S. aureus* isolated from cow milk harbored at least one of *sea, seb, sec-1, sed, see* and *tsst-1,* and 35.6% of the isolates harbored multiple enterotoxin

genes. Similarly, Rall *et al.* (2008) and Saka and Gulel (2018) found that, , 68.4% and 12% respectively of *S. aureus* from milk and dairy products were positive for at least one of staphylococcal enterotoxin (SE) genes. Likewise, Klotz *et al.* (2003) identified the enterotoxins in 47.3% of 93 *S. aureus.* Wu *et al.* (2018) searched the distribution of classic enterotoxin genes (*sea-see*) in isolates of *S. aureus* from retail vegetable in China, and found at least one of them in 23.26% of the isolates. However, Aragão *et al.* (2019) did find none of the enterotoxin genes (*sea, seb, sec, sed, see*, or *tsst-1*) in *S. aureus* isolates from cheese of goat milk. Allaion *et al.* (2022) reported that among 136 isolates of the *S. aureus*, 94% of them harbored at least one enterotoxin genes. Nazari *et al.* (2014) identified that 80.7% of *S. aureus* from milk and milk products were positive to at least one enterotoxin genes. Filipello *et al.* (2019) found 67% of *S. aureus* isolates from traditional dairy products were to be positive to at least one of the 29 different types of enterotoxins including *sea* and *seb*. Rosengren *et al.* (2010) reported enterotoxin genes in 70% of *S. aureus* obtained from cheese.

Furthermore, Al-Ashmawy *et al.* (2016) indicated that the antibiotic resistance of *S. aureus* could be associated with enterotoxogenicity of the strains. Studies have determined that *sea, seb, sed, seg, sei, sej* and *eta* (exfoliative toxin A gene) are the 7 most frequently detected enterotoxin genes in MRSA isolates; whereas *tsst-1* and *sec* are most frequent in methicillin sensitive *S. aureus* (MSSA) (Sila *et al*., 2009). According to Al-Ashmawy *et al.* (2016), all (100%) of the 106 MRSA isolates from milk and milk products were positive to *sea, seb*, and *sec*. Similarly Kwon *et al.* (2005) determined all (100%) of 14 MRSA strains obtained from bovine milk harbor sed, sei and sej enterotoxin genes. More importantly, studies have estimated that more than 90% of LA-MRSA ST9 strains hold at least one enterotoxin genes, and also high rate of *tsst-1* gene with negative expression rate (Butaye *et al*., 2016). Similarly Rodríguez-Lázaro *et al.* (2017) revealed that 61.6% of MRSA strain from dairy product and 11.5% of them from meat harbor at least one of eight enterotoxin genes (*sea, seb, sec, sed, seg, seh, sei, sej*). The *sea* was detected in 23.8% and 20% of MRSA from dairy product and meat respectively; whereas, *seb* was determined in 14.3% and 20% of the strain from dairy product and meat respectively. Other studies have also identified enterotoxin genes in MRSA strains obtained from dairy products (Arcuri *et al*., 2010; Günaydın *et al*., 2011; Murphy *et al*., 2010; Neder *et al*., 2011). Doulgeraki *et al.*, (2017) and Khairullah *et al.* (2020) also stated that MRSA strains are commonly known in carrying enterotoxin genes.

S. aureus strains has been studied producing more than one enterotoxin (Omoe *et al*., 2005). The current study revealed that some of the isolates contain more than one toxin genes such that 1(4.2%) held *sea* and seb, and 3(12.5%) harbored *sea* and *tsst-1*. But none of them were found holding seb and tsst-1 genes simultaneously. In agreement with the current findings, studies have found multienterotoxin genes in strains of *S. aureus*. Rall *et al.* (2008) also determined that 64%, 23%, 5% and 7.7% of the *S. aureus* harbored one, two and three (*sea* + *sec* + *seh*) and four (*sea* + *seg* + *sei* + *sej*, *sea* + *sed* + *seg* + *sei* and *seb* + *seg* + *seh* + *sei*) enterotoxin genes respectively. Seyoum *et al.* (2016) also found that 35.6% of the strains of *S. aureus* isolates from bovine milk harbored more than one enterotoxin genes that 7.3% harbor *sea+seb*, and 0.92% *sea+seb+sed+tsst-1*, 1.8% *sea+sec+sed+tsst-1* and 0.92% *sea+sec+see+tsst-1*. Allaion *et al.* (2022) reported that 80.7% more than one enterotoxin genes. Blaiotta *et al.* (2004) also determined that 11 of 109 (10%) of *S. aureus* strains harbored SEs and/or TSST1 genes. Similarly Chen *et al.* (2018) reported that the *S. aureus* isolates from food and human patients harbored *sea* at rate of 48.28%, *sea+sec* at rates of 31%, *sea+ seb* at rates of 13.8%, *sea+seb + sec* at rates 3.4% and *sea, seb* and *sed* at rates of 3.4%. Similarly, Fooladi *et al*. (2010) reported that 31.1% of the *S. aureus* isolated from dairy products were positive to at least one of *sea* and seb genes that 15.6% harbor *sea*, 9.3% harbor seb and 6.2% both *sea* and *seb*. Klotz *et al.* (2003) in their study on 93 isolates of *S. aureus*, determined that 37 (39.8%) were positive to only one enterotoxin, 4(4.3%) were positive to two genes (*sea + seb*, *sea + sec1*, *seb + sec1*, and *sec1+sed*), 1(1.08%) were positive to three genes (*seb+sec1+sed*). Gencay *et al.* (2010) found *sea* genes in only 2.9% of *S. aureus* obtained from poultry meats but none of seb-see and seg-sej genes. Filipello *et al.* (2019) determined that *sea+seb* in 0.91% of 110 of *S. aureus* from traditional dairy products. As Nazari *et al.* (2014) reported, of 52 *S. aureus* isolates, 53.8% carried one enterotoxin, 15.38% harbored two enterotoxin genes, 11.53% held three enterotoxin genes.

Regarding the types of the sample in the current study, 5(50%) of isolates from milk obtained directly from lactating cows, 1(16.7%) of isolates from bulk milk, 1(100%) of isolates from yogurt, and 6(85.7%) of isolates from cheese harbored at least one enterotoxin genes. Toubar *et al.* (2018) found that 33.3%, 50%, and 40% of *S. aureus* from cheese, milk and yogurt respectively harbored at least one of *sea* and *seb* genes. Rahimi (2013) tested enterotoxogenicity of *S. aureus* isolated from traditional and commercial dairy products and found that 80% of 20

isolates were positive to at least one of nine (*sea-see* and *seg-sej*) enterotoxin genes. Aydin *et al.* (2011), enterotoxin genes in *S. aureus* isolates from meat and meat product (100%), raw milk (49.2%), dairy products (66.67%), and bakery products (55.56%) and ready-to-eat foods (50%).

In the current study, the *sea* gene was identified in isolates obtained from lactating cows, bulk milk, yogurt and cheese at rates of 50%, 16.7%, 100% and 71.4% respectively. Similarly, tsst-1 gene was found harbored by 10% of isolates of lactating cows, and by 42.9% of that of cheese samples. Fooladi *et al.* (2010) also identified 15.6% and 9.3% of the *S. aureus* isolates possessed the *sea* and *seb* genes respectively. However, seb was determined only in the isolates obtained from lactating cows. Hoque *et al.* (2018) identified *sea* in 11.72% (8.33% in isolates of clinical mastitis and 24.71% in isolates of subclinical mastitis) and *seb* in 11.03% (6.67% in isolates of clinical mastitis and 14.12% in isolates of subclinical mastitis) of *S. aureus* obtained from bovine mastitis. Aydin *et al.* (2011) found *sea* genes in 7.7%, 9.7%, and 11.1% in *S. aureus* strains isolated from meat, raw milk and dairy product respectively. Ahmed *et al.* (2021) reported that none of the meat isolates and 10% of cheese isolates of *S. aureus* harbored *seb* gene but could not find the same gene in poultry meat. Allaion *et al.* (2022) have also determined that 11.6% and 3.1% of the enterotoxin gene positive isolates contained *sea* and *seb* respectively. However, Günaydın *et al.* (2011) reported *tsst-1* in 5.4% of but did not find *sea* and *se*b in *S. aureus* isolates from subclinical mastitis.

The secretion of the enterotoxins by the strains of the *S. aureus* is considered as the important virulence factor in the zoonotic characteristics of the bacterium. As the result, the bacterium has been incriminated in causing food poisoning as an important zoonotic pathogen (Aragão *et al*., 2019; Feng *et al*., 2008; Islám *et al*., 2016; Lowy, 2003).

Milk is considered as an important nutrient for growth, and enterotoxin production of *S. aureus* (Rall *et al*., 2008). In addition, Butaye *et al.* (2016) reviewed that the cows are the main sources of SEs positive strains of *S. aureus* than the dairy owner's hands. Cebeci *et al.* (2021) also stated that the main sources of food intoxications are dairy products even though meat and meat products can be the sources. It has been repeatedly state that bovine mastitis might be associated with enterotoxins or superantigens production of *Staphylococcus* species (Artursson *et al*., 2016; Rall *et al*., 2014; Schabauer *et al*., 2018). Fursova *et al.* (2020) and Moges *et al.* (2012) reviewed

that *S. aureus* isolates from mastitic cow can produce enterotoxins. Researcher have mentioned *sea* as one of the enterotoxin gene associated with bovine mastitis. The studies determined the gene in 23 to 50% of *S. aureus* isolates from mastitis cow (Rall *et al*., 2014). However, Akineden *et al.* (2001) and Aragão *et al.* (2019) could not found the enterotoxin genes in the *S. aureus* isolated from mastitis cow and artisanal goat cheese respectively.

5.3.3. Antibiotic resistance characteristics and resistance genes

In the current study *S. aureus* isolates were found to be resistant to several antibiotics. High resistance was seen to penicillin (100%), amoxacillin (100%), oxacillin (100%), tetracycline (100%), oxy-tetracycline (100%), ampicillin (83.3%) and to norfloxacin (75%). However, they were highly susceptible to clindamycin (100%), cefoxitin (100%) and ciprofloxacin (100%). They also shown relatively high susceptibility to gentamycin (91%), trimethoprim/ sulfamethoxazole (80%), and erythromycin (75%). The isolates were seen strongly resisting against antibacterial groups, which are commonly used in both veterinary and public medications in the study area. Among these drugs, the penicillin and tetracycline groups account for the front line antibiotics in the veterinary medication in Ethiopia in general and in the study area in particular.

In accordance with the current study, there were studies conducted in Ethiopia which reported that *S. aureus* isolates from dairy animals, and milk and milk products were highly resistant (more than 70%) to different β-lactam class of antibiotics (Aberá *et al*., 2010; Aki *et al*., 2017; Balemi *et al*., 2021; Emeru *et al*., 2019; Gırma & Tamir, 2022; Girmay *et al*., 2020; Grimá *et al*., 2021; Marama *et al*., 2016; Tesfáye *et al*., 2021; Tibebu *et al*., 2021).

In the current study, a total of 34 isolates were tested for *balZ* (penicilinase coding) and *nuc* (thermo-endonuclease coding) genes and all contained *nuc* gene but none of them harbore *blaZ* gene. The gene mediates β-lactame antibiotic resistance.i.e. its product hydrolyze beta-lctam ring of the beta-lactum antibiotics (Harkins *et al*., 2017; Lowy, 2003). However, as indicated above, all the tested isolates were resistant to peninicillin but did not harbore the gene. Such phenomenon might happen when there is change in the sequences of the primer to target the region properly. That is the target gene might be altered due to mutation of the gene (Lucas *et al*., 2021).

Various researchers in Ethiopia with highly variable rates have also reported the tetracycline group resistance patterns of *S. aureus* isolates from different animal origin samples. Girmay *et al*. (2020) reported 47.61% oxy-tetracycline resistant. In addition, Belachew (2016) Mekonnen *et al.* (2018) and Emeru *et al.* (2019) reported 100%, 54% and 15% respectively resistance to tetracycline.

The antibiotic resistance characteristics of *S. aureus* have also been reported by previous researches from different parts of the world. Islam *et al.* (2016) reported that majority of *S. aureus* isolated from raw milk in Bangladesh were resistant to tetracycline (73.33%), and erythromycin (73.33%) but susceptible to gentamicin (100%) and ciprofloxacin (93.33%). Ahmed *et al.* (2021) from Sudan determined that isolates of *S. aureus* from cheese, milk, meat, and fish were found susceptible to gentamycin at rates of 100%, 87.3%, 86.7% and 100% respectively. The same study reported also that the isolates from aforementioned samples were sensitive to ciprofloxacin at rates of 100%, 98.4%, 100% and 100% respectively. Likewise, the findings of Thaker *et al.* (2013) who have conducted a susceptibility test on *S. aureus* isolates from milk and milk products in India, agreed with the current study. The research determined that 100% of the isolates were resistant to penicillin but 80% were susceptible to ciprofloxacin. In contrast the study was not in line with the current study in that only 20% of the isolates were resistant to oxytetracycline. Mekuria *et al.* (2013) also assessed the drug resistance profile of *S. aureus* recovered from milk samples of dairy cows and nasal swab from dairy farm workers in Ethiopia, and reported that highest resistance was observed against penicillin (92.2%) followed by tetracycline (66.7%) but less against lindamycin (17.6%), rythromycin (23.5%), entamycin (19.6%) and against sulphamethoxazole-timethoprim (21.6%). Kelman *et al.* (2011) reported a result that *S. aureus* from ground meats in Nepal had a resistance pattern of 0% to ciprofloxacin, 4.5% to clindamycin, 8% to erythromycin, 1.5% to gentamicin, 26% to penicillin, and 69% to tetracycline.

Moreover, *S. aureus* isolates from different environmental and plant product samples have been assessed for their susceptibility against different antibacterial agents. The results of the studies revealed varying degree of resistance to the antibiotics. Accordingly, Wu *et al.* (2018) determined that the isolates from vegetables were resistant to penicillin (93.3%), Gentamicin (16.7%), Erythromycin (40%), Tetracycline (43.3%), Ciprofloxacin (23.3%),

Trimethoprim/sulphamethoxazole (0.00) and Clindamycin (23.3). A research in Ethiopia conducted on isolates of *S. aureus* obtained from cockroaches showed that all were resistant to penicillin (100%), but susceptible to tetracycline, ciprofloxacin, gentamicin, ciprofloxacin, and erythromycin (Moges *et al*., 2016). Similarly the study conducted by Solomon *et al.* (2018) in Ethiopia revealed that 53.3% of the *S. aureus* isolates from cockroaches were resistant to Oxacillin (MRSA), and 33.3% to Vancomycin.

The current study screened the *S. aureus* isolates for methicillin resistance by using cloxacillin supplemented manitol salt agar (MSA). The modified medium was found successfully screened MRSA that all isolates, which were able to grow on the medium, were further tested by using oxacillin disc and PCR, and were found resistant to oxacillin and harbored the *mecA* gene.

Accordingly, Ninety-two (92) *S. aureus* were checked on the modified MSA and twelve (13%) grew on the plate. All of the MRSA suspected isolates were found resistant to oxacillin and harbored *mecA* gene. In this regard, the result is in agreement with the report of Croes *et al.* (2009) who identified MRSA specific *mecA* gene in 100% of MRSA strains.

MRSA strains from milk and milk products as well as mastitis cow have been reported at range of 8% to 98% of *S. aureus* isolate in Ethiopia. For example, Borena *et al.* (2023) reported MRSA at rate 98.48% in isolates from mastitis cow in Ambo and Bako towns, and Aki *et al.* (2017) reported at rate of 74.11% in isolates from mastitic lactating cows in and around Assosa town. Other studies also determined the proportion of MRSA at higher proportion than the current report (Girmay *et al*., 2020; Lemma *et al*., 2021; Tesfáye *et al*., 2021). However, Tibebu *et al.* (2021) in Bishoftu dairy farms reported at less rate (8%) than the current finding. Similarly MRSA in mastitic dairy cow, bulk milk and milk products has been reported from various countries at different prevalence rates (Badua *et al*., 2020; Hamid *et al*., 2017; Haran *et al*., 2012; Hoque *et al*., 2018; Shrestha *et al*., 2021)

In total, 46 of the isolates were tested *mecA* (gene responsible for methicillin resistance) gene and 10(21.74%) of them were found bearing the gene. The current finding is in accordance with the report of Khán *et al.* (2020) which determined 20% of *mecA* gene in 20% of the *S. aureus*. Mec A gene can also be found in other species of staphylococcus such as *S. lugdunensis* and *S. pseudointermedius (MRSP)* (Buller *et al*., 2014; CLSI, 2009).

5.3.4. Multi drug resistance characteristics of S. aureus

The current study determined that all the tested *S. aureus* isolate were resistant to at least one antibacterial agent. It was determined that 33 (71.74%) of the 46 isolates were found resistant to three antibiotics and 5(10.87%) were resistant to nine of the ten antibiotics. The multiresistant characteristics of the *S. aureus* is great warning for the medical fields that the pathogen may be in the ways of loosing effective antibiotics against the infection caused by the microbes.

The multidrug resistance (MDR) index is an important value calculated to determine the antibiotic resistance pattern and health risk of the pathogen. The MAR-index value of ≥0.2 indicates multidrug resistance of the tested pathogen (Afunwa *et al*., 2020). In the current study all tested isolates have MAR-index of >0.2. which ranged between 0.33 and 0.90 and the values are greater in MRSA strains which were 0.7 to 0.9. Studies have also revealed that the MAR-index values were greater in MRSA strains of *S. aureus* isolates (Sadat *et al*., 2022).

S. aureus uses several resistance mechanisms for different classes of antibiotics. The pathogen enzymatically hydrolyses β-lactam rings in β-lactam antibiotic, and modifies dalfopristin in quinupristin-dalfopristin (Q-D) antibiotics, reduces affinity to penicillin binding proteins in β-lactam antibiotic (particulary in methicillin resistant), to vancomycin, and to dihydrofolate reductase (DHFR) in oxazolidinones, and to enzyme-DNA complex in quinolones , and traps the antibiotics (particularly Vancomycin) in Glycopeptides antibiotics. In addition, the bacterium resists to aminoglycosides (eg. gentamicin) by modifying the antibiotics using acetylating and/or phosphorylating enzymes. Some strains of the bacterium perform over production of p-aminobenzoic acid by enzyme for resisting to trimethoprim- sulfamethoxazole, mutations in the QRDR region for resisting to quinolones, and reducing the binding of the antibiotic to the 23S ribosomal subunit for Quinupristin- dalfopristin (Q-D) resistance (Lowy, 2003). Furthermore, *S. aureus* develop resistance to tetracyclines using various mechanisms including, protection and modification of binding site, active efflux pump, and drug modification (Yılmaz & Aslantas, 2017).

6. CONCLUSION AND RECOMMENDATIONS

Based on the results and discussion of the study, it is concluded that mastitis continued to be one of the major constraints milk production in the study area. As age of the animal adivances the animal is likely to develop subclinical mastitis than clinical mastitis. The clinical mastitis more occurs in association with tick infestation. Bovine mastitis was found to be considerably distributed in the study area, regardless of the agro-ecological variation of the areas. The breeds and ages of the animals could not be the risk factors for t the prevalence mastitis in the lactating cows. The intensive farming system might be one of the determinants for the high prevalence of the mastitis due to unhygienic management systems. The tick infestation could not only exacerbate the development of clinical mastitis but it might also be one of the major risk factors for the high prevalence of masititis in general in the lactating cows. Washing milkers' hands and the udder of the animal before milking were found to be good practices to reduce the prevalence rate of mastitis. Furthermore, it has been determined that poor hygienic milking practices such as hand milking, using unwashed milkers' hands, and the absence of udder cloth for drying wet udders play significant roles in the distribution of the mastitis-causing pathogens among the quarters and animals.

S. aureus was significantly prevalent in mastitis positive than in the mastitis negative lactating cows that the pathogen might be one of the major etiology of mastitis. In addition, the isolation of the *S. aureus* from non-mastitis cows indicated that the bacterium is the normal flora of the teat canal and skin. The presence of *S. aureus* particularly in fermented milk (yogurt and cheese) can either be due to environmental contamination or to the fact that the bacterium can resist the acidic pH of the products. The unsafe milk collection and storage practices such as using plastic utensils for milk collection and storage, and unsmoked containers might enhance the crosscontamination and multiplication of the pathogens in milk and milk products.

A considerable number of *S. aureus* isolates were found to produce enzymes such as staphylokinase, lipase, and protease, and some of them produced two enzymes. Moreover, majority of the isolates tested for the susceptibility of commonly used antibiotics showed resistance to the antibiotics particularly to betalactams and tetracycline group. This might be one

of the main indicators for the high virulence of the pathogen to cause serous and invassive infections in the host, and the commonly used antibiotics might not cure the infections.

Moreover, the isolates were determined to harbor the enterotoxin genes tested *(sea*, *seb* and *tsst-1)*. This summed up with the unhygienic milk collection and storage could also be the major warning for the possibility of foodborne poisoning in the study area.

A significant prevalence of MRSA was also obtained in this study. The multidrug resistance characteristics of the isolates revealed that all the tested isolates had >0.2 MDR index values, which is indicative of the Multidrug resistance (MDR) characteristic. The index value is higher in the MRSA strains than in the methicillin-susceptible *S. aureus* (MSSA). In both MRSA and MSSA strains, gentamycin was found to be the drug of choice. However, in MSSA, ciprofoxacin, trimethoprim/sulfamethoxazole, cefoxitin, erythromycin, and clindamycin were the antibacterial agents that showed good effectiveness against *S. aureus*.

Based on the above conclusion, the following recommendations can be forwarded:

- A combination of mastitis prevention and control methods should be practiced.
- The local farmers should be trained on the economic importance of mastitis and prevention methods.
- Subclinical mastitis diagnostic methods should be familiarized with the local professionals and, if possible, in the farmers.
- Subsequent research should be conducted on the *S. aureus* isolates to determine the genetic and phenotypic characteristics associated with the *blaZ* gene and penicillin resistance.
- Antibiotics that are most effective against the isolates should be used both in veterinary and public medicine.
- Alternative antibacterials, particularly non-chemical antibiotics such as bacteriophage and bacteriocins, need to be formulated.
- The local community should be aware of hygienic practices to reduce mastitis prevalence in bovine and contamination of milk and milk products.
- Training with a focus on the safety and handling of dairy products should be given.

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ANNEXES

Annex 1: Structured Questionnaire of milking and milk storage practices

Addis Ababa University

College of Natural and Computational Science

Department of Biology

A. English Verssion

I. Questionnaire for Milk Handlers

This questionnaire is prepared to assess the knowledge of milk handlers about safe handling of milk and/or milk products in aspect of food safety. You are kindly requested to listen or read (for veterinary professionals) the questions carefully and respond clearly in the manner indicated for each question. The information you provide is very crucial for the success of the study. You are, therefore, kindly requested to be honest toward all the items provided. Your responses will only be used for the research purpose and therefore be kept confidential.

Date: _____ Sample ID: _____

1. Demographic Characteristics of the Owners

Address: _____

Name _____ Business Name (Where applicable)

1. 1. Sex: Male □ Female □

1.2. Age _____

1. 3. Education Status: Illiterate □ Elementary □ Secondary □ College Diploma and above □

2. Economic and dietary importance of Milk and Milk Products

2.1. What is your major means of income? Dairy farm□ Other □

2.2. Which milk products do you sell? Raw milk□ Yogurt□ Cheese□ None□

2.3. To whom do you sell the milk and/or milk products? To processing plants□ To the local consumers□ To restaurants□ To others (specify) _____

2.4. Do you and/or your family consume raw milk? Yes □ No □

2.5. Do you and/or your family consume yogurt and/or cheese? Yes □ No □

3. Hygienic and preservation practices

3.1. Which milking methods do you use to milk your cow? Hand □ Machine□

3.2. Does milker wash his/her hands before milking? Yes □ No □

3.3. Does the milker wash udder before milking? Yes □, No□ ,

3.4. Does milker wash his/her hands with interval of milking each cow? Yes □ No □

3.5. Has milker milking clothes clothed only during milking time? Yes □ No □

3.6. How often does milker washes cloth(s) used for milking? Daily □ , 2-4 per week□ , weekly □, More than week□

3.7. Do you use cloth/fabrics to dry the udder? Yes □No □

3.8 If your answer for Question 3.8 is “Yes” do you use the clothe for more than a cow? Yes □ No□

3.9. If your answer for Question 3.9 is “Yes” how frequent do you wash the cloth?

With each milking interval □; Twice daily □; Daily □;More than two days □; Not at all □

3.10. If you are using the towel for individual cow, how often do you wash it?

Twice per day □; Daily □; More than two-day interval □; Not at all □

3.11. What types of Milking Utensils do you often use for milk collection from the cow?

Plastic □; Metal □; Wooden or Calabash □; Other _____

3.12. What types of Milking Utensils do you often use for storage milk and milk products?

Plastic □; Metal □; Wooden or Calabash □; Clay □; Other_____

3.13. Do you clean the milk equipment? Yes □;No □

3.14. If your answer for Q. 3.14 is yes how often do you clean them? Twice per day□; Daily□; Once per week □

3.15. Do you smoke the milk equipments? Yes □; No □

3.16. If your answer for Q. 3.16 is yes, at what interval do you smoke? Daily □;Two days per week□; 3-4 days per week□; Weekly □

3.17. Do you use warm water to clean milk equipments ? Yes □; No □

3.19. Do you use soap to wash milk equipments? Yes □; No □

3.19 Have you ever observed food poisoning symptoms following consuming milk and/or milk products? Yes □; No □

B. Afan Oromo version of the questionnaire for Milk Handlers

I. Gaaffilee Namoota aannan qabaniif dhiyaatu

Gaaffileen kun hubannoo dhimma qulqullina annanii fi bu'aa annanii irratti namootni aannan qaban qorachuudhaaf qophhaa'e. Eyyamamoo ta'uu keessaniif sin galateeffachaa gaaffilee kana sirriitti dhageeffachuun akka deebiftan kabajaan sin gaafanna. Odeeffannoon isin nuuf laattan fiixaan ba'insa qoranichaaf baayyee barbaachisaa dha. Kanaafuu, gaaffilee ka'aniif amanamummaa fi dhugaan akka deebiftan kabajaan sin gaafanna. Deebbiin sin laattan dhimma qorannoo kana qofaaf akka ooluu fi icciitiin keessan akka eegamu asumaan siniif mirkaneessina.

Guyyaa: _____ Lakk. Sumuuda: _____

1. Seenaa abbaa qabeenyichaa

Teessoo: _____

Maqaa _____ Maqaa hojii (yoo danda'ame)

1. 1. Saala: Dhiira □ Dhalaa □

1.2. Umurii _____

1. 3. Sadarkaa Barnootaa: Kan hin baratiin □ Sad. 1ffaa (1-6) □ Sad. 2ffaa □ Dipploomaa Kollejii fi isaa ol □

2. Gahee annan fi bu'aa aannanii dinagdee fi nyaata keessatti qabu

2.1. Maddi galii keessan inni gudaa maali? Oomisha aannanii□; Ka biraa □

2.2. Bu'aa aannanii keessaa kam gurgurtaaf oolchitu? Aannan Dhedhii□; Ittittuut□; Baaduu□; Fillannoowwan kana keessa hin jiru□

2.3. Bu'aalee annanii yoo ni gurgurtu ta'e, eessatti/eenyutti gurgurtu? Warshaa oomisha annaniitti□; Fayyadamtoota naannootti □; manneen daldala nyaatatti □; ka biraa (adda baasaa) □

2.4. Isin yokaan maatiin keessan aannan dheedhii soorattuu? Eyyee □ Laaki □

2.5. Isin yokaan maatiin keessan itittuu ykn baaduu soorattuu? Eyyee □ Lakki □

3. Hojiwwan qulqullinaa fi kusaa nyaataa

3.1. Loon annanii kee mala kamiin elemta? Harkaan □ Maashinaan□

3.2. Elemtuun/toonni elemuu dura harka dhiqatuu? Eyyee □ Laakki □

3.3. Elemtuun elemuun dura mucha loonii dhiquu? Eyyee □, Lakki □ ,

3.4. Elemtoonni tartiiba loon elemuu gidduutti harka dhiqatuu? Eyyee □ Laaki □

3.5. Uffata elemaa yeroo elmaa qofa uffatamu qabduu? Eyyee □ Lakki □

3.6. Uffatni elemaa ammam ammamitti miicama? Guyyaa guyyaatti □ Torbee keessatti guyyaa 2-4 □ Torbeetti takkaa □, Torban tokkoo ol □

3.7. Wayyaa ittiin mucha qoorsan qabduu? Eyyee □Lakki □

3.8. Deebbiin Gaff. 3.7 yoo "Eyyee" ta'e, qoorsituu muchaa tooko loon tookkoo oliif itti fayyadamtuu? Eyyee □Lakki□

3.9. Deebbiin gaaffii 3.8 yoo “Eyyee” yoo ta’e qoorsituu kana ammamitti miiccu?

Elmaa gidduutti□ guyyaatti al lama □ Guyyaatti tokko □ Guyyaa lamaa olitti□ Hin micaamu □

3.10. Erbee qoorsituu tokkoon tokkoo looniif kan fayyadamtan yoo ta’e, ammam ammamitti miiccu? Guyyaati lama □ guyyaatti takkaa □ Guyyaa lamaa fi isaa olitti □ Hin micamu □

3.11. Gosa meshaa annan itti elmamu Pilastika □ Sibila □ Muka or Migira □ Kan biraa (Adda baasaa) _____.

3.12. Meshaalee annanii fi bu’aan annanii itti kuufamu

Pilastika □ Sibilaa □ Muka or Migira □ Suphee □ Kan biraa (adda baasi)_____

3.13. Qodaa annanii miiccuu? Eyyee □Lakki □

3.14. Deebbiin Gaaff.. 3.13 yoo “Eyyee” ta’e, ammam ammamiiti dhiqxu? Guyyaatti al lama□ Guyyaa guyyaatti□ Torbanitti takkaa □

3.15. Qodaa aannanii aaraan ultuu? Eyyee □Lakki □

3.16. Deebbiin Gaaff. 3.15 “Eyyee” yoo ta’e, ammam ammamitti diqxa? Guyyaa guyyaatti □; Torbanitti guyya lama□; Torbanitti guyyoota 3-4 □; Torban torbanitti □

3.17. Qodaa annanii dhiquuf bishaan ho’aa fayyadamtaa ? Eyyee □;Lakki □

3.18. Qodaa aannanii saamunaan miiccaa? Eyyee □;Laaki □

3.19. Mallattoo dhukkuba summaa’uu nyaata argitee beektaa? Eyyee □;Lakki □

MALDI-ToF Machine with attached computer

Bruker MALDI Biotyper
Identification Results

BRUKER

Run Identifier: 220428-1519-1011027836 Run Creation Date/Time: 2022-04-28T15:26:00.442

Run Info:

Run Identifier:	220428-1519-1011027836
Comment:	Negasa Staph. aureus second round
Operator:	tof-user@MBT-WIN10
Run Creation Date/Time:	2022-04-28T15:26:00.442
Number of Tests:	45
Type:	Standard
BTS-QC:	passed
BTS-QC Position:	D9:0
Instrument ID:	8604832.05381
Server Version:	4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+++) (A)	16386 (Standard)	Staphylococcus aureus	2.27	Staphylococcus aureus	2.19
A2 (+++) (A)	16381 (Standard)	Staphylococcus aureus	2.28	Staphylococcus aureus	2.24
A3 (+) (B)	16380 (Standard)	Staphylococcus aureus	1.87	Staphylococcus aureus	1.83
A4 (+++) (A)	16379 (Standard)	Staphylococcus aureus	2.13	Staphylococcus aureus	2.20
A5 (+++) (A)	16378 (Standard)	Staphylococcus aureus	2.28	Staphylococcus aureus	2.25
A6 (+) (B)	16377 (Standard)	Staphylococcus aureus	1.82	Staphylococcus aureus	1.80
A7 (+) (B)	16375 (Standard)	Staphylococcus aureus	1.99	Staphylococcus aureus	1.96

Result overview table--continued on next page

Result overview table--continued from previous page

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A8 (+) (B)	16374 (Standard)	Staphylococcus aureus	1.90	Staphylococcus aureus	1.74
A9 (+) (B)	16373 (Standard)	Staphylococcus aureus	1.94	Staphylococcus aureus	1.92
A10 (+++) (A)	16372 (Standard)	Staphylococcus aureus	2.08	Staphylococcus aureus	2.04
A11 (+++) (A)	16371 (Standard)	Staphylococcus aureus	2.07	Staphylococcus aureus	2.07
A12 (+++) (A)	16370 (Standard)	Staphylococcus aureus	2.01	Staphylococcus aureus	1.98
B1 (+) (B)	16369 (Standard)	Staphylococcus aureus	1.88	Staphylococcus aureus	1.86
B2 (+++) (A)	16368 (Standard)	Staphylococcus aureus	2.11	Staphylococcus aureus	2.04
B3 (+) (B)	16367 (Standard)	Staphylococcus aureus	1.84	Staphylococcus aureus	1.83
B4 (+++) (A)	16385 (Standard)	Staphylococcus aureus	2.09	Staphylococcus aureus	2.01
B5 (+) (B)	16366 (Standard)	Staphylococcus aureus	1.91	Staphylococcus aureus	1.86
B6 (-) (B)	16384 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B7 (+++) (A)	16383 (Standard)	Staphylococcus aureus	2.15	Staphylococcus aureus	2.09
B8 (+) (B)	16382 (Standard)	Staphylococcus aureus	1.96	Staphylococcus aureus	1.90
B9 (-) (C)	16391 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B10 (+) (B)	16392 (Standard)	Staphylococcus aureus	1.73	Staphylococcus aureus	1.71
B11 (-) (C)	16402 (Standard)	**No Organism Identification Possible**	1.37	**No Organism Identification Possible**	1.36

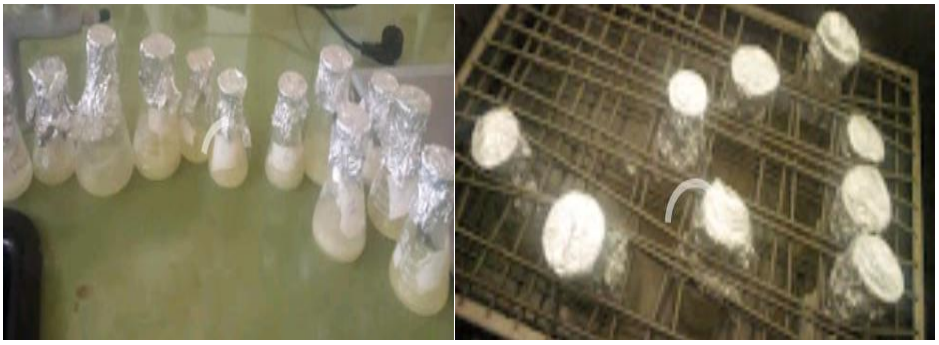
Result overview table--continued on next page

Results obtained from MALDI-ToF analysis

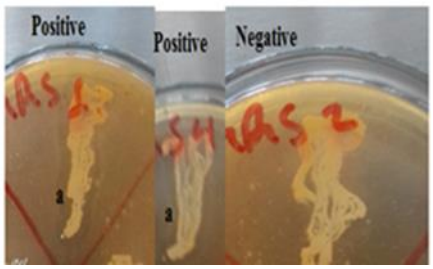
Annex 2: Staphylokinase test on heated plasma agar

Annex 3: Protease production test on Skimmed milk Agar

Annex 4: SAK production broth



Annex 5: Lipase production test on 1% v/v butter fat supplemented nutrient agar

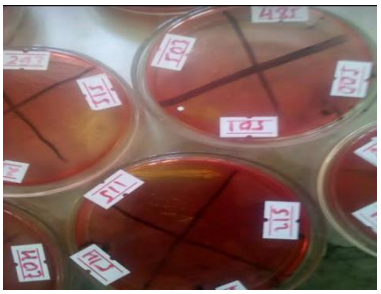


Annex 6: Cloxacillin (500mg) tablet used incorporated into the MSA for screening MRSA

The working solution was prepared as:

$$c_1v_1 = c_2v_2$$

where c_1 and v_1 were the concentration and volume of the stock solution respectively, and c_2 and v_2 were the concentration and volume of working solution respectively. Accordingly, 10ml with 6 µg/µl concentration working solution was prepared by constituting 1.2 ml (1200 µl) of the stock solution in 8.8ml (8800 µl) sterile distilled water. The culture was, therefore, prepared by supplementing 1 µl of the working solution of cloxacillin solution per 1ml of the mannitol salt agar melt.



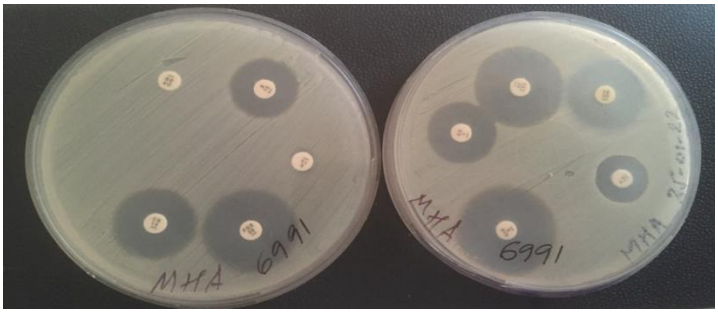
S. aureus on Cloxacillin Supplimented MSA after 18 hours

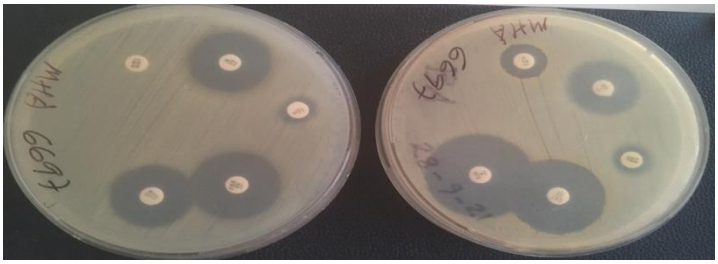
Mcfarland Densitometer

MacFerland

Antibiotic Dispenser

Mesuring the antibiotic inhibition zone





Annex 7: Publications from the research

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Isolation, identification, and determination of antibiogram characteristics of *Staphylococcus aureus* in cow milk and milk products (yoghurt and cheese) in West Showa Zone, Ethiopia

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ABSTRACT

Cross-sectional research aimed to identify *Staphylococcus aureus* from milk, yoghurt, and cheese collected from selected towns in the west Showa zone was conducted from May 2020 to March 2021. The isolates were identified based on colony characterisation, Gram stain, and coagulase tests, and matrix assisted laser desorption/ionisation time-of-flight biotyper. The drug susceptibility test was conducted using the disk diffusion method. The presence of *mecA*, *blaZ*, and *nuc* genes was assessed by polymerase chain reaction. The data were analysed using SPSS Ver.20. The result revealed that *S. aureus* was identified in 20.3%, 14.9%, and 4.8% of milk, cheese, and yoghurt samples, respectively. The rate was significantly higher in milk (OR = 5.04), and in cheese (OR = 1.46) than in yoghurt ($p < 0.05$). All 24 tested isolates were resistant to penicillin, tetracycle, and oxytetracycline. The *nuc* gene was found in all 24 tested isolates but no *mecA* and *blaZ* genes were tested.

1. Introduction

Dairy products play a significant economic role in Ethiopia. Mainly, they serve as a source of food and income that milk and milk products support about 50–70% of the rural community (Desta, 2002). Butter, which is among the dairy products, is used as cosmetics for hairdressing, particularly by women and children (Duguma, 2022).

Generally, there are lowland pastoral, rural highland (smallholder), and urban and peri-urban dairy production systems in Ethiopia (Desta, 2002). Traditionally, dairy products have been widely supplied by rural farmers who are practicing the extensive farming system in the country. Recently, however, the most commercialised urban and peri-urban semi-intensive and intensive cattle dairy production systems including small and big private and state farms, have been progressing in and around urban areas where the demand for the products is high (Gwandu et al., 2018). Over the last two decades, the dairy industry has been showing significant development in the country being expanded at a rate of 3% (Ahmed, Ehui, & Assefa, 2015).

In Ethiopia, more than 80% of milk for human consumption is obtained from cattle. Other animals including camels, goats, and sheep contribute about 6%, 8%, and 5%, respectively. Of these milk types, cow milk is the most common type consumed in all agro-ecological areas. Cow milk is also the sole milk type from which milk products such as cheese, yoghurt and butter are made. The camel, goat and sheep milk are mostly used up in postural low land areas and are consumed in their raw state (Hussen, Tegegne, Kurtu, & Berhanu, 2008).

Milk and milk products are perishable as the fact that they are particularly susceptible to microbial contamination, and they contain protein and carbohydrates, which are important for microbial multiplication. Bacterial contamination of milk and milk products is among the important difficulties in the dairy industry, especially in developing countries where the products are processed by traditional methods. The majority of the sources of the contaminations are associated with less hygienic handling of the products and less attention given to animal health. Bacteria can contaminate milk and milk products either from primary sources as in the case of milk from mastitis cows or secondary sources, which are linked to less hygiene in the chains of production of milk and milk products. Microbially contaminated milk spoils rapidly or the

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pathogens may infect the consumers or produce toxic biochemicals to cause milk-borne illnesses (Gwandu et al., 2018).

Pathogenic bacteria such as *Brucella abortus*, *Escherichia coli* 0157: H7, *Mycobacterium bovis*, *Campylobacter jejuni*, *Salmonella* spp., *Clostridium* spp., and *Staphylococcus aureus* (*S. aureus*) are responsible for nearly 90% of infections associated with dairy products (Gwandu et al., 2018). Of the pathogens, *S. aureus* is the main culprit behind most milk and milk product-associated staphylococcal disease outbreaks. As the consequence, foods of milk and milk product origins are frequently implicated in staphylococcal poisoning epidemics.

S. aureus is a Gram-positive clustered cocci bacteria that lives on both human and animal skin and in mucosal cavities of the hosts as microflora. It has been isolated from various body parts including skin, urogenital, nasal, and buccal openings of humans and animals. In some special cases of immunocompromised individuals like infants, elders, and immune-deficient patients, *S. aureus* can be an opportunistic pathogen to cause infectious diseases. In such circumstances, it can result in a variety of illnesses, from minor ailments like boils and pimples to serious infections like wound infections, pneumonia, and/or septicaemia which can result in life-threatening illnesses (Torrence & Isaacson, 2008).

The virulence factors including drug resistance, enterotoxin production, biofilm development, and other virulence factors, such as adhesion factors, nucleases, proteases, lipases, hyaluronidase, and collagenase secretions, are linked to the pathogenicity of *S. aureus* (Shettigar & Murali, 2020). The antibiotic resistance characteristics of the bacterium have been the main concern of the world as far as the control of infections caused by pathogenic strains of *S. aureus* is concerned. *S. aureus* has developed resistance to antibacterial drugs since the beginning of the formulation of penicillin (the first antibiotic formulated) in 1942. The penicillin-resistant strain of the bacterium was then determined in 1959. Since then numerous attempts had been made to formulate antibiotics that can kill and/or inhibit penicillin-resistant *S. aureus,* and eventually methicillin, a synthetic β-lactam antibiotic was developed. At the time, methicillin, which is resistant to the hydrolysis of the β-lactamase enzyme, successfully treated infections caused by penicillin-resistant *S. aureus* (Guo, Song, Sun, Wang, & Wang, 2020). Recently, however, methicillin-resistant *S. aureus* (MRSA) strains have emerged and have led to lacking effective treatment drugs against infection caused by the MRSA strain (Eshetie et al., 2016).

Milk and milk products are marketed both formally and informally in Ethiopia. Formal marketing systems involve fixed locations, recognised sources of the products, and legally responsible sellers. The systems are conducive to consistent quality control procedures. On contrary, in an informal marketing system, the sellers could not have fixed locations and the sources of the products are not well known. Most of the time, marketing in informal types is performed based on bargaining. In Ethiopia, the majority of the milk and milk products have been supplied to consumers through an informal marketing system. In such marketing systems, quality control would be a major challenge in the value chains of dairy products. The milk is processed into milk products such as yoghurt, butter, and cottage cheese by farmers at the household level. The methods expose the products to staphylococcal and other bacterial contaminations. The products, which are prepared in such less hygienic conditions, are used for the consumption of the family members, and/or commercially supplied to the markets for sale to the public consumption (Duguma, 2022).

As milk and milk products are commonly consumed in almost all nations, contamination of the product by pathogens including *S. aureus* implies multidimensional public health risks (Kardas et al., 2016). Therefore, the identification and determination of antibiotic susceptibility of *S. aureus* are crucial. The current study focused on the isolation and identification of *S. aureus* from cow milk and its products (yoghurt and cheese) in four selected towns (Holeta, Ambo, Guder, and Bako) of West Showa Zone of Oromia Regional State, Ethiopia. The study also determined the drug susceptibility patterns of the isolates. The research was conducted with the null hypothesis that the isolation rates of *S. aureus* did not significantly differ with the type of dairy products, and they were susceptible to the commonly used antibiotics in the study area.

The current study is important for the dairy industry in designing strategies and modifications for processing cow milk and milk products. It pointed out the *S. aureus* contamination level of the cow milk and milk products (cheese and yoghurt) in the study area, which is important in the value chain of the industry. The research also gave important information about public and animal health hazards in the dairy industry in the study area.

2. Material and methods

2.1. Study area

The study was conducted in purposively selected towns of Especial Zones surrounding Finfine (Holeta) and West Shawa Zone (Ambo, Guder, and Bako) of Oromia, Ethiopia (Fig. 1).

2.2. Sampling design and sample size determination

A cross-sectional study to isolate, identify, and determine the antibiogram characteristics of *Staphylococcus aureaus* from cow milk and milk products was conducted from May 2020 to March 2021. These towns were selected from the fact that raw milk and traditionally processed milk products are sold in local markets. Particularly cheese has been prepared by the local farmers and supplied for sale informally. Even though yoghurt is not prepared and supplied to local markets or restaurants and hotels, the raw milk supplied by the farmers was fermented and commonly sold in restaurants without the application of further decontamination techniques. Furthermore, the towns vary agro-ecologically from one another and are accessible to transportation services for quick transportation of the samples to the laboratory.

Since there were very few dairy farms in the study area, they were selected based on the voluntariness of the farm management. However, the smallholders and cheese sellers at the market level were selected randomly.

The sample size was calculated according to the formula given by (Thrusfield & Christley, 2007) as

$$n = \frac{Z^2}{d^2} P(1-p)$$

where n = sample size, Z = 1.96 (z value at 95% CI), p = expected prevalence, d = 0.05 (absolute precision)

$$n = \frac{1.96^2}{0.5^2} 0.5(1-0.5) = 385$$

The sample size was estimated using a 0.5 prevalence rate at a 95% confidence interval (CI) since investigations to isolate and identify *S. aureus* from milk products have not been done, and because fermented milk products may impact the prevalence of the bacteria in the products. However, only 354 out of the 414 samples were accepted for bacterial isolation and identification in the laboratory.

Fig. 1. Map of the study area.

2.3. Sample collection

Milk and yoghurt samples were collected at the household level; whereas the cheese samples were collected from the local market level, except for Bako town where the samples were collected from the households.

About 25 mL milk sample was collected from the bulk milk container after agitation to homogenise the content. The sample was taken from the top of the bulk tank using a sanitised dipper. The sample was added into a sterile screw-caped milk collection vial. In the case of yoghurt samples, the owners were asked to add about 25 mL to sterile screw-caped wide-mouthed sample collecting containers directly from the container. The cheese samples were collected in such a way that approximately 25 g of cheese was taken with sterile spoons used separately for each sample. The samples were then added to a screw-capped wide-mouthed tube. The collecting containers were labelled with sample ID, date of collection, and sample types, and were transported to the Zoonosis and Food Safety Research Laboratory of Ambo University. At the arrival of the laboratory, the samples were either processed as soon as possible or stored at +4 °C for a maximum of 12 h until processed.

2.4. Bacterial isolation and identification

In the laboratory, the samples were enriched with brain heart infusion (BHI) broth (Accumix, Vena, India). Briefly, 100 mL of BHI broth was added directly to 25 mL of milk or yoghurt samples. The cheese samples were enriched in such a way that the sample (about 25 g) was first suspended in 100 mL sterile saline water (0.8%, w/v, NaCl). About 5 mL of the suspension of the cheese sample was mixed with 5 mL of the BHI broth. The enriched samples were incubated at 37 °C for 24 h (4% CO_2). A loop full of the culture was streaked on Mannitol Salt Agar (MSA) (Accumix) plate, and incubated at 37 °C for 24 h (4% CO_2). The growth of the bacterium on MSA was checked and repeated subculturings would be performed in the cases of mixed and undispersed colonies. *S. aureus* was suspected if the bacteria could resist the high salt content (7.5%) of MSA to grow and ferment mannitol rapidly within 24 h. The mannitol fermentation was determined by observing the yellowish-colored colony and surrounding medium. The typical presumptive colonies were transferred to BHI agar plates and incubated for 24 h at 37 °C (4% CO_2), and the purity of the colony was visually checked for its uniformity in color and forms. The isolates on the BHI agar plates were then tested by Gram staining for the cell morphology and arrangements.

The presumptive isolates were further tested using primary biochemical tests including catalase and coagulase tests. The catalase test was performed by mixing a loop full (using a plastic loop) of the overnight cultured bacteria on a nutrient agar plate with two drops of 3% H_2O_2 (Sheba Pharmaceuticals PLC). The immediate production of babble was considered catalase positive. The tube coagulase test method was used to test the coagulase enzyme production of the isolates. It was carried out in such a way that 0.5 mL of a 24 h culture in BHI broth was mixed with an equal amount of rabbit plasma [obtained from National Veterinary Institute (NVI), Bishoftu, Ethiopia]. The mixture was incubated at 37 °C and checked for the presence of clots at every 30 min interval for 4 h and if no clotting was observed within 4 h the sample would wait for 24 h. The presence of the clot was considered coagulase-positive, and the isolates with no clot within 24 h were recorded as coagulase-negative.

The coagulase-positive isolates were further confirmed as *S.aureus* by matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) biotyper mass spectrometry identification standard method (Singhal, Kumar, Kanaujia, & Virdi, 2015) at the National Animal Health Diagnostic and Investigation Centre (NAHDIC), Sabata, Ethiopia. The extended direct transfer (EDT) method was used (Chen et al., 2018). Briefly, a single colony of the 24 h BHI agar culture was picked by wooden application sticks and smeared as a thin film directly onto a spot on a MALDI target plate. The smeared material was overlaid with 70% formic acid and allowed to dry at room temperature. The material was then overlaid with matrix (α-cyano-4-hydroxycinnamic acid (HCCA portion) and allowed to dry at room temperature. *E. coli* (ATCC 8739) was used as quality control for the reading of the machine. The plate was then inserted into the MLD-ToF machine, run, and the result was displayed on the computer connected to the machine.

2.5. Antibiotic susceptibility test

The Kirby-Bauer disk diffusion antibiotic sensitivity test (Khalili, Soltani, Negahban, Abdollahi, & Gholami, 2012) was carried out in the National Animal Health Diagnostic and Investigation Centre (NAHDIC) laboratory. The test was performed to check the susceptibility of the isolates to ciprofloxacin (5 μg), penicillin G (10 units), trimethoprim/sulfamethoxazole (1.25/23.75 μg), cefoxitin (30 μg), oxytetracycline (30 μg), gentamycin (10 μg), erythromycin (15 μg), tetracyclin (30 μg), clindamycin (2 μg) and novobiocin (5 μg).

The test was performed in such a way that *S. aureus* isolates were first cultured on BHI agar plates, at 37 °C for 24 h and checked for purity. Two to three colonies of the fresh (24 h) culture were mixed with 5 mL sterile saline (0.85% NaCl) water. The concentration of the bacterium was measured at 0.5 Mcfarland using a Mcfarland Densitometer. The swab was dipped into the suspension and streaked evenly on a Muller-Hinton agar plate of 90 mm diameter in such a way that the surface of the plate was sufficiently covered by the inoculum. The antibiotic discs were then placed on the surface of the plate using the disk dispenser. The discs were gently pressed by sterile thumb forceps taking care not to touch the surface of the plate. The plates were incubated at 37 °C (4% CO_2). The diameter of the inhibition zones of the antibiotics was measured in centimeters using a caliper after 18 h incubation at 37 °C (4% CO_2). The width of the inhibition zone was then compared with the standards provided by the Clinical and Laboratory Standard Institute (CLSI, 2013).

2.6. Molecular detection of antibiotic-resistant genes in Staphylococcus aureus

2.6.1. DNA extraction

DNA extraction was performed using Qiagen DNA extraction kit (Qiagen, German) protocol for bacterial culture (Thermo Scientific, Germany). Briefly, newly isolated *Staphylococcus* species isolates were grown overnight at 37 °C on BHI agar (52 g L^{-1}) (Himedia, India); HM infusion powder 12.5 g L^{-1}, BHI powder 5 g L^{-1}, protease peptone 10 g L^{-1}, dextrose 2 g L^{-1}, NaCl 5 g L^{-1}, Na2HPO4 2.5 g L^{-1}, and agar, 15 g) and final pH (at 25 °C) 7.4 ± 0.2. One to two loop full of distinct colonies of bacterial isolates was taken and suspended in 600 μL sterile PBS buffer (at a pH of 7.4 ± 0.2). The suspended bacterial culture was centrifuged at 20,000×*g* for 5 min. The supernatant was discarded, and the pellet was harvested. The pellet was suspended in 180 μL of enzymatic lysis buffer and vigorously vortexed for 10–20 s and the cells were incubated at 37 °C for 30 min in the water bath. After incubation 25 μL of proteinase K and 200 μL of lysis (AL) buffer were added to the tube and briefly vortexed. The tubes were incubated at 56 °C for 30 min in the water bath. After incubation 200 μL of 100% (v/v) ethanol was added and vortexed. Using a micropipette the entire contents of the extracted sample were transferred to the labeled spin column tube and the column was centrifuged at 10,000×*g* for 1 min at 25 °C. The column was removed from the collection tube and placed in a new collection tube, then 500 μL of washing (AW1) buffer was added to the column and centrifuged at 10,000×*g* for 1 min at 25 °C. The column was removed from the collection tube and the column was placed in a new collection tube. A 500 μL of washing (AW2) buffer was then added to the column and centrifuged at 20,000×*g* for 3 min at 25 °C. The tubes were carefully removed from the centrifuge. The column was then transferred to a 1.5 mL tube and 200 μL of elution (AE) buffer was added to the column. The column was stood at room temperature for 1min. Finally, the column was centrifuged 10,000×*g* for 1min at 25 °C and the pure DNA was collected and stored at −20 °C until used for further analysis.

2.6.2. PCR amplification

The genomic DNA of *Staphylococcus* species isolates was subjected to PCR to amplify the resistance genes using a PCR master kit (Qiagen). The kit contained 25 μL mixtures including 2 μL of each primer, 1.5 μL 25 mM $MgCl_2$, 5 μL PCR buffer, 0.5 μL dNTP, 0.2 μL Taq polymerase enzyme, 13.8 μL RNase free water and 2 μL extracted DNA. Sterile water was added instead of nucleic acids for the negative control. ATCC 25923 was used as a positive control for all selected primers. The PCR tubes containing the mixture were tapped gently and the PCR tubes with all the components were transferred to (Flex cycler, Biometra Gmbh, Germany) thermal cycler. Three sets of primers were used for amplification of targeting antibiotic resistance and thermonuclease genes. The genes are designated as *mec*A, *nuc*, and *blaZ* genes. These primers are commercially synthesised by Eurofins genomics India Pvt. Ltd.

The forward and reverse primers (oligonucleotides) used to target oxacillin resistant (mecA) gene were *mecA*-F (GTAGAAA TGACTGAACGTCCGATGA) and *mecA*-R (CCAATTCCACATTGTTTC GGTCTAA) (Hoque, Das, Rahman, Haider, & Islam, 2018).

The forward and reverse primers (oligonucleotides) to target the penicillin resistance (*blaZ*) gene were *blaZ* −F (TACAACTGTAA-TATCGGAGGG) and *blaZ*-R (CATTACACTCTTGGCGGTTTC) (Okiki et al., 2020). In addition, the forward and reverse primers (oligonucleotides) used to target the thermonuclease (nuc) gene were *nuc*-F (GCGATTGATGGTGATACGGTT) and *nuc*-R (AGCCAAGCCTT-GACGAACTAAAGC), respectively (Salisbury, Sabatini, & Spiegel, 1997). The amplification program of the above three genes was performed under the following conditions: initial denaturation at 95 °C for 5 min followed by annealing at 55 °C for 30 s, the extension at 72 °C for 1 min per cycle and a final extension of 72 °C for 10 min. The cycle was repeated for 37 cycles.

2.6.3. Gel electrophoresis and visualisation

The PCR products were separated on 1.5% (w/v) agarose gel containing ethidium bromide (2 μL) that intercalates into the DNA and is used for visualisation. An 8 μL amplification product of each sample with 2 μL loading dye was applied into the slots of agarose gel. DNA marker of 100 bp (Qiagen; Germany) was used to estimate the molecular weight of the DNA fragments. Electrophoresis (Apelex Midigel XL, France) was performed in 1 × TBE buffer at 120 V for 1 h. The products were visualised with UV illumination (Gel Doc™ XR^+, BioRAD; Germany) and imaged with a gel documentation system.

2.7. Data management and analysis

The data obtained from field and laboratory works were recorded and coded on Excel Microsoft 2016 worksheets. The data was exported to SPSS ver. 20 statistical software for data analysis. The descriptive statistics including percentages were used and displayed on tables. Univariable logistic regressions were used to determine the association between the prevalence of *S. aureus* contamination of dairy products, and associated risk factors. The significance of the association was determined at 95% CI and *p*-value <0.05.

3. Results and discussion

3.1. Isolation rate of S. aureus in milk, yoghurt, and cheese

Out of the total 354 samples of milk and milk products (123 milk, 148 cheese, and 83 yoghurts), 51 (14.4%) were *S. aureus* positive (Table 1). The result is in accordance with the result of Gebremedhin et al. (2022) who isolated 10.7% of the milk and milk products in Holeta Town of Ethiopia, is less than the reports of Ayele

Table 1
Univariate logistic regression analysis of *S. aureus* prevalence based on sample types.[a]

Sample	Tested	Positive	Pearson χ2	p of χ2	OR (95% CI)	*p* of Bin Log
Milk	123	25 (20.3)			5.04 (1.683–15.080)	0.004
Cheese	148	22 (14.9)	9.706	0.008	3.45 (1.146–10.379)	0.028
Yoghurt	83	4 (4.8)			Reference	1.00
Total	354	51 (14.4)				

[a] Positives are reported as a number with % in parenthesis. Abbreviations are: OR, odd ratio (95% CI given in parenthesis); Bin Log, binary logistic regression.

et al. (2017) and Lemma, Alemayehu, Stringer, & Eguale (2021) who isolated the bacterium in 23%, and 20.3% of milk and milk products in Sebata town and Addis Ababa city of Ethiopia respectively. Elsayed, Abdelkader, Mahmoud, and El- Shafey (2018) with an isolation rate of 46% in Egypt, Pervin, Rahman, Zereen, & Ahmed (2019) with 69% in Bangladesh, and Saka and Terzi Gulel (2018) with 28% in Turkey also found higher isolation rates of *S. aureus* in milk and milk products than the current result. However, the current result is higher than the findings of Thaker, Brahmbhatt, and Nayak (2013) who determined *S. aureus* in 6.25% of milk and milk products in India.

The prevalence of the bacteria was highest in milk (20.3%) followed by cheese (14.9%) in the current study. The lowest isolation rate was determined in yoghurt (4.8%). The rate was significantly higher in milk and cheese than in yoghurt (OR = 5.04 and 3.45 respectively). However, statistically, the prevalence rate was similar both in milk and cheese ($p > 0.5$) though figuratively the bacterium was more frequently isolated from milk than in cheese (Table 1). In agreement with the current findings, previous studies have determined significantly high *S. aureus* isolation rate in raw milk than in fermented dairy products (Al-ashmawy, Sallam, & Abd-elghany, 2016; Lemma et al., 2021; Sadek & Koriem, 2020; Tarekgne, 2016; Tarekgne, Skeie, Rudi, Skjerdal, & Narvhus, 2015; Thaker et al., 2013). However, Gebremedhin et al. (2022) and Zakary, Nassif, and Mohammed (2011), determined that raw milk was found less contaminated by *S. aureus* than yoghurt and cheese.

The organic acids (lactic, acetic, propionic, and butyric acids) produced by lactobacilli bacteria in fermented dairy products like cheese and yoghurt are responsible for lower pH values of the products than of raw milk (Girma & Aemiro, 2021; Nyamakwere, Esposito, Dzama, & Raffrenato, 2021). The acids are expected to inhibit the growth of bacteria in the fermented milk products though some pathogens could survive in the acidic condition of the products (Girma & Aemiro, 2021).

The isolation rate of *S. aureus* was found to be significantly higher in cheese than in yoghurt. This variation might be associated with the difference in the processes through which the products have been prepared and packaged (Andualem & Geremew, 2014). Cheese has been processed from sour milk and remained after churning. The milk is heated approximately to 40–70 °C for 15–25 min and left stat for 3–5 h to cool. The whey is removed through a strainer and the solid part (called Badu) is harvested. The product is divided into different portable-sized containers to take it to the market. In the market, cheese is, in turn, transferred to the container of the purchaser (Asresie, Yilma, Seifu, Zemedu, & Eshetu, 2018; Berhe, Vogensen, Ipsen, Seifu, & Kurtu, 2017; Mattiello et al., 2018; O'Connor, 1993). However, yoghurt (Itittu) is prepared directly from milk without removing the butter. Usually, the raw milk is put at room temperature for 12–24 h to ferment (Andualem & Geremew, 2014). The product is consumed without removing the whey or removing only a little portion, which makes it less thick than cheese. Yoghurt is usually used directly from the utensil in which it has been prepared except poured into clean cups. Apart from their variation in content, the repeated transfer of the cheese to different containers might expose the product to more bacterial contamination than yoghurt.

Fermented milk products such as cheese and yoghurt were expected to affect the survival of *S. aureus* (Abd El-Gawad, El-Sayed, El- Zeini, Hafez, & Saleh, 2014; Felicio et al., 2015; Soliman & Ahmed, 2019). However, *S. aureus*, and other pathogenic bacteria such as *E. coli*, *Bacillus cereus*, *Klebsiella* spp., and *Enterobacter* spp., have been repeatedly isolated from traditional Ethiopian cheese (Andualem & Geremew, 2014; Berhe et al., 2017). Andualem and Geremew (2014) reviewed that the high bacterial load in raw milk which subsequently decreases in cheese due to low PH and heat, can in turn increase the product due to bacterial entrance from the packaging, flavours, and handlers.

The prevalence rate of *S.aureus* in the samples with respect to other associated risk factors was as depicted in Table 2. Accordingly, the rate per sample type did not significantly differ with the towns ($p > 0.05$). However, it was found to vary with the sources of the samples (large dairy farm, small householder, and market/restaurant). The pathogen was highly prevalent in milk samples collected from the large dairy farm (46.7%) than from the smallholder (17.6%) and markets (restaurants) (0.0%). In contrast, it was isolated only from cheese and yoghurt obtained from the market at the rate of 22.2% and 14.8% respectively ($p < 0.05$). That is it was not identified in cheese and yoghurt samples collected from large dairy farms and smallholders. In agreement with the current study, Tarekgne et al. (2015), Gebremedhin et al. (2022) and Tarekgne (2016) found that *S. aureus* has been isolated with a higher rate in milk from large size dairy farms than the small dairy farms or householders.

3.2. *Antibiotic sensitivity of the isolates of* S. aureus

Twenty-four of the 51 *S. aureus* isolates (11 from the milk, 11 from the cheese, and 2 from the yoghurt) were tested for their susceptibility to 10 antibiotics (9 commonly used antibiotics and one, novobiocin, to which *S. aureus* is genetically susceptible; Table 3). All of the isolates were found susceptible to ciprofloxacin, trimethoprim/sulfamethoxazole, cefoxitin, gentamycin, clindamycin, novobiocin, and 87.5% of them to erythromycin. However, all of the 24 isolates were resistant to pencillin G, oxytetracycline, tetracyclin, and 1 of them was resistant to erythromycin; a further 2 were intermediate susceptible. However, none of the isolates tested was found resistant to cefoxitin as a consequence of which there was no MRSA strain found among the tested isolates (Table 3).

Regarding the multidrug resistance pattern of the isolates, all of the isolates (100%) were determined resistant simultaneously to two classes of antibiotics (penicillin and tetracyclin). However, one of the isolates was found resistant to three classes of the drugs (penicillin, tetracyclines, and erythromycin), which is considered multidrug-resistant (Table 4).

Penicillin is the most widely antibiotic followed by tetracyclines prescribed to treat staphylococcal infection in human and animal medicines in many countries (Ruhe & Menon, 2007; Trzcinski, Cooper, Hryniewicz, & Dowson, 2000). *S.aureus* has repeatedly been reported highly resistant to penicillin. Regarding this, the findings the current results agreed with those of previous studies (Ayele et al., 2017; Bonko et al., 2021; Cebeci & Gündoğan, 2021; Hallabjaiy, Darogha, & Hamad, 2014; Jahan et al., 2015; Kalayu et al.,

Table 2
The isolation rates of *S. aureus* in milk, cheese, and yoghurt with associated risk factors.

Sample type	Factor	Number of samples	Positive (%)	$\chi 2$	*p*-value
Towns					
Milk	Holata	23	7 (30.4)	3.058	0.409
	Ambo	23	6 (26.1)		
	Gudar	54	8 (14.8)		
	Bako	23	4 (17.4)		
Cheese	Holata	51	13(25.5)	7.643	0.057
	Ambo	44	5 (11.4)		
	Gudar	45	4 (8.9)		
	Bako	8	0(0.0)		
Yoghurt	Holata	14	0 (0.0)	2.830	0.502
	Ambo	31	3 (9.7)		
	Gudar	31	1 (3.2)		
	Bako	7	0 (0.0)		
Source of samples					
Milk	Small holder	102	18 (17.6)	8.409	0.024
	Market	6	0 (0.0)		
	Dairy farm	15	7 (46.7)		
Cheese	Small holder	44	0 (0.0)	12.790	0.003
	Market	99	22 (22.2)		
	Dairy farm	5	0 (0.0)		
Yoghurt	Small holder	49	0 (0.0)	8.265	0.022
	Market	27	4(14.8)		
	Dairy farm	4	0 (0.0)		

Table 3
Antibiogram pattern of *S. aureus* isolates.[a]

Antibiotic	Susceptibility		
	Susceptible	Intermediate	Resistant
CIP	24 (100)	0	0
CN	24 (100)	0	0
DA	24 (100)	0	0
E	21 (87.5)	2 (8.3)	1 (4.2)
FOX	24 (100)	0	0
NV	24 (100)	0	0
OT	0	0	24 (100)
P	0	0	24 (100)
SXT	24 (100)	0	0
TE	0	0	24 (100)

[a] Values for susceptibility are the number with the percentage in parenthesis where value > 0 (n = 24). Abbreviations are: CIP, ciprofloxacin; CN, gentamycin; DA, clindamycin; E, erythromycin; FOX, cefoxitin; NV, novobiocin; OT, oxytetracycline; P, pencilin G; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline.

Table 4
Multidrug resistance characteristics of *S. aureus* isolates.[a]

Antibiotics		Resistant isolates
Number	Identity	
Two	OT, P	24 (100)
	E, P	1 (4.2)
	P,TE	24 (100)
	E, OT	1(4.2)
	TE, OT	24 (100)
	E,TE	1(4.2)
Three	E, OT,P	1(4.2)
	OT, P, TE	24 (100)
	E, P, TE	1(4.2)
	E,OT,TE	1(4.2)
Four*	E, OT, P, TE	1(4.2)

[a] Abbreviations are: E, erythromycin; OT, oxytetracycline; P, pencilin G; TE, tetracycline. *Values for resistant isolates are the number with the percentage in parenthesis where value > 0 (n = 24). An asterisk indicates multidrug resistant isolate.

2020; Mekuria, Asrat, Woldeamanuel, & Tefera, 2013; Pervin et al., 2019; Thaker et al., 2013). However, few studies determined less proportion of *S. aureus* isolates resistant to penicillin than the current findings (Bendahou, Lebbadi, Ennanei, Essadqui, & Abid, 2008; Dabele, Borena, Admasu, Gebremedhin, & Marami, 2021; Dittmann et al., 2017; Fursova, Sorokin, Sokolov, & Dzhelyadin, 2020).

Tetracycline groups are structurally related antibiotics prescribed to treat both Gram-positive and Gram-negative bacterial infections. The classes are broad-spectrum bacteriostatic that inhibit protein synthesis of the bacteria (CLSI, 2009; Nigussie, Melaku, Tadese, Belete, & Kebede, 2021). Tetracyclines are among the commonly used antibiotics both in veterinary and human health practices in Ethiopia. The class includes tetracycline, oxytetracycline, chlortetracycline, doxycycline, and minocycline (Mohammed, Mengistu, Abdurehman, Belina, & Mengistu, 2022). The tetracyclines resistance pattern of *S. aureus* found in the current study is in agreement with some previous studies that have reported more than 50% of *S. aureus* isolates have been resistant to the tetracyclines (Bonko et al., 2021; Islam, Kabir, & Rahman, 2016; Mekuria et al., 2013). However, the rate is seen as much higher than the findings of many previous studies conducted in different parts of the world. According to the studies, less than 50% of *S. aureus* isolates were found resistant to tetracyclines (Bendahou et al., 2008; Gebremedhin et al., 2022; Pervin et al., 2019; Yakubu, Abdullahi, Whong, & Olayinka, 2020). The high rate of penicillin and tetracyclines resistance pattern of the *S.aureus* isolates in the current study might be associated with the irrational use of the drugs in animal treatments (Gebremedhin et al., 2022).

3.3. Detection of genes

All of the tested isolates were found to hold the *nuc* gene. However, none of them was found to contain *mecA* and *blaZ* genes. Fig. 2 presents the PCR result of the tests. The *nuc* gene codes the thermonuclease enzymes that enables the bacteria to resist heat and produce heat-stable toxins (Medveov & Valk, 2012). The presence of the gene in the isolates confirmed that the isolates were *S. aureus* (Bagcigil et al., 2012). Though identification of the nuc gene in a bacterium confirms that the isolate is *S. aureus,* not all *S. aureus* contain the gene (Blaiotta et al., 2004). Therefore, the presence of the gene in the *S. aureus* isolates indicated that either the isolates themselves or the toxins they produce resist heat

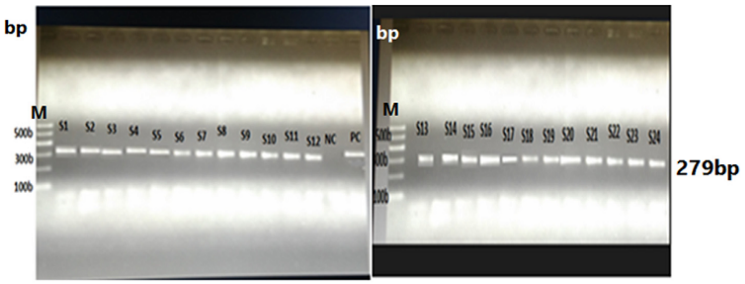

Fig. 2. PCR product electrophoresed in agarose gel; the PCR uses specific primers for *mecA*, *blaZ* and *nuc* genes in *S. aureus*, the length of *mecA* is 163bp, *blaZ* is 279 bp and *nuc* is 846 bp. M is the ladder, S1–S24 are the tested samples, NC is the negative control, PC is the positive control.

treatments (Sutejo et al., 2017). The result of the current study is in line with the findings of Jahan et al. (2015), Saka and Terzi Gulel (2018) and Sutejo, Amarantini, and Budiarso (2017) who determined the *nuc* gene in *S. aureus* isolated from milk and milk products.

It has been repeatedly reported that the penicillin resistance of staphylococcus species is mediated by *blaZ* gene that encodes the β-lactamase enzyme that hydrolyses β-lactam antibiotics (Harkins et al., 2017; Lowy, 2003). However, in the current study, all the penicillin-resistant *S.aureus* tested for the presence of blaZ gene were not found to contain the gene. Studies have revealed that there might be the possibility of missing *blaZ* gene amplification in β-lactam antibiotic-resistant *S. aureus* (Lucas et al., 2021; Zuniga et al., 2020). Lucas et al. (2021) hypothesised the occurrence of such conditions with the change in the sequences of the primer to target the region properly. The result of the gene detection also revealed that all 24 isolates were not found to harbour *mecA* genes that code the methicillin resistance enzyme (PBP 2a) (Gizaw et al., 2020). None of the isolates was found resistant to cefoxitin, which would have indicated methicillin resistance.

4. Conclusions

The study concluded that *S. aureus* was isolated from milk and milk products, and raw milk was highly contaminated with the bacterium than fermented milk products (cheese and yoghurt). However, none of the dairy products was devoid of the isolates. It was also determined that *S. aureus* isolates were found resistant to the commonly used antibiotics such as penicillin, tetracycline, and oxytetracycline that have been widely used in veterinary medicine in the study area. The *nuc* gene harbouring *S. aureus* or the toxin they produce could be heat-resistant.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Research Article

Isolation and Determination of Antibacterial Sensitivity Characteristics of *Staphylococcus aureus* from Lactating Cows in West Shewa Zone, Ethiopia

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Staphylococcus (S.) aureus is one of the etiologies of bovine mastitis, hindering milk production and productivity in dairy farms. This study was aimed at assessing the distribution of bovine mastitis and the isolation rate of *S. aureus* in milked cows of West Shewa Zone. The clinical mastitis was diagnosed by physical methods including observation and palpation, whereas the subclinical mastitis was tested by the California mastitis test (CMT). All of the cows tested for mastitis were aseptically sampled (teat-milk) for bacteriology. The bacterium was primarily identified based on colony characterization, catalase, coagulase tests, and Gram stain reaction. Finally, MALDI-TOF Biotyper confirmed the species. The antibacterial sensitivity characteristics of the isolates to different antibacterial drugs were tested by the disk diffusion method. The drugs were selected based on the frequent usage in veterinary medicine in the study area. By using particular primers, the presence of the resistance (*mecA* and *blaZ*), and thermonuclease (*nuc*) genes were determined by polymerase chain reaction (PCR). The data were analyzed by R statistical software. The associations between the dependent variables (prevalence of mastitis and *S. aureus*) and the explanatory variables were analysed by chi-square (χ^2) and logistic regression tests at a 95% confidence interval (CI). Accordingly, 258 lactating cows were examined, of which 97 (37.6%) were mastitis positive. Of these mastitis positive cows, 59 (60.8%) were subclinical and 38 (39.2%) were clinical. Among the 258 milk samples, 43 (16.7%) were positive for *S. aureus*. According to the results of the current investigation, subclinical mastitis was significantly more prevalent than clinical mastitis ($p < 0.05$). The disease was found varied with the lactation stage of the animal, milking with washed hand, udder washing before milking, and tick infestation of the teat. In comparison to animals from farms with lower number of lactating cows, the prevalence of the bacteria was significantly higher in animals managed in farms with large (OR = 12.58, 95% CI = 2.33–68.54, and $p < 0.05$) and medium (OR = 12.58, 95% CI = 2.33–68.54, and $p < 0.05$) population of lactating cows per herd. The isoation rate of the bacterium was also found significantly higher in tick-infested cows (OR = 27.69, 95% CI = 9.71–93.01, and $p < 0.05$) than tick free cows. The antibiogram tests revealed that the isolates resisted penicillin G and tetracycline group drugs (oxytetracycline and tetracycline). Moreover, the nuc gene was confirmed to be present in all of the examined isolates. However, they were not found harboring *blaZ* and *mecA* genes. We concluded that *S. aureus* is sustaining as a main causative agent of bovine mastitis, and they were resistant to the frequently used antibiotics in public and veterinary medicines in the study areas. Therefore, research-based interventions need to be taken in action to combat the pathogen.

1. Introduction

The dairy products are among the resources of the livestock sector, which play important domestic economic roles in Ethiopia. However, the production and productivity of milk, which is the raw material for dairy products, are hindered by various factors, among which diseases, feed, and the genetic variety of the animals are the most frequently incriminated [1, 2]. Mastitis has been mentioned among the major obstacles to affect the dairy industry by incuring the dairy farms to unnecessary expenses worldwide in general and in developing countries in particular [3].

According to the symptoms, mastitis can be classified as clinical and subclinical. The clinical and subclinical mastitis are assigned whether the signs of the disease are determined by a tentative diagnosis or not. Although only the abnormalities of the milk (changes in color and/or consistency) might be determined in the early stage of clinical mastitis. However, when the illness worsens, the udder begins to show signs of inflammation, such as swelling, redness, heat, and pain. In subclinical mastitis (SCM), however, only a reduction in milk yield is apparent without obvious observable alteration in milk or udder of the animal; hence, it remains undetected for a long period of time [4]. As a result, SCM has been considered as a more istractive and prevalent type of the disease than clinical mastitis. It has been estimated that SCM alone can cause more than 90% of total milk losses of dairy farms in Ethiopia [2].

Bovine mastitis is caused by either trauma or infections. Bacteria, fungi, and viruses are implicated in causing mastitis in bovines. Among the bacterial pathogens, S. aureus has been detemined to cause mastitis in animals, and food poisoning in dairy products (particularly in yogurt and cheese) [5, 6].

One of the unique characteristics of *S. aureus* in causing mastitis is that it is difficult to cure once it has developed. This might be attributed to the fact that, it possesses a wide variety of virulence and pathogenicity factors including quorum sensing, which enable it to escape immune defense mechanisms, and then causes infections in the availability of immune cells [7]. The other virulence factors include swift development of antibiotic resistance, enterotoxigenicity, and production enzymes such as staphylokinase, lipase, and hemolysins. The antibiotic resistance development nature of the pathogen might be linked to acquiring mobile genetic elements through gene transfer [6, 8]. For example, the methicillin-resistant S. aureus (MRSA) strains of the bacterium have been evolved by acquiring genetic components known as staphylococcal cassette chromosome mec (SCCmec), [9]. SCCmec contains various virulence genes of which mecA, codesfor a modified penicillin-binding protein (PBP-2a). Since PBP-2a has less affinity for methicillin, *mecA positive S. aureus* has developed resistance to methicillin which is drug of choice to treat beta-lactam antibiotic resistant S. aureus infections. In addition the genetic cassette may also contain genes encoding resistance to other groups of antibiotics, and various other virulence factors, including enterotoxins [6, 8]. Nevertheless, penicillin resistance of *S. aureus* might be attributed to penicillinase enzyme production. Penicillinase enzyme, which is coded by blaZ gene,is responsibe for the hydrolysis of penicillin and penicillin derivatives by breaking the -lactam ring of penicillin [10–12]. Such characteristics of the pathogen might lead to fatal infections caused by the strains because of the lack of alternative antibiotics [6, 13].

Furthermore, the thermostability nature of *S. aureus* is linked with the *nuc* gene, which encodes the heat-stable enzyme known as thermonuclease. Therefore, *nuc* gene-positive *S. aureus* can survive in heat-processed foodstuffs, and/or the toxins they produce might be thermostable. However, although the *nuc* gene detection in *S. aureus* has been conducted for species identification [14, 15], the *nuc* gene is not harbored by all strains of *S. aureus* [6, 16].

Therefore, since *S. aureus* is an important disease-causing organism in animals and humans through infection or toxin production in foods [6], assessment of the prevalence and antibiotic susceptibility characteristics of the bacterium in lactating cows can play significant roles in the application of treatment and control procedures against the pathogen. The detection of the genes (*mecA*, *blaZ*, and *nuc*) also helps us to have better understanding of the types of antibiotics intended to be used and/or expect the type of enterotoxins produced (if any) by the isolates. Although few previous studies have been conducted in the study area, they have not detected the resistance or the thermonuclease gene in *S. aureus.* The current study was, therefore, carried out to isolate, identify, and detect resistance genes in *S. aureus*.

2. Materials and Methods

2.1. Study Area. West Shewa zone is expected to hold the total population of 2,058,700, of whom 1,028,500 are male and 1,030,200 are female. There are about 1.23 million cattle, 0.43 million sheep, 0.19 million goats, 0.207 million equines, and 1.2 million chickens reared in the zone (Figure 1).

For the current study, four districts of the West Shewa zone, namely, Walmara, Ambo, Toke Kutaye, and Bako Tibe, were randomly selected. The agroecological features, and geographical situations of the districts are illustrated in Table 1.

2.2. Study Animal. Inclusion Criteria: the study included lacating cows of the selected districts. That is to be included in the study, the cow should have been lactated at the time samples were collected. Both the local and crossbred cows (crossed between the Zebu and exotic breeds) were included.

Exclusion Criteria: cows, which were not lactating at the sampling time, were excluded from the study.

The ages of the animals were recorded according to the information obtained from the owners. The management systems of the farms were assigned based on the criteria set, and judged by observation.

2.3. Study Design. The investigation was conducted between May 2020 and March 2021 using cross-sectional types of the study design.

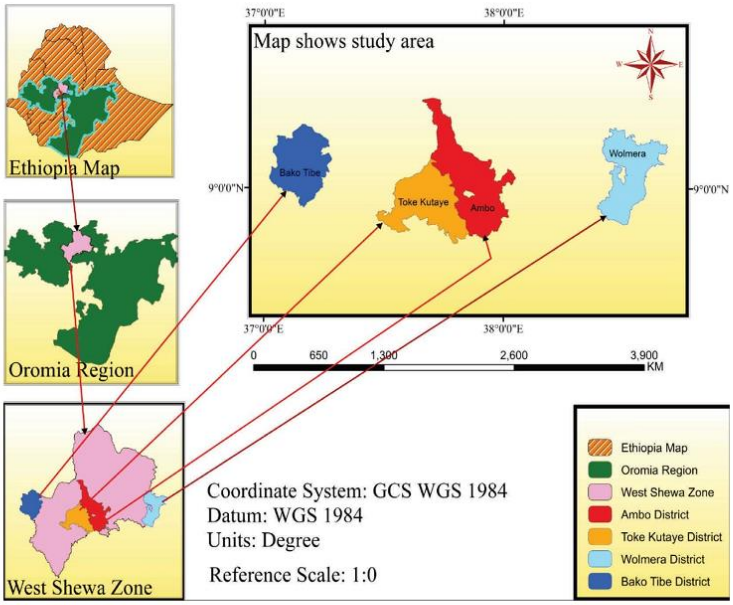

Figure 1: Map of the study area.

Table 1: General characteristics and agroecological features of the selected districts.

Characteristics	Districts			
	Wolmera	Ambo	Toke Kutaye	Bako Tibe
Administrative town	Holeta	Ambo	Guder	Bako
Distance from Addis Ababa in km	34	114	123	260
Direction	West	West	West	West
Latitude	8°50′–9°15′N	8°47′–9°21′N	9°11′N–9°18′N	9°06′N
Longitude	38°25′–38°45′E	37°32′–38°3′E	38°20′E–38°33′E	37°09′E
Average temperature (range) in °C	16 (9.13–19.66)	22 (15–29)	22 (16–29)	29 (28–31)
Altitude range in masl	2060–3380	1800–3200	1600–3194	1570–2600
Rain fall in mm	1,100	900	900	887

2.4. Sample Size Determination. Based on Thrusfield's [17] earlier formula, the number of animals that were sampled was estimated as follows:

$$N = \frac{z^2}{d^2} p(1-p), \quad (1)$$

where N is for sample size, z is the z value at 95% confidence intervals (1.96), p stands for predicted prevalence, and d stands for absolute precision, which is 0.05 at 95% CI.

$$N = \frac{1.96^2}{0.05^2} 0.71(1-0.71) = 318. \quad (2)$$

Since the earlier studies performed in some localities of the area reported the prevalence of mastitis to be 70.6%, the number of samples was then calculated with the expected prevalence of 0.71. Therefore, a total of 318 animals were expected to be tested for mastitis. However, only 258 animals were checked for mastitis. The pooled milk samples from all functional teats per animal were collected from all mastitis-checked animals for bacteriology.

2.5. Sampling Techniques. The districts, the peasant associations (PAs) in each district, and the farmers who had lactating cows at the time of sampling were randomly selected using a lottery system. However, since the advanced (semi-intensive and intensive) dairy farms were few in number, all of them were purposefully selected for the study. The clinical- mastitis-positive animals were sampled purposively. However, randomly chosen cows that did not exhibit obvious indications of inflammation on their teats or udders were examined for subclinical mastitis using the California mastitis test (CMT).

The number of milked cows in each farm was divided into three categories as: small (1–5 heads), medium (6–10 heads), and large (>10 heads). The lactating stage of the animals was classified in such a way that "early" from 0 to 3 months, "mid" from 3 to 6 months, and "late" longer than 6 months since gestation. The cows were divided into three age groups as: young < 4 years old, medium from 4 to 6 years, and adults > 7 years old. The cows were also grouped according to their parity level as few (1-2 calving), medium (3–5 calving), and many (>5 calving) [18].

Physical observation and palpation (when needed) of the animal were made to determine the body condition of the animal, tick infestation of the teat area, and presence of clinical mastitis. The body condition of the animal was recorded as good, moderate, or poor according to the criteria set by Metaferia et al. [19]. The clinical mastitis was considered as positive whenever there were changes in color and/or consistency of milk, or the cardinal signs of udder inflammation were observed.

The CMT was carried out in accordance with instructions provided by Quinn et al. [20]. Milk from each functional teats was squirted in each CMT paddle cup and then combined with roughly the equal amounts of 3% CMT reagent. The puddle was moved gently to agitate the mixture. The production of jelly (viscous) consistency within 3–5 minutes was considered CMT positive and then recorded as subclinical mastitis positive.

Strict aseptic practices were applied to prevent contamination from milker hands, the barn surroundings, and skin of the udder and teats. Briefly, the sample collector's hands were protected with gloves and disinfected with 70% ethanol. The teat were washed, disinfected with 70% ethanol, and the foremilk (initial jets) was expelled. A milk sample of 3 to 5 mL was drawn into a sterile universal bottle. The bottle was then labeled (with the animal ID, district, and the date of collection), and transported to the laboratory (Zoonosis and Food Safety Research Laboratory of Ambo University). The milk samples for bacteriology were collected as the pooled sample that milk from all functional quarters of a cow was squirtedinto a single test tube for bacterial identification. Therefore, the prevalence rate of S. aureus was expressed as per cow.

2.6. Isolation and Identification of S. aureus. In the laboratory, each sample was enriched with approximately 5 mL of sterile brain heart infusion (BHI) broth and incubated for 24 hours at 37°C. Consequently, the enriched culture was then spread onto a mannitol salt agar (MSA) plate and incubated at 37°C for 24 hours. Bacterial growth and mannitol fermentation within 24 hours were checked. The single colony was chosen and subcultured on brain-heart-infusion (BHI) agar. The pure colony on BHI agar was then stained by Gram stain, and observed under a light microscope. The morphology and arrangements of the bacterial cells was determined. Gram positive (purple blue) cocci with grape-like cellular arrangement were presumptively considered as staphylococci. Primary biochemical tests such as catalase and coagulase tests were carried out on the presumptive isolates [6].

Finally, the suspicious isolates were confirmed as S. aureus by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Biotyper mass spectrometry using the extended direct transfer (EDT) approach in accordance with the procedure described in Feyissa et al. [6].

2.7. Antibiogram Characteristics of S. aureus Isolates. To assess the isolates' susceptibility to antibacterial agents, we used disk diffusion techniques [21]. Initially, the purity of the isolates was checked on BHI agar. Second, 3-4 distinct pure colonies (24 hours culture) were suspended in a sterile 0.85% NaCl solution (to a concentration level of 0.5 McFarland turbidity). The turbidity was measured by using the McFarland Densitometer. The cotton swab on wooden stick was deeped into the suspension and streaked onto Muller–Hinton agar plates until the inoculum had sufficiently covered the plate's surface. Using a disk dispenser, the antibiotic disks were then applied to the agar plate and incubated at 37°C for 18 hours. The zones of inhibition of each antibiotic were measured (in mm), and the diameter was compared to the guidelines provided by the CLSI manual [22]. The drugs were chosen based on their availability in the laboratory and distribution in local veterinary clinics and pharmacies. As a result, the following antibiotics were chosen:

(1) Erythromycin (15 mg)
(2) Trimethoprim/sulfamethoxazole (1.25/23.75 mg)
(3) Ciprofloxacin (5 mg)
(4) Oxytetracycline (30 mg)
(5) Penicillin G (10 units)
(6) Cefoxitin (30 mg)
(7) Gentamycin (10 mg)
(8) Tetracyclin (30 mg)
(9) Novobiocin (5 μg)
(10) Clindamycin (2 mg).

2.8. Antibiotic Resistance and Thermonuclease Gene Determination

2.8.1. Bacterial DNA Extraction. Using the Qiagen DNeasy extraction kit (Thermo Scientific, Germany), the isolates' DNA was extracted in accordance with the manufacturer's instructions [6].

2.8.2. Gene Amplification Using PCR. In the current study, mecA, nuc, and blaZ genes of *S. aureus* isolates were attempted to be amplified by PCR. The procedure used a 31 μl master kit mixture which contains the following ingredients:

(1) 2 μL (2 μL $*$ 2 $*$ 3 = 12 μL) each primer
(2) μl of 25 mM $MgCl_2$
(3) 5 μl of PCR buffer

(4) 0.5 μl of dNTP

(5) 11.8 μl of RNase-free water

(6) 0.2 μl of Taq polymerase enzyme

An equal amount of 2 μl of extracted DNA from each sample and 2 μl of the master mix were mixed separately. Sterile water was used as the negative control. The specific primers of *mecA*, *nuc* [15], and blaZ [12] (Eurofins Genomic India Pvt. Ltd) were employed to target the oxacillin-resistant, thermonuclease, and penicillinase genes, respectively (Table 1).

The amplification of the genes was carried out in such a way that the above mixture was added to the PCR tubes and labelled gently. The PCR tubes with all the components were transferred to a Flex cycler thermal cycler (Biometra Gmbh, Germany). The thermal cycler was programmed as initial denaturation 95°C for 5 min and then the final denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min repeating for 37 cycles [23].

For the PCR amplification of the genes, the following specific primers were used:

5′GTAGAAATGACTGAACGTCCGATGA (mecA-F) and 5′CCAATTCCACATTGTTTCGGTCTAA3′ (mecA-R) with product length of 163 bp [15].

5′GCGATTGATGGTGATACGGTT (nuc-F) and 5′AGCCAAGCCTTGACGAACTAAAGC (nuc-R) with product length of 279 bp [15].

5′TACAACTGTAATATCGGAGGG (blaZ–F) and 5′CATTACACTCTTGGCGGTTTC (blaZ-R) with product length of 846 bp [11].

2.8.3. Gel Electrophoresis and Visualization of Amplicons. The PCR products that were stained with ethidium bromide were separated by electrophoresis in a 1.5% (w/v) agarose gel. The PCR product mixed with loading dye (bromophenol blue) was applied in the slots of the agarose gel. A maximum of 600 bp of DNA markers (Qiagen; Germany) with 100 bp interval DNA fragments were used to estimate the length of the amplicons. Electrophoresis was then run for one hour at 120 V with 1X TBE buffer. The separated PCR products in electrophoresis were then photographed using UV illumination and recorded by a gel documentation system (Gel DocTM XR+, BioRAD; Germany).

2.9. Data Analysis. Data from the field and lab were collected, coded, and entered into Microsoft Excel 2016 files.

R statistical analysis software was used to analyze the data. The association between the dependent variables (the prevalence of mastitis and S. aureus) and the risk factors was determined using the chi-square (χ^2) test and univariable and multivariable logistic regressions at 95% confidence intervals and a p value <0.05.

3. Results

3.1. S. aureus Isolation Rate and Risk Factors. A total of 258 milk samples from lactating cows were collected; 97 (37.6%) of these samples were from mastitis-positive cows, of which 59 (60.82%) were subclinical and 38 (39.5%) were clinical cases.

Out of the total 258 milk samples collected, 43 (16.67%) were *S. aureus* positive. The bacterium was isolated at a rate of 34/97 (35.05%) in mastitis-positive animals and 9/161 (5.59%) in mastitis-negative animals. Of the 34 *S. aureus* isolates from mastitis-positive cows, 19 (55.88%) were from clinical and the rest 15 (44.12%) were from subclinical mastitis. The chi-square (χ^2) test analysis revealed a significant correlation between the cows' mastitis positivity and the rate of *S. aureus* isolation. Similarly, the rate was found significantly varied with the type of mastitis that the bacterium was highly significant in clinical mastitis compared with subclinical mastitis ($p < 0.05$). This might make it evident that bovine mastitis which is caused by *S. aureus* is mostly clinical.

The logistic regression analysis of *S. aureus* and associated risk factors is displayed in Table 2. The univariate logistic regression analysis revealed that the districts, farm type, animals' body conditions, number of lactating cows in the herd, washing of the milker's hands and udder before milking, tick infestation, and mastitis positivity were associated significantly with the isolation rates of *S. aureus*. In the analysis of multivariate logistic regression, *S. aureus* was isolated at a significantly higher rate in the animals from farms with a large number of lactating cows in the herd and those infested with ticks than in the animals from farms with a small number of lactating cows and free of tick infestation, respectively ($p < 0.05$).

Also, we made an effort to identify the prevalence of mastitis and other risk variables among lactating cows in the research area. Out of 258 cows examined, 97 (37.6%) had mastitis, with 38 (39.18%) having clinical mastitis and 59 (60.82%) having subclinical mastitis. The occurrence rate of the disease was found to significantly vary with the farm type, lactation stage, milker's hand washing before milking, udder washing before milking, and current tick infestation of the teat. Hence, the occurrence of the disease was significantly higher in mid and late than early lactation stage and in cows managed under an extensive management system than in an intense management system. Furthermore, the disease occurred more frequently in animals milked with unwashed hands than in animals milked with washed hands, in animals whose udders were not washed before milking than in animals washed before milking, and in tick-infested animals than in tick-free animals ($p < 0.05$) (Table 3).

The chi-square test model revealed that SCM was generally significantly higher than clinical mastitis in the population. The disease types were discovered to vary with the age group; current tick infestation of the teat indicated that the animals in the younger age group were developing subclinical mastitis more frequently than the medium-aged groups. Tick-infested teats, on the other hand, were found to develop clinical types of the disease more frequently than tick-free teats ($p < 0.05$).

3.2. Antibiogram Characteristics of S. aureus. The antibiotic susceptibility profiles of 10 of the 43 *S. aureus* isolates were examined using 9 antibiotic disks. In the current study susceptibility of the isolates to Novobiocin was also

Table 2: Study of *S. aureus* isolation rate and related risk factors using binomial logistic regression.

Characteristics	Category	Samples	*S. aureus* positive (%)	Univariate		Multivariate	
				OR (95% CI)	*p* value	OR (95% CI)	*p* value
Districts	Toke kutaye	51	4 (7.84)	1.0		1.0	
	Wolmera	77	18 (22.22)	3.36 (1.16–12.20)	0.039	3.85 (0.92–19.96)	0.081
	Ambo	81	16 (20.78)	3.08 (1.05–11.30)	0.057	3.20 (0.66–18.40)	0.164
	Bako tibe	49	5 (10.20)	1.34 (0.33–5.70)	0.681	0.92 (0.14–6.10)	0.931
Agroecology	Lowland	49	5 (10.20)	1.0			
	Midland	128	20 (15.62)	1.63 (0.61–5.14)	0.358		
	Highland	81	18 (22.22)	2.51 (0.92–8.07)	0.089		
Breeds	Cross	115	21 (14.69)	1.0			
	Local	143	22 (19.13)	1.37 (0.71–2.66)	0.342		
Farm types	Intensive	27	1 (3.70)	1.0		1.0	
	Semiintensive	49	36 (12.24)	3.63 (0.58–70.5)	0.245	0.89 (0.08–23.35)	0.930
	Extensive	182	36 (19.78)	6.41 (1.3–116.3)	0.073	1.77 (0.22–40.61)	0.642
Age group of the animal	Adult	87	17 (13.93)	1.0			
	Medium	122	16 (18.39)	1.39 (0.66–2.95)	0.385		
	Young	49	10 (20.41)	1.58 (0.65–3.71)	0.297		
Lactation stage	Late	67	8 (11.94)	1.0			
	Mid	95	17 (17.89)	1.61 (0.67–4.17)	0.304		
	Early	96	18 (18.75)	1.70 (0.71–4.39)	0.246		
Parity level	Medium	167	24 (14.37)	1.0			
	Few	86	18 (20.93)	1.58 (0.79–3.09)	0.186		
	Many	5	1 (20.00)	1.49 (0.07–10.61)	0.727		
Animal's body condition	Moderate	47	22 (12.43)	1.0		1.0	
	Poor	34	11 (23.40)	2.15 (0.93–4.77)	0.063	1.20 (0.33–4.24)	0.778
	Good	177	10 (29.41)	2.94 (1.20–6.86)	0.014	2.67 (0.69–10.41)	0.152
Number of lactating cows in the herd	Few	224	33 (14.73)	1.0			
	Medium	16	4 (25.00)	1.93 (0.52–5.93)	0.279	9.99 (1.70–57.56)	0.009
	Large	18	6 (33.33)	2.89 (0.95–8.02)	0.047	12.58 (2.33–68.54)	0.003
Washing hands before milking	Yes	154	16 (10.39)	1.0		1.0	
	No	104	27 (25.96)	3.02 (1.55–6.07)	0.001	0.46 (0.11–1.69)	0.262
Washing udder before milking	Yes	158	12 (7.59)	1.0		1.0	
	No	100	31 (31.00)	5.47 (2.71–11.68)	0.000	2.12 (0.50–9.04)	0.304
Current tick infestation of the teat	No	208	11 (5.29)	1.0		1.0	
	Yes	50	32 (64.00)	31.84 (14.2–76.7)	0.000	27.69 (9.71–93.01)	0.000
Mastitis	Negative	161	9 (5.59)	1.0		1.0	
	Positive	97	34 (35.05)	9.11 (4.29–21.24)	0.000	2.34 (0.53–10.86)	0.263

OR = odds ratio and 1.0 = reference group.

Table 3: Binomial logistic regression analysis of association between bovine mastitis and risk factors.

Characteristics	Category	Mastitis positive (%)	Univariate		Multivariate	
			OR (95% CI)	*p* value	OR (95% CI)	*p* value
Districts	Wolmera	28 (34.57)	1.0			
	Ambo	33 (42.86)	1.42 (0.75–2.71)	0.285		
	Toke kutaye	19 (37.25)	1.12 (0.54–2.33)	0.754		
	Bako tibe	17 (34.69)	1.01 (0.47–2.11)	0.988		
Agroecology	Highland	28 (34.57)	1.0			
	Midland	52 (40.62)	1.30 (0.73–2.32)	0.381		
	Lowland	17 (34.69)	1.01 (0.47–2.11)	0.988		
Breeds	Local	42 (36.52)	1.0			
	Cross	55 (38.46)	1.09 (0.65–1.81)	0.749		
Farm types	Intensive	4 (14.81)	1.0		1.0	
	Semiintensive	18 (36.73)	3.34 (1.07–12.75)	0.051	2.45 (0.31–22.64)	0.40672
	Extensive	75 (41.21)	4.03 (1.48–14.17)	0.013	8.20 (1.33–68.25)	0.03451
Age group of the animal	Adult	43 (35.25)	1.0			
	Medium	33 (37.93)	1.12 (0.63–1.99)	0.691		
	Young	21 (42.86)	1.38 (0.70–2.71)	0.353		
Lactation stage	Early	28 (29.17)	1.0		1.0	
	Mid	41 (43.16)	1.84 (1.02–3.38)	0.045	5.03 (1.48–19.27)	0.01275
	Late	28 (41.79)	1.74 (0.91–3.37)	0.096	6.12 (1.55–27.32)	0.01229
Parity level	Moderate	51 (30.54)	1.0		1.0	
	Few	43 (50.00)	2.27 (1.33–3.90)	0.003	2.32 (0.78–7.16)	0.13117
	Many	3 (60.00)	3.41 (0.55–26.49)	0.186	0.12 (0.00–2.80)	0.19550
Animal's body condition	Moderate	57 (32.20)	1.0		1.0	
	Good	17 (50.00)	2.11 (1.00–4.45)	0.049	0.64 (0.12–3.20)	0.58828
	Poor	23 (48.94)	2.02 (1.05–3.89)	0.035	1.14 (0.30–4.22)	0.84861
Number of lactating cows in the herd	Small	80 (35.71)	1.0			
	Medium	7 (43.75)	1.40 (0.48–3.90)	0.520		
	Large	10 (55.56)	2.25 (0.85–6.11)	0.101		
Hand washing before milking	Yes	18 (11.69)	1.0		1.0	
	No	79 (75.96)	23.88 (12.6–47.8)	0.000	15.98 (5.6–51.5)	0.0000
Udder washing before milking	Yes	14 (8.86)	1.0		1.0	
	No	83 (83.00)	50.22 (24.4–111.3)	0.000	36.90 (12.2–135.6)	0.0000
Current tick infestation of the teat	No	55 (26.44)	1.0		1.0	
	Yes	42 (84.00)	14.60 (6.77–35.34)	0.000	34.24 (5.9–253.3)	0.00020
S. aureus	Negative	63 (29.30)	1.0		1.0	
	Positive	34 (79.1)	9.11 (4.29–21.24)	0.000	1.15 (0.22–6.09)	0.86272

OR = odds ratio and 1.0 = reference group.

Table 4: Antibiogram characteristics of *S. aureus*.

Antibacterials	Total tested	Susceptibility		
		Susceptible no (%)	Intermediate no (%)	Resistant no (%)
Ciprofloxacin	10	10 (100)	00 (0.00)	00 (0.00)
Penicillin G	10	00 (0.00)	00 (0.00)	10 (100)
Trimethoprim/sulfamethoxazole	10	10 (100)	00 (0.00)	00 (0.00)
Cefoxitin	10	10 (100)	00 (0.00)	00 (0.00)
Oxytetracycline	10	00 (0.00)	00 (0.00)	10 (100)
Gentamycin	10	10 (100)	00 (0.00)	00 (0.00)
Erythromycin	10	10 (100)	00 (0.00)	00 (0.00)
Tetracycline	10	00 (0.00)	00 (0.00)	10 (100)
Clindamycin	10	10 (100)	00 (0.00)	00 (0.00)
Novobiocin	10	10 (100)	00 (0.00)	00 (0.00)

determined. However, it has been performed as the default method for identifying *Staphylococcus* species from other Gram-positive cocci. Therefore the drug was used as supplementary test for S. aureus identification methods. The suceptibility test revealed that all the 10 isolates of S.aureus were resistant to Oxytetracycline, Tetracycline, and Penicillin G. In contrast, all of the isolates were shown to be susceptible to the antibiotics Clindamycin, Gentamycin, Ciprofloxacin, Cefoxitin, and Trimethoprim/Sulfamethoxazole (Table 4). Research has also investigated that the antibiotics Penicillin G, ox Tetracyclin, and Trimethoprim/Sulfamethoxazole are the most common drugs used for bovine treatment in the study area.

3.3. Antibiotic Resistance and nuc Genes. The antibiotic susceptibility tested *S. aureus* isolates were also checked if they have been harboring the drug resistance genes-*blaZ* and *mecA*-e, and thermonuclease (*nuc*) genes. The results of the conventional PCR revealed that all the tested *S.aureus* isolates bore the 279 bp long *nuc* gene. But none of them were found possessing the 163 bp and 846 bp long *mecA* and *blaZ* genes, respectively (Figure 2).

4. Discussion

In the current study, *S. aureus* was found in 43 (16.67%) of 258 total milk samples. The rate was significantly higher in mastitis-positive animals (35% versus 5.6%) ($p < 0.05$). Many previous studies have also isolated *S. aureus* at various rates from mastitis-positive cows. This result agreed with Emeru et al. [24] and Girmay et al. [25] reports. However, it is less when compared with the findings of Seyoum et al. [26], Birhanu et al. [2], and Abebe et al. [3], and it exceeded the findings of Bekele et al. [27], Etifu and Tilahun [28], and Lakew et al. [29].

S. aureus isolation rates were also found to differ between clinical and subclinical mastitis, with the pathogen being significantly more common in clinical mastitis-positive cows (OR = 2.93) than in subclinical mastitis-positive cows if other variables remained constant. In dissimilarity to the current study, Mekibib et al. [30] and Madut et al. [31] determined a higher *S. aureus* isolation rate in subclinical than clinical mastitis.

S. aureus has been identified as one of the primary etiologies of infectious mastitis in cattle [32]. The bacterium resides on superficial surfaces of the udder and the teats, as well as in the teat canal [33–35]. It is among the microflora and hence sometimes infects the animal as an opportunistic pathogen as the consequence of mechanical injury to the teat and other stress factors [36].

In the current study, increase in the number of lactation cows in the herd and tick infestation were linked with the higher isolation rates of *S. aureus*. The number of the lactating cows might affect the hygienic practices such as washing of milker's hands and teats of the animals before milking unless there is an increase in labor resources in the farm. In line with the current findings, Gebremedhin et al. [37] found a direct correlation between tick infestation and the prevalence rate of *S. aureus* in milk samples, as well as an inverse correlation between teat washing before milking and the isolation rate of *S. aureus*. Vouraki et al. [38] also reviewed that there have been reports of *S. aureus* isolation from *Rhipicephalus* spp. tick. *S. aureus* can spread from infected to uninfected udder quarters during milking if the cow's udder or teats are not cleaned before milking since it lives on the udder or teat surface of cows.

The prevalence of *S. aureus* did not significantly vary across animal parity levels in the current investigation ($p > 0.05$). Birhanu et al. [2], Garedew et al. [39], and Tasse et al. [40], however, reported the increment of the isolation rate of *S. aureus* with the parity number of cows.

It has been determined that 37.6% (97/258) of the tested cows were mastitis positive. This result was consistent with that of Sarba and Tola [41], who found that the prevalence of bovine mastitis in the Ambo district was 41.7%. However, Dabele et al. [18] found a higher prevalence rate (30.5%) of the disease in selected districts of the zone. The findings of most of the previous studies regarding bovine mastitis conducted in other parts of the country have reported the prevalence rate ranging between 46% and 73% which were higher than the current finding [3, 23, 25–28, 41, 42].

We found that the animals at the medium and late lactation stages were about 5 and 6 times more mastitic, respectively, than the early lactation stage. Similarly, the management systems were found significantly affecting the prevalence rate of the disease. Accordingly, the cows managed under an extensive farming system were found

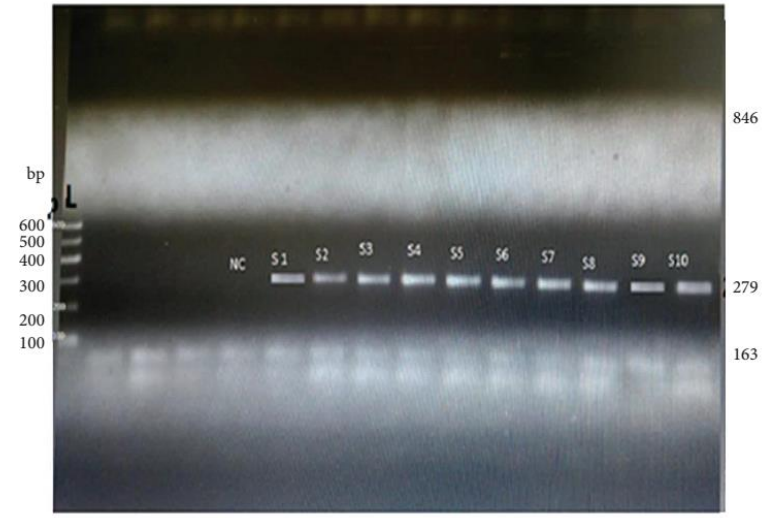

Figure 2: UV-illuminator imaged PCR product after electrophoresis in 1.5% agarose gel. L = ladder, S1–S10 denote samples, and NC stands for negative control. Description: the length of mecA = 163 bp, nuc = 279 bp, and blaZ = 846 bp. Note: bp represents base pairs.

about 8 times more attacked by mastitis than the cows managed under an intensive farming system. This might be because the extensive management system exposes the animal to trauma and tick infestation during grazing in the field, which, in turn, exposes the teats to pathogenic microorganisms including *S. aureus*. The rate of isolation of *S. aureus* in the current study was higher in tick-infested animals (OR = 27.69, 95% CI: 9.71–93.01, and $p < 0.001$) than in tick free lactating cows. The findings are consistent with previous research on the relationship between tick infestation and mastitis in Ethiopian cattle [43–46]. The positive association between tick infestation and bovine mastitis has been reported from different parts of the world [30, 37, 46, 47]. However, very few research studies, for example, Tolosa et al. [49] and Dabele et al. [18] in Ethiopia, reported that there is no significant variation in prevalence mastitis between tick-infested and tick free cows. Since ticks damage the tissues of teats and can be vectors for pathogenic microorganisms, they are strongly associated with udder lesions and mastitis in cattle [50–52]. However, little has been understood about the association between the parasite infestation and mastitis in Ethiopia [1].

In this research, lactation stage was found associated with bovine mastitis and the finding was in accordance with the previous findings [53, 54]. The finding agrees with Baraki et al. [55], Etifu and Tilahun [28], Abebe et al. [3], and Sarba and Tola [41] reports. According to Abera et al. [42], the rate of bovine mastitis was higher in cows with above 6 months of lactation than the early lactating cows. Similarly, Abebe et al. [3] and Dabele et al. [18] determined that the animals with early lactation stage have less probability to be affected by mastitis than those in the above middle lactation stage. Kitila et al. [45] and Argaw [53] stated, however, that bovine mastitis is more prevalent in cows with early stage than the middle lactation stages.

In our study, we also determined that washing hands before milking played a significant role in reducing mastitis in lactating cows. Our determination agreed with the finding of Shittu et al. [56] who found that the cows in the farms where hand washing before milking had been practiced were less affected by mastitis than the counterpart. Milker's hands might serve as the main vehicle for the spread of pathogenic microorganisms among the teats of the same animal and/or different animals. It is, therefore, crucial that washing hands within the interval of milking each animal can reduce the transmission chances among the animals [3].

Udder washing is an important hygienic practice to reduce not only the contamination of milk by pathogens of animal origin, but it also reduces the distribution of mastitis-causing microorganisms from animal to animal. Washing udders prior to milking can also remove dirt, organic matter, and microorganisms, promote milk let-down, and improve milk quality. The evidence found in this study confirms the practice to reduce the occurrence of mastitis in bovine. Previous studies also pointed that udder washing was found reducing the prevalence rate of mastitis in bovine [18, 30]. In contrast, however, Abebe et al. [3] did not find the udder washing associated with the reduction of mastitis in cows. But it should be underlined that the practice is not solely used to prevent mastitis since mastitis is a multifactorial disease in dairy animals [2].

The costly nature of mastitis in the dairy industry might be attributed to reduction in milk yield and quality and increment of treatment costs [2, 3, 27, 28]. The disease can also affect the responses of the ovarian follicules in cows as a consequence of which the conception rate can significantly decrease [57].

Furthermore, we have found that subclinical mastitis was significantly prevalent than the clinical type of the disease in the study area. About 61% of the mastitis-positive cows were

subclinical, and the rest 39% were clinical mastitis ($p < 0.05$). It has been reported that subclinical mastitis is the more common type of the disease than clinical mastitis [2]. In this aspect, the current finding is in accordance with the findings of most studies [29, 57–58]. Argaw [53] estimated the prevalence of clinical and the subclinical mastitis in Ethiopia to be 23 and 85%, respectively. The occurrence rate of clinical mastitis in this study was 14.73% (38/258), which is greater than the rates reported by Abera et al. [42] (10%), Abebe et al. [3] (3.4%), and Bekele et al. [27] (10%). The prevalence rate of subclinical mastitis (26.81%) is less than the reports of Abera et al. [42], Abebe et al. [3], Birhanu et al. [2], Baraki et al. [55], and Bekele et al. [27] who have reported the rates as 36.7%, 59.2%, 40%, 45.5%, and 47%, respectively.

All the 10 isolates checked for the antibiotic susceptibility were determined resistant to the three of the most commonly used drugs in the veterinary drugs in the study area: Tetracycline, Penicillin G, and Oxytetracycline. However, all of the ten *S. aureus* isolates were susceptible to Trimethoprim/Sulfamethoxazole, Gentamycin, Ciprofloxacin, Erythromycin, Clindamycin and. Cefoxitin, The observational analysis revealed that Pen-streptomycine, Penicillin G, Oxytetracycline, and sulfa drugs such as Sulfadimidine and Trimethoprim/Sulfamethoxazole are the most commonly used antibiotics in veterinary medicine. The disadvantages of using the sulfa drug as a treatment in practical applications are that they are needed in high doses and are less effective against inflammation-causing infections. The current study is in line with the assessments of Bekele et al. [27], Seyoum et al. [26], Mekonnen et al. [60], and Girmay et al. [61] who determined majority of mastitis cows isolated *S. aureus* resistant to Oxytetracycline and/or. Penicillin G.

The augmentation of the resistance pattern of the mastitis-causing microbes including *S.aureus* to commonly used antibiotics poses an additional burden on the industry [3]. It should also be underlined that the resistant microorganisms obtained locally can be disseminated to another area through the transportation of food materials.

All the tested isolates have harbored the *nuc* gene. However, the *blaZ* gene was not observed although the isolates were 100% Penicillin G resistant. *BlaZ* gene is responsible for hydrolysing the beta-lactam antibiotics such as penicillin and ampicillin because it encodes the β-lactamase enzyme [62]. The lack of *mecA* in the isolates was confirmation for susceptibility of the *S. aureus* isolates to cefoxitin, and the availability of *nuc* indicated that the isolates were thermostable [62].

5. Conclusions

Based on the study's findings, we deduced that *S. aureus* continues to be the primary cause of mastitis in dairy cows. Clinical mastitis was shown to have a greater prevalence of *S. aureus* than subclinical mastitis. Also, it was discovered that the study area has a high distribution of bovine mastitis. The antibacterial susceptibility traits of *S. aureus* showed us that they were extremely resistant to the locally prevalent antibiotics used in veterinary cares. There may be other genetic components or methods by which the bacteria resist penicillin, which could account for the absence of the blaZ gene in the examined *S. aureus* isolates. Therefore, the prevention and control of the pathogen, and as a consequence, the reduction of the prevalence of the disease, need to be designed. Antibiotics with better treatment responses should also be introduced to the area. Furthermore, the penicillin-resistant mechanism of *S. aureus* should be studied in depth.

Data Availability

The data used to support the findings of the study are available on request from the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

At Ambo University's Zoonosis and Food Safety Research Laboratory, *S. aureus* was initially isolated and identified. The authors consequently have the utmost regard for the administration of Ambo University in general and the head and lab staff of the Department of Veterinary Laboratory Technology in particular for granting the laboratory authorization for this work. The authors would also like to thank the head of the National Animal Health Diagnostic and Investigation Center (NAHDIC) and all the workers. Their gratitude should especially go to Mr. Abebe Olani, who supported them in facilitating the laboratory work in NAHDIC.

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Annex 8: Curriculum Vitae of the Aughter

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PERSONAL DETAIL

Date of Birth	:	June 6th 1977
Gender	:	Male
Marital Status	:	Married
Nationality	:	Ethiopian
Religion	:	Christian
Languages	:	English, Amharic, Afan Oromo

PERSONAL ATTRIBUTES

I am a mature & born again Christian committed to making a difference, excited by challenge and embrace diversity. I am enthusiastic, creative and rigorous who hungers for success. Given the opportunity to serve, I shall adhere to the rules, terms and conditions stated under the terms and reference of the vocation with utmost regard to the management.

KEY SKILLS

Time skills are excellent, organized and efficient in excelling at my work, this include:

- Flexibility/adaptability/managing multiple priorities
- Teamwork: I believe strongly in team work to manage work related issues: who excels at building trusting relationships with customers service and colleagues and with organization as whole.
- Possess good communication and presentation skills by consistently exhibiting high standards of professional conduct.
- Ability to take control and caution to avoid loses and reputation damage to the organization.

PERSONAL VALUES

A conscientious go-getter, who is highly organized, dedicated and committed to professionalism. I am dependable, responsible contribute committed to excellence and success

- Creative/Innovative: I believe that I am pro-active about fixing problems and finding solutions.
- Intelligence: I am always willing to learn and continue learning new things each and every day. I don't fear asking questions and figuring out the "in the mud" details.
- Self-Disciplined: I run in the 100-degree heat when nobody else is out there with you. I am able to get up for work when there is no one to hold you accountable.
- Self-Aware: I'm always able to analyze my decisions and beliefs. It helps me know why I made the decision and why I believe what I believe.
- Honesty/Integrity: I'm an all seasoned professional whose honesty and integrity provide for effective leadership and optional business relationships.

CAREER AND PERSONAL OBJECTIVES

- To serve in a capacity that would utilize my potential and give me enough exposure besides enhancing peace.
- To achieve excellence in working with highly changing technology and dynamism of people
- Ability to work in any given environment and produce good results.
- To pursue self-development and being an example to the community and population at large.
- To further gain hands-on experience on secretarial skills to the ever changing responsibilities

WORK EXPERIENCE

2015 to Date : **Ambo University College**

Position : Lecturer in Agriculture and Veterinary Science, Department of Veterinary Laboratory

March 2011 – September 2013 : **Ambo University College**

Position	**:**	Graduate Assistant in Veterinary Laboratory Agriculture and Veterinary Science Department
2006 –2010	:	Abe Dongoro District
Position	**:**	Head of the District Office; Oromia, Animal Production and Veterinary Services office
2000-2008	:	Abe Dongoro District
Position	:	Veterinary service Team Leader, Agricultural Office, Oromia, Ethiopia
1998 – 2000	**:**	Abe Dongoro District
Position	**:**	Animal health assistant, Agricultural office

EDUCATION AND TRAININGS

2017 to Date	:	Pursuing PhD in Applied Cellular and Molecular Microbiology Addis Ababa University, college of Natural and Computational Science
Sept 2012 – Sept 2014	:	Addis Ababa University, college of Veterinary Medicine and Agriculture Pursued MSc. in Veterinary Microbiology
Nov 2011 – Feb 2012	:	Basic Computer; Attained Computer skills and Journal Reviewing
2005 – 2010	:	Addis Ababa University, college of Veterinary Medicine and Agriculture Pursued BSc. in Veterinary Laboratory Technology, Faculty of Veterinary Medicine and Agriculture
1996 – 1998	:	Addis Ababa University, Faculty of Veterinary Medicine and Agriculture Animal Health Assistance
1985 – 1996	:	Gidda Ayana High School, Ethiopian School Leaving Certificate Exam (ESLCE)

PUBLICATIONS

- Review on vaccine design strategies against cancer: published in Journal of Cancer Research and Experimental Oncology (Volume 7, No. 1, pp.1-12, 2015
- Toxoplasmosis in camels (Camelus dromedaries) of Borana Zone, Oromia Region, Ethiopia: Seroprevalence and Risk Factors. Published in jornal of Tropical animal health and production (doi:10.1007/s11250-016-1133-3, pp. 1-8)
- Isolation and Characterization of Aerobic Bacteria and Influenza Virus 3 from Nasal Passageway of Camels (Camelus dromedaries) in Borana, Southern Ethiopia. Published in JJ Vet Sci Res 2019; 4(3):052
- Review on Phage Therapy as Alternative Treatment for Meticillin- Resistant S. Aureus (MRSA) Infections: Identification and Application of Phage. Published in J Sports Sci Physical Ther: JSSPT-104. 2019
- The Distribution and Drug Resistance Characteristics of Methicillin Resistant Staphylococcus aureous to be Public and Animal Health Burdon in Ethiopia: Systematic Revie. Published in J Nur Healthcare, 2022, 7(4), 47-67
- Isolation, identification, and determination of antibiogram characteristics of Staphylococcus aureus in cow milk and milk products (yoghurt and cheese) in West Showa Zone, Ethiopia. Published in International Dairy Journal. 137 (2023) 105503. www.elsevier.com/locate/idairyj.
- Isolation and Determination of Antibacterial Sensitivity Characteristics of Staphylococcus aureus from Lactating Cows in West Shewa Zone, Ethiopia. Published in Veterinary Medicine International. Volume 2023, Article ID 3142231, 12 pages. https://doi.org/10.1155/2023/3142231
- Sero-prevalence study and molecular detection of Brucella in pigs from sero-positive animal blood in central Ethiopia. Publication in Int. J. Adv. Res. Biol. Sci. 2023, Volume: 10, Issue: 5, page: 139-150: DOI:

http://dx.doi.org/10.22192/ijarbs.2023.10.05.011

MSC. THESIS

Isolation and Identification of Viruses and Aerobic Bacteria, and antibiotic susceptibility profile of selected bacteria from Respiratory Tracts of Borana Camels, Southern Ethiopia

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Declaration
I declare to the best of my knowledge that the information I have provided above is correct and reliable.

Date
30th May, 2023