**Thesis Ref. No________________**

**ADDIS ABABA UNIVERSITY**

**COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE**

# "EVALUATION AND IN VITRO ANTIMICROBIAL EFFECT ASSESSMENT OF ETHYL PYRUVATE'S EFFICACY AGAINST MAJOR PATHOGENS ASSOCIATED WITH BOVINE MASTITIS"

**MSc THESIS**

**BY:**

**TSEGANESH ASEFA**

**MSc PROGRAM IN VETERINARY CLINICAL MEDICINE**

**JUNE, 2025**

**BISHOFTU, ETHIOPIA**

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# “EVALUATION AND IN VITRO ANTIMICROBIAL EFFECT ASSESSMENT OF ETHYL PYRUVATE'S EFFICACY AGAINST MAJOR PATHOGENS ASSOCIATED WITH BOVINE MASTITIS”

**A Thesis Submitted to The Department of Clinical Studies, College of Veterinary Medicine and Agriculture in Partial Fulfillment of the Requirements for The Degree of Master of Science in Veterinary Clinical Medicine**

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**DEPARTMENT OF CLINICAL STUDIES**
**MSc PROGRAM IN VETERINARY CLINICAL MEDICINE**

**JUNE, 2025**
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As members of the examining board for MSc open defense, we have carefully read and reviewed Tseganesh Asefa thesis, titled “**Evaluation and in Vitro Antimicrobial Effect Assessment of Ethyl Pyruvate's Efficacy Against Major Pathogens Associated with Bovine Mastitis**" Based on our careful examination of the thesis, we accepted that it be approved as satisfying the requirement for the thesis of a Master of Science in Veterinary clinical medicine degree.

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**SIGNED DECLARATION SHEET**

First, I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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## AKNOWLEDGMENTS

First and for most I would like to thank the creature of all things, God, who have been helping me throughout my life. This work and the whole of my life's success would not have been possible without the will of almighty God.

I was deeply grateful to my advisor Prof. Alemayehu Lemma (PhD,) for his unwavering mentor-ship and encouragement during the preparation of this thesis. His willingness to share his time and knowledge has been instrumental in shaping this thesis into a well-defined and feasible endeavor.

I also want to extend my deepest gratitude and appreciation to my co-advisor Dr. Bethel Befekadu, for giving me her time and advising the thesis paper work. Her support, guidance and encouragement have been invaluable.

I would like to express my gratitude to the Animal Health Institute (AHI) for offering me a conducive environment and the necessary facilities to successfully complete this thesis. A special word of thanks goes to Dr. Gizat Almaw from the Bacteriology Laboratory of the Institute who was truly helpful in the laboratory work of this thesis. His help was truly appreciated. Additionally, I would like to extend my special appreciation to the professionals at the Institute, namely Mekdes Tamiru and Abebe Olani for their exceptional technical support.

I would like to thank Dr. Selam Meseret from International Livestock Institute and Behailu Beyene from Animal Development Institute (ADI) for provided LACTOSCAN SCC machine for SCC and guide me the procedures.

Finally, and most importantly, I am glad for my family's unconditional, unequivocal, and loving support.

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## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ADI | Animal development institute |
| AHI | Animal health institute |
| AMR | Antimicrobial resistance |
| ARF | Acute renal failure |
| ATP | Adenosine tri phosphate |
| CLSI | Clinical and laboratory standard institute |
| CMT | California Mastitis test |
| CNS | Central nervous system |
| CSA | Agricultural Sample Survey |
| DNA | Deoxyribonucleic acid |
| EMB | Eosin methylene blue |
| EP | Ethyl pyruvate |
| FAO | Food and Agriculture Organization |
| HF | Holstein Friesian |
| IMI | Intramammary infection |
| MALDI-TOF-MS | Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass spectrometry |
| NMC | National Mastitis Council |
| NO | Nitric oxide |
| ROS | Reactive oxygen species |
| SAP | Severe acute pancreatitis |
| SCC | Somatic cell count |
| TNF | Tumor necrosis factor |
| WHO | World Health Organization |

## ABSTRACT

The treatment of bovine mastitis is complex due to its varied etiology and the growing challenge of antimicrobial resistance, which limits the efficacy of conventional antibiotic therapies, particularly in persistent infections or those caused by certain pathogens like *Staphylococcus aureus*. Ongoing research explores alternative treatments such as plant-based therapies. However, the efficacy and practical application of these alternatives are still under investigation. Cross sectional study was conducted in Bishoftu, Ethiopia, from November 2024 to May 2025. This study aimed to evaluate the antimicrobial effects of ethyl pyruvate (EP) on major pathogens of bovine mastitis and assess them in vitro susceptibility profiles. Bacteria were isolated and identified using selective media and MALDI-TOF MS from 90 milk samples of cows confirmed to have mastitis using CMT and SCC which resulted 59.3% were "strongly positive," 30.5% "distinct positive," and 10.2% "weakly positive," with a statistically significant correlation ($p<0.01$; $r=0.653$) with SCC result. Culturing revealed that 65.5% of the samples contained primarily *Staphylococcus aureus* (28.8%), *Escherichia coli* (21.1%) and *streptococcus agalactiae* 6.7%. Sensitivity tests were carried out with different concentration (100%, 75%, 50% and 25%) of ethyl pyruvate with conventional antibiotic disks to see resistance pattern. The antibacterial effects of EP were found to be concentration-dependent. EP75 was the most effective at a 29.82 mm zone of inhibition, while EP25 had the lowest efficacy at 16.89mm. A significant difference was found in susceptibility among bacterial species, with *E. coli* showing the highest susceptibility at 25.63± 0.53 mm and *S. aureus* the lowest at 17.65mm. Furthermore, 100% multi-drug-resistant to Tetracycline, Ampicillin, and Amoxicillin showed across all tested species. In vitro analyses indicate EP possesses a significant antimicrobial property against major pathogens of bovine mastitis. Further *in vivo* investigation is warranted to ascertain its therapeutic efficacy and comprehensive dose-response studies to establish the optimal dosage regimens and administration routes for ethyl pyruvate practical application in field settings

**Key words**: B*acteria, Bovine mastitis, Concentration, Ethyl pyruvate, susceptibility*

# 1. INTRODUCTION

Ethiopia has one of the largest livestock populations in Africa (CSA, 2019). Despite having more cattle than any other African nation, Ethiopia's per capita milk consumption was lower than that of other nations in the region (FAO, 2009). This is partially caused by the native Zebu cattle's low genetic potential for milk production and the main cause is a number of diseases that could infect and negatively impact the health of the livestock population.

Mastitis is the most prevalent and costly disease, resulting in decreased milk yield, treatment expenses for dairy farmers and the culling of animals at an unsuitable age (Mekonnin *et al.*, 2016). It is a mammalian gland inflammation caused by pathogenic microorganisms and their toxins that invade the mammary gland (Abegewi *et al*., 2022). It is characterized by physical, chemical, and bacteriological abnormalities in the milk, as well as pathological changes in the udder glandular tissue. Mastitis can be caused by a variety of microorganisms, including viruses, fungus, algae, and both infectious and ambient bacteria. Bacterial pathogens pose a significant risk to the mammary gland among infectious agents; the most prevalent pathogens linked to mastitis are *coliforms*, *streptococci*, and *staphylococci* (Perez *et al.*, 2022).

It is one of the most economically damaging diseases due to its high incidence, which affects all dairy herds globally. Mastitis can account for up to 70% of decreased milk production, 9% of milk that is thrown out after treatment, 7% of veterinary care costs, and 14% of premature culling, according to several research findings (Bradley, 2002; Demme and Shimeles, 2015). In addition, some udder infections, such as *Staphylococcus aureus*, impact food safety and public health by producing toxins that cause food poisoning (Dego and Tareke, 2020). Identification of the types of organisms and selection of an effective antimicrobial agent against the organism is critical to the successful care of animals and public health (Griffioen *et al.*, 2021).

Antibiotic medication is the primary means of mastitis management, which includes both curative and preventive approaches (Benic *et al*., 2018). Many dairy farms across the world employ antibiotics, such as penicillin, ampicillin, tetracycline, and gentamycin, as part of the preventative management of mastitis and for dry cow therapy (Hossain *et al.*, 2017). However, the emergence and spread of microorganism's resistant to multiple anti-microbial agents is great challenge to the medicine. Furthermore, the ability of certain microorganisms, like *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*, to form a biofilm presents significant challenges in the medicine (Donlan, 2002). Additionally, antibiotic residues in milk pose health risks to consumers (Oliver and Murinda, 2012; Kurjogi *et al*., 2019). With declining antibiotic discovery rates, there's an urgent need for novel antimicrobial therapies capable of eradicating microbes.

Ethyl pyruvate (EP) shows promise as a novel antimicrobial agent, addressing the urgent need for new therapies. Initial studies demonstrated its ability to reduce injury during ischemia and endotoxemia by scavenging reactive oxygen species and suppressing pro-inflammatory cytokines (Kao and Fink, 2010). EP has also shown anti-inflammatory and antibacterial effects in various models (Ulloa *et al*., 2002; Venkataraman *et al*., 2002; Yang *et al*., 2002; Sappington *et al.*, 2003; Song *et al.*, 2004; Johansson *et al.*, 2008). Notably, EP exhibits antimicrobial properties against biofilm-associated bacteria like *Staphylococcus epidermidis* and *Staphylococcus aureus*, commonly isolated from mastitis (Debebe *et al*., 2016).

EP is believed to directly interfere with glucose metabolism causing depletion of cellular ATP and affect mitochondrial oxidation via accumulated toxic methylglyoxal (Baunacke *et al*., 2014). Its anti-inflammatory nature and use as a food additive makes it an interesting candidate to reduce severity without milk withdrawal times and environmental safety (WHO, 2001; Bennett-Guerrero *et al.*, 2009). EP addresses both infection and inflammation, making it potentially beneficial for treating mastitis without contributing to antibiotic resistance. However, its application in bacteria causing bovine mastitis has not been explored. Therefore, the objectives of this work are:

**General objectives**

- ✓ To evaluate the antimicrobial efficacy of ethyl pyruvate against major bacteria pathogens in bovine mastitis and assess its potential inhibitory effect depending on concentration and bacterial species.

**Specific objectives**

- ✓ To isolate and identify major bacteria associated with bovine mastitis from raw milk, using standard screening and advanced methods of identification
- ✓ To evaluate the *in vitro* antimicrobial effects of ethyl pyruvate on major pathogens of mastitis
- ✓ To determine the *in vitro* dose dependent susceptibility profiles of key mastitis pathogens to ethyl pyruvate using different dilutions
- ✓ To assess resistance profile of identified bacteria species for standard antimicrobial agents used in treating bovine mastitis

## 2. LITERATURE REVIEW

### 2.1. Background of Major Pathogens Associated with Mastitis

Mastitis is recognized as a classic example of a complex disease, which is known to be caused by the interaction of three bio-systems: the host, the infection, and the animal's environment. Breed, anatomy of the teat canal and the existence of a teat lesion are examples of host factors. The ability to live in the animal's immediate surroundings, colonize the teat duct, stick to the mammary epithelium, and resist being flushed away with milk flow are all examples of agent factors. Environmental factor includes milking practice, housing system and bedding (Makovec and Ruegg, 2003).

Bovine mastitis has been associated to about more than 135 different microorganisms, the most prevalent of which are categorized as either contagious or environmental mastitis pathogens (Bradley *et al.*, 2002). Intra-mammary infections brought on by microorganisms whose primary reservoir is the cow's surroundings are collectively referred to as environmental mastitis. Those pathogens comprise a diverse group of organisms, such as environmental *streptococci (Streptococcus uberis* and *Streptococcus dysgalactiae*), *coliforms (Escherichia coli, Klebsiella, Enterobacter, and Citrobacter), Pseudomonas, Proteus, Listeria and yeast* (Cameron *et al.,* 2016; Bobbo *et al*., 2017). Among them *E. coli* is the most prevalent cause of bovine mastitis (Zhang *et al*., 2018).

From contagious pathogens *Staphylococcus aureus* has been extensively recognized as a prevalent pathogen in Intramammary infections in dairy cows due to its high transmissibility and ability to induce chronic infections (Monistero *et al*., 2018). Numerous surface proteins that serve as virulence factors, such as adherence to surfaces, induction of biofilm formation, invasion of epithelial cells and immune evasion are expressed by it. *Streptococcus agalactiae* is also among the major mastitis pathogens which have a considerable impact on cow health, milk quality and productivity (Mungube *et al*., 2004). The primary means of transmission is during milking include infected milking equipment, clothing, and the hands of milkers or machine operators (Abera *et al*., 2010).

## 2.2. Current Treatment Strategies for Mastitis

Over the past few decades up to now, cows with mastitis (clinical or sub-clinical) are more likely to be treated with antibiotics, such as Fluoroquinolones, aminoglycosides (including gentamycin, amikacin) (Cameron and McAllister, 2016), ampicillin, tetracycline etc., which can be given by intra-mammary infusion, intramuscular or intravenous injections (Hossain *et al*., 2017). In addition, streptomycin and cloxacillin have also been applied for treating IMI, (Petrovski *et al*., 2015). Cephalosporin's such as third-generation ceftiofur and fourth-generation cefquinome have also been used (Dong *et al.,* 2022). Alternative approaches to mastitis treatment, aside from antibiotics, encompass animal-derived products like lactoferrin and chitosan as well as microbial-derived substances like bacitracin (Saeed *et al.,* 2024). Others include herbal antimicrobial substances, antimicrobial peptides, bacteriophages, and nanomaterials. There are vaccines for mastitis, but they should be used in conjunction with a mastitis prevention program because they only lessen the severity of the illness and do not prevent future infections (Radostits *et al.*, 2007).

## 2.3. Antibiotic Resistance in Mastitis

Around 60–70% of the antimicrobial drugs used on dairy farms are solely used to treat and prevent mastitis (Metaferia *et al*., 2011). The causal pathogen's resistance to the selected antimicrobial agent is the main problem of treatment and control program of mastitis worldwide (Barlow, 2011). Antimicrobial resistance in microorganisms and the rise of multidrug-resistant bacteria may be primarily caused by selection pressure and the abuse of antimicrobial drugs in animal production. Worldwide, a number of studies have documented AMR bacteria from cow's milk, particularly those that are resistant to penicillin G. Dairy cows have been treated with penicillin and beta (β)-lactam antibiotics for more than 50 years, which may account for the rise of resistance to these drugs (Molineri *et al.,* 2021). One of the earliest AMR bacteria was *S. aureus,* which was discovered in 1948, only a few years after penicillin manufacturing and use (Pantosti *et al.*, 2007; Economou, and Gousia, 2015).

### *2.3.1. Causes of antimicrobial resistance in mastitis*

**Bacterial biofilm formation:** Microbes can form highly structured communities known as biofilms, which are encased in a self-produced protective extracellular matrix. This phenomenon poses significant challenges for the medical industry (Donlan, 2002). Biofilms consist of microbial communities that adhere to biological or non-biological surfaces, embedded in a polymer matrix of extracellular DNA, proteins, polysaccharides, and mineral crystals (Costa and Silva, 2020). The protective nature of biofilms hinders the penetration of antibiotics, facilitates delayed growth, alters metabolism, and allows the emergence of persisted cells. These adaptations enable bacteria to evade host immune responses and antimicrobial effects, allowing them to thrive in hostile environments (Costa and Silva, 2020). Notable examples include *S. aureus*, *P. aeruginosa*, and *S. epidermidis* (Wu, 2015). Consequently, infections associated with biofilms are notoriously resistant to both host defenses and conventional antimicrobial treatments (Jabra-Rizk, 2004).

**Intracellular localization of bacteria:** Many pathogenic bacteria, such as *S. aureus*, can invade and persist within eukaryotic cells such as bovine mammary epithelial cells and endothelial cells (Kamaruzzaman *et al.*, 2018). These bacteria utilize specific adhesion proteins to enter host cells and employ mechanisms like the zipper mechanism or endocytosis for invasion. Once inside, they can establish chronic infections, complicating treatment options (Leon *et al.*, 2015). The challenge of treating intracellular bovine mastitis arises from the limited ability of conventional antibiotics to penetrate host cells effectively (Goormaghtigh and Van Bambeke, 2024). Furthermore, some bacteria reside within lysosomes and phagolysosomes, which maintain very acidic conditions. This acidic environment contributes to the bacteria's defense, as many antimicrobials are ineffective under such conditions (Collier *et al.*, 2014).

**Genetic mutation:** Through horizontal gene transfer and gene mutations, bacteria develop resistance (McInnes *et al*., 2020). Bovine mastitis has been linked to a number of antimicrobial resistance genes, including ermA, ermB, and ermC (erythromycin resistance genes), norA (fluoroquinolone resistance gene), tetM and tetK (tetracycline resistance genes), and blaZ and blaTEM (β-lactam resistance genes) (Monistero *et al*., 2020).

### *2.3.2. Effects of antimicrobial resistance in mastitis*

The emergence and spread of microorganism's resistant to multiple anti-microbial agents is a great challenge to the medicine. As antibiotics become less effective, certain infections are becoming harder, and even impossible, to treat. Apart from AMR, misusing antimicrobials negatively affects gut microbiota of dairy cows (Dong *et al*., 2022). Furthermore, indiscriminate use and not following the treatment regiments increases the risk of the presence of antibiotic residues in the milk, which renders it unfit for processing and consumers (Pascu *et al*., 2022). However, while resistance rates continue to rise, the rate of antibiotic discovery has dropped substantially. Sadly, only a few new agents have recently been approved and are available. Therefore, there is an urgent need for new approaches in the field of antimicrobial therapy, having the capacity to eradicate biofilms as well (Tomanić *et al.*, 2023).

## 2.4. Ethyl Pyruvate as A Potential Treatment

Ethyl pyruvate is a simple aliphatic ester that produced from pyruvic acid (CH3COCOOH) of an endogenous metabolite. It has a molecular weight of 116.5 and the formula CH3COCOOC2H5. Additionally, EP is a translucent, colorless liquid. At room temperature, ethyl pyruvate has a fresh flowery scent and is soluble in water. It is also a very weak acidic chemical. Its stability, low toxicity, and affordability have made it a popular choice in biomedicine (Lu, *et al*., 202).

Its biological effects were first noted in research that demonstrated in reduce intestinal and hepatic damage during experimental ischemia, reperfusion, and endotoxemia by suppressing pro-inflammatory cytokines and scavenging reactive oxygen species. The positive effects of EP in mammalian cells have been attributed to a variety of potential targets (Kao and Fink, 2010). In numerous preclinical models of critical illnesses, EP treatment has been demonstrated to increase survival and improve different organ dysfunctions (Fink, 2008). About 100 new articles released after 2010 have not yet been compiled and reviewed, out of the approximately 340 EP-related publications that have been published to date. Recent research has demonstrated that EP has anti-oxidative, anti-inflammatory, anti-tumor, and anti-trypanosome properties in addition to other pharmacological effects (Muller *et al*., 2010; Worku *et al*., 2015; Lu *et al*., 2021).

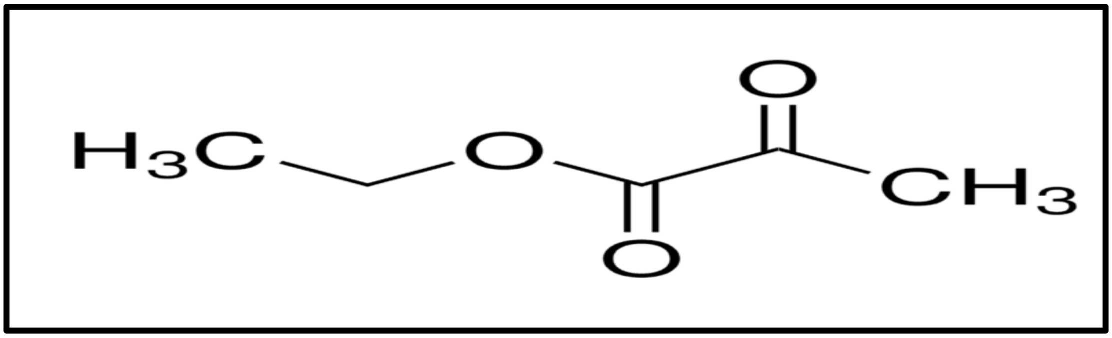


**Figure** 1:Chemical structure of Ethyl pyruvate

## 2.5. Previous Studies On the Pharmacological Actions of Ethyl Pyruvate (EP)

### *2.5.1. Anti-Inflammatory Activity*

In experimental animal models, this novel anti-inflammatory drug can treat systemic inflammation and numerous inflammatory organ damage including hemorrhagic shock (Di *et al*., 2010), arthritic CIA in mice (Di *et al*., 2010), and fatal sepsis and systemic inflammation (Kim *et al*., 2008). It reduces the tissue damage associated with severe acute pancreatitis (SAP) (Yang *et al*., 2004; Cheng *et al.*, 2007; Yang *et al.*, 2008; Luan *et al*., 2013), sepsis-induced acute renal failure (ARF) (Miyaji *et al.*, 2003). It also prevented neutrophils from adhering to lung epithelial cells and suppressed the production of inflammatory-regulating cytokines like IL-8, thereby, EP was found to be effective in treating acute pulmonary problems (Johansson and Palmblad, 2009).

### *2.5.2. Antioxidant Activity*

By reducing oxidative stress and nitric oxide release in a range of in vitro and in vivo model systems, EP's antioxidant and ROS scavenger properties have been effectively demonstrated (Kenneth and Mitchell, 2010). EP infusion lowers oxidative stress and nitric oxide release, it enhanced hemodynamic stability and alleviated chronic endotoxemia-induced acid-base derangements in pigs (Hauser *et al*., 2005). By reducing the harmful effects of reactive oxygen species (ROS) in rats with severe abdominal infections, it lessens the damage to the intestinal mucosa (Li *et al.,* 2006). In rats given paraquat, EP has also demonstrated antioxidant effects on the liver and lung tissues (Lembert *et al.*, 2001).

### *2.5.3. Anticancer Activity*

EP has the ability to stop the growth of some tumors. This anti-tumor action might be related to its anti-inflammatory properties. EP inhibit the growth of tumors, perhaps by causing a change in the manner of cell death model from tumor promoting necrotic cell death to tumor suppressive apoptotic cell death (Lim *et al*., 2007). In a liver tumor model, EP exhibits strong anti-tumor action (Liang *et al*., 2009). Through its mechanism of targeting alveolar macrophages and respiratory epithelial cells, intranasal administration of EP dose-dependently inhibited the release of tumor necrosis factor alpha in Broncho alveolar lavage fluid and lung inflammation in mice (van Zoelen *et al*., 2007). By down-regulating the HMGB1-RAGE axis, EP administration on cultured cancer cells were also prevents gallbladder cancer cells from growing and invading (Li *et al.,* 2012).

## 2.6. Antimicrobial Activity of Ethyl Pyruvate and Its Mechanism

EP possesses antimicrobial properties against bacteria, fungi, molds, and parasites in *vivo* and *vitro* model that exhibit clinically significant resistance (Worku *et al*., 2015; Cetin *et al.,* 2019). Treatment with vaporized EP produced strong antibacterial activity against both Gram-positive and Gram-negative bacteria (Zeki *et al*., 2012). Additionally, EP can become a powerful agent that promotes matrix breakdown and inhibits microbial adhesion, biofilm colonization, and cell proliferation. Because of this, EP possesses antimicrobial properties against pathobiont bacteria and fungi biofilms, including *Salmonella spp., Clostridium spp., Gram positive cocci*, non-spore producing anaerobes, Gram negative oxidase positive bacteria and *Candida albicans (*that the common antifungal medication Amphotericin B (AmpB) was unable to inhibit) (Debebe *et al*., 2016).

### *2.6.1. Mechanism of action*

By direct interference with glucose metabolism, EP efficiently suppresses the development of cells that primarily rely on glycolysis (Baunacke *et al.*, 2014). According to target studies, EP inhibits pyruvate kinase (PK) and glyoxalase 1 (GLO 1), two enzymes involved in the glycolytic and para-glycolytic pathways (Hollenbach *et al*., 2008; Worku *et al*., 2015). Consequently, EP depletes cellular ATP and influences mitochondrial oxidation by causing the hazardous methylglyoxal to accumulate. Many microorganisms and cells that primarily rely on glucose oxidation ought to respond well to EP therapy. Given that practically every microorganism under investigation has a highly conserved glycolytic chain. EP is less harmful to cells when they obtain their energy via gluconeogenesis rather than glycolysis. The toxicity of EP increased when microorganisms are metabolically activated (Baunacke *et al.*, 2014).

### 2.7. Importance of Implementing Ethyl Pyruvate in Treatment Protocols

Human, veterinary, and technological industries could all benefit greatly from this possibly novel antimicrobial, antifungal, anti-inflammatory and antibiofilm chemical. As of currently, EP is superior to other antibiotic agents for a number of reasons: it has not yet been shown to cause any side effects in clinical trials (Bennett *et al*., 2009), it is tissue protective (Kao and Fink, 2010), it inhibits a wide range of pathobionts (bacteria, fungi, molds, and parasites) and it is innocuous to symbionts (*Lactobacillus spp),* it dissolves the biofilm matrix, prevents biofilm adhesion and maturation, and it environmentally safe (Debebe *et al.,* 2016). Since EP addresses several targets, the possibility of resistance developing is minimal. Clinical investigations have already used EP infusion and no adverse effects have been documented in people (Bennett *et al.*, 2009). It is safe for human consumption as a food additive (FAO, 2001). Due to its low cost and low toxicity, EP has been considered for potential use in a wide range of areas within the biomedical field. Ethyl pyruvate may be a particularly interesting option to lessen the severity of bovine mastitis without significantly altering milk withdrawal times.

## 3. MATERIAL AND METHODLOGY

### 3.1. Description of Study Area

The study was carried out in Bishoftu, Oromia, Ethiopia's East Shoa zone. The town of Bishoftu is situated 45 kilometers southeast of Ethiopia's capital, Addis Ababa. With an elevation of 1850 meters above sea level (masl) and an annual rainfall of 866 mm, 84% of which falls during the lengthy rainy season (June to September), the region is situated at latitude 9ºN and longitude 40ºE. The dry season extends from October to February. With a mean relative humidity of 61.3%, the average annual maximum and lowest temperatures are 26°C and 4°C, respectively (Metages, 2018). There is also an extensive animal population in the Bishoftu area, with over 160,697 cattle, 22,181 sheep, 37,510 goats, 1,660 horses, and 191,380 fowl. The livestock production system in the area is both intensive and extensive type (CSA, 2023).

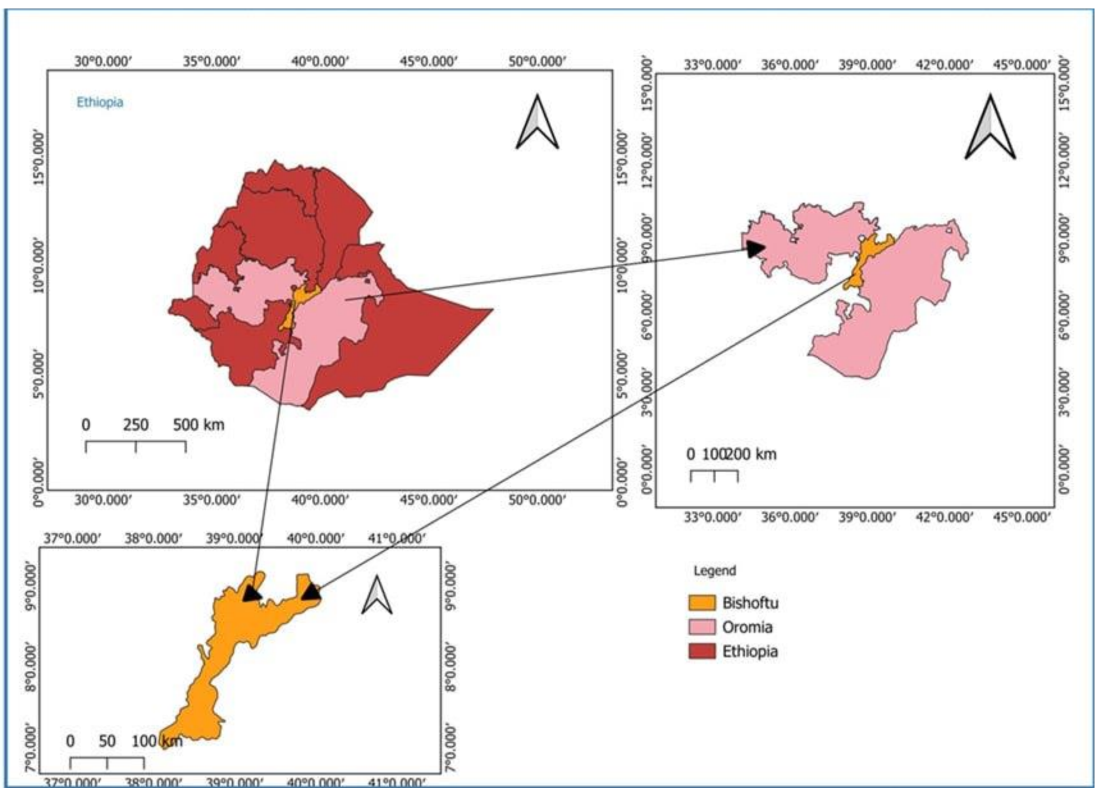


**Figure 2**: Map of study area, Bishoftu town, Oromia region. Ethiopia

## 3.2. Study Population and Husbandry Practices

The study animals were lactating Holstein Friesian (HF) breed dairy cows in the selected dairy farms. Every animal was identified by the farm's name with a particular age group, lactation stage, parity, milk production, and body condition that had both clinical and subclinical mastitis instances. All the farms examined were practiced intensive management system and there milking system was manual (by hand).

## 3.3. Study Design and Sample Size

Cross sectional study design was conducted during the study period from November 2024 to April 2025. A total of 10 dairy farms from intensive management systems were selected and assessed based on willingness and cooperation. In addition, 90 milk samples were collected from 30 individual lactating cows through systematic purposive sampling.

## 3.4. Data Collection Methods

### *3.4.1. Clinical Examination of Udders*

A thorough clinical examination of the mammary glands was performed on each animal, encompassing both visual inspection and careful palpation. The udders were assessed for indicators of inflammation, fibrosis, tick infestation, visible trauma, and swelling of the supramammary lymph nodes. In cases of clinical mastitis, rectal temperature was recorded to evaluate systemic involvement. Any observed anomalies or alterations in milk characteristics (e.g., watery secretions, clots, blood, flakes, pus), udder consistency, and size were meticulously investigated, adhering to established clinical examination guidelines (Quinn *et al*., 2011). The collected data for each animal will be utilized for subsequent analyses. Before the collection of milk samples, surfaces of the teats and udder were thoroughly cleaned with tap water, dried, and then swabbed and soaked in 70% alcohol. During scrubbing with alcohol, teats on the far side of the udder were washed with alcohol first, then those on the near side to prevent re-contamination of teats (NMSA, 2004).

*3.4.2. California Mastitis Test (CMT)*

In cows without clinical mastitis, milk samples were collected from each udder quarter and screened for sub-clinical mastitis using the CMT as per Quinn et al. (1999). Reactions were scored from 0 (negative) to 3 (strongly positive) based on coagulation and viscosity, indicating infection presence and severity. CMT-positive samples were selected for Somatic Cell Count (SCC) and bacteriological culture following Quinn et al. (1999). A cow was considered positive for mastitis if at least one quarter yielded a positive CMT result.

*3.4.3. Milk Sample Collection, Handling and Storage*

Using standard milk-sampling methods, additional 90 milk samples were taken from CMT-positive quarters. To minimize contamination of the teat ends during sample collection, the near teats were sampled first, followed by the distant. A sterile sample cup was filled with 10 mL of milk after the first three milking streams were disposed of. After being kept in an icebox, the samples were taken to the Animal Development Institute (ADI) for SCC and then to the Animal Health Institute (AHI) lab for additional bacteriological testing. Before being inoculated in a conventional bacteriological medium, samples were kept in the lab at 4°C for a maximum of 24 hours (NMC, 2004).

*3.4.4. Somatic Cell Count (SCC) Test*

The examination of somatic cell count (SCC) in milk samples was conducted within 3-6 hours of collection using the LACTOSCAN SCC LIT reference method as per standards set by Milkotronic Ltd. Initially, the milk sample was vortexed, and 100 microliters of milk was added to a micro-tube containing SOFIA GREEN lyophilized dye, followed by further vortexed. Subsequently 8 microliters of this mixture were pipetted into the microfluidic camera of the LACTOCHIP X4 and inserted into the cartridge of the LACTOSCAN SCC machine. Finally, the results were analyzed on the LACTOSCAN computer. A result up to 200,000 cells/mL was taken to be a low SCC, since this is generally considered healthy mammary gland. A high SCC was associated with trace (200,000–500,000 cells/mL), weakly positive (500,000–1,500,000 cells/mL), distinctly positive (1,500,000–5,000,000 cells/mL) and strongly positive (greater than 5,000,000 cells/mL) mastitis findings (Williamson et al., 2022).

### *3.4.5. Bacterial Isolation and Identification*

Milk samples were enriched in peptone water for 24 hours, followed by streaking onto selective media: Mannitol salt agar (for *Staphylococci*), Edwards's media (for *Streptococcus*), MacConkey agar for Gram-negatives, and eosin methylene blue (EMB) agar for *E. coli*. Plates were incubated aerobically at 37°C for 24-48 hours. *Staphylococci* were identified by growth on mannitol salt agar, with *S. aureus* fermenting mannitol to produce yellow colonies (Quinn, 1999). Suspected *Staphylococcus* colonies with grape-like clusters and golden-yellow color were further cultured on nutrient agar. *Streptococci* were isolated on Edwards's media based on colony morphology and hemolysis. Suspect colonies were sub-cultured on Tryptic Soy Agar with 5% sheep blood, and Gram-positive cocci exhibiting hemolysis were considered *Streptococci* (Chuang, 2019). Pure isolates were obtained on 5% sheep blood agar. Gram-negative bacteria, primarily *E. coli*, *Klebsiella*, and *Pseudomonas*, were targeted on MacConkey agar based on lactose fermentation. Mucoid, dome-shaped lactose-fermenting colonies suggested *Klebsiella*. Presumptive *E. coli* colonies from MacConkey were sub-cultured on EMB; metallic green sheen colonies were selected. Non-lactose fermenting pale colonies on MacConkey producing greenish pigment on nutrient agar were suspected *P. aeruginosa* (Quinn *et al*., 2011). Representative pure colonies were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

### *3.4.6. MALDI-TOF-MS based Identification of Pathogens*

The method for species identification was carried out using MALDI-TOF MS, (Bruker Daltonik) according to the company's instructions (Bizzini *et al*., 2010). Smear bacteria (single colony) as a thin film directly on to a spot on a MALDI target plate after all putative isolates were sub-cultured on nutrient agar (HIMEDIA, INDIA) and incubated at 37 °C for 24 hours overnight. Following a 2-minute the pellet was reconstituted using a solution of 25 µl formic acid (70%). The plate was then left to air dry at room temperature. For every designated BTS QC spot, 1 µl of BTS was applied, and it was left to air dry. After covering the entire area with a droplet of 1μl of matrix solution (CHCA), the region was once more fully dried at room temperature. Lastly, analysis was carried out using biotyper software after inserting the MALDI target into the MALDI-TOF mass spectrometer.

### 3.5. Ethyl Pyruvate Disk Preparation

Ethyl pyruvate discs were prepared by dissolving varying volumes of ethyl pyruvate in deionized water to obtain specific concentrations: 100%, 75%, 50%, and 25%. In a sterile tube, 3 mL of ethyl pyruvate was combined with 1 mL of deionized water for the 75% solution, while subsequent dilutions involved mixing 2 mL of ethyl pyruvate with 2 mL of deionized water for the 50% solution, and 1 mL of ethyl pyruvate with 3 mL of deionized water for the 25% solution. All dilution including the 100% (4ml of EP) concentration was prepared in a safety cabinet to maintain a sterile environment and prevent contamination. Small pieces of filter paper were then impregnated with the resulting solutions of ethyl pyruvate and deionized water.

### 3.6. Antimicrobial Susceptibility Test Using Disc Diffusion Method

#### *3.6.1. Ethyl pyruvate (EP) Susceptibility Test*

After identifying bacterial isolates with MALDI-TOF (Bruker Daltonik), antimicrobial susceptibility for ethyl pyruvate was done using disk diffusion method (Kirby Bauer technique) (Khalili *et al.,* 2012). Representative colonies of isolates were suspended in sterile 5ml saline water solution and adjusted to a turbidity of 0.5 McFarland units using a densitometer. Sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the surface of Muller- Hinton agar (Himedia, M173) plate within a sterile safety cabinet. For *streptococcus* 5% sheep blood were mixed in Muller- Hinton agar. The plates were held at room temperature for 15 minutes to allow drying followed by placing ethyl pyruvate-impregnated Small paper disks on every plate that prepared in different concentration ranged 25%, 50%, 75% and 100%.  The plates were incubated at 37°C for 18 hours. During incubation, the EP diffused from the disk into the surrounding agar. The diameters of the zones of inhibition were measured in millimeters using a digital caliper (ExGizmo, 12 Inch-300mm).

### *3.6.2. Antimicrobial Susceptibility Test using commercial antibiotic disks*

Antimicrobial testing was also done using commercial drugs to show resistance pattern using the same procedure on EP Susceptibility Test. Antimicrobial discs were prioritized including penicillin G (10µg), Ciprofloxacin (5µg), Erythromycin (15µg), Trimethoprim-Sulphamethoxazole (25µg), Ampicillin (10µg), Amoxicillin clavulanic acid (30µg), Tetracycline (30µg), Gentamycin (10µg), Cefoxitin (30µg), Streptomycin (10µg) and vancomycin (30µg) based on their availability and distribution to nearby veterinary clinics and pharmacies. Zone diameters were measured and interpreted as sensitive, intermediate and resistant according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2022). Isolates considered multi-drug resistant were found to be resistant to three or more antibiotics from different families (Magiorakos *et al*., 2012).

## 3.7. Data Management and Analysis

Data collected from field and laboratory investigations were recorded, screened and coded using Microsoft Excel 2016 program and analyzed using SPSS version 20 software. Antibiotic efficacy of Ethyl pyruvate determined by zone of inhibition result and other drugs was determined by the CLSI standard. Descriptive statistics were used to compute the proportions of each bacteria species isolate and mean zone of inhibition. Chi-Square. and ANOVA were used to compare proportions and averages. Pearson’s r was computed to find out correlations between variables. P-value was held at 0.05 to determine statistically significant differences.

## 3.8. Ethical Consideration

A request explaining the purpose of the study was submitted to the Addis Ababa University College of Veterinary Medicine and Agriculture's animal research ethics and review committee prior to the study's commencement. The research was carried out following receipt of an acceptance certificate from the committee (Annex 13), with reference number VM/ERC/04/17/025/2025.

# 4. RESULTS

## 4.1. Milk Sample Screening Test Result (SCC and CMT)

Analysis of CMT results revealed a high prevalence of mastitis with 59.3% exhibiting a "strongly positive" reaction, 30.5% a "distinctly positive" reaction, and 10.2% a "weakly positive" reaction. Furthermore, a strong correlation was observed between CMT scores and SCC (Table 1) demonstrating a significant positive association between higher CMT scores and elevated SCC. These findings underscore the utility of both CMT and SCC as critical indicators in the assessment of bovine mastitis.

**Table** 1:Correlation Between California Mastitis Test Scores and Somatic Cell Count

| | | **California Mastitis Test** | **Somatic Cell Count** |
|---|---|---|---|
| **California Mastitis Test** | Pearson Correlation | 1 | 0.653** |
| | Sig. (2-tailed) | | 0.000 |
| | N | 60 | 60 |
| **Somatic Cell Count** | Pearson Correlation | 0.653** | 1 |
| | Sig. (2-tailed) | 0.000 | |
| | N | 60 | 60 |

## 4.2.Prevalence of Major Identified Bacteria Species in Milk Sample

Following culture and isolation on selective media, bacterial pathogens from milk samples of cows with clinical and sub-clinical mastitis were identified using MALDI-TOF mass spectrometry. Identified bacterial growth was observed in 65.5% (59/90) of the samples. *S. aureus* was the predominant species of the total isolates followed by *E. coli* the second most prevalent bacteria spp. *Klebsiella* and *Pseudomonas* were less frequent next to *Streptococcus*. This distribution highlighting the diversity and dominance of specific pathogens in the mastitis milk samples.

**Table 2**: Isolation rate of bacterial Species from Mastitis in the Study Areas

| Bacterial species | Frequency | Percent [%] |
|---|---|---|
| *Escherichia coli* | 19 | 21.1 |
| *Pseudomonas aurgonisa* | 4 | 4.4 |
| *Klebsiella* | 4 | 4.4 |
| *Staphylococcus aureus* | 26 | 28.9 |
| *Streptococcus agalactiae* | 6 | 6.7 |
| **Total** | 59 | 65.5 |

### 4.3. Concentration-Dependent Antibacterial Effects of Ethyl Pyruvate

Antibacterial effect of the EP across identified bacterial species reveal a clear concentration-dependent. At the highest concentration of EP (100), *E. coli*, *P. aeruginosa* and *Klebsiella* showed strong susceptibility. In contrast, *S. aureus* and *S. agalactiae* exhibited much lower sensitivity. At EP75, *E. coli* displayed the highest zone of inhibition indicating very strong susceptibility followed by *S. agalactiae* and *P. aeruginosa*. With EP50, moderate inhibition was observed on *E. coli*, *Klebsiella* and *P. aeruginosa*, while *S. agalactiae* and *S. aureus* continued to show substantial susceptibility. At the lowest concentration of EP25 most bacterial strains exhibited smaller zones of inhibition, with the exception of *S. agalactiae*, which still responded relatively well.

**Table 3**: Antibacterial activity for identified bacterial species at varying EP dilutions

| EP dilution | Bacteria species | N | Mean (±SD) |
|---|---|---|---|
| **EP100** | *Escherichia coli* | 10 | 29.4±4.2 |
| | *Klebsiella variicola* | 4 | 25.75±0.96 |
| | *Pseudomonas aeruginosa* | 4 | 29.50±3.4 |
| | *Staphylococcus aureus* | 10 | 8.40±0.79 |
| | *Streptococcus agalactiae* | 6 | 8.00±0.0 |
| **EP75** | *Escherichia coli* | 10 | 36.80±10.67 |
| | *Klebsiella variicola* | 4 | 26.75±2.8 |
| | *Pseudomonas aeruginosa* | 4 | 28.50±2.75 |

| | | | |
|---|---|---|---|
| | *Staphylococcus aureus* | 10 | 26.70±5.3 |
| | *Streptococcus agalactiae* | 6 | 30.33±1.9 |
| **EP50** | *Escherichia coli* | 10 | 19.00±5.1 |
| | *Klebsiella variicola* | 4 | 20.75±4.99 |
| | *Pseudomonas aeruginosa* | 4 | 18.50±1.8 |
| | *Staphylococcus aureus* | 10 | 19.90±0.96 |
| | *Streptococcus agalactiae* | 6 | 26.33±0.67 |
| **EP25** | *Escherichia coli* | 10 | 17.30±4.1 |
| | *Klebsiella variicola* | 4 | 11.25±5.4 |
| | *Pseudomonas aeruginosa* | 4 | 16.00±4.1 |
| | *Staphylococcus aureus* | 10 | 15.6±5.4 |
| | *Streptococcus agalactiae* | 6 | 24.33±2.2 |

### 4.4. Comparative Susceptibility of Bacterial Species to Ethyl Pyruvate

*E. coli* displays the highest mean of 25.63±0.53mm, suggesting it is the most susceptible to ethyl pyruvate followed by *P. aeruginosa* follows with a mean of 23.13±0.83mm, indicating moderate susceptibility. *S. agalactiae* has a mean of 22.25±0.7 also reflecting considerable susceptibility. In contrast, *Klebsiella* shows a lower mean of 21.1±0.83mm, indicating reduced susceptibility compared to the others. Notably, *S. aureus* has the lowest mean of 17.65±0.5 suggesting it is the least susceptible among the tested species. These findings highlight significant differences in susceptibility, with *E. coli* being the most affected by ethyl pyruvate.

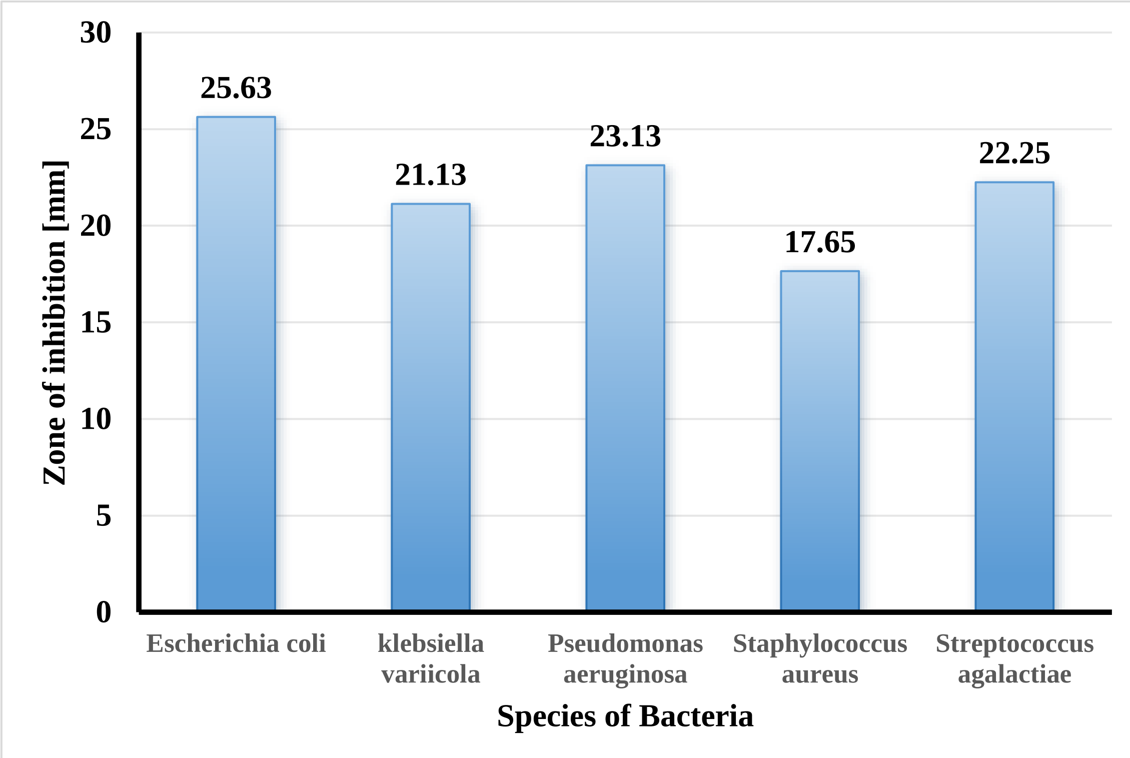


**Figure** 3: Varying levels of susceptibility among bacterial species to ethyl pyruvate,

### 4.5. Evaluating the Antimicrobial Efficacy of Ethyl Pyruvate Dilutions

The results for the different ethyl pyruvate dilutions reveal varying levels of antimicrobial effectiveness as indicated by the mean values for the zone of inhibition. EP100 shows a mean of 20.21±0.621. However, EP75 stands out with the highest mean of 29.817± 0.621 indicating the strongest level of inhibition among the tested dilutions and demonstrating robust antimicrobial activity. EP50 has a mean of 20.897±0.621, reflecting moderate effectiveness, while EP25 records the lowest mean of 16.897±0.621, suggesting reduced efficacy compared to the higher concentrations. EP75, significantly enhance the zone of inhibition with strong antimicrobial potential (Figure 4).

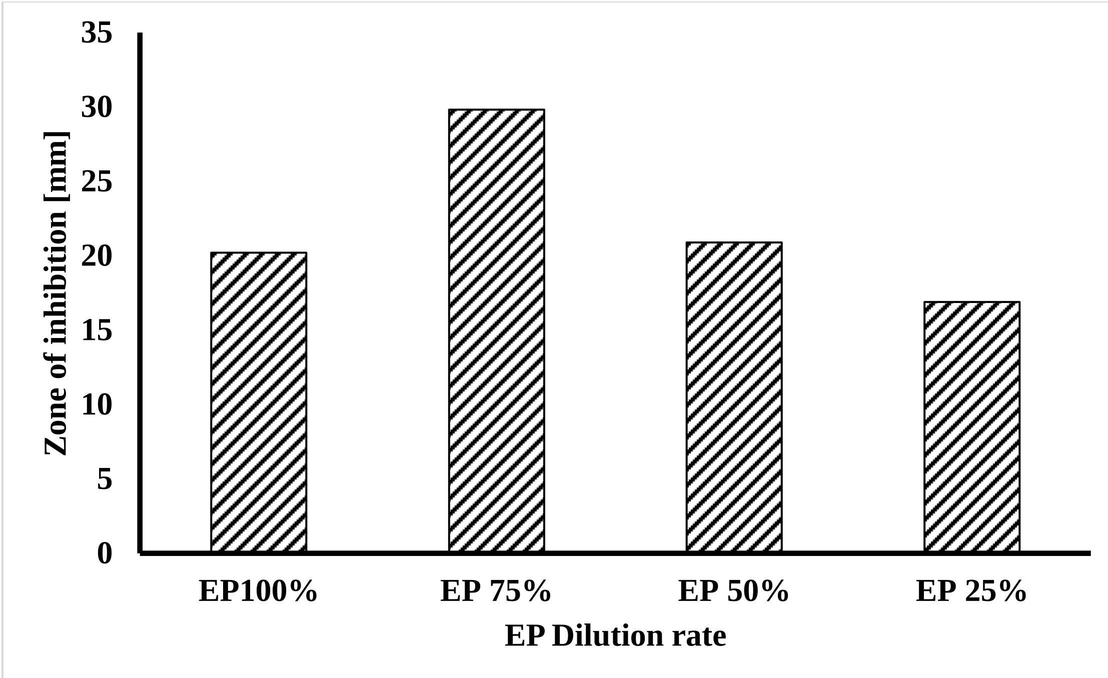


**Figure** 4: Mean antibacterial activity across ep dilutions

### 4.6. Effects of Ethyl Pyruvate Dilution and Bacterial Type on Zone of Inhibition

Zone of inhibition is significantly influenced by both the concentration of ethyl pyruvate (EP dilution) and the type of bacterial species. This means that not only do different concentrations of ethyl pyruvate produce significantly different antibacterial effects, but also that the response varies significantly among different bacterial species (Table 4).

**Table** 4: Correlation between zone of inhibition with EP dilution and bacteria species

| **Correlation** | **Rho** | **P-value** |
|---|---|---|
| Zone of inhibition-EP concentration | 0.868 | <0.000 |
| Zone of inhibition-bacteria species | 0.747 | <0.000 |
| EP concentration- bacteria species | 0.847 | <0.000 |

***Note****: Rho: Spearman rank correlation coefficient*

### 4.7. Antimicrobial Resistance Profiles of Bacterial Isolates

Isolates of *S. aurous* were tested for susceptibility against different antimicrobials. The bacteria isolates demonstrated varied levels of phenotypic resistance rates which range from 0% to 100%. Tetracycline, Ampicillin and Penicillin G displayed the highest resistance, with 100% of all isolates as indicated in Figure 5.

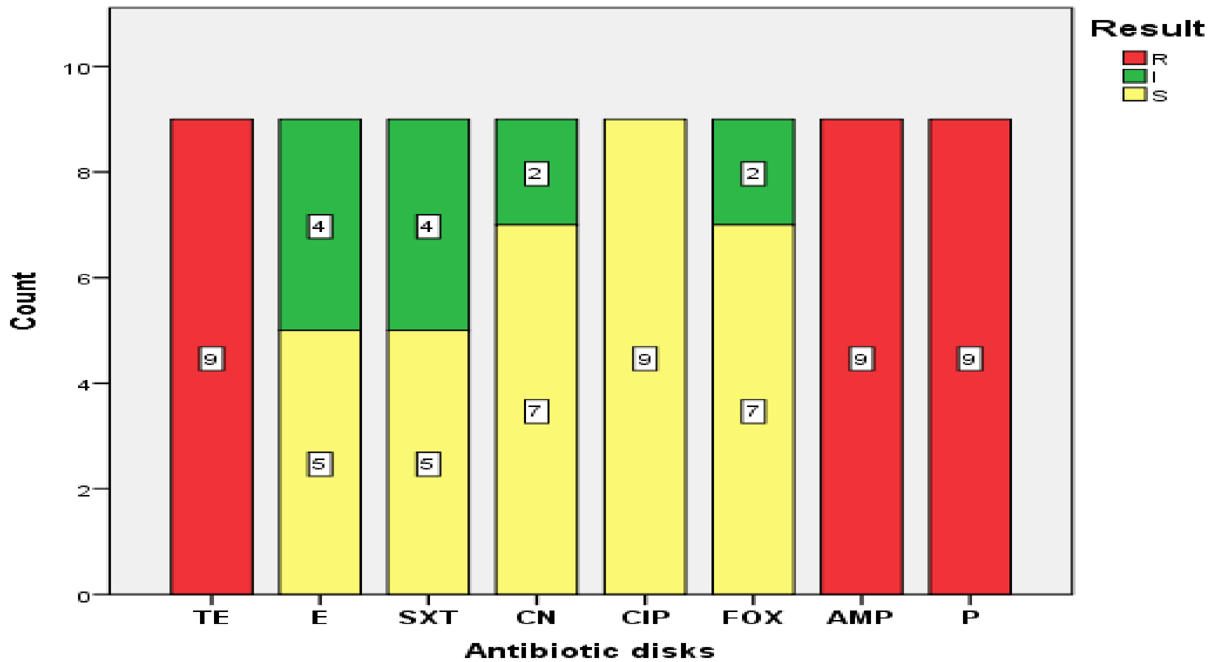


**Figure** 5: Antimicrobial resistance profile of *S. aureus* (n=9)

*R=resistance; I: intermediate; S: susceptible; P: penicillin G; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole; E: erythromycin; CN: gentamicin, CIP: ciprofloxacin; FOX: Cefoxitin, AMP: ampicilin*

*E. coli* shows a high level of susceptibility to the tested antibiotics, with resistance percentages ranging from 0% to 100%. The highest susceptibility was observed for Ciprofloxacin, which recorded no resistance in all bacteria, while Tetracycline and Amoxicillin exhibited the highest resistance rate at 100% (Figure 6).

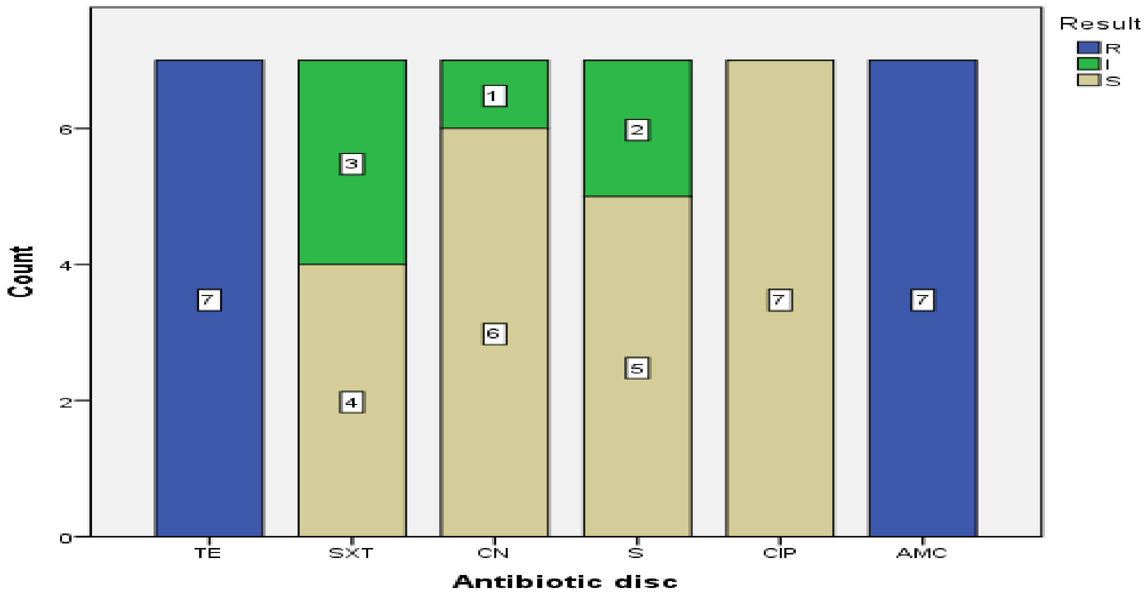


**Figure** 6: Antimicrobial resistance profile of *E. coli (n=7)*

*TE: tetracycline; SXT: trimethoprim/sulfamethoxazole; CN: gentamycin, CIP: Ciprofloxacin; S: streptomycin, AMC: amoxicillin clavulanic acid*

*Streptococcus* shows significant resistance to most tested antibiotics. Tetracycline showed greater resistance (100%) followed by Vancomycin (83.3%) and Penicillin (33%). However, Erythromycin had the highest susceptibility rate at 66% (Figure 7).

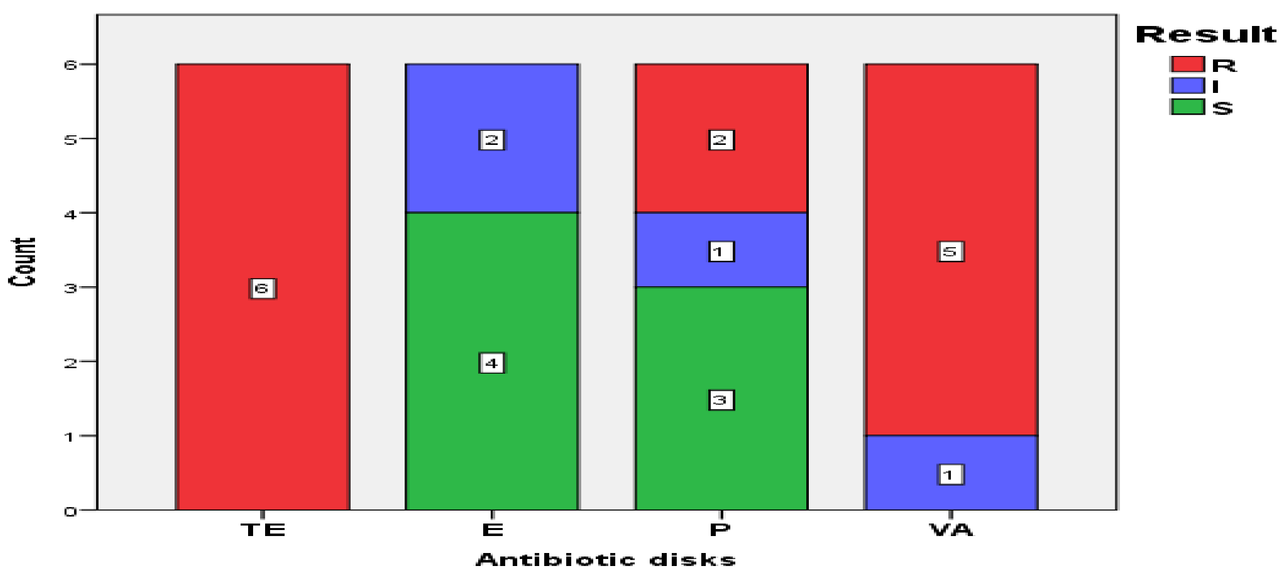


**Figure** 7: Antimicrobial resistance profile of *S. agalactiae(n=6)*

*P: penicillin G; TE: tetracycline; E: erythromycin; VA: vancomycin*

*Klebsiella* reveal a mixed response to various antibiotics. Tetracycline, penicillin and Amoxicillin shows a highest resistance rate of 100%, followed Sulfamethoxazole-Trimethoprim and streptomycin with a percentage of 50. And gentamicin demonstrates 25% susceptibility (Figure 8).

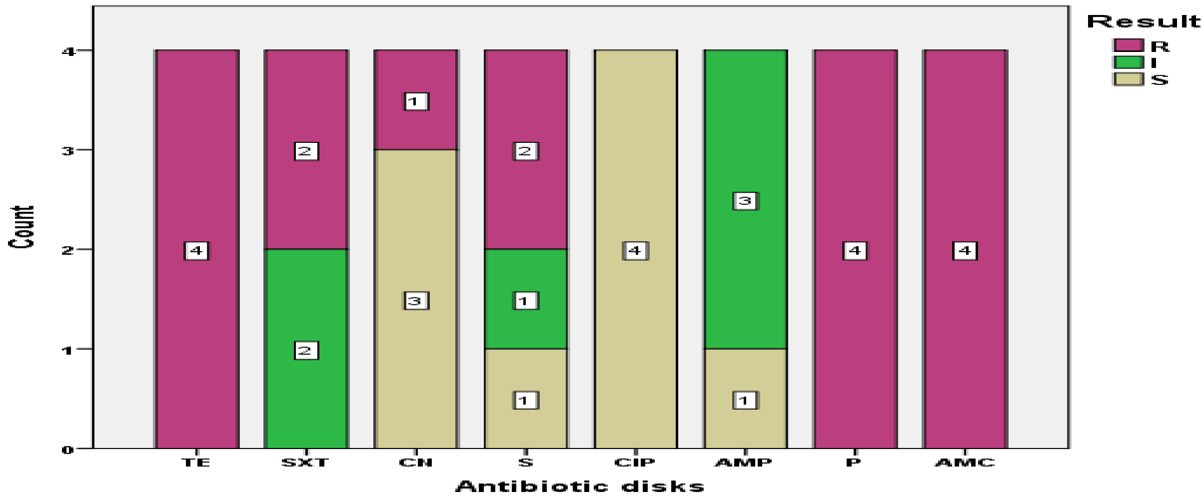


**Figure** 8:Antimicrobial resistance profile of *Klebsiella(n=4)*

*TE: tetracycline; CN: gentamicin; CIP: ciprofloxacin; S: streptomycin; SXT: trimethoprim/sulfamethoxazole; AMP: ampicillin, AMC: amoxicillin clavulanic acid*

*Pseudomonas* shows a remarkable resistant to most antibiotics, with 100% susceptibility across nearly all tested isolates. The only exception is Ciprofloxacin, which reveals complete susceptibility. This pattern indicates that *Pseudomonas* is generally resistant for most antibiotics.

### 4.8. Multidrug Resistance Patterns

From the six isolates of *Str. agalactiae* multidrug resistance (MDR) was shown by half of the isolates with resistance for three antibiotic classes. The MDR isolates were also resistant to three, four and five number of antibiotics with 25%, 50% and 25 respectively for *Klebsiella.*

With regard to *P. aeruginosa* all of the four isolates were MDR with resistance to seven antimicrobial classes with percentage of 100% (Table 5). These findings indicate a worrying trend of multidrug resistance development among the bacteria isolates, posing a significant effect on dairy cattle.

**Table 5**: Multidrug resistance pattern of identified bacteria isolates in the study area

| **Bacteria spp** | **No. of AM Class** | **Resistance pattern** | **No. of isolates** | **Percent** |
|---|---|---|---|---|
| *S. agalactiae* | 3 | TE, P, VA | 3 | 50% |
| *Klebsiella* | 2 | TE, AMP, AMC | 1 | 25% |
| | 3 | TE, S, AMP, AMC | 1 | 25% |
| | 3 | TE, SXT, AMP, AMC | 1 | 25 |
| | 4 | TE, SXT, S, AMP,AMC | 1 | 25% |
| *P.auroginosa* | 5 | TE,SXT,CN,S,FOX,AMP, AMC | 4 | 100% |

***Note**: AM: antimicrobial; MDR: multidrug resistance; P: penicillin G; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole; VA: vancomycin*;

## 5. DISCUSSION

A study on bovine milk samples was conducted to identify the primary bacterial etiologies of mastitis. Following CLSI standards, 65.5% of samples were positive for mastitis. This prevalence is consistent with the findings of 63.11% Kassa *et al*., (2014) in the Hawassa and Wando Genet areas. However, the observed prevalence was higher than reported in several other studies within Ethiopia; 46.7% by Abera *et al*., (2013) in Adama town and 53.25%; by Biniam *et al*., (2015) in Dire Dawa town, but lower than documented by Mekibib *et al*., (2010) 71.05% in Holeta town in Central Ethiopia.

The predominant mastitis-causing agent isolated was *Staphylococcus aureus*, exhibiting a prevalence of 28.9%. This finding is comparable to the reported 29.9% by Tora *et al*., (2022) in Ethiopia. However, the current prevalence was notably higher than that reported in studies from other Ethiopian regions, ranging from 10.69% to 15.64% (Ayano *et al.*, 2013; Abunna *et al.,* 2016; Gebremedhin *et al*., 2022; Borena *et al*., 2023), and also exceeded prevalence of 46.5% documented by Seyoum *et al.,* (2018). Variations in the prevalence of *S. aureus* can be attributed to different sampling methods, hygienic milking practices, handling techniques, and the use of shared towels to dry udders. Observations during the farm-level sample collection process also revealed unsanitary milking and raw milk processing practices. Since S. aureus is typically found on the udders or teat surfaces of infected cows, this is the primary means of transfer between uninfected and infected udder quarters, often occurring during milking (Abebe *et al*., 2016).

The present investigation identified *Streptococcus agalactiae* as responsible for 6.7% of the mastitis cases. This finding aligns with previous reported 7.2% by Mekibib *et al*., (2009) in Holeta Town, Central Ethiopia. But higher than the report of Tora *et al*., (2022). Although it is notably lower than the documented 10% by Tsegaye *et al*., (2019) in and around Haramaya district, eastern Ethiopia. *S. agalactiae* survives poorly outside the udder, and established infections are eliminated by frequent use of penicillin and other antibiotics (Fesseha *et al*., 2021). The comparatively lower incidence of *Streptococcus* may be attributed to the widespread use of Penstrip for mastitis treatment in the field.

*Escherichia coli* constituted a significant proportion of the isolates, accounting for 21.1% of the total. Closely similar reports have been documented from Maya City, Eastern Hararghe Zone, Oromia, Ethiopia (Isayas *et al.,* 2025) and lower prevalence reported from (Birhanu *et al*., 2017). A much higher finding, that exceed 40%, was reported from Bishoftu and Addis Ababa (Megersa *et al.,* 2019; Abunna *et al.,* 2019). Variations in farm hygiene practices and stable management could be the source of such discrepancies in *E. coli* prevalence as fecal contamination, a primary source of *E. coli*, can occur directly or indirectly through bedding, calving stalls and udder wash water (Belay *et al.,* 2022). On the other hand, the prevalence of *P. aeruginosa* and *Klebsiella* consistent were relatively lower compared to previous findings (Workeneh *et al.,* 2002; Mekibib *et al.,* 2009). But higher than the report of Abuna *et al.,* (2013). Notably, the contagious pathogen *Staphylococcus aureus* exhibited a greater frequency compared to other identified species.

This investigation assessed the *in vitro* antimicrobial efficacy of EP across a panel of bacterial species implicated in bovine mastitis, revealing a concentration-dependent response. EP exhibited significant inhibitory activity against both Gram-positive (*Staphylococcus aureus* and *Streptococcus agalactiae***)** and Gram-negative ***(****Escherichia coli***,** *Klebsiella* and *Pseudomonas aeruginosa*) pathogens. These findings corroborate previous research by Azime and Selcen (2024), which demonstrated EP's broad-spectrum antibacterial properties against food-borne microorganisms, and Debebe *et al.,* (2016), who reported EP's growth-inhibitory potential against clinically relevant pathobionts.

Quantitative analysis revealed a direct correlation between EP concentration and antibacterial activity. The highest tested concentration (75%) demonstrated the most pronounced inhibitory effects across all bacterial species, followed by the 50% concentration. Consistent with these findings, studies by Durak *et al*., (2012; Cetin et al. (2019) and Azime and Selcen (2024) have similarly shown that increasing EP concentrations correlate with enhanced antimicrobial activity in both *in vitro* and food preservation models, suggesting a dose-dependent mechanism of action that may contribute to extended product shelf life.

The study revealed a counter-intuitive finding: undiluted EP exhibited lower *in vitro* antibacterial activity compared to diluted ones. This contrasts with reports of strong vaporized EP activity (Tornuk & Durak, 2015), highlighting the influence of application method and physical factors like diffusion in agar, potentially hindered by high EP concentration (Bonev *et al.,* 2008; Durak *et al*., 2012; Ali *et al.,* 2021). In essence, the reduced efficacy of a 100% EP solution compared to 75% likely involves a complex interplay of solvent effects on EP's ability to diffuse on the agar and penetrate bacterial cells (Muteeb *et al.*, 2023). For that the aggregation and viscosity of ethyl pyruvate at high concentrations may hinder diffusion through the agar, leading to smaller inhibition zones and potentially lower apparent antimicrobial activity.

EP generally demonstrated greater efficacy against Gram-negative bacteria ***(****E. coli > Pseudomonas aeruginosa > Klebsiella****)*** compared to *Staphylococcus aureus*. This aligns with some studies (Cetin *et al.,* 2019) and may relate to the thinner outer membrane of Gram-negative bacteria facilitating EP penetration. However, other research indicates comparable or strong EP activity against both Gram-positive and Gram-negative bacteria in different contexts (Tornuk and Durak, 2015; Azime and Selcen, 2024). These discrepancies underscore the complex interplay of EP concentration, application method, bacterial species, and cell wall characteristics in determining its antimicrobial efficacy against bovine mastitis pathogens.

Antimicrobial susceptibility testing revealed widespread resistance among identified bacteria to commonly used antibiotics. *Staphylococcus aureus* exhibited high resistance (100%) to tetracycline, ampicillin, and penicillin G, consistent with findings in central Oromia (Marami *et al.,* 2022; Gebremedhin *et al.,* 2022; Borena *et al.,* 2023) and tetracycline resistance also reported (Feyissa *et al.,* 2023). Penicillin G resistance was also high (94.4%-100%) across multiple studies (Abera *et al*., 2013; Ayele *et al.*, 2017; Marami *et al.,* 2022; Borena *et al.,* 2023; Feyissa *et al*., 2023). However lower tetracycline resistance was reported (Ayele *et al.,* 2017; Borena *et al.*, 2023). Alarming resistance levels in *S. aureus* to these antibiotics have been noted in past research (El-Jakee *et al.,* 2008; Daka *et al*., 2012), potentially due to prolonged and indiscriminate use.

*Pseudomonas aeruginosa* isolates displayed 100% resistance to seven of eight tested antimicrobials (tetracycline, trimethoprim/sulfamethoxazole, gentamicin, cefoxitin, streptomycin, amoxicillin, and ampicillin). These result aligning with Decimo *et al*., (2016). However, varying range of resistance from 51–100% was reported by (Meng et al., 2020). Notably, all *P. aeruginosa* isolates in this study were multidrug-resistant, potentially due to mechanisms like efflux pumps and target modification (Depardieu *et al*., 200).

Antimicrobial susceptibility testing revealed high resistance among ***E.** coli* isolates (100%) to amoxicillin and tetracycline, consistent with some studies (Yang *et al.,* 2018) but contrasting with lower resistance reported elsewhere (Ababu *et al*., 2020); Dule *et al*., 2025). *Streptococcus agalactiae* showed high resistance to tetracycline (100%) and vancomycin (85%), and with moderate penicillin resistance (50%), showing variability compared to European data (Hendriksen *et al.*, 2008).

Variations in resistance patterns may stem from pathogen-specific resistance gene expression influenced by agro-ecological factors (Reuben and Owuna, 2013). Multidrug resistance observed in this study could be attributed to frequent or irrational antimicrobial use, self-medication and reliance on older drug classes (Mohammed *et al.*, 2021) fostering the development of resistant bacteria. The global increase in multidrug-resistant bacteria is a major concern (Magiorakos *et al*., 2012; Esther *et al.*, 2017).

## 6. CONCLUSION AND RECOMMENDETIONS

This comprehensive investigation into bovine mastitis in the studied region reveals a very high prevalence, with *Staphylococcus aureus* identified as the predominant bacterial pathogen. Alarmingly, antimicrobial susceptibility testing revealed widespread resistance to commonly used antibiotics across all major isolates. High resistance to tetracycline, ampicillin, and penicillin G in *S. aureus*, extensive multidrug resistance in *P. aeruginosa* and *Klebsiella* and significant resistance to tetracycline and vancomycin in *S. agalactiae* underscore the critical challenge of managing bovine mastitis in this context. Notably, the *in vitro* evaluation of EP demonstrated promising antimicrobial activity against key mastitis-causing bacteria and showed greater efficacy against bacteria that were resistant to the common antibiotic is worth considering. Diluted concentrations generally exhibiting greater efficacy than undiluted EP. EP's activity also showed greater inhibition of Gram-negative bacteria. While, the observed multi-drug resistance emphasizes the urgent need for antimicrobial stewardship programs, and improved diagnostic practices to guide targeted therapies, further exploration of alternative treatments like ethyl pyruvate to combat this economically significant disease in the dairy sector is imperative. Based on the findings of this study, the following recommendations are proposed to further investigate the potential of ethyl pyruvate (EP) in bovine mastitis management:

- Implement well-designed field trials to evaluate the efficacy of EP as a therapeutic agent for mastitis in naturally infected cows
- Explore the potential for synergistic effects EP in combination with conventional antimicrobial agents.
- Conduct comprehensive dose-response studies to establish the optimal dosage regimens and administration routes for ethyl pyruvate in bovine mastitis treatment, aiming to maximize therapeutic efficacy while minimizing potential adverse effects.
- Undertake further mechanistic studies to fully elucidate the specific pathways through which EP exerts its antimicrobial activity and to assess its long-term impact on health, including potential effects on udder health and milk quality.

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## 8.ANNEXES

**Annex 1:** Type and preparation of bacteriological medias used

1. Mannitol salt agar

| Ingredients | g/liter |
|---|---|
| Peptone | 5 |
| Tryptone | 5 |
| HM Peptone B | 1 |
| Sodium chloride | 77 |
| D-Mannitol | 10 |
| Phenol red | 0.025 |
| Agar | 15 |
| pH after sterilization | 7.4±0.2 |

Preparation: Suspend 111.02 grams in one liter of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri dishes.

**2.** Tryptic soya agar (Himedia)

| Ingredients | g/liter |
|---|---|
| Tryptone | 17 |
| Soya peptone | 3 |
| Sodium chloride | 5 |
| Dextrose (Glucose) | 2.5 |
| Dipotassium hydrogen phosphate | 2.5 |
| Agar | 15 |
| Final pH ( at 25°C) | 7.3±0.2 |

Preparation: Suspend 45 grams in one liter of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-

50°C. Mix well and pour into sterile Petri dishes

3. Mueller Hinton agar (Himedia)

| Ingredients | g/liter |
|---|---|
| Beef infusion | 300 |
| Casein and hydrolysate | 17.5 |
| Starch | 1.5 |
| Agar | 17 |
| Final pH ( at 25°C) | 7.3±0.2 |

Preparation: Suspend 38 grams of the medium in one liter of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121oC for 15 minutes. Mix well before pouring.

**Annex 2***:* Data collection sheet

1. Kebele_____3. Farm Name/No.__________ Tel. ____________________Date: __________________

2. Name of respondent____________________, Position 1) manager, 2) owner, 3) attendant 4). Other
3. Educational level of the respondent? ________________
5. Herd structure
6. What is the total milk yield of the farm __________?
7. How many cows got mastitis in your farm every year? ________
8. Do you treat cows with mastitis? 1) Yes 2) no, how? By a vet or traditional
9. What is your opinion on the cure rate of mastitis when treated? 1) 25% 2) 50% 3) 100% 4) other____
10. If traditional treatment is used, please name the types and route/method_________________________
11. No of cows with recurring mastitis (more than once) in every year in your farm? _______
12. What do you do for cows with recurring mastitis? ______________________________
13. If you have culled cows in your farm, what was the main reason for culling? (rank them) 1st____________________ 2nd ____________________ 3rd ________________ 4th _________________
14. Price of medication per cow ________________; and Veterinary service _______________

15. Type of housing A) loose house allowing free movement B) loose house with tie, C)

housing with tie in cubicle, C) housing without tie in cubicle, D) housing shared with people, E) other (specify)________

16. Hygiene score of the farm _____________

17. Any isolation pen for sick animals? A) Yes, B) No

18. Manure and waste management? A) Poor (floor is levelled prohibiting one side flow or waste flows, and not disposed far away), B) Satisfactory (slant floor, waste easily drains, waste disposed far away), C) Very good (slant floor, waste easily drains, wastes disposed into disposal pit or septic tank, tolerable odor)

**Annex 3:** Animal level-data collection

| No | Farm No/ID | Animal ID | Age (mth) | Parity | Breed | Stage of Lacta. | Pregnancy status | Affected Udder/ Teat | CMT result |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |

**Annex 4:** Informed consent form

Good morning/good afternoon!

I am Tseganesh Asefa, a post graduate student at Addis Ababa University College of Veterinary Medicine and Agriculture in the field of MSc in Veterinary clinical medicine. Now I am conducting research on ""**Evaluation and in Vitro Antimicrobial Effect Assessment of Ethyl Pyruvate's Efficacy Against Major Pathogens Associated with Bovine Mastitis**". I will appreciate your participation in this study. The general objective of this study is to evaluate the antimicrobial efficacy of ethyl pyruvate against major bacteria pathogens in bovine mastitis and assess its potential inhibitory effect in vitro. To effectively attain the objective of the research, I am requesting your help. For your participation in the study no payment will be granted or has no any special privilege to you. All information you give will be kept confidential and won't be accessible to any third party. You have the right not to participate from the beginning, or you may stop participating at any time after starting the participation. If you agree to participate, you will be asked to: answer a few questions regarding your general farm management and presence of mastitis in your farm and provide a sample (raw milk)

Respondent statement: I have understood the above statements:

□ Yes (Agree to participate) □ No (Not agree to participate)

Informed consent: I have read this form, or it has been read to me in the language I understand all conditions stated above. Therefore, I am willing to participate in this study.

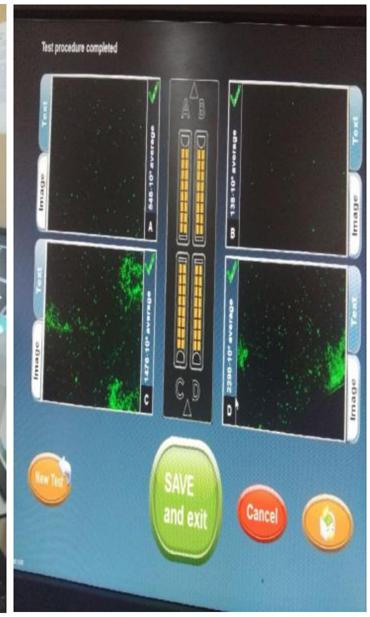
Name of respondent: ____________________________________________

Signature: ____________________________________________________

Date: ________________________________________________________

**Annex 5:** Milk sample collection and screening

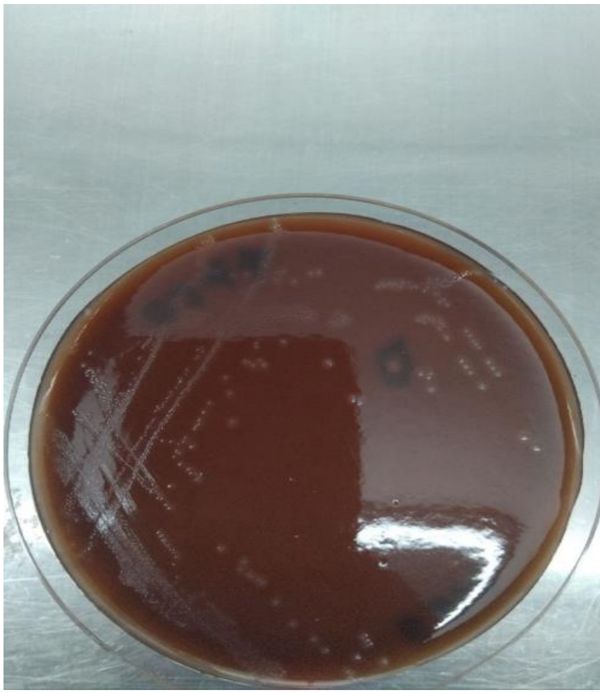
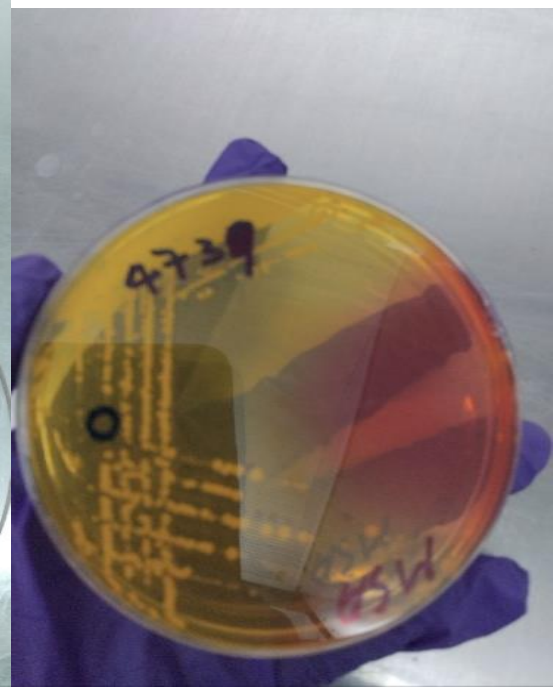
Milk sample and CMT test




CMT result C. LACTOSCAN machine and SCC result

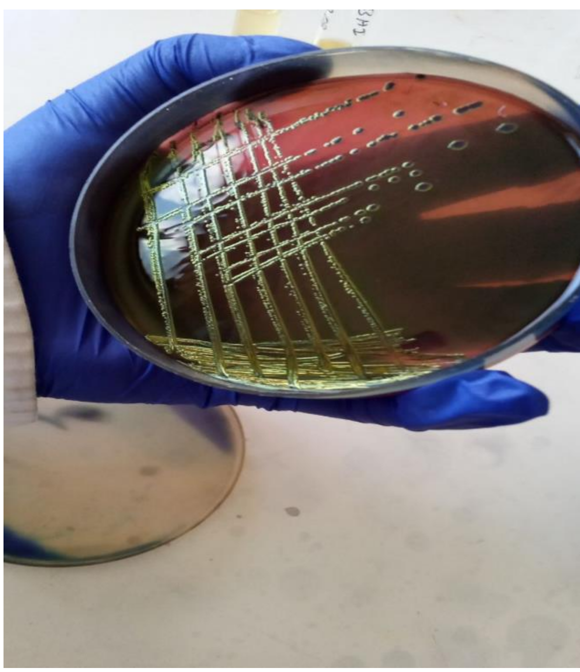
**Annex 6:** Media preparation and Bacteria Inoculation


**Annex** 7: : Result of bacteria species on their selective medias


F. characteristics of S. agalactiae

G.characteristics of S. aureus on MSA





H. E. coli on EMB

I. P. aeruginosa on nutrient agar media

**Annex 8:** Standard protocol bacteria identification with MALDI_TOF

1. Smear bacteria (single colony) as a thin film directly on to a spot on a MALDI target plate

2. For five minutes, the target plate was allowed to air dry at ambient temperature.

3. Following the addition of 25 µL of 70% formic acid, the pellets were reconstituted.

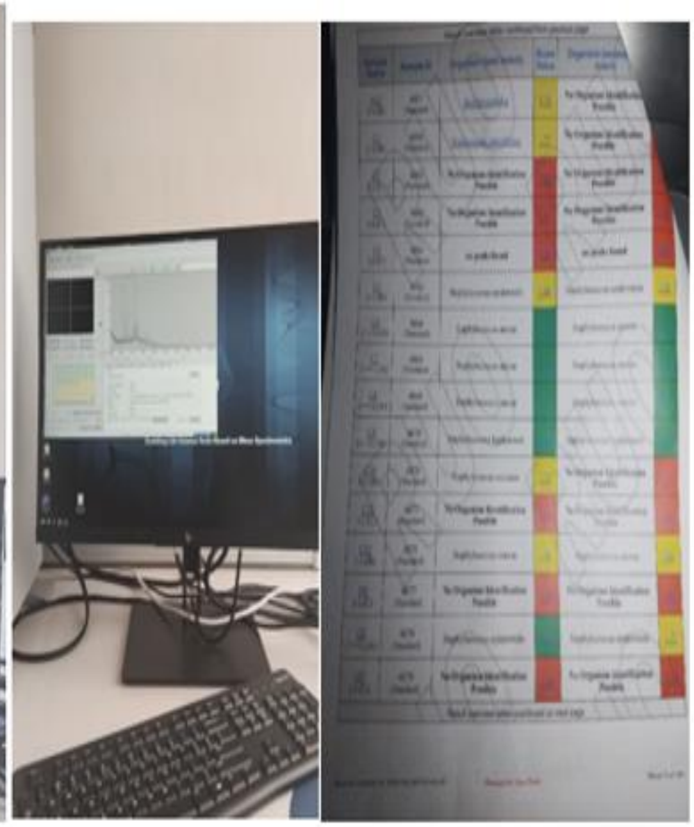
4. At the designated BTS QC point, 1 µL of BTS was applied, and the mixture was allowed to dry completely.

5. Every sample position and BTS QC position had 1µL of CHCA matrix solution applied over them.

6. The areas were allowed to air dry at ambient temperature.

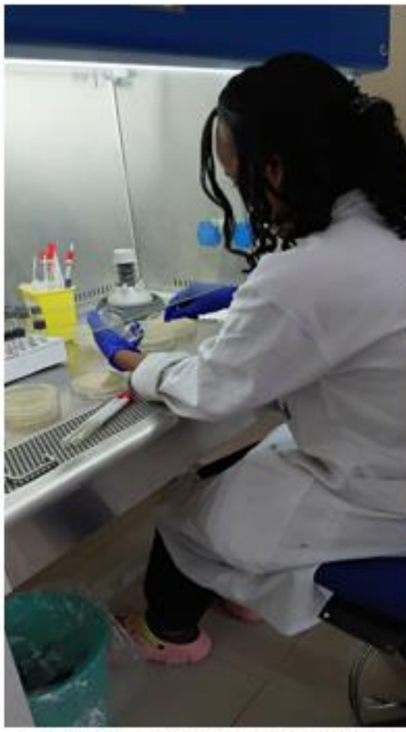
7. A unique bar code was scanned, and then the target plate was placed into the mass spectrometer.

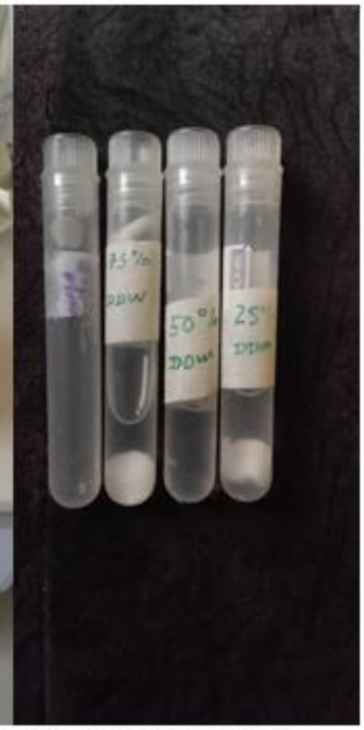

J. MALD-TOF MS



K. MALDI- TOF output results

**Annex 9:** Antimicrobial susceptibility test



**L.**Inoculation of isolates on Muller agar **M.** McFarland Densitometer **N.** Ethyl pyruvate at different concentration


N. EP impregnated paper DIffusin O. Measuring the inhibitory zone using calliper

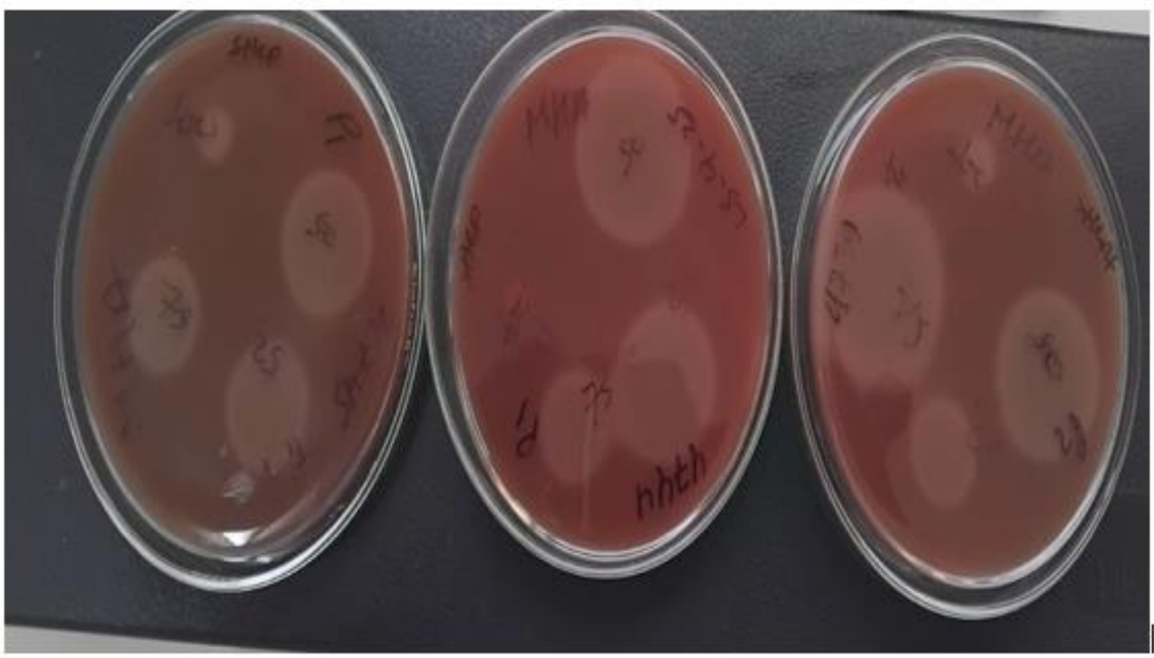
**Annex 10**: The antimicrobial disc used to measure the zone of inhibition of bacteria susceptibility, together with its interpretations

| Types antimicrobial discs | Symbol | Bacteria | Susceptible(mm) | Intermediate | Resistance(mm) |
|---|---|---|---|---|---|
| Tetracycline | TE (30 µg) | S. aureus | ≥19 | 15-18 | ≤14 |
| | | E.coli | ≥15mm | 12-14 | ≤11 |
| | | S. Agalactiae | ≥23 | 19-22 | ≤18 |
| | | Klebsela&Pseudomona | ≥15 | 12-14 | ≤11 |
| Erythromycin | E (15 µg) | S. aureus | ≥23 | 14-22 | ≤13 |
| | | S.agalactiae | ≥21 | 16-20 | ≤15 |
| Amoxicillin-clavulanic acid | AMC(30 µg) | E.coli | ≥18mm | 14-17 | ≤13 |
| | | Klebsela&Pseudomona | >18 | 14-17 | <13 |
| Trimethoprim Sulfamethoxazole | SXT (25 µg) | S. aureus | ≥16 | 11- 15 | ≤10 |
| | | E.coli | ≥16mm | 11-15 | ≤10 |
| | | Klebsela&Pseudomona | ≥16 | 11- 15 | ≤10 |
| Gentamycin | CN (10 µg) | S. aureus | ≥15 | 13-14 | ≤12 |
| | | E.coli | ≥15mm | 13-14 | ≤12 |
| | | Klebsela&Pseudomona | ≥18 | 15-17 | ≤14 |
| Streptomycin | S ((10 µg) | E.coli | ≥15mm | 12-14mm | ≤11mm |
| | | Klebsela&Pseudomona | >15 | 12-14 | <11 |
| Ciprofloxacin | CIP(5 µg) | S. aureus | ≥21 | 16-20 | ≤15 |
| | | E.coli | ≥26mm | 22-25 | ≤21mm |
| | | Klebsela&Pseudomona | ≥26 | 22-25 | ≤21 |
| Cefoxitin | FOX (30 µg) | S. aureus | ≥22 - | | ≤21 |
| | | Klebsela&Pseudomona | ≥18 - | 15-17 | ≤14 |
| Ampicillin | AMP (10 µg) | S. aureus | ≥29 - | | ≤28 |
| | | Klebsela&Pseudomona | ≥17 | 14-16 | ≤13 |
| Penicillin G | P (10 µg) | S. aureus | ≥29 - | | ≤28 |
| | | Strep.agalactiae | ≥24 - | | ≤0.12 |
| Vancomycin | VA (30µg) | Strep.agalactiae | ≥17 | | ≤1 |

Source : CLSI, 2024

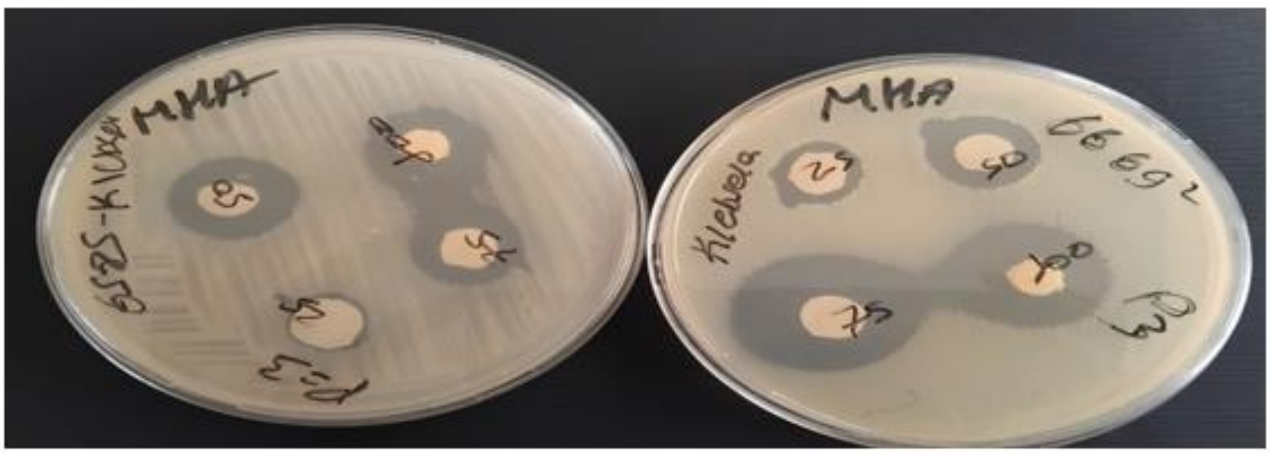
**Annex 11:** Result of Ethyl pyruvate susceptibility tests

P. Ethyl pyruvate susceptibility test results on E. coli



Q. Ethyl pyruvate susceptibility test result on S. agalactiae


R. Ethyl pyruvate susceptibility test results on P. aeruginosa

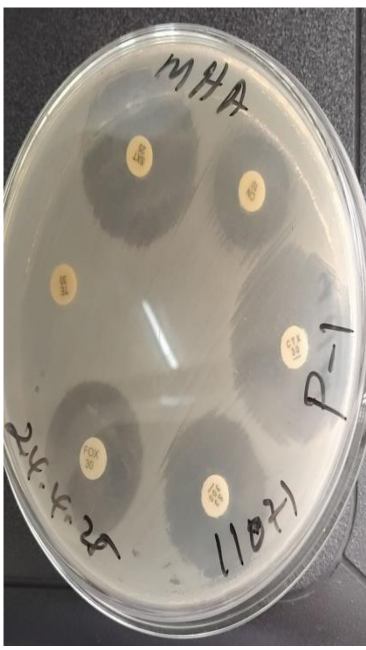



S. Ethyl pyruvate susceptibility test results on Klebsiella


T. Ethyl pyruvate susceptibility test results on S. aureus

**Annex 12:** Result of antimicrobial susceptibility test of commercial antibiotics disks


Klebsiella P.auruginosa



E. coli S. agalactiae S. aureus

**Annex** 13: Animal Ethical clearance certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

*Ethical clearance certificate*

**Certificate Ref. No: VM/ERC/04/66/17/2025**

Name of Applicant: **Tseganesh Assefa** (DVM, MSc student)

Address*:* Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Evaluation of antimicrobial effect and in vitro assessment of Ethyl pyruvate efficacy against major pathogens associated with bovine mastitis*

| | |
|---|---|
| Date of application: | **December, 2024** |
| Nature of the project: | In vitro **experimental trial** |
| Target animal species: | **dairy cattle** |
| Number of animals involved: | **30** |
| Study area: | **Bishoftu, Ethiopia** |

Minutes No. and date of review: **VM/ERC/04/17/025, 25/02/2025**

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

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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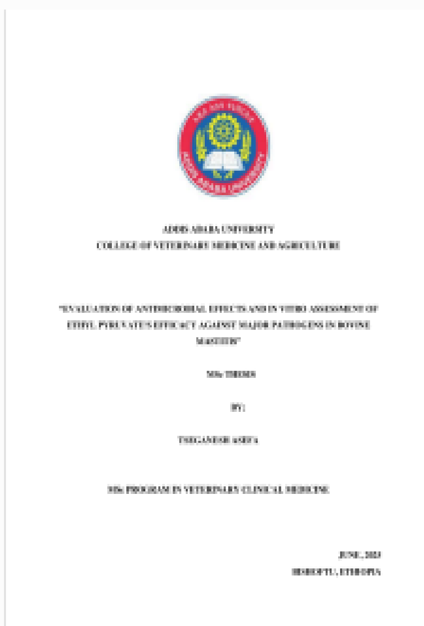
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