In Vitro Characterization of Lactic Acid Bacteria (Lab) Strains and Their Suppression Potential against Bovine Mastitis Causing Bacterial Pathogens

Asrat Yitagesu Abera

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Biotechnology

Office of Graduate Studies

Adama Science and Technology University

December, 2024

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master's Thesis "*In vitro characterization of Lactic Acid Bacteria (LAB) strains and their suppression potential against Bovine Mastitis causing bacterial Pathogens*"is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citations.

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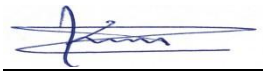
RECOMMENDATION

I/we,the advisor(s) of this thesis, hereby certify that I/we have read the revised version the thesis entitled “In vitro characterization of Lactic Acid Bacteria (LAB) strains and their suppression potential against Bovine Mastitis causing bacterial Pathogens” prepared under my/our guidance by Asrat Yitagesu submitted in Partial Fulfillments of the Requirement for the Master’s Science in Biotechnology. Therefore, I/we, recommend the submission of revised version of the thesis to the department following the applicable procedures.

Ketema Tafess Tulu(PhD)		Dec, 2024
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APPROVAL PAGE

I/we, the advisors of the thesis entitled "*In vitro characterization of Lactic Acid Bacteria (LAB) strains and their suppression potential against Bovine Mastitis causing bacterial Pathogens"* and developed by *Asrat Yitagesu* hereby certify that the recommendation and suggestion made by the board of examiners are appropriately incorporated into the final version of the thesis.

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We, the undersigned, members of the Board of Examiners of the thesis by Asrat Yitagesu have read and evaluated the thesis entitled "In vitro characterization of Lactic Acid Bacteria (LAB) strains and their suppression potential against Bovine Mastitis causing bacterial Pathogens"and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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Final approval and acceptance of the thesis is contingent upon submission of its final Copy to the office of postgraduate studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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LIST OF ABBREVIATION

LAB	Lactic acid bacteria
MRS	de Man Rogosa and Sharp
CFU	Colony forming unit
GRAS	Generally, recognized as safe
BCP	Bromocrysol purple
PBS	Phosphate buffered saline
BATS	Bacterial adhesion to solvent

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ABSTRACT

Mastitis is a disease known to cause a great deal of loss of production and has a major economic impact. The purpose of the present study was aimed at In vitro characterization of Lactic Acid Bacteria (LAB) strains and their suppression potential against Bovine Mastitis causing bacterial Pathogens. This study investigated the in vitro probiotic potential of seven lactic acid bacterial strains (Lactiplantbacillus pentosus BB26, Lacticasebacillus casei BB28, Lactobacillus plantarum BB31, Lactiplantibacillus plantarum BB64, Lactiplantibacillus plantarum DZ11, Lactiplantbacillus plantarum DZ31, Lacticasei bacillus paracasei GB15 against mastitis-causing pathogens. In this study the pH and temperature tolerance, carbohydrate fermentation, hemolytic and antibiotic susceptibility profiles, cell surface hydrophobicity properties and antimicrobial activity of the strains were characterized. The results revealed that 25°C was the optimum temperature and that more growth occurred at a pH of 4. Strains / isolates Lactobacillus plantarum BB31, Lactiplantbacillus plantarum DZ31, Lactiplantbacillus pentosus BB26 and Lactiplantibacillus plantarum BB64 showed the highest activity against all tested pathogens. The selected strains also showed best out-aggregation, cell surface hydrophobicity and co-aggregation potential. The Study concluded that LAB from the bovine environment has the potential to be used as a non-antibiotic formulation to treat mastitis-causing pathogens.

***Keywords**: Mastitis, probiotics, economic loss, prevention, LAB starins*

1. INTRODUCTION

In Ethiopia, dairy farming is mostly managed through an extensive system that includes smallholder farmers in rural areas. Currently, semi-intensive and intensive dairy pro-duction systems are gaining popularity among farmers with good market access. However, important impediments to dairy production include the low genetic potential of indigenous cattle breeds diseases, insufficient feed and water, and slow progress in dairy development technologies. Mastitis is one of the diseases that are known to be prevalent in various dairy production systems around the country, resulting significant economic losses (Girma, 2022).

Bovine mastitis is defined as inflammation of the mammary gland, where the mammary tissue is severely affected. Bovine mastitis is an important disease in the dairy industry and the leading cause of economic loss in milk production worldwide ((Morales-ubaldo *et al*., 2023). It is considered as the major endemic and prevalent disease of dairy cattle (Girma, 2022)and is generally caused by microorganisms. In most cases, infection occurs when bacteria enter the udder through the teat canal and multiplies, causing inflammation with or without clinical signs (clinical or subclinical mastitis, respectively) (Maity & Ambatipudi, 2021). Recently, this disease has caused major economic losses, considerable distress to animals and decreased milk production in dairy sectors (Cheng & Han, 2020).

Conventional methods for the control of bovine mastitis, such as diagnosis, segregation of the animals and improved hygiene, have been developed (Hamadani *et al*., 2010). These practices are not effective for the appearance and prevalence of the disease. Hence, the control of bovine mastitis still relies heavily on the use of therapeutic and preventive protocols with antibiotics. Although antibiotic therapy to control bovine mastitis is effective in most cases, it can also be detrimental, because of the emergence of antibiotic resistance and the occurrence of antibiotic residues in milk and meat (Prescott, 2008). In addition, this widespread use of drugs increases the risk of antibiotic residues in milk and dairy products and the risk of transmission of antibiotic resistance to both commensal bacteria and opportunistic pathogens. Owing to these weaknesses, the development of complementary and natural alternatives to prevent this disease is highly desirable (Armas et al., 2017)

Probiotic lactic acid bacteria with a variety of applications have great potential to control

bovine mastitis, and they are able choices for treating many infectious diseases in humans (Pellegrino et al., 2019). The use of LAB, which are generally recognized as safe (GRAS), and their antimicrobial peptides (bacteriocins) has recently been proposed for the control of bovine mastitis (Espeche *et al*., 2012). Nisin, currently the only bacteriocin widely used as a food preservative, exhibits antimicrobial activity against a wide range of gram-positive bacteria, including foodborne and mastitis-causing pathogens. Formulations containing nisin administered to bovine teats by dipping or intramammary infusion have been shown to be effective in restricting or treating *staphylococcal* and *streptococcal* infections (Chae *et al*., 2021).

These probiotics may exert beneficial effects on host health through several mechanisms: adhesion to epithelial cells, colonization, production of bio-surfactants, auto-aggregation and co-aggregation of pathogens, production of antagonistic metabolites (organic acids, hydrogen peroxide, and bacteriocins), competition for nutrients and production of enzymes, and/or immunomodulation(Armas *et al*., 2017). LAB are the main components of the indigenous microbiota of the teat canal, and because of this, they are optimal candidates for designing species-specific probiotic products to prevent mastitis (Armas *et al*., 2017).

1.1. Statement of the problem

Bovine mastitis, a common and costly inflammatory condition of the udder in dairy cows, poses significant economic challenges to the dairy industry. In the case of Ethiopia, the control of this disease still relies heavily on the use of therapeutic and preventive protocols with antibiotics. However, the emergence of antibiotic resistance and the occurrence of antibiotic residues in milk and meat cause major problems in the dairy sector. The role of LAB as potential probiotics offers a promising alternative to traditional antibiotic treatments for preventing and managing this condition. However, there is a lack of comprehensive characterization of LAB strains isolated from fermented milk that can effectively inhibit mastitis pathogens and enhance udder health. This study aims to investigate and characterize the probiotic potential of LAB isolated from fermented milk to identify effective strains for the prevention of bovine mastitis, addressing the urgent need for sustainable and health-promoting solutions in dairy production.

1.2. Objectives of the study

1.2.1. General Objective

To assess the beneficial properties of LAB strain to select good candidates for inclusion in a probiotic cocktail for checking their suppression potential against Bovine mastitis causing Bacterial pathogen in the mammary.

1.2.2. Specific Objectives

- To assess the probiotic properties of LAB strains isolated from fermented milk
- To characterize the beneficial properties of LAB to select good candidates for inclusion in a probiotic cocktail against infectious mastitis in the mammary tract.

2. LITERATURE REVIEW

2.1.Bovine mastitis

2.1.1. Definition

The term mastitis refers to inflammation of the mammary gland, regardless of the cause. The classic meaning of the word mastitis is derived from the Greek word “Matos”, meaning breast or udder, and the suffix “itis”, meaning inflammation. It is defined as inflammation of the mammary gland, nearly constantly due to the impacts of bacterial or mycotic pathogens. It is characterized by physical, chemical and usually bacteriological changes within milk, as well as pathological changes within glandular tissue (Pellegrino *et al*., 2019).

2.1.2. Etiology and types of Mastitis

Mastitis can be caused by a single pathogen or combination of two pathogens. According to the US National Mastitis Council Guidelines for diagnosis of mastitis, isolation of more than three pathogens in a milk sample is considered contamination. About 137 microbes have been isolated from milk (Cheng & Han, 2020). Depending on the causative agent, mastitis in cow can be categorized into three main types: contagious, environmental and summer mastitis (Pascu *et al*., 2022).

2.1.2.1. Contagious Mastitis

It is caused by bacteria living on the skin of the teat and inside the udder. Contagious mastitis can be transmitted from one cow to another during milking and can further be classified into as (Pascu *et al*., 2022).

A. **Clinical mastitis**

Clinical mastitis is characterized by the presence of gross inflammation signs such as swelling, heat, redness, and pain. This is caused by visual clots or discoloration of the milk, often in combination with tender and swollen udder, sometimes in combination with fever, loss of appetite, etc (Sears *et al*., 1993). Clinical mastitis can again be divided into acute mastitis, which is characterized by gross inflammation, a reduction in milk yield and changes in milk composition, and systemic signs such as fever, depression, shivering and loss of appetite and loss of weight; acute mastitis, which is similar to per-acute mastitis but with fewer systemic

signs such as fever and mild depression; and subacute mastitis, in which the signs of mammary gland inflammation are minimal and no visible systemic signs (Sears *et al*., 1993).

B. **Subclinical mastitis**

Subclinical mastitis is characterized by changes in milk composition (leukocytes and epithelial cells, changes in milk pH and ion concentration) with no clinical signs of gross inflammation or milk abnormalities (Lakew *et al*., 2019). Subclinical mastitis is characterized by a lack of visible signs in the milk or in the udder(Cobirka et al., 2020). It nevertheless results in decreased milk production. As reported by Shearer and Harris.(2003) and Seegers et al. (2003), subclinical mastitis occurs 15 to 40 times more often than the clinical form, and its duration is longer. Subclinical mastitis is therefore more difficult to detect, and infection serves as a reservoir of pathogens that spreads the udder infection among animals within the herd (Cobirka et al., 2020).

C. **Chronic mastitis**

This is an inflammatory process that occurs for months, and may continue from one lactation to the other. It is subclinical but may present as a periodic flare- up sub-acute or acute form that lasts for a short period of time (Hamadani *et al*., 2010). It can result in loss of quarter or teats. Cows with blind quarter's produce less milk. It is an inflammatory process that exists for months, and may persist from one lactation stage to another. It exists for the most part as subclinical, and may flare up periodically as sub-acute or acute form for a short period of time. The results are hard lumps in the udder from the walling off of bacteria, and the forming of connective tissue(Cobirka et al., 2020)

2.1.2.2. Environmental Mastitis

Environmental conditions and management practices of the herds have decisive effects on animal health and welfare. Keeping the herd clean and comfortable can reduce the incidence and severity of mastitis(Cheng & Han, 2020). It is caused by organisms such as Escherichia coli, which do not normally live on the skin or in the udder but enter the teat canal when the cow comes in contact with a contaminated environment. Pathogens are normally found in feces bedding materials, and feed (Hamadani *et al.,* 2010). High stocking density, contaminated floor, wet bedding, poor ventilation, and hot and humid climate can promote

growth of mastitis pathogens and increased exposure of cows, resulting in higher occurrence of mastitis(Cheng & Han, 2020)

2.1.2.3. Summer mastitis

A third type of mastitis, referred to as summer mastitis, is an acute illness of dry cows and heifers that causes extensive and painful damage to the udder. The infected quarter is permanently damaged, resulting in early culling of the cow. Infection is more likely to occur when cows are in environments where the teats can easily be exposed to damage and high fly populations. Although these all factors are involved in bovine mastitis, the current research focuses on bovine mastitis, which is caused mainly by bacterial infections (Girma, 2022).

2.2. Major mastitis causing pathogens

2.2.1. Causative Bacterial Agents of Mastitis

Bacterial intra-mammary infection (IMI) is considered to be the main cause of bovine mastitis. Many bacterial species have been identified as causative agents for bovine mastitis. These bacterial infections can be classified into 2 types based on the bacterial origin; Contagious and environmental (Lakew *et al*., 2019).

2.2.1.1. Streptococcal Mastitis

Streptococcal species frequently reported to cause mastitis are *Streptococcus agalactiae, dysgalactiae,* and *uberis,* with the former being more prevalent (MK, 2017). The incidence of infection varies from herd to herd and may exceed staphylococcal mastitis. *Streptococcus agalactiae* was a major cause of chronic mastitis in pre-antibiotic era and is still a serious cause of chronic mastitis in some herds, although it can be eradicated readily by proper antibiotic therapy and management(MK, 2017)

Agalactiae is multiplies in the milk and on the mammary epithelial surfaces, an obligate parasite of the udder and, unlike coliform organisms, it does not survive in the environment of the cow; hence, it is relatively easy to eradicate. New infections in a *Str. agalactiae*-free herd generally are attributed to introduction of infected cows, to transmission of the organism

through milking personnel as a carrier, and to heifer calves raised on raw milk containing *Str. agalactiae* and permitted to suckle the teats of penmates(MK, 2017)

2.2.1.2. Staphylococcal Mastitis

Staphylococcal mastitis is associated with udder infection by *aureus*. It became prominent as herds were freed of *Str. agalactiae* infection. *Aureus* mainly produces subclinical and chronic mastitis, but it also may cause peracute mastitis and lead to gangrene of the quarters. Bacterial toxins and toxic products are thought to be involved in the causation of mastitis and gangrene. The a-toxin is potentially the most damaging, because it causes vasoconstriction leading to ischemic necrosis of affected tissues and gangrene. This type of Gangrenous mastitis is most often common for young cows after calving(Bouchard et al., 2013)

2.2.1.3. Coliform Mastitis

The coliform organisms commonly involved in mastitis are *E. coli, Klebsiella* sp, and *aerogenes*, the former being most prevalent. Their pathogenic effect is an attribute of endotoxin contained in bacterial cell wall. Coliform bacteria are ubiquitous in the environment of the dairy cow; manure and infected quarters are the primary source of *E. coli.* Coliform mastitis is typically acute or per acute, but chronic and subclinical infections accompanied with periodic acute flareups also occurs. Cows suffering from per acute mastitis may die within a few days due to end toxemia but usually overcome acute mastitis(Abegewi et al., 2022)

Staphylococcus spp. are the most prevalent causative agent of bovine mastitis in dairy cows, followed by coagulase-negative staphylococci (CNS), Streptococcus spp., *E.coli*, *Bacillus* spp., *pneumoniae*, *Enterobacter* spp., *Corynebacterium* spp., *Micrococcus* spp., *Pseudomonas* spp., and *Arcanobacterium pyogenes* (Girma, 2022).

2.3. Bovine mastitis risk factors

There are several risk factors known to be associated with the incidence of bovine mastitis that play significant role, including pathogen, host, and environmental factors. All these factors were taken into consideration in the mastitis control programs (Cheng & Han, 2020).

2.3.1. Bovine mastitis control and treatment

2.3.1.1. Five-point plan

Five-point plan introduced by National Institute for Research in Dairying (NIRD) since 1960s is effective in controlling contagious mastitis pathogens (Lee *et al*., 2023). The five points are: i) identify and treat clinical cases; ii) post milking teat disinfection; iii) Dry cow therapy(DCT); iv) cull chronic cases; v) routine maintenance of milking machine. The five-point plan is not very effective against the environmental pathogens and hence, is coupled with other appropriate strategies to control mastitis infections (Down *et al*., 2016).

2.3.1.2. Antibiotic therapy

The main strategy to treat mastitis is by the use of antibiotics, such as penicillin, ampicillin, tetracycline, gentamycin, etc., which can be given by intra-mammary infusion, intramuscular or intravenous injection (MK, 2017). Dry period is an important stage in lactation cycle; any infection during dry period will affect the next lactation, and therefore, it is very important to take care of the cow's health before the next milking cycle. Before drying off the cows, they were checked for any sign of mastitis; chronic mastitis cases, which are hard to detect by naked eyes, were checked via the California mastitis test (CMT) (Bhutto *et al*., 2012).

2.4. Challenges of mastitis control and antimicrobial resistance in dairy cattle

Mastitis, the inflammation of the mammary gland, which is usually a consequence of the adhesion, invasion, and colonization of the mammary gland by mastitis pathogens, occurs in three forms: clinical, subclinical, and chronic mastitis. Among these forms, subclinical mastitis is more common and results in reduced milk production without observable clinical signs or milk abnormalities (Zeryehun & Abera, 2017). For this reason, it is difficult to diagnose and persists longer in the herd (Abrahmsén *et al*., 2014).

The main treatment or mastitis is intra-mammary infusion or parenteral administration of antibiotics, such as streptomycin, ampicillin, cloxacillin, penicillin, and tetracycline (Naranjo-Lucena & Slowey, 2023). Although effective treatment or bovine mastitis depends on the antimicrobial susceptibility of the pathogen, the type of mastitis, the cattle breed, and the

treatment regimen, the emergence of drug resistance is a serious challenge for mastitis control.

2.5. Mechanism of drug resistance development in microorganisms

The treatment of infectious diseases caused by pathogenic microbial strains is one of the most traditional problems in the clinical field (Tenover, 2006). This necessity encouraged investigators to synthesize novel and more potent inhibitor compounds, such as antibiotics. However, the emergence of bacterial or fungal resistance has persuaded investigators to find other options. The increasing emergence of new forms of multidrug resistance among human pathogenic bacteria, coupled with the consequent increase in infectious diseases, urgently requires the discovery and development of novel antimicrobial drugs with new modes of action.

It was not until 1940 with the discovery of penicillin, the first, best-known and most widely used antibiotic (Fleming, 1928). In 1928, an English bacteriologist, Sir Alexander Fleming, conducted the first clinical trials of penicillin in humans. This antibiotic was obtained from a blue-green mould of the soil called *Penicillium notatum*. This discovery marked the beginning of the development of antibacterial compounds produced by living organisms(Tenover, 2006)

Most antibiotics in use today originated from the phylum Actinobacteria, with nearly 80% of actinobacterial-derived antibiotics produced by soil-dwelling bacteria of the genus Streptomyces (Barka *et al.*, 2016). With the discovery of streptomycin, the golden age of antibiotic discovery and development (1940–1990) ensued. This involved the efforts of many academic institutions and major pharmaceutical companies in the United States and other countries. Currently, antibiotics affect almost every process in bacterial cells. On the basis of their structure and mode of action, at least seven major groups of antibiotics have been described. These include β-lactams (inhibits cell wall synthesis), aminoglycosides (protein synthesis), macrolides (protein synthesis), tetracyclines (protein synthesis), daptomycin (cell membrane function), platensimycin (fatty acid biosynthesis), and glycopeptides (cell wall synthesis(Barka et al., 2016)

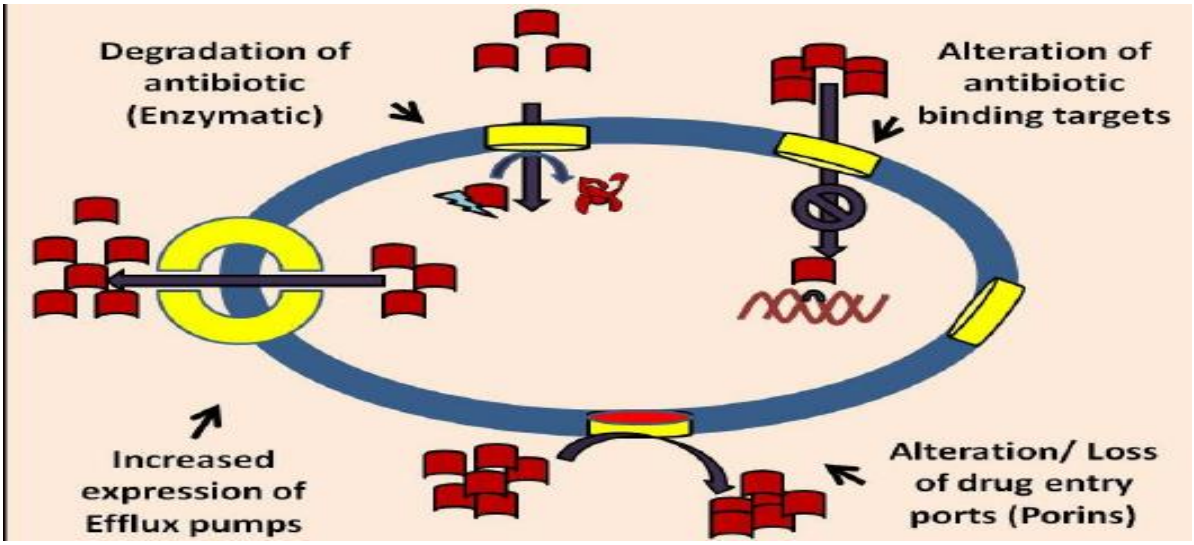

Figure 1. Bacterial antibiotic resistance mechanisim (Barka et al., 2016)

It is only natural that organisms that produce antibiotics also contain self-resistance mechanisms against their own antibiotics. In addition, the coexistence of producer and non-producer bacteria is also believed to have resulted in the coevolution of resistance mechanisms in non-producing environmental bacteria. Resistance determinants found in these two groups of bacteria have garnered significant attention in recent years because of their possible link with the emergence of resistance in pathogenic clinical isolates (Marinelli *et al*., 2008).

2.5.1. Self-Resistance Mechanisms in Producer Organisms

Antibiotic producing bacteria involve a variety of sophisticated mechanisms for self-defense against their own antibiotics. They often involve multiple mechanisms simultaneously to ensure complete protection from the biologically active molecules produced by them. Interestingly, the genetic determinants of self-resistance are almost always clustered together with antibiotic biosynthesis genes, and their expression is coregulated (Mak *et al*., 2014).

2.5.2. Antibiotic Modification or Degradation

Antibiotic modification is a commonly used strategy for rendering an antibiotic ineffective, especially in the case of aminoglycoside antibiotics (for example, kanamycin, gentamycin, and streptomycin), chloramphenicol, and β-lactams (Wright, 2005). Many aminoglycoside modification enzymes (AMEs), including N-acetyltransferases(AACs), O-phosphotransferases

(APHs), and O-adenyltransferases (ANTs), which acetylate, phosphorylate, or adenylate aminoglycoside antibiotics, respectively, are known to exist in producer bacteria. This enzyme may not be directly involved in resistance in producers, but instead may perform other metabolic functions. One example is that β-lactamases produced in most Streptomyces species are not related to the resistance or synthesis of β-lactams. However, it helps to destroy the β-lactam rings of penicillin, which are extremely important antibiotics for treating human infection(Wright, 2005)

2.5.3. Antibiotic Efflux

The efflux of antibiotics is another commonly used mechanism for self-resistance, although it usually occurs in conjunction with other mechanisms, such as modification of the antibiotic or the target. Some bacteria can produce pumps that sit on their membrane cell wall to pump out antibiotics from their cells (Marinelli *et al*., 2008). In some cases, mutations in bacterial DNA can help bacteria produce more pumps, which in turn increase resistance. These efflux pumps can transport a variety of compounds, such as signal molecules and nutrients. Some of these pumps can also transport antibiotics from bacteria, lowering the antibiotic concentration inside bacterial cells.

The best studied example of antibiotic efflux among producers is found in *Streptomyces* peucetius, which produces two closely related anticancer antibiotics, daunorubicin (Dnr) and doxorubicin (Dox). These two antibiotics intercalate with DNA, preventing further rounds of replication. The efflux of these antibiotics in *S. peucetius* occurs via the ABC (ATP-binding cassette) family transporter *DrrAB*, which is encoded by the *drrAB* genes embedded within the gene cluster responsible for the biosynthesis of these antibiotics(Nishino *et al*., 2021). The DrrAB system has been studied in significant molecular and biochemical detail. The *DrrAB* pump is assembled from two subunits, each of the ABC proteins *DrrA* and the integral membrane protein *DrrB*. The *DrrA* protein functions as the catalytic nucleotide binding domain (NBD). The *DrrB* protein functions as the carrier protein and forms the transmembrane domain (TMD(Wen et al., 2018)

Interestingly, *OtrC*, which is found in the oxytetracycline producer Streptomyces rimosus, is another example of a self-resistance efflux system that exhibits multidrug specificity. Self-

resistance in S. rimosus is conferred by two efflux proteins: *OtrB* (previously known as TetB), which is located in the biosynthesis cluster, and *OtrC*, which is located outside of the cluster (Mak *et al*., 2014). *OtrB* belongs to the major facilitator superfamily (MFS) of transport proteins, but little is known about its mechanism of action or substrate specificity. The *OtrC* protein is an ABC family protein, and like *DrrAB*, it also confers resistance to multiple antibiotics and MDR substrates, including ampicillin, bromide, doxorubicin, ethidium, ofloxacin, oxytetracycline and vancomycin(Wen et al., 2018)

2.5.4. Antibiotic sequestration

Sequestration involves the function of drug-binding process, which prevent the antibiotic from reaching its target. Among producers of the *bleomycin* family of antibiotics, the primary mechanism of resistance involves sequestration of the metal-bound or the metal-free antibiotic by binding proteins *TlmA*, *BlmA*, and *ZbmA* in S.behindustanus ATCC 31158, and *Streptomyces flavoviridis*, respectively (Sabnis *et al*., 2018). Each bleomycin-family producer member has one or more genes related to ABC transporters in their biosynthesis clusters, which may be used to remove the antibiotics bound to binding proteins (Xiang *et al*., 2021).

2.5.5. Target Modification/Bypass/Protection Mechanisms

Target modification acts as a self-resistance mechanism against several classes of antibiotics, including *β-lactams*, *glycopeptides*, *macrolides*, *lincosamides*, *streptogramins* and *aminoglycosides* (Wilson *et al*., 2020). The β-lactam antibiotic has a similar structure to PBP substrates (*peptidoglycan* precursors), thus allowing the antibiotic to associate and cause acylation of the active site serine resulting in its inhibition. The producer *Streptomyces* species, despite being Gram-positive, are highly resistant to penicillins, which is due to either overproduction of PBPs or synthesis of low-affinity PBPs (Zhang and Cheng, 2022).

Three classes of PBPs (A, B, and C) are found in bacteria. Analysis of the biosynthesis clusters of β-lactam producing bacteria revealed that they often contain genes encoding PBPs, suggesting their role in self-resistance. Interestingly, *Streptomyces* species contain on average more than 10 PBPs, including both Classes A and B, a number much greater than that found in other *Actinobacteria*. Some of these PBPs indeed have low affinity for β-lactams, most likely due to the absence of a serine/threonine protein kinase domain (STPK) (renamed PASTA) that

binds β-lactams (Peterson and Kaur, 2018). Glycopeptides, such as vancomycin and teicoplanin, inhibit cell wall transpeptidation and transglycosylation by associating with peptidoglycan precursors (*D-Ala-D-Ala*) (Binda et al, 2014). Antibiotic resistance results from a change in the peptidoglycan precursor from *D-Ala-D-Ala* to *D-Ala-D-Lac* or *D-Ala-D-Ser*, which results in a 1000- and 6-fold reduction in affinity for glycopeptides, respectively (Peterson & Kaur, 2018)

2.6. Probiotic lactic acid bacteria

Fermented foods have been a significant part of the human diet for many years (Abdel Tawab et al., 2023). These foods, which can be made from plants, meat, or milk, have the advantage of being preserved for a longer time compared to fresh raw foods. Fermented foods are also a rich source of probiotic bacteria. Probiotics are strains of microorganisms that provide positive health benefits when consumed by humans and animals. The majority of probiotics consist of lactic acid bacteria (LAB), particularly from the genera *Lactobacillus* and *Bifidobacterium*. However, other genera such as *Bacillus, Enterococcus, Pediococcus,* and various yeasts can also serve as probiotics. LAB are primarily gram-positive, anaerobic rods that originate from the human gastrointestinal tract (Abdel Tawab et al., 2023)

3. MATERIALS AND METHODS

3.1. Sample collection and description of the study sites

The experiment was conducted at Holeta National Agricultural Biotechnology Research Centre (NABRC), Microbial Biotechnology Laboratory, which is located 29 km to the west of the capital city of Ethiopia. The mean annual rainfall in Holeta, Ethiopia is 1037mm and the mean annual temperature is 22.2^{o}C. The laboratory experiments were conducted using a completely randomized design (CRD).

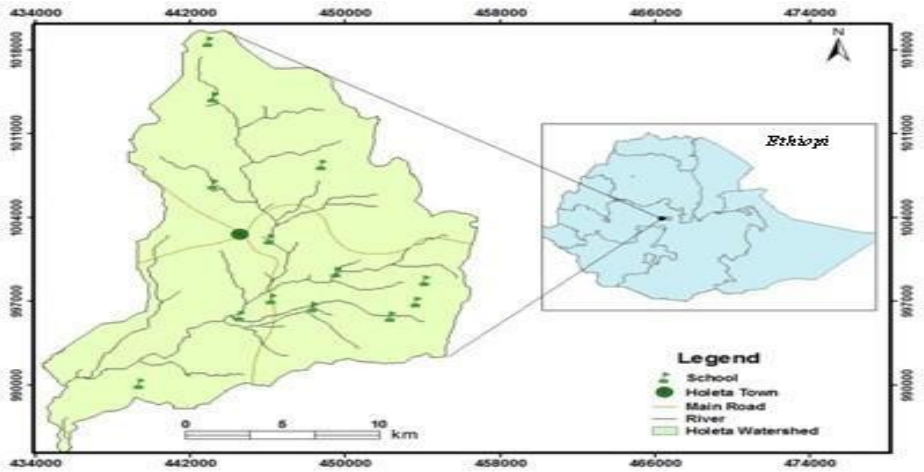

Figure 2. **Map of study area (adapted from GIS desktop 10)**

3.2.Bacterial Strains and Culture Conditions

The LAB strains analysed in this study were collected from the National Agricultural Biotechnology Research Centre (NABRC), Microbial Biotechnology Laboratory. The strains were previously isolated from the fermented milk of healthy local and Holstein cattle. They were screened for functional properties (Kebede et al., 2021), probiotic function (Workie and Kebede, 2022) and were genetically identified via 16S rRNA gene sequencing.

Seven LAB strains *pentosus (BB26), casei (BB28), plantarum (BB31), plantarum (LB64), plantarum (DZ11), plantarum (DZ31), paracasei (GB15)]* were obtained. Approximately 0.1 ml of the culture was inoculated into 1 ml of sterilized de Man, Rogosa sharp agar (composed of tryptic digest of casein, beef extract, yeast extract, glucose, sorbitan monooleate, di-potassium hydrogen orthophosphate, magnesium sulfate, manganese (II) sulfate, ammonium citrate, sodium acetate, agar), and Sharpe broth (MRS, Britania) and incubated at 37°C. The

strains were sub-cultured on MRS agar two to three times.

Mastitis causing pathogen strains were previously isolated from diseased animals found around Holeta town. The isolates were screened by an animal biotechnology team and preserved for further testing. The isolates were confirmed via PCR and preserved at deep freez (-20°C). The target strains (*Staphylococcus aureus, E. coli, L. monocytogenes* and *Salmonella* spp.) were isolated on common medium such as blood agar, MacConkey agar (composed of Bile salts: Crystal violet dye, lactose and Neutral red), brain heart infusion agar(composed of Agar: 15g/l Brain extract: 7.8 g/l, Dextrose: 2.0 g/l and Disodium phosphate: 2.5 g/l and mannitol agar.(composed of Sodium chloride: 75 g/L, D-mannitol: 10 g/L, Phenol red: 0.025 g/L, Agar: 15 g/L, Enzymatic digest of casein: 5 g/L, Enzymatic digest of animal tissue: 5 g/L, Beef extract: 1 g/L).

LAB strains were grown in de Man, Rogosa, and Sharpe (MRS, Britania) broth, and incubated and stored as described by Frola et al., (2012). Before performing additional studies, bacteria were sub-cultured three times, every 24 hrs at 37°C in MRS broth. Mastitis -causing pathogens were grown on nutrient broth (containing yeast extract, peptone, sodium chloride and other basic nutritional requirements) and incubated at 37°C for 24 hrs. The isolates were sub-cultured three times on nutrient agar.

3.3. Preliminary characterization of LAB strains

3.3.1. Acid stress test

The LAB isolates that survived the preliminary screening were subjected to lower pH broth. The isolates were inoculated into MRS broth, and the pH was adjusted to 3, 3.5 or 4. Growth was monitored spectrophotometrically on a microliter plate at 600 nm and compared with that of the control culture set at 37°C and pH 6.0±0.02.

3.3.2. Temperature stress test

LAB isolates were inoculated into MRS broth and incubated at 15, 25, 45, and 55°C anaerobically. Absorbance measurements were recorded at 600 nm, and their respective growth values were compared to those of the control culture set at 37°C and pH 6.0±0.02. The viability of the surviving isolate will be checked on MRS agar media.

3.3.3. Sugar utilization test

LAB isolates from the preliminary screening were tested for the utilization of various carbohydrates as the sole carbon source using deep well microliter plates, and the results were read at 600 nm via a spectrophotometer and compared with those of the control culture. All the test isolates were inoculated in triplicate into wells containing six carbohydrates broth consists of trypticase or protease peptone 10 g/l, sodium chloride (NaCl): 5 g/l, beef extract (optional): 1 g/l, phenol red (7.2 ml of 0.25% phenol red solution): 0.018 g/l, carbohydrate source: 10 g/l. The tested carbohydrate includes glucose, fructose, Cellobiose, sucrose, xylose and lactose. Phenol red was used as an indicator to demonstrate significant phenotypic diversity among the tested LAB strains. The cell mass of the strains was then read at 600 nm using a spectrophotometer.

3.3.4. Antibiotic resistance test

Antibiotic resistance of the isolated strains was determined using the Kirby–Bauer disk diffusion method (according to the CLSI document M2-A9 suggestions). Seven antibiotic impriginated disks, such as ampicillin, chloramphenicol, ciprofloxacin, gentamycin, kanamycin, tetracycline, and streptomycin, were used for determination of the antibiotic resistance of bacteria to antimicrobial agents. After the strains were activated on MRS broth, 100 µl of each strain was cultivated on Mueller Hinton agar, after which the antibiotic discs were placed using forceps after incubation for (24 hrs, at 37°C), the bacterial strains were evaluated as resistant, medium-grade sensitive and sensitive according to the criteria of the NCCLS document M2-A9 by measuring the inhibition zone diameters in mm around the antibiotic discs.

3.3.5. Hemolytic Activities.

Hemolytic activity was examined using the use of blood agar supplemented with 5% defibrinated sheep, horse or human blood. The isolates were streaked onto agar plates and incubated at 37°C (Roldán-Pérez et al., 2023).The plates were then examined for hemolysis patterns surrounding the colonies and were designated as β, α or γ hemolysis depending on the nature of the hydrolysis zone observed (Yasmin et al., 2020).

3. 4. Secondary characterization of LAB strains

3.4. 1. Auto-aggregation test

Overnight -cultured fresh isolates were harvested, washed and suspended in 2 ml phosphate -buffered saline PBS(137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4).. The absorbance of each sample was read at 600 nm at 0, 1st, 2nd, 3rd, 4th, 5th and 24th hours of incubation(Urcan et al., 2024).Auto-aggregation was determined via the following formula: -

$$A_{agr}\% = 1 - \left(\frac{A_{t_x}}{A_{t_0}}\right).100$$

Where:

A_{agr} = Auto aggregation percentage, A_{tx} = Absorbance at 0, 2nd, 4th, 6th and 24th hour of incubation, A_{t0} = Absorbance at 0 time of incubation.

3.4.2. Co-aggregation test

Each mastitis causing bacterial strain was grown according to the methods and materials described previously to assess the interaction between LAB strains and Mastitis causing bacterial strains (Pellegrino et al., 2019). Both the pathogenic and LAB isolates were washed and suspended in 2 ml PBS buffer salane. The absorbances of the individual and mixed (1:1 ratio) suspensions of the pathogens and LAB isolates were measured at 0, 2nd, 4th, 6th and 24th hours of incubation. Co-aggregation was determined using the following formula described in (Li et al., 2015).

$$Coaggregation\ \% = \frac{\left[\frac{A_{pth} + A_{iso}}{2}\right] - A_{mix}}{\frac{A_{pth} + A_{iso}}{2}}.100$$

Where: A_{pth}= absorbance of the pathogenic isolate,

A_{iso}= absorbance of the test isolate,

A_{mix}= absorbance of the mixture of the pathogen and the test isolate.

3.4.3. Antimicrobial activity assay

The bacteriocin production potential of the LAB strains was screened using the agar well diffusion method as described in (Herreros et al., 2005). Total Fresh cultures of LAB strains were spotted onto Muller Hinton agar plates (which contains beef extract, acid hydrolysate of casein, starch, and agar and overlaid with indicator (pathogenic) microbes. Mastitis causing pathogens (S. aureus, E. coli, L. monocytogenes and Salmonella typhi.) were used as indicator (pathogenic) microbes. The antimicrobial activity of the isolates was determined by measuring the inhibition zone surrounding the spot. Antibiotic disks and buffer saline were used as positive and negative controls, respectively.

3.4.4. Cell surface hydrophobicity

The *in vitro* cell surface hydrophobicity of the LAB strains was evaluated by measuring the microbial cell adhesion to hydrocarbons according to the method described by (Rokana *et al*. 2018). Briefly, overnight cultures of LAB strains grown in MRS broth were separately harvested via centrifugation (8000 rpm, 4°C for 10 min) and washed twice with 0.85% NaCl before being resuspended in 1ml of 0.85% NaCl buffer. Finally, the absorbance (A0) was measured at 600 nm. Approximately 3 ml of the cell suspension was blended with 1 ml of hydrocarbon (xylene) and incubated at 37 °C without shaking for 1 hrs for separation of the aqueous and organic phases. The aqueous phase (1 ml) was removed carefully, and the absorbance (A1) was measured at 600 nm. The percent hydrophobicity of the isolate was determined by the decrease in the level of absorbance and calculated via the following formula:

$$\% \text{ cell surface hydrophobicity (CSH)} = \left(\frac{a-b}{a}\right)\times 100$$

Where: a= initial cell concentration of the aqueous phase,

b= concentration of cell in the aqueous phase after partitioning,

3.4.5. Bacteriocin production assay

The strains was inoculated on MRS agar plate and incubated for 16 hrs at 37 °C.The colony were detected and over laid with appropriate soft agar seeded with an over-night culture of the

indicator strains (LAB).Finally,the formation of growth inhibition zone around the colony were examined.

3.4.6. Agar well diffusion method

Overnight cultures of LAB strains grown in Muller Hinton agar (which contains beef extract, acid hydrolysate of casein, starch, and agar) were separately centrifuged at (8000 rpm, and 4°C for 10 min). The cell-free supernatants were poured into Muller Hinton Agar wells prepared using cork barrier and overlaid with indicator microbes (Herreros et al., 2005). Antibiotic disk were used as positive control while sterilized distilled water were used as negative control. The plate was then incubated at 37°C, and the bacteriocin activity was calculated by measuring the inhibition zone diameter in mm surrounding the spot.

3.4.7. The kinetic growth curves of seven LAB strains

The growth curves of the isolates were determined using the use of broth colony forming units. Isolates were inoculated into MRS broth medium and incubated at 37°C. The isolates were then standardized on a spectrophotometer using McFarland standard solution. The samples were drowned every 12 hrs and enumerated by colony forming units (CFUs), which provide a direct measurement of bacterial cell mass counts using a spectrophotometer.

3.5. Statistical analysis

The physiologic and probiotic data are presented as the mean values and standard deviations (means ± SDs), which were calculated from triplicate trials. The data were analyzed using one-way ANOVA and Tukey's multiple range tests ($P < 0.05$) via SPSS 10.0.7 software and R-software version 3.3.1.

4. RESULTS

4.1. Screening of the strains

Morphological, biochemical and microscopic examination revealed that some of LAB strains were rods, whereas others were cocci in shape, negative according to the oxidase test, and were gram-positive, catalase negative and non-motile. The acid production abilities of all the strains were screened on sterilized reconstituted skim milk (10%, w/v). The results revealed that four isolates (*Lpb. plantarum* (LB64), *Lpb. plantarum* (DZ31*), Lpb. pentosus* (BB26) and *L. plantarum* (BB31)) reduce the pH to less than 4.90 after 24 hrs of incubation (Table 1).

Table 1: Morphological, macroscopic and biochemical characterization of selected isolates of LAB.

	Colony morphology				**Biochemical test**			
Code	**Shape**	**Colony size**	**Color**	**Surface/ texture**	**Gram staining**	**Catalase test**	**Motility**	**pH value**
Lpb. plantarum (LB64)	Cocci	Small	White	Smooth/ Shiny	+ve	-ve	-ve	4.90
Lpb.plantarum (DZ31)	Rode	Medium	White	Smooth	+ve	-ve	-ve	4.76
Lpb.plantarum (DZ11)	Rode	Small	Yellow	Smooth	+ve	-ve	-ve	5.30
Lcb. paracasei (GB15)	Cocci	Medium	White	Smooth	+ve	-ve	-ve	5.18
Lbp. casei (BB28)	Cocci	Medium	White	Smooth	+ve	-ve	-ve	5.60
Lb. pentosus (BB26)	Cocci	Small	White	Smooth/ Shiny	+ve	-ve	-ve	4.80
*L. plantarum (*BB31)	Cocci	Small	White	Smooth/ Shiny	+ve	-ve	-ve	4.63

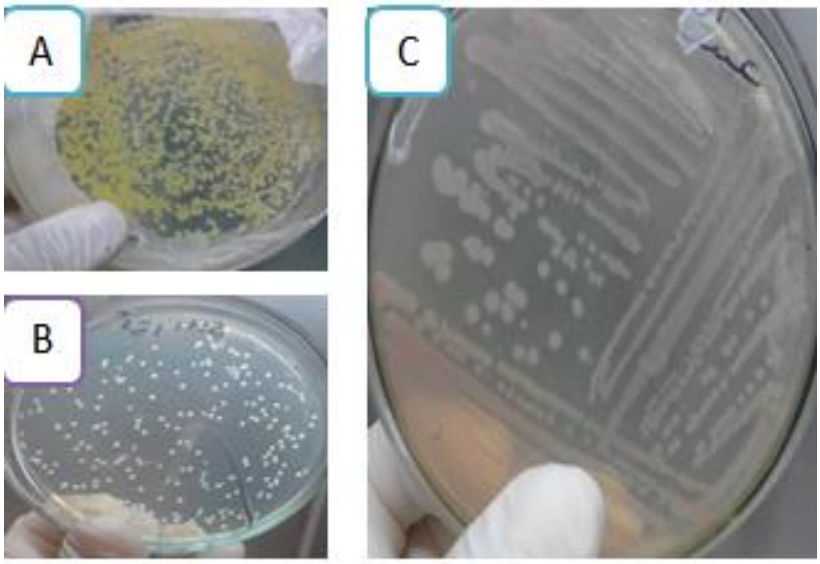

Figure 3: morphological characteristics of lactic acid bacteria strains

A: yellow raised, B; white smooth raised, C: white rough and raised colonies of lactic acid bacteria on (LAB) on MRS agar media

4.2. Tolerance to pH

The isolates significantly varied for each pH, as depicted in Figure 4. The graph shows that some of the strains survived at pH 3 and 3.5 (as the scatter plot in the figure shows that some of the strains were above the od-mean value), whereas all of them grew above the od-mean value at pH 4.0 (Fig. 4).

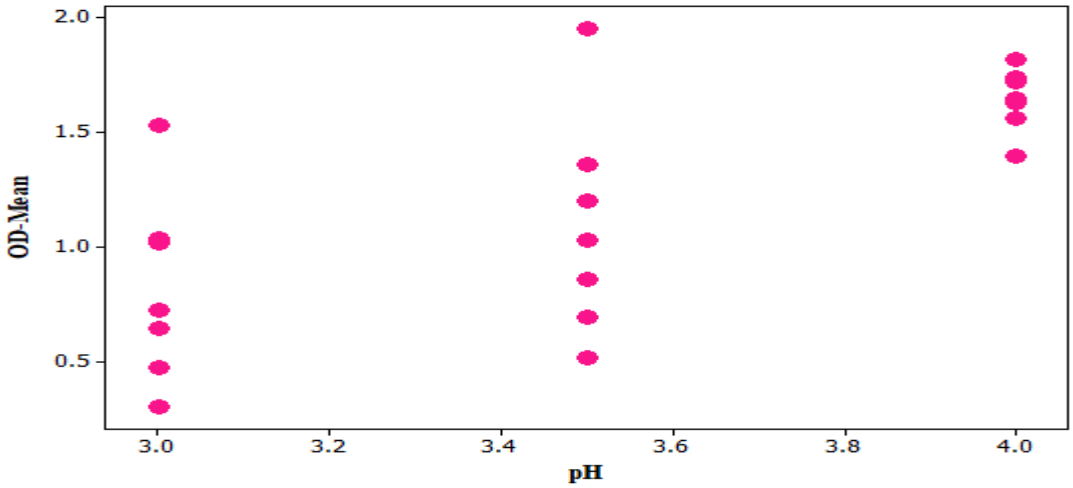

Figure 4: pH tolerance test of seven LAB strains

4.3. Growth at different temperatures

The strains significantly varied at each temperature during the 24 hr incubation period. The significant difference in the graph below shows that the optimum temperature is 25°C (Fig. 5). Some of the strains survived at 45°C (the line graph shows that some of the strains were above the mean value), whereas all of the tested strains grew less at 15 and 55°C (below the mean values).

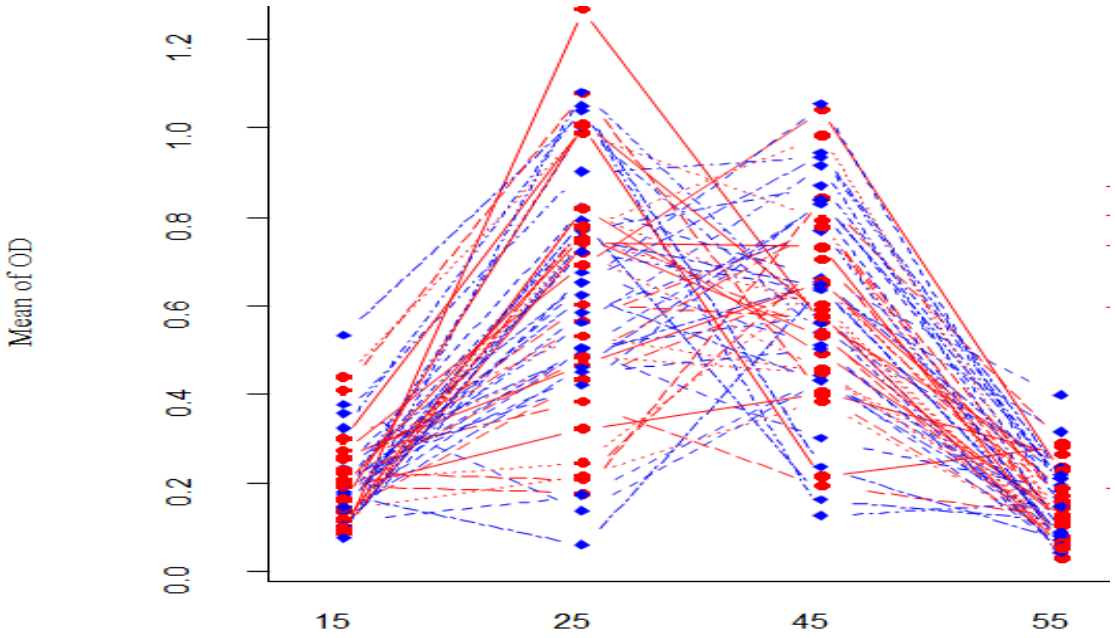

Figure 5: Temperature tolerance testing:

Low microbial growth at 15°C and 55°C. High microbial growth and optimal temperature tolerance at 25°C, followed by 45°C.

4.4. Sugar utilization

Seven LAB strains, including L. pentosus (BB26), Lcb. casei (BB28), Lbp. plantarum (BB31), Lbp. plantarum (LB64), Lpb. plantarum (DZ11), Lbp. plantarum (DZ31), and Lcb. paracasei (GB15), were tested for their ability to ferment glucose, xylose, fructose, lactose, cellobiose, and sucrose (Table 3). The results revealed that all the tested strains highly fermented glucose, sucrose, fructose, and cellobiose. L. plantarum (BB31), Lpb. plantarum (LB64), and Lp. paracasei (GB15) exhibited high performance in utilizing lactose, whereas Lpb. Pentosus, Lpb.plantarum (BB26), Lcb. casei (BB28)), Lpb.plantarum (LB64), and Lpb. plantarum

(DZ11) exhibited moderate performance when lactose was used as the main carbon source. Similarly, L. plantarum (BB31) and Lpb. plantarum (DZ31) performed well in the utilization of xylose. The strain Lpb. plantarum (DZ11) was the least efficient for fermenting xylose (Table 2).

Table 1: Fermentation ability of the LAB strains toward the 6 carbohydrates

Lactic acid bacterial strain ID	**Carbohydrate**					
	Glucose	**Fructose**	**Lactose**	**Xylose**	**Cellobiose**	**Sucrose**
L.pentosus (BB26)	++	++	+	+	++	++
Lcb. casie (BB28)	++	++	+	+	++	++
L. plantarum (BB31)	++	++	++	++	++	++
Lpb .plantarum (LB64)	++	++	+	+	++	++
Lpb. plantarum (DZ11)	++	++	+	N	++	++
Lpb . plantarum (DZ31)	++	++	++	++	++	++
Lcb. paracasei (GB15)	++	++	++	+	++	++

Note: ++: Highly ferment, +: moderate, N: no fermentation

4.5. Bacteriocin production

All LAB strains demonstrated potential inhibition against *E. coli, L. monocytogenes, S. aureus,* and *Salmonella thphi*. Among them, *L. plantarum* (BB31) showed the highest activity against *E. coli* with an average inhibition zone of 16.93 ± 1.210 mm, followed by *Lpb. plantarum* (LB64) and *L. pentosus* (BB26) with average inhibition zones of 15.33 ± 0.577 mm and 14.67 ± 1.528 mm, respectively. *Lcb.Paracasei* (GB15) exhibited the least activity against *E. coli* with an average inhibition zone of 6.93 ± 0.116 mm.

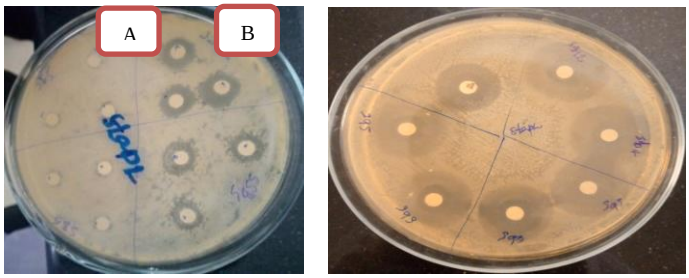

Figure 6**:** Bacteriocin production by the disc diffusion assay;

A: the control, B: Supernatant from LAB strains producing antimicrobial against *S. aureus.*

L. plantarum (BB31) also showed the highest activity against *L. monocytogenes*, *S. aureus*, and *Salmonella typhi*, whereas all six strains presented the highest activity against *L. monocytogenes* except *Lcb. paracasei* (GB15), which exhibited the lowest activity, with an average inhibition zone of 8.03 ± 1.34mm. Two strains, *Lpb. plantarum* (BB31) and *L. plantarum* (DZ31), demonstrated the highest activity against *S. aureus*, with average inhibition zones of 17.13 ± 0.07 mm and 17.17 ± 2.02 mm, respectively. The strain *Lcb. paracasei* (GB15) exhibited the least activity against *S. aureus*, with an average inhibition zone of 8.80 ± 0.43 mm. *plantarum* (BB31) also exhibited the highest activity against Salmonella, with an average inhibition zone of 17.13 ± 1.12 mm, followed by *Lpb.plantarum* (LB64) and *L. pentosus* (BB26), with average inhibition zones of 15.08 ± 0.07 mm and 14.4 ± 0.6 mm, respectively. The strain Lcb. *paracasei* (GB15) exhibited the least activity against *Salmonella thyphi*, with an average inhibition zone of 7.00 ± 2.64 mm (Fig. 4).

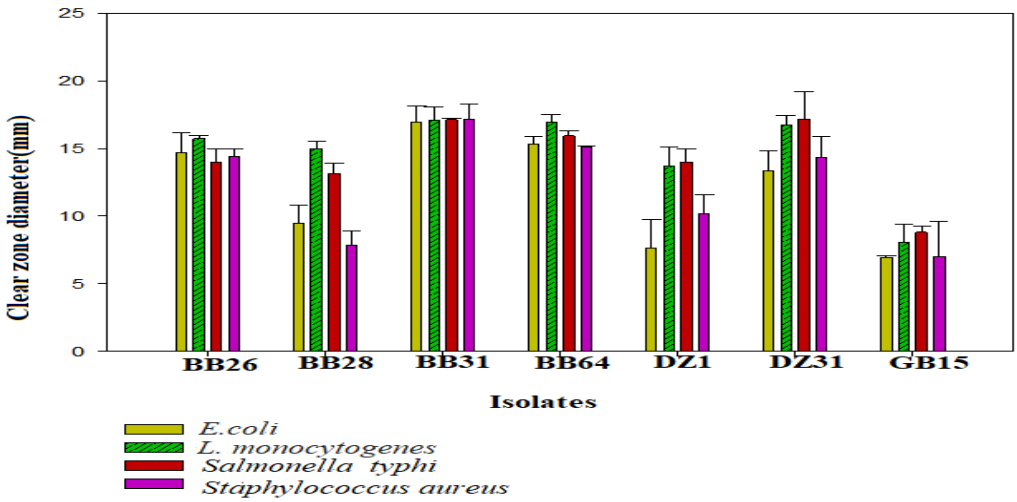

Figure 7: Antimicrobial activities of 7 LAB strains against mastitis pathogens

4.6. Safety evaluation of LAB strains

4.6.1. Antibiotic susceptibility test

The susceptibility and resistance profiles of the selected lactic acid bacterial strains were tested against seven antibiotics, including chloramphenicol, ciprofloxacin, kanamycin, ampicillin, gentamycin, streptomycin, and tetracycline (Table 3). All of the strains were susceptible to the provided antibiotics.

Table 2: Antibiotic susceptibility and hemolytic activity of 7 selected LAB strains

Strains ID	Zone of diameter (mm) for some antibiotic disc							Hemolytic Activity
	TE30	CHL	CIP	KA	Amp	GM	S25	
L.pentosus (BB26)	S	S	S	S	S	S	S	γ-hemolysis
Lcb. casie (BB28)	S	S	S	S	S	S	S	γ-hemolysis
L. plantarum (BB31)	S	S	S	S	S	S	S	γ-hemolysis
Lpb .plantarum (LB64)	S	S	S	S	S	S	S	γ-hemolysis
Lpb. plantarum (DZ11)	S	S	S	S	S	S	S	γ-hemolysis
Lpb . plantarum (DZ31)	S	S	S	S	S	S	S	γ-hemolysis
Lcb. paracasei (GB15)	S	S	S	S	S	S	S	γ-hemolysis

Note: CHL=chloramphenicol, CIP = ciprofloxacin, KA= kanamycin, Amp = ampicillin, GM = gentamycin, S25=streptomycin, TE30= tetracycline.

4.6.2. Hemolytic activity

The hemolytic activities of the 7 tested strains were evaluated on blood agar supplemented with 5% sheep blood. Alpha (α)-hemolysis: A partial damage to red blood cells (RBCs) that causes a green or brown discoloration around bacterial colonies on a blood agar plate,

Beta (β)-hemolysis a complete lysis of RBCs that causes a clear or transparent zone around bacterial colonies on a blood agar plate.

Gamma(γ)-hemolysis: No hemolysis occurs, and there is no clearing or lysis on the Columbia blood agar plates were evaluated .None of the tested strains showed α-hemolytic or ß-hemolytic activity when they were grown on Columbia blood agar plates. The tested strains showed γ hemolysis, i.e., negative, or no hemolytic activity (Table 3).

4.6.3. Auto-aggregation

All LAB strains exhibited the potential for auto-aggregation and had a survival rates ranging from 40.69% to 121.47%. Among the tested strains, *pentosus* (BB26) and *plantarum* (DZ31) showed the most potential for auto-aggregation, whereas, *paracasei* (GB15) had the lowest auto-aggregation potential with approximately 40.69% auto-aggregation potential. The mean auto-aggregation of the tested strains significantly varied at $P \leq 0.05$, as shown in Table 4.

4.6.4. Co-aggregation

For their ability to co-aggregate with specific pathogens such as *S. aureus, E. coli, L. monocytogenes,* and *Salmonella*. The co-aggregation ratio of *S. aureus* ranged from 36.01% to 126.79%. The strain *L.plantarum (BB31)* exhibited the highest co-aggregation potential, while *Lpb. plantarum* (BB28) had the lowest co-aggregation potential at 36.01%. Two LAB strains, *L. plantarum* (BB31) and *L. pentosus* (BB26), showed the highest co-aggregation with *E. coli*. The co-aggregation ratio of L. *monocytogenes* was 76.94%, and the strain *Lpb. plantarum* (DZ31) had the highest co-aggregation potential. *L. plantarum* (BB31) showed the highest potential for co-aggregation with Salmonella (139.12%), while *Lcb. paracasei* (GB15) exhibited the least co-aggregation ratio at 31.52%.

Table 3: Auto-aggregation and hydrophobic potential of the 7 LAB strains

Strain ID	**Auto-aggregation**	**Hydrophobicity**
L.pentosus (BB26)	121.47 ± 2.97[a]	75.68 ± 1.02[b]
Lcb. casie (BB28)	88.17 ± 2.52[b]	49.05 ± 6.51[de]
L. plantarum (BB31)	88.59 ± 0.52[b]	80.25 ± 0.48[b]
Lpb .plantarum (LB64)	83.51 ± 1.68[b]	66.16 ± 0.02[bc]
Lpb. plantarum (DZ11)	83.24 ± 0.87[b]	39.47 ± 2.40[e]
Lpb . plantarum (DZ31)	99.82 ± 1.56[a]	99.02 ± 0.75[a]
Lcb. paracasei (GB15)	40.69 ± 1.61[c]	59.31 ± 1.93[cd]

Table 4: Co-aggregation properties of LAB strains

	Co- aggregation			
LAB strain ID	*S.aureus*	*E.coli*	*L. monocytogen*	*Salmonella*
L.pentosus (BB26)	82.42 ± 1.82c	66.12 ± 0.71[a]	54.56 ± 0.09[bc]	91.56 ± 0.44[b]
Lcb. casie (BB28)	36.01 ± 0.52d	41.05 ± 3.99[c]	35.63 ± 2.81[e]	77.07 ± 0.60[c]
L. plantarum (BB31)	126.79 ± 1.62a	69.03 ± 1.04[a]	51.03 ± 3.16[bc]	139.12 ± 4.40a
Lpb .plantarum (LB64)	77.52 ± 1.28c	55.37 ± 0.33[b]	63.49 ± 0.59[b]	86.63 ± 0.81[ab]
Lpb. plantarum (DZ11)	31.91 ± 0.84d	27.71 ± 3.01[d]	39.07 ± 4.06[de]	67.91 ± 3.17[cd]
Lpb . plantarum (DZ31)	99.16 ± 2.28b	61.92 ± 2.80[ab]	76.94 + 2.67[a]	81.84 ± 1.80[b]
Lcb. paracasei (GB15)	45.28 ± 0.54d	19.16 ± 4.07[e]	43.00 ± 0.11[d]	31.52 ± 3.66[e]

4.6.5. Cell surface hydrophobicity

The hydrophobicity of the cell surface was demonstrated by its high adherence to xylene, with

isolates showing a hydrophobicity percentage greater than 49.05%. Among all the strains, Lbp. plantarum (DZ31) had the highest adherence capacity, with a 99.02% adherence rate which indicate this strain is the potent to compete the surface area against pathogens. How ever, Lcb. casie (BB28) exhibited the lowest cell cerface hydrophobicity. On the other hand, Lpb. plantarum (DZ11) (39.47 ± 2.40) exhibited the lowest cell-surface hydrophobicity. The mean value of hydrophobicity significantly varied at P≤ 0.05 (refer to Table 4).

4.6.6. The growth kinetics of the strains

The growth kinetics or curves of seven lactic acid bacteria were determined using spectrophotometer measurements after 36 hrs of incubation. The strains showed a gradual growth curve at 12 hrs of incubation and highly increased until 24 hrs of incubation (Figure 8). The strains showed similar growth kinetics after 36 hrs of incubation.

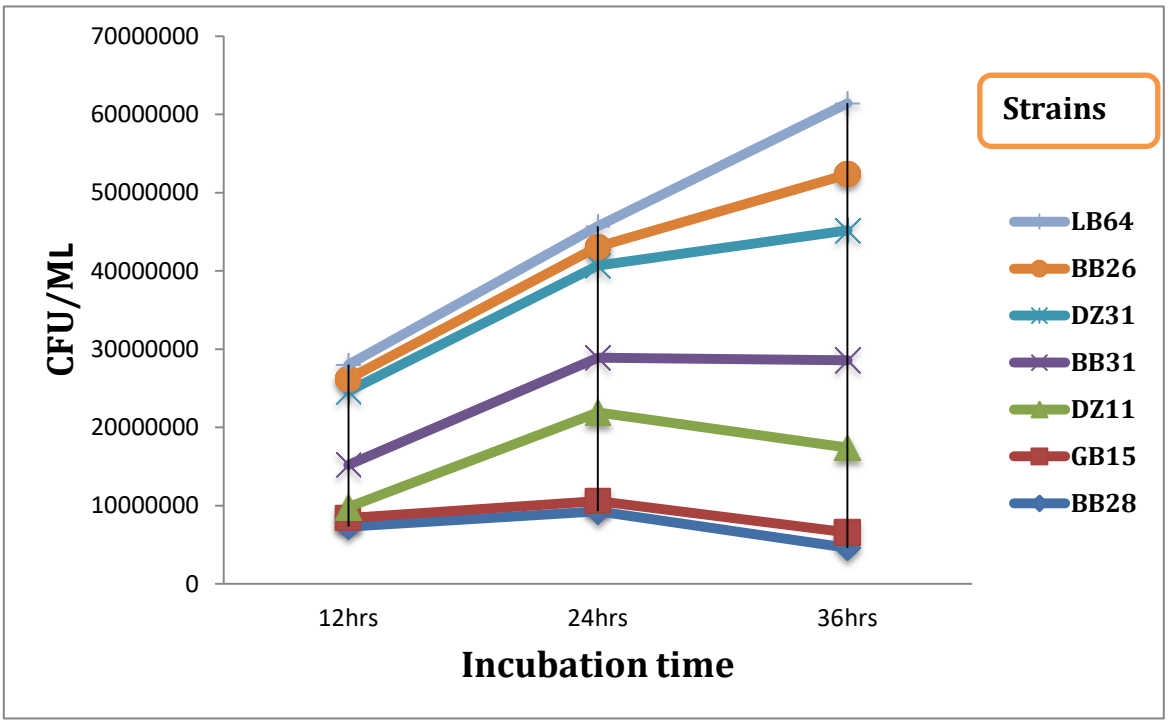

Figure 8: Growth dynamics of the 7 LAB strains

5. DISCUSSION

The focus of this study was to assess the beneficial properties of LAB strain to select good candidates for inclusion in a probiotic cocktail for checking their suppression potential against Bovine mastitis causing Bacterial pathogen in the mammary and to evaluate their probiotic functions as potential alternatives to antibiotics in fighting mastitis-causing pathogens. The strains were previously isolated, and purified from raw and fermented milk samples on de-Man Rogosa Sharpe agar (MRS) medium. In this study the probiotic potential of all seven LAB strains were evaluated on the basis of several factors, including acid-tolerance, sugar utilization capacity, bacitracin production capacity, survival rate under different incubation conditions, and safety. All seven LAB strains exhibited promising tolerance rates and were able to grow at a pH of 4. Similarly, all of the isolates were able to grow at 25°C, whereas only a few were capable of growing at 45°C. These results are consistent with the findings of (Azadnia & Khan Nazer, 2009). (Kebede *et al.*, 2021) also reported similar results, where lactic acid bacteria isolated from raw and fermented milk can grow at temperatures of 25 and 45°C.

One of the key indicators of a good probiotic is its ability to withstand an acidic environment. The ability to tolerate specific acidic conditions is an essential characteristic of isolates that prevents the formation of crucial metabolites. High-quality probiotics should be able to resist low pH levels, even those as low as pH 2.0, and tolerate high acid levels. Thus, the acid-tolerance of seven lactic acid bacterial strains was evaluated. The isolates showed significant variation for each pH, as depicted in Figure 4. In a recent study, all LAB strains tasted, including *L.pentosus* (BB26), *Lcb. casei* (BB28), *L. plantarum* (BB31), *Lpb. plantarum* (LB64), *Lpb. plantarum* (DZ11), *Lpb. plantarum* (DZ31), and *paracasei* (GB15), survived at pH 4.0. Furthermore, *plantarum* (DZ31), (BB26), *Lcb.casei* (BB28), and *Lp. plantarum* (BB31) continued grow to pH 3.5. These results are consistent with those reported by (Mandhania et al., 2019), which showed the acid tolerance of *E. lactis* and *L. plantarum* at pH 2.0 and 4.0 respectively.

This study investigated the antimicrobial activity of *pentosus* (BB26), *casei* (BB28), *Lbp plantarum* (BB31), *Lpb. plantarum* (LB64), *Lpb.plantarum* (DZ11), *Lpb. plantarum* (DZ31), and *Lcb. paracasei* (GB15) against mastitis-causing pathogens. *L. Pentosus* (BB26), *L.*

plantarum (BB31), *Lpb .plantarum* (LB64) and *Lpb. plantarum* (DZ31) were exhibited antagonistic properties towards four of the pathogens tested, as proven by the agar diffusion test (Fig 4). The observed antimicrobial activity is most likely due to the production of nisin and amino acid peptide, which exhibits a broad spectrum of inhibitory activity against several Gram-positive bacteria. (Dinev *et al.*, 2017) found that *L. plantarum* strains are effective against various bacterial pathogens, including methicillin-resistant *S.aureus* and multidrug-resistant enter-aggregative *Escherichia coli*.

LAB-produced organic acids, such as lactic acid and acetic acids, are considered to exert strong inhibitory effects on Gram-negative bacteria, particularly those produced by *L. plantarum* (Makras & De Vuyst, 2006). The findings of (Chae *et al*., 2021) concerning the activity of *L. plantarum* against pathogenic skin microbiota are also in line with the present results. (Zhu & Zhang, 2020) reported that lacidophilin from *L.pentosus* has the best antibacterial ability against *S. aureus* and *E. coli* under acidic conditions and has a low antibacterial ability at high concentrations of metal ions LAB strains exert their probiotic effect in vivo not only by secreting antimicrobial substances but also by adhering to epithelial cells and modulating the host immune response. According to (Bouchard *et al*., 2013), probiotic strains must meet several criteria, including antagonistic activity against pathogens, safety requirements, adhesion and persistence on the cell surface, survival, and ability to function in the target tissue. While hydrophobicity and electron-donor/electron-acceptor properties play crucial roles in microbial colonization, these cell surface characteristics are not sufficient to explain these phenomena (Armas *et al*., 2017).

Although there are several limitations regarding the exact determination of cell surface hydrophobicity mechanisms, the present study used a rapid method involving xylene, which allows conclusions to be drawn. *Lpb.plantarum* (DZ31) has high cell surface hydrophobicity. This study evaluated the cell surface hydrophilicity properties of seven bacterial strains, namely *pentosus* (BB26), *Lcb.casei* (BB28), *L.plantarum* (BB31), *Lpb.plantarum* (LB64), *Lpb .plantarum* (DZ11), *Lpb .plantarum* (DZ31), and *Lcb. paracasei* (GB15). Specifically, the study examined their ability to auto aggregate and co-aggregation. These abilities are important in selecting a potential probiotic strain because they facilitate bacterial adhesion to epithelial cells (auto aggregation) and prevent colonization by pathogenic microorganisms (co-

aggregation) (Armas *et al*., 2017) *L.pentosus* (BB26) showed high auto aggregation and low co-aggregation abilities oward any of the tested pathogens whereas *L.plantarum* (BB31) showed low auto-aggregation and high co-aggregation abilities toward any of the tested pathogens. The literature shows a discrepancy in the auto-aggregation and co-aggregation abilities of LAB. The present results are in line with the reports of (Amenu and Bacha, 2023).

6. CONCLUSION AND RECOMMENDATIONS

The beneficial properties of LAB strains for checking their suppression potential against Bovine mastitis causing Bacterial pathogen and their probiotic functions as potential alternatives can be concluded that, the LAB strains under investigation showed antimicrobial effects against mastitis -causing pathogens, such as *S. aureus, E. coli, L. monocytogenes* and *Salmonella* species. *Lactobacillus* strains presented the highest auto and cell hydrophobicity. These compounds also presented the best coaggregation activity against indicator bacteria. Therefore, LAB from the bovine environment may have the potential to be used as nonantibiotic formulations to treat mastitis-causing pathogens.

On the basis of the results and conclusions of this study, the following recommendations are proposed: since LAB strains belonging to the *Lactobacillus* genus present the highest autoaggregation, cell hydrophobicity and antimicrobial activity against mastitis causing bacteria, additional investigations are proposed, such as analyses of, the metabolites produced (bacteriocin, organic acids, diacetyl, and hydrogen peroxid), probiotic features and in vivo probiotic effects of those LAB strains

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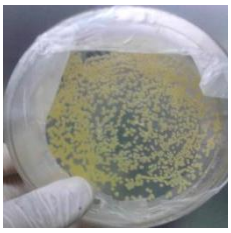
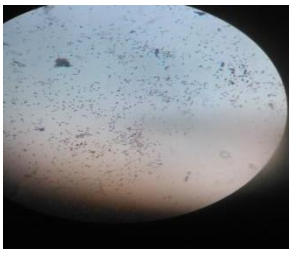
APPENDEXIES

Appendixes 1: Morphological and Macroscopic characterization of selected strains of LAB





Appendixes 2: Summary of temperature ANOVAs for LAB strains

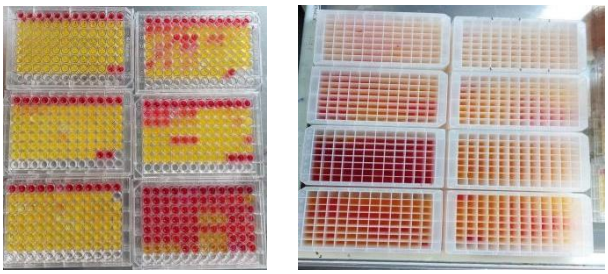
Class	**Levels**	**Values**
Strain	7	LB64 BB31 BB26 DZ11 DZ31 GB15 BB28
Temperature	4	15 25 45 55

Dependent Variable: OD

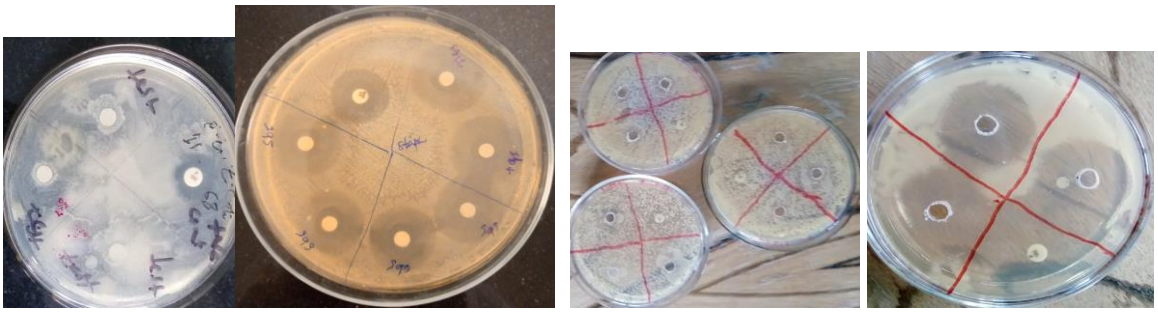
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	110	24.25399764	0.25712795	4.68	<.0001
Error	124	7.42526733	0.01751242		
Corrected	235	31.67926497			

Source	DF	ANOVA SS	Mean Square	F Value	Pr > F
Strain	6	4.97703597	0.09571223	5.47	<.0001
Temperature	3	2.17416718	10.72472239	612.41	<.0001
Strain * Temperature	18	7.10279449	0.10963330	6.26	<.0001

Appendixes 3: Simple carbohydrate fermentation by 7 LAB strains



Appedixes 4: Disc and well diffusions assay of LAB strains test



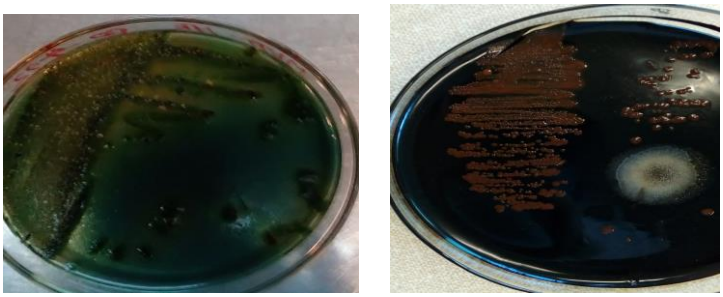
Appendix 5: The summary of Descriptive statistics of Bacteriocin productions of LAB strains

Dependet Variable: Cz diameter in (mm)

strains	Pathogene	Mean	Std. Deviation
BB15	*E.coli*	13.5000	1.32288
	L.monocy	11.3333	1.52753
	S.aures	13.9667	3.21299
	Salmonel	311.9333	518.20224
	Total	87.6833	259.06476
BB26	*E.coli*	11.5667	1.25033
	L.monocy	9.0000	0.00000
	S.aures	8.8333	.76376
	Salmonel	12.1333	1.66533
	Total	10.3833	1.81350
BB28	*E.coli*	9.4667	1.36137

	L.monocy	15.0000	.50000
	S.aures	13.1667	.76376
	Salmonel	7.8333	1.04083
	Total	11.3667	3.09173
BB31	*E.coli*	14.3333	.97511
	L.monocy	17.2000	2.22711
	S.aures	16.5133	1.18006
	Salmonel	15.0833	.41932
	Total	15.7825	1.66120
DZ11	*E.coli*	9.0333	1.05040
	L.monocy	8.9333	1.83394
	S.aures	8.8133	.54455
	Salmonel	7.7667	1.66233
	Total	8.6367	1.28465
DZ31	*E.coli*	15.5967	1.35758
	L.monocy	13.1200	.85510
	S.aures	14.8800	1.63805
	Salmonel	12.9667	2.48261
	Total	14.1408	1.86112
LB64	*E.coli*	15.3333	.57735
	L.monocy	16.9667	.55076
	S.aures	15.9333	.40415
	Salmonel	15.0833	.07638
	Total	15.8292	.84919
Total	*E.coli*	12.6900	2.74081
	L.monocy	13.0790	3.47753
	S.aures	13.1581	3.25982
	Salmonel	54.6857	196.07082
	Total	23.4032	97.98471

Appendix 6: The haemolytic activity of selected LAB strains



Appendix 7: Antimicrobial activities of probiotic LAB strains against mastitis pathogens

Strain Id	***Pathogens***			
	E.coli	***L.monocytogenes***	***S.aures***	***Salmonella***
***pentosus* (BB26)**	14.67 ± 1.528	15.73 ± 0.25	14 .00 ± 1.00	14.4 ± 0.6
***casei* (BB28)**	9.47 ± 1.361	15.00 ± 0.50	13.17 ± 0.76	7.83 ± 1.04
***plantarum* (BB31)**	16.93 ± 1.210	17.07 ± 1.01	17.13 ± 0.07	17.13 ± 1.12
***plantarum* (LB64)**	15.33 ± 0.577	16.97 ± 0.55	15.93 ± 0.4	15.08 ± 0.07
***plantarum* (DZ11)**	7.67 ± 2.082	13.73 ± 1.37	14.00 ± 1.00	10.17 ± 1.44
***plantarum* (DZ31)**	13.33 ± 1.528	16.77 ± 0.68	17.17 ± 2.02	14.33 ± 1.52
***paracasei* (GB15)**	6.93 ± 0.116	8.03 ± 1.34	8.80 ± 0.43	7.00 ± 2.64

Appendix 8: During media preparation in laboratory

Appendix 9: During antimicrobial assay of the selected strains

Appendix 10: During incubation using anaerobic culture jar.

Appendex 11: During morphological characterization