Probiotic Properties of Lactic Acid Bacteria Isolated from *Tella* Produced in Adama City and Asella Town, Oromia, Ethiopia

Msganaw Mola Zeleke

A Thesis Submitted to the Department of Applied Biology
School of Applied Natural Science

Presented in PartialFulfillment of the Requirement of the Degree of Master's in Applied Microbiology

Office of Graduate Studies
Adama Science and Technology University

May, 2024
Adama, Ethiopia

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DECLARATION

I hereby, declare that this Master Thesis "Probiotic Properties of Lactic Acid Bacteria Isolated from *Tella* Produced in Adama City and Asella Town, Oromia, Ethiopia" 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 citation.

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We the advisors of this thesis hereby, certify that we have read the revised version of this thesis "Probiotic Properties of Lactic Acid Bacteria Isolated from *Tella* Produced in Adama City and Asella Town, Oromia, Ethiopia" prepared under our guidance by Msganaw Mola submitted in partial fulfillment of the requirements for the degree of Masters in Microbiology. Therefore, we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

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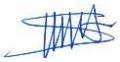
Seid Mohammed (Ph. D)		02/08/2024
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LIST OF ACRONYMS OR ABBREVIATIONS

CFCS- Cell Free Cultural Supernatant

DPPH- 2,2-diphenyl-1-picrylhydrazyl hydrate

EFSA- European Food Safety Authority

GRAS- Generally recognized as safe.

LAB-Lactic acid bacteria

MALDI-TOF- Matrix-assisted laser desorption ionization–time-of-flight

MRS-de Man Rogosa Sharpe

NaCl-Sodium chloride

QPS- Qualified Presumption of Safety

ABSTRACT

Probiotics are live microorganisms when taken in sufficient amounts, provide unlimited benefits to the host. Thus, this study aimed to evaluate the probiotic properties of LAB isolated from Tella produced in Adama City and Asella Town, Oromia, Ethiopia. The collected Tella samples were processed for isolation of LAB on suitable culture media following standard methods. Of 90 isolates, six LAB isolates were screened and identified as P. pentosaceus LABADI6, L. mesenteroides LABADI17, L. lactis LABADI31, L. paracasei LABASI49, L. plantarum LABASI54, and L. mesenteroides LABASI83 using MALDI-TOF-MS system. The isolates were tested for their probiotic properties, including acid and bile salt resistance, auto-aggregation, co-aggregation, hydrophobicity, antibacterial activity, antioxidant activities, and antibiotic susceptibility. The data was analyzed using spss version 27 and version 27 excel spreadsheet. Among LAB isolates, specifically L. lactis LABADI31 showed a higher degree of pH tolerance that ranged from 2 to 3 for 0, 6, and 24 h, with survival rates of 76, 66, 58, 77.6, 72.7, 66, 85, 79, and 77.3%, respectively. All the isolates survived well at lower bile salt concentrations (0.75, & 0.3% ox gal). Nevertheless, the four isolates (L. lactis LABADI31, L. paracasei LABASI49, L. plantarum LABASI54, and L. mesenteroides LABASI83) tolerated even the highest bile salt concentration content of 1.0% ox gall with the survival rate of ≥69.67 %. Among the isolates, L. lactis LABADI31 demonstrated the highest auto-aggregation with survival rates of 74.2, 85.26, and 93.23% at 0, 6, and 24 h, respectively. The co-aggregation values of the LAB isolates ranged from 44.9% (L. mesenteroides LABADI17 with P. aeruginosa ATCC 43895) at 0 h to 96.56% (L. lactis LABADI31 with E. coli ATCC 25923) at 24 h. The cell-free supernatant of the isolates exhibited antibacterial activity against foodborne pathogens of E. coli ATCC 25922, P. aeruginosa ATCC 27853, S. pyogenes ATCC 13311, and S. aureus ATCC 25923. Each isolate exhibited resistance, intermediate, and susceptibility levels to six antibiotics. Resistance was observed against two antibiotics tested, erythromycin and kanamycin. The six isolates were potentially useful in producing probiotic products for health applications. Finally, more investigation is required to assess their probiotics potential and possible in vivo effects.

Keywords: Antimicrobial, Antioxidant, Beverage, Fermented foods, LAB, Pathogens, Probiotic

1. INTRODUCTION

1.1. Background of the Study

Ethiopian culture boasts a wealth of tradition and historical richness characterized by a unique fusion of cultures and customs. This diversity results from Ethiopia's geographical location since it is situated at the crossroads of Africa, the Middle East, and the Horn of Africa. This positioning has given rise to a multitude of distinct traditional cultures (Birhanu *et al*., 2021). A notable facet of Ethiopian culture pertains to its traditional beverages and foods. Within Ethiopian culture, fermented foods are not only a vital part of the dietary customs, but alcoholic drinks also hold a significant place in people's lives, forming an inseparable connection with their history. The consumption of alcoholic beverages serves both nutritional and social functions, enriching the cultural and social aspects of human life (Lee *et al*., 2015).

In Ethiopia, a range of traditional fermented beverages can be found; among them, one is known as *Tella*. *Tella* is a conventional Ethiopian beverage that shares similarities with commercial beer. It is crafted from various cereal crops like barley, maize, sorghum, finger millet, and a shrub or woody small tree called *gesho* (*Rhamnus prinoides*) as a traditional substitute for hops. Like commercial beer, *Tella* is made up of malted barley and other grains (Birhanu *et al*., 2021). It is widely consumed throughout Ethiopia, with a particular concentration of production and consumption in the central and northern regions of Ethiopia (Lee *et al*., 2015).

Tella holds significant cultural importance in both rural and urban areas of Ethiopia. It is frequently enjoyed during special occasions such as holidays, wedding ceremonies, and other social gatherings (Wedajo, 2020). Among rural communities, *Tella* serves as a fundamental beverage during busy farming seasons to provide refreshment and an energy role. It's worth noting that *Tella* goes by various names among different ethnic groups in Ethiopia, including "*Tella*" in Amharic, *Farsoo* in Afaan Oromoo, and *Siwa* in Tigrigna. Tella's ingredients and process methods vary in Ethiopia (Hotessa & Robe, 2020). Within Ethiopian rural households, *Tella* holds nutritional significance due to its composition characterized by low alcohol content, important levels of LAB, and probiotic dietary fiber. The beverage is typically cloudy with

suspension and falls within an alcohol range of 2 to 4%, resulting from fermentative microbes (Tekle *et al*., 2019).

LABs are widely acknowledged as safe microorganisms and hold significance in the fermentation and preservation of food and feed, serving as inherent microflora or as starter cultures in controlled settings. The preservative impact of LAB primarily arises from the generation of organic acids, particularly lactic acid, leading to a reduction in pH levels (Yang *et al*., 2012). Due to their ability to preserve numerous food and feed formulations for extended periods without causing any harmful consequences, different LAB species have obtained the designation of being considered safe, known as "Generally Recognized as Safe" (GRAS) by the US FDA and "Qualified Presumption of Safety" (QPS) by the European Food Safety Authority (EFSA) (Ström *et al*., 2002).

LABs are sourced from diverse fermented food/beverage groups, particularly those exhibiting notable success and heightened resilience, and are harnessed as probiotics. Probiotics are living microorganisms with health-promoting characteristics for the host when consumed in adequate quantities since they contribute to the overall balance of organisms within the body (Bromley & Uchman, 2003). In recent decades, researchers have directed their attention towards probiotics due to the countless benefits they offer to human/animal health. These advantages encompass improvements in gut health, alleviation of lactose intolerance, prevention of diarrhea, relief from constipation, reduction of serum cholesterol levels, prevention of vaginitis and intestinal diseases, management of diabetes mellitus, safeguarding against hepatic infection, reducing colon inflammation, reducing the risk of bowel tumors and cancer, minimizing oxidative stress, and enhancing overall immune system function (Nyanzi *et al*., 2014).

Cell surface hydrophobicity (CSH) is a biophysical measurement of a cell’s affinity for a hydrophobic versus hydrophilic environment. Cell surface hydrophobicity of a probiotic strain is a measure of its intestinal colonization, that is, adhesion and persistence once they have entered the intestinal cavity. Cells with higher CSH prefer a hydrophobic environment while those with lower CSH will preferentially remain in an aqueous environment(Souza *et al*., 2018).

Auto aggregation is macroscopically observed as the formation of bacterial clumps that settle at the bottom of culture tubes. In auto aggregation, bacteria of the same type, e.g. in pure culture, form these clumps. This contrasts with co-aggregation, where bacteria of different strains or even different species are associated. Thus, auto aggregation can be regarded as a kind of self-recognition process. This is a widely observed phenomenon among both environmental and pathogenic species. Aggregation is associated with the surface of the bacterial cells and secreted substances, such as exopolysaccharides. These factors may play a significant role in the strength and speed of interaction between cells (Rajab *et al*., 2020). The ability to high auto-aggregation may also affect the longer duration of these strains in the digestive tract (Rajab *et al*., 2020). Rajab *et al*., (2020) suggest that auto- and co-aggregation properties may also depend on the length of bacterial cells.

Co-aggregation helps in the fast adaptation to antibiotics in aquatic systems resulting in an enhanced survival rate of bacterial cells. Although in no direct correlation with the pathogenicity of these bacterial species, this study shows yet another example of aggregation-dependent tolerance against antimicrobial resistance which could be a mechanism to evade host defenses during bacterial infection. Consistent with this, co-aggregation of oral bacteria increased resistance to phagocytosis and promoted abscess formation in a mouse subcutaneous infection model. Co-aggregation may also play a role in the formation of late-stage dental biofilms. Longer cells present bigger surface areas; therefore, their aggregation is greater than in bacteria with short cells or spherical shapes (Rajab *et al*., 2020). Some researchers have also reported that adherence and auto-aggregation of *Lactobacillus* cells are closely associated (Celebioglu & Svensson, 2018). On the other side, some scientists described, that strains with a low ability to aggregate and co-aggregation may be characterized by a high degree of adhesion, which is opposite to the generally prevailing opinion (Alp & Kuleaşan, 2020).

The existence of an excess of reactive oxygen species (ROS) such as hydroxyl radicals (OH), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2) produced during cellular metabolism in the absence of adequate antioxidants can result in vivo oxidative harm to biomolecules like proteins, lipids, and chromosomes (Li *et al*., 2012). Reactive oxygen species (ROS), comprising superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (. OH), are naturally generated as byproducts of cellular metabolism. They play essential roles in processes such as

cell signaling, apoptosis, gene expression, and ion transport (Li *et al*., 2012). Nevertheless, if an excessive quantity of these ROS molecules is produced or if cellular defenses are insufficient, biomolecules like proteins, lipids, and nucleic acids may suffer damage because of the oxidative stress process. This damage can pose various age-related degenerative conditions that encompass cancer, Alzheimer's, and Parkinson's disease (Afonso *et al*., 2007).

In general, various microorganisms participate in fermentation processes, but only a few of them determine the final product's quality. Under suitable environmental conditions, a specific microbial community plays a crucial role in shaping the quality of a particular food. Therefore, the isolation and characterization of the microorganisms engaged in the fermentation of *Tella* are employed to anticipate the end product's desired quality. Previous research has primarily concentrated on the identification and characterization of fermentative microbes with probiotic properties under laboratory conditions.

However, limited knowledge exists regarding the collective impact of microbes obtained from fermented foods/beverages for their application in health biotechnology particularly in developing countries like Ethiopia (Earnshaw, 1992). The initial screening is done to assess the cumulative probiotic properties of LAB recovered from the target fermented product. This process aids in advancing the probiotic properties of microbes to develop suitable and cost-effective technology to modernize *Tella* production. Consequently, there is a need for comprehensive information regarding the systematic isolation and characterization of LAB that are involved in *Tella* fermentation. The objective of this study is to isolate LAB from ready-to-consume *Tella* and further assess their probiotic properties.

1.2 Statement of the problem

Probiotic bacterial strains help maintain health through illness prevention, control, and treatment, which supports the promotion of a healthy diet. Probiotics play a significant role in promoting digestive health, enhancing immune function, and inhibiting pathogenic bacteria. Investigating the LAB in Tella could validate its health benefits and potentially identify strains with significant probiotic properties (Gupta *et al*., 2021; Holzapfel, 1997; Hotessa & Robe, 2020; Leroy & De Vuyst, 2004). It is possible to create functional beverages by adding

probiotics to food matrices. These probiotic microbes must, however, demonstrate tolerance to a range of stressors experienced throughout the production process. *Tella* may be the most practical substitute and easily accessible source of alcohol-tolerant LAB strains with useful qualities, especially probiotic properties in the context of developing probiotic traditional beer. The preceding investigation on *Tella* mostly dealt with characterizing its physicochemical properties, microbial profiles during fermentation, and conventional processing techniques (Tadesse *et al*., 2017; Tekle *et al*., 2019).

Characterizing the LAB from *Tella* can also inform efforts to improve the safety and quality of the beverage. Understanding the probiotic and antimicrobial activities of these LAB strains can help in developing standards and practices for safer *Tella* production. With the increasing global interest in probiotics, identifying and commercializing LAB strains from *Tella* can open new economic opportunities for local producers. It can enhance the market value of Tella and promote it as a healthy beverage. Interestingly, little research was done on the selection of LAB for use as a starter culture in industrial applications for probiotic qualities of LAB isolates. Thus, the main goal of this study was to assess the probiotic properties of LAB isolated from *Tella* produced in Adama City and Asella Town, Oromia, Ethiopia.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this study was to evaluate the probiotic properties of LAB isolated from the Ethiopian traditional fermented beverage (***Tella)*** obtained from Adama City and Asella Town, Oromia, Ethiopia.

1.3.2 Specific objectives

- ✓ To isolate and characterize LAB from *Tella* samples.
- ✓ To identify the potent and selected LAB based on protein profile.
- ✓ To determine the antimicrobial, antioxidant activities, and antibiotic resistance patterns of the LAB isolates.
- ✓ To study the probiotic properties of the LAB isolates.

1.4 Significance of the study

In the current reality, there is a growing prevalence of emerging diseases and antibiotic-resistant pathogens. This research can enhance our comprehension of the probiotic capabilities of LAB in *Tella*. The identification of strains with probiotic attributes could lead to the development of health-promoting probiotic products, integrating them into dietary practices to improve gut health. The investigation into the antibiotic resistance patterns of these LABs is crucial given the global concerns surrounding antibiotic resistance. Recognizing which strains exhibit resistance or susceptibility can guide the responsible use of antibiotics and inform strategies to combat antibiotic-resistant pathogens.

The study also offers insights into the safety of *Tella* and similar fermented beverages by assessing the antimicrobial properties of the LAB involved. This information contributes to the formulation of food safety guidelines and quality control measures. Examining the antioxidant activities of these bacteria can have implications for developing functional foods and dietary supplements with potential health benefits, particularly in managing conditions related to oxidative stress. Considering the cultural and historical significance of *Tella* in Ethiopia, the study plays a role in preserving and promoting traditional food and beverage practices by ensuring their safety, quality, and beneficial attributes. The findings may have implications for public health and wellness by providing insights into the consumption of *Tella* and related products for potential health benefits, contributing to healthier dietary choices. The study adds to broader scientific knowledge about LAB, its diverse applications, and the management of antibiotic resistance in microbial populations. In summary, this research holds significance in terms of health promotion, food safety, cultural preservation, and advancing our understanding of the intricate relationship between LAB and human health.

2. LITERATURE REVIEW

In human diets, traditional fermented foods, and beverages (TFFB) play a significant role. Vessels found in archaeological sites from Asia date back to approximately 8000 B.C., providing the oldest indication of the usage of fermented foods and beverages (McGovern *et al*., 2004). "Foods or beverages produced through controlled microbial growth with conversion of food components through enzymatic action" (Dimidi *et al*., 2019) is the current definition for fermented foods and beverages. Foods that have undergone fermentation are consumed globally. According to reports, this group comprises 5 to 40% of the food consumed by humans (Tamang *et al*., 2016).

Significantly, the health benefits of fermented foods have made them indispensable in human diets. The presence of useful bacteria and their capacity to change the chemical components of plant and animal raw materials is how fermented meals and beverages function in the human body. Food fermentation improves a food's nutritional qualities and health benefits by increasing the bioavailability of nutrients and promoting probiotic and prebiotic activities (Tamang *et al*., 2016). The bacteria must remain fictile during gastrointestinal transit to reach the site of action and provide this effect. For microorganisms to carry out their many tasks, such as fermentation and altering the gut microbiota, they must enter the intestinal tract alive.

In several developing countries, food processing uses fermentation as its primary biotechnology application because it is a low-cost technology. Africa produces a vast array of fermented dishes and drinks from foods like milk, grains, fruits, and starchy root crops (Franz *et al*., 2014; Gadaga *et al*., 1999; Simatende *et al*., 2015). Fermentation has several advantages, such as extending food shelf life, reducing harmful and antinutritional elements, and improving the sensory qualities and nutritional value of the food. Ethiopia is a nation rich in diverse cultures. This diversity is reflected in the range of food and beverages that the different ethnic groups prepare and consume. Among the countries where a vast array of traditional fermented drinks is made and enjoyed is Ethiopia. The several types of traditional fermented drinks are often made locally and on a modest scale for consumption. Varieties of *Tella*, *Tej*, *Brode*, *Areki*, *Keribo*, and *Korefe* are among the fermented drinks enjoyed in Ethiopia (Tafere, 2015).

Traditional fermented food and beverage products are a staple of both developed and emerging societies' meals. As a result, microbes are used in the preparation of the animal and plant components used to make these food products, altering the materials' physical and nutritional attributes. Yeast and LAB are abundant in raw vegetative and animal materials from Africa. According to Christoph *et al*., (2017), the predominance of certain yeast species appears to be primarily product-specific. One of the biggest and most conventional methods for making and storing food is the fermentation of row plant and animal elements. For this purpose, fermented foods are given as weaning foods during festivities like marriage, naming, and rain-making ceremonies. Moreover, fermentation offers a natural means of lowering the volume of material to be carried, eliminating unwanted components, enhancing the food's nutritional content and appearance, lowering the cooking energy needed, and producing a less dangerous item (Simango, 1997). Various techniques, fresh ingredients, and microbes are used worldwide in the production of fermented food and beverage products. Accordingly, there are only four primary types of fermentation: alkali, lactic, acetic, and alcoholic (Sathe & Mandal, 2016).

In developing nations such as Ethiopia, the benefits associated with fermented products are particularly salient during the production process. Reduced raw material waste, shortened cooking times, improved protein and carbohydrate digestibility, increased micronutrient bioavailability, and elimination of harmful and anti-nutritional elements were the outcomes of these effects (Sanni, 1993). Furthermore, fermented foods and beverage products have probiotic effects (resistant to low pH and antibacterial activity) and a low rate of pathogenic bacteria, which are particularly significant in developing nations where fermented foods have been shown to lessen the severity of diarrhea (Kimmons *et al*., 1999) Therefore, deeper comprehension of the gut microbiota will aid in the creation of fresh approaches for the prevention and/or management of various illnesses (de Almada *et al*., 2015).

2.1 Preparation of *Tella*

Tella, a customary fermented beverage in Ethiopia, bears considerable cultural and traditional significance in the nation. It is created by blending malted barley with *Kita*, a thick flatbread crafted from the flour of sorghum, ***dagusa,*** corn, and other grains, baked on a hot metallic griddle. This beverage is highly valued for its contributions to enjoyment, medicinal purposes,

and its connection to extraordinary events such as holidays and weddings. The production of *Tella* is a time-consuming process that requires careful mindfulness. The traditional Ethiopian *Tella* preparation-specific steps (Figure 1).

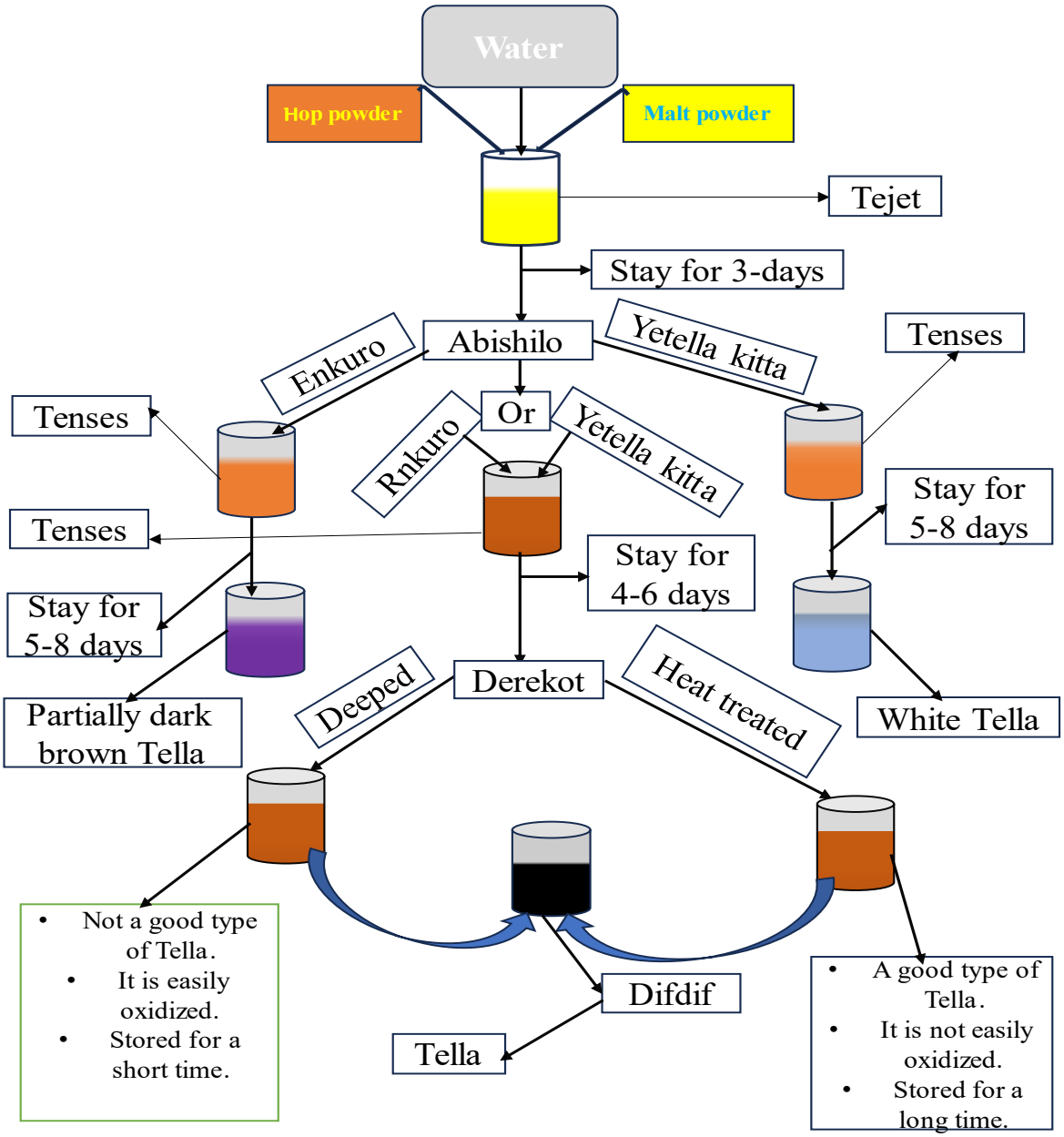

Figure 1: *Tella* processing flow chart(Talema & Nega, 2022).

2.1.1 Nutritional values and safety of Tella

Fermentation processes enhance the nutritional quality of raw ingredients by improving the digestibility of nutrients and inactivating anti-nutritional factors(Chelule *et al*., 2010). They also improve the acceptability of the food by destroying undesirable flavors of the raw ingredients (Meilgaard *et al*., 1991). Lowering the pH of food products through fermentation is a form of food preservation. This is a self-limiting process in that further reduction of pH may be unfavorable to the producing organisms. Therefore, fermentation technology is of great importance in ensuring food safety, preservation, and food flavoring (Nout, 1991).

Traditional fermentation is a form of food processing, where microbes, for example, LAB are utilized. The bacteria use food as a substrate for their propagation. This is a form of food preservation technology, used from ancient times. Over the years, it has become part of the cultural and traditional norm among the indigenous communities in most developing countries, especially in Africa. The rural folk have come to prefer fermented over unfermented foods because of their pleasant taste, texture, and color. This popularity has made fermented foods one of the main dietary components of the developing world (Mosha & Vicent, 2004).

2. 2 Lactic acid bacteria

LAB encompasses a varied category of Gram-positive bacteria, existing as either rods or cocci, without spore formation, and exhibiting a negative catalase reaction. These bacteria possess similar traits in terms of metabolism and physiology. Lacking cytochromes, they commonly thrive in non-aerobic environments but display aerotolerance. These bacteria are meticulous, tolerant to acidity, and exclusively engage in fermentation. They are present in diverse fermented beverages and foods, including sorghum beer, milk, sourdough bread, cassava, and pickled (fermented) vegetables (Lelise *et al*., 2014).

LABs can maintain their internal pH, enabling them to thrive in acidic surroundings. Their growth persists until they deplete all essential nutrients and resources for survival, or until the accumulation of toxic or growth-inhibiting substances, like hydrogen peroxide, reaches a threshold. Once the concentration of hydrogen ions becomes too low for the bacteria to tolerate,

growth ceases. As an example, *L. lactis* sub-species and *S. thermophilus* exhibit growth in milk until the p^H level reaches 4.5, irrespective of nutrient availability (Khalid, 2011).

Due to their incapacity to synthesize specific compounds, LAB thrives in environments abundant in amino acids, vitamins, purines, and pyrimidines. In laboratory conditions, these bacteria are commonly cultivated in media containing tryptone, yeast extract, and lactose, although lactose-free strains may be grown in glucose-containing media. While some LABs function as free-living organisms, others establish associations with vertebrate animals and may even pose potential health risks, as seen with *S. pneumoniae* and *S. pyogenes* in humans and animals. Although most LAB strains cannot synthesize vitamins, some can produce certain water-soluble vitamins, including vitamin B (such as folates, riboflavin, and vitamin B12). For example, *S. thermophilus* and *L. delbrueckii* sub-species *lactis* can generate folates, while *L. reuteri* can biosynthesize cobalamin (Zukiewicz *et al*., 2014).

Due to their adaptable metabolic characteristics and resilience to environmental challenges, LAB finds application in the industrial synthesis of lactic acid, vitamins, and food constituents. LAB serves as a starter culture in fermented food production, with certain strains classified as probiotics. The taste, texture, and nutritional content of fermented foods are molded by LAB through their engagement in glycolysis, lipolysis, and proteolysis metabolic processes (Wu *et al*., 2017). Various species within the LAB genera are employed in the food industry as starter cultures, notably *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, and *Leuconostoc*. In the food industry, starter cultures serve not only for lactic acid production but also contribute to the generation of advantageous metabolites influencing the flavor and attributes of food products. These metabolites include organic acids, alcohols, aldehydes, esters, sulfur compounds, polyols, exopolysaccharides, and antimicrobial agents (Bintsis, 2018).

2.2.1 Methods of Isolation, Identification, and Characterization of LAB

2.2.1.1 Isolation of LAB

LAB can be isolated from myriad sources viz., traditional or homemade pickles, kimchi, dry-cured meats; silage of plants; milk, milk products, cheese; fermented beverages, jams, fishes; fruits like cupuaçu, strawberry, cherry tomato, blueberry, blackberry, cherry, apple, and flowers;

intestinal tracts of honey bee, Mediterranean trout, wild boar; seed meal; ferments of cocoa beans, cricket powder, cassava, cereal foods, and sourdoughs, etc. Both novel and traditional plant/animal materials can serve as promising sources for the isolation of LAB. The de Mann Rogosa Sharpe (MRS) agar medium supplemented with 1% CaCO3 is commonly and widely used as a defined growth medium developed for culturing of LAB, as it can provide sustenance and support the growth of almost all LAB genera viz., *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Weissella*, when it's made selective through reduction of the pH to 5.7 and addition of sorbic acid (0.14%). It is chemically comprised of ammonium citrate, beef extract, di-potassium hydrogen orthophosphate, glucose, magnesium sulfate, manganese (II) sulfate, sodium acetate, sorbitan monooleate, casein tryptic digest, yeast extract, and agar added with distilled/deionized water. MRS agar can give a perfect colony count and determine the characteristic size and morphologies of LAB strain colonies, whilst the other microbes can be excluded through specific colonial appearance and confirmation tests (Monika *et al*., 2017).

2.2.1.2 Identification, and Characterization of LAB

Identification of isolated LAB can be conducted through polyphasic approaches, taking into consideration dependent factors like the sample source, the number of isolated strains, and genotypic, phenotypic, and biochemical features of strains. Regular protocols followed for clinical isolate identification use phenotypic tests, while food isolates and vegetable/fruit isolates demand molecular approaches and molecular typing methods respectively. Universal and well-established LAB identification by genotypic methods is 16S rRNA gene sequencing where conserved genes represent adequate variability serving as phylogenetic markers for genus and species level of identification. The sequenced nucleotides are analyzed for similarities and aligned for the construction of phylogenetic trees using programs like BLAST of NCBI, (Di Cagno *et al*., 2013; Emerenini, 2013; Moraes *et al*., 2013). Biochemical and physiological characterization of LAB isolates can be done by testing for cell/colony morphology and characteristics, Gram staining affinity, catalase, and oxidase production (Menconi *et al*., 2014), carbohydrate fermentation, tolerance to NaCl contents (1.5–10%), their growth at variable

ranges of pH (3.0–8.5) and temperature (15–45°C), arginine hydrolysis, utilization of citrate (Monika *et al*., 2017), and lactic acid production(Ahire *et al*., 2021).

2.2.2 Roles of LAB in food preservation

The primary benefits of LAB in traditional fermented foods involve their role in enhancing flavor and conducting safe metabolic activities. LAB utilizes the available sugars within foods to produce organic acids and other metabolites while proliferating in the fermentation process (Nigatu, 1998). To prevent the proliferation of unwanted microorganisms, starter culture bacteria generate lactic acid in traditional fermented products like yogurt (Daeschel, 1993).
In recent times, the prevalence of foodborne illnesses has risen, attributed to expanded manufacturing facilities and the adoption of process control methods like Hazard Analysis and Critical Control Points (HACCP) in the food industry, leading to the damage of chemical preservative microbes. LAB serve as natural preservatives, drawing increased human interest in preserving food through LAB due to their safe association with fermented foods. LAB produces a variety of metabolic products, with antimicrobial activities being a common feature, including organic acids, fatty acids, hydrogen peroxide, and diacetyl. Notably, the global community has shown interest in LAB's ability to produce specific proteinaceous substances, bacteriocins, which inhibit the growth of pathogens such as *Bacillus spp*., *Clostridium*, *Enterococcus spp*., *Listeria*, and *Staphylococcus* thereby extending the shelf life of food (Soomro *et al*., 2002).

2.2.3 Lactic acid bacteria as probiotic

Probiotic strains belonging to the genera *Lactobacillus* and *Bifidobacterium* are known to encompass various species and have been utilized in foods, beverages, and therapeutic formulations. These probiotic strains are selected due to their Generally Recognized as Safe (GRAS) status. *Lactobacillus* strains such as *L. acidophilus*, *L. casei*, *L. gasseri*, *L. reuteri*, and *L. rhamnosus*, play a significant role as probiotics (Nyanzi & Jooste, 2012).

Probiotics are living, reduced-scale microorganisms that when taken in sufficient amounts into the gastrointestinal tract, boost the host's health. Probiotics are defined as microorganisms that, when taken as directed, are known to create favorable conditions for healing. In addition to the more peripheral genera of *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*,

Porolactobacillus, Tetragenococcus, Vagococcus, and *Weissella*, the core genera of the LAB include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (FAO/WHO, 2002).

2.2.3.1 Nanoencapsulation and Health Benefits of Probiotics

The largest portion of the food industry's potential for nanotechnology is centered on nanoencapsulation (Sekhon, 2010). With nanotechnology, the quantity of active ingredients can be decreased. Probiotic bacteria can be prepared for distribution at a particular location within the gastrointestinal tract via nanoencapsulation (Altenhoefer *et al*., 2004). Longer shelf lives and actual usability of the product are achieved by components in the encapsulated form (Meijerink *et al*., 2010). To produce customized probiotic bacterial arrangements that could be transported to regions of the gastrointestinal tract where they interact with receptors, nanoencapsulation is preferred. The stability of oil-in-water emulsions can be increased by a starch-like nanoparticle preventing lipids from oxidizing (Vidhyalakshmi *et al*., 2009).

Gainful probiotic microorganisms are better encapsulated in nanoparticle form because they can target a specific area of the gastrointestinal system and support the healthy function of probiotic bacteria (Yang *et al*., 2014). Additionally, to strengthen immunity, these nano-encapsulated designer bacterial arrangements might be used as a component of vaccination planning(Chikindas, 2014). Probiotics have numerous health advantages (Figure 2) for people, pets, and plants. While some of these benefits have been substantially realized and developed, others have shown promise, necessitating human testing to support their claims (Le`ne *et al*., 1997). The benefits of probiotic microscopic organisms are remarkably strain-specific, meaning that no single strain may provide all suggested benefits, and not all strains of a given species are effective against defined health issues (Le`ne *et al*., 1997). When probiotic food products reach the end of their shelf life, they should contain up to 10^7 CFU/g (Kobayashi *et al*., 2011).

Currently, human usage of a few highly characterized strains of *Bifidobacteria* and *Lactobacilli* is possible to reduce the risk of GI disorders. Probiotic LAB must overcome several growth inhibitors to ensure that they reach the necessary population sizes in the target location to produce their effects. The human gastrointestinal system comprises three major areas: the stomach, which is mostly home too high-impact gram-positive microbes ($<10^3$ CFU/g) (Mills

et al., 2011). The genera *Bacteroides, Bifidobacterium, Lactobacillus,* and *Streptococcus* (10^3–10^4 CFU/g) are found in the tiny digestive tract. Additionally, the genera *Bacteroides, Bifidobacterium, Eubacterium, Fusobacterium,* and *Lactobacillus* are well represented in the big intestine (10^{11}–10^{12} CFU/g).

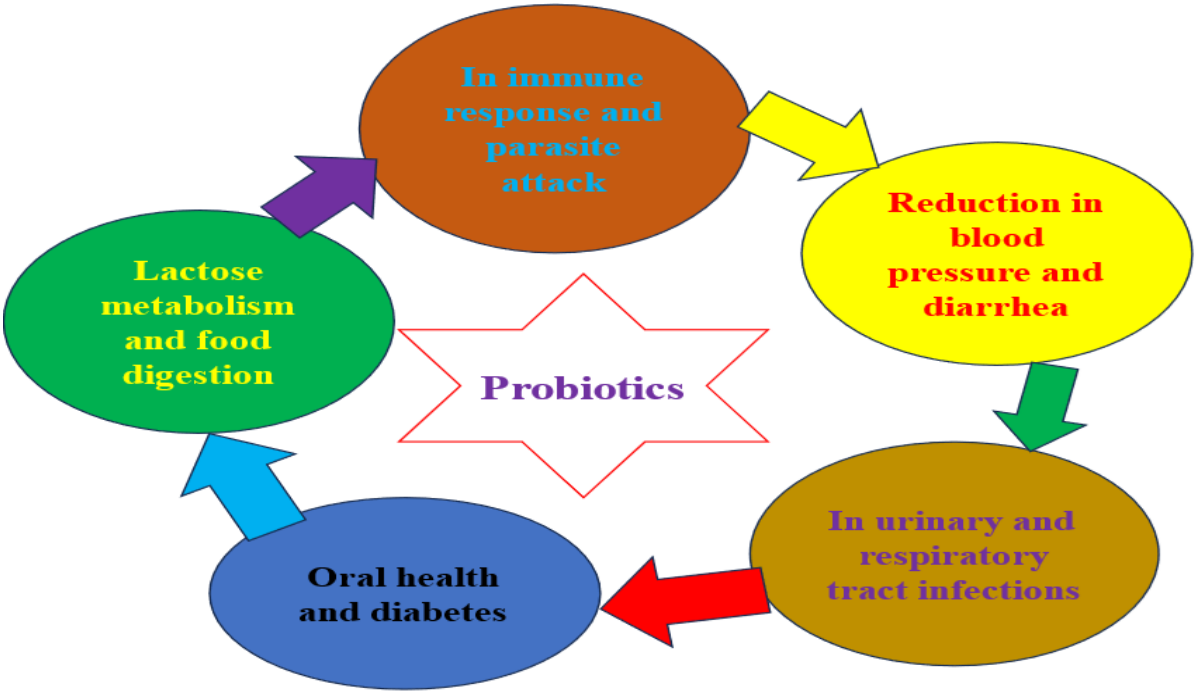

Figure 2: Probiotics health beneficial application(Kumar *et al*., 2018).

2.2.4 Factor affecting the growth of LAB

LABs are subjected to different environmental stresses such as temperature, pH changes, and osmotic and oxidative stresses. Metabolism and many cell functions can be affected and, finally, cell survival may be compromised. The impact of the main stressors on LAB was characterized. A deep understanding of the influence of selected physicochemical stressors on LAB can be complicated by the coexistence of various stressors.

2.2.4.1 Effect of Temperature

The optimal growth temperature for most LAB ranges from 30-45°C(Terpou *et al*., 2019). Some thermophilic strains show viability at temperatures up to 60°C. The homofermentative *L. acidophilus* and *S. thermophilus* can grow at temperatures as high as 45°C with optimum growth

from 40-45℃(Oberg *et al*., 2022). When the temperature reduces beyond the optimal range for exponential colony growth, the bacterial cells are subjected to thermal stress. Even small temperature differences can have a significant impact on the LAB viability. In the study of Neffe-Skocińska *et al*., (2011) the *L. casei* LOCK900 strain added to loins has a better survival rate during the 21-day ripening of fermented pork at 20℃ (from 10^7 to 10^8 cfu/g) compared to samples aged at 16℃ (from 10^4 to 10^6 cfu/g). Low temperatures above 0℃ used in the fermentation, ripening, and storage of some products are generally not harmful to LAB. However, cellular metabolism decreases, and enzyme activity and protein synthesis drop, which in turn can inhibit LAB cell growth. LAB can adapt to decreased temperature and with a reduced rate can grow at a temperature below 20℃ and even at 15℃, but not at 7℃(De Angelis & Gobbetti, 2004).

2.2.4.2 Effect of pH

In the biochemical transformations carried out by the LAB, the main end product of sugar fermentation is lactic acid, which lowers the pH of the food matrix. Thus, LABs that are acid-tolerant produce a bacteriostatic and even bactericidal environment harmful to many competitive microorganisms, including putrefactive and pathogenic bacteria. The LAB cell membrane like that of other acidophiles is highly impermeable to proton influx into the cytoplasm(Baker-Austin & Dopson, 2007). Unfortunately, prolonged exposure to acidic conditions or if the pH is too low can compromise the lifespan of LAB (Papadimitriou *et al*., 2016). For the growth of LAB during food production and storage, a neutral or slightly acidic pH is optimal (5.5–6.0). However, *Lactobacillus* strains have good tolerance to acidic environments and grow in the pH range of 3.7 and 4.3, while Bifidobacterium spp. do not tolerate a drop in pH below 4.0 (Tripathi & Giri, 2014). At a low pH, undissociated lactic acid and acetic acid passively diffuse across the membrane into the cell, leading to a decrease in intracellular pH (pHi) (De Angelis & Gobbetti, 2004). Intracellular acidification significantly reduces enzyme activity and causes permanent damage to the LAB proteins. Foods with high acidity (pH < 4.6), such as fruit juices and yogurts, are challenging for LAB survival, but at the same time, due to the inactivation of undesirable microorganisms in an acidic environment, they

may require milder processing methods than products with low acidity (pH > 4.6)(X. Gao et al., 2022).

2.2.4.3 Effect of Oxygen

LABs are anaerobic or relatively anaerobic bacteria, lacking catalase and superoxide dismutase activity, and susceptible to adverse effects and oxygen toxicity. The exposure to oxidative stress for LAB is harmful and their range depends on the species and strain. It can lead to irreversible damage to the bacterial cell. Generally, LAB tends to be more tolerant to oxygen than bifidobacteria (Gao *et al*., 2021). Several LAB strains can metabolize oxygen in reactions of flavin oxidases(Zotta *et al*., 2016). Oxygen exerts a toxic effect on anaerobic LAB, acting toxically through peroxides and free radicals called reactive oxygen species (ROS) containing at least one oxygen atom and one or more unpaired electrons. This group of oxygen byproducts includes oxygen free radicals, e.g., superoxide anion radical, hydroxyl radical, hydroperoxyl radical, singlet oxygen, reactive nitrogen species (RNS), and reactive sulfur species. As a result of the biochemical oxidation reaction, ROS can damage nucleic acid, leading to its fragmentation, cellular lipids within the plasma membrane, and proteins with the reduction of ATP and depletion of energy resources(Feng & Wang, 2020).

2.2.4.4 Effect of Osmotic Stress and Water Activity

As a result of drying or the addition of water-binding solutes, the amount of free water in the food is significantly reduced. The bacterial cell wall is responsible for the shape of the cell and plays a key role in maintaining osmotic pressure. Both too high (hypoosmotic stress) and low water content (hyperosmotic stress) in food products is not favorable to the growth rate of LAB. The relationship between water content and water activity (a_w) is non-linear and depends on the interaction of food components with water. The lower limit of aw for bacterial growth is about 0.91(Brauer *et al*., 2023). The effective ion concentration in cytoplasm is defined as the ionic strength. With the activity of specific ion channels and solute transporters, it maintains the cell volume. Another factor is macromolecule crowding. Bacterial proteins along with ribosomal RNA are space-consuming and therefore reduce cell volume(Liu *et al*., 2019). When cells are under environmental osmotic stress osmoregulatory transporters are activated and ionic

concentration changes as well as ionic strength. Dehydration and the associated osmotic stress led to a decrease in the bacterial cell volume, an increase in ionic strength and crowding, and a decrease in cell turgor(Poolman, 2023). The effect of induced osmotic stress may be a decrease in growth rate, a decrease in metabolic activity, and finally increased cell mortality during irrigation(Fonseca *et al*., 2019). In the study of Teixeira et al. a non-linear relationship was found in the viability of spray-dried strains of *L. delbrueckii* ssp. *bulgaricus* with water activity, showing the highest survival rates at medium and low aw values in the range of 0.11 to 0.23(Teixeira *et al*., 1995).

2.3 Fermentation with LAB

Microbial fermentation has played a crucial role in food and beverage processing for millennia. Through this process, food products are not only safeguarded but also show improvements in both quality and safety, while simultaneously reducing the energy needed for cooking. The transformative effects of microbial fermentation led to the development of novel aromas, flavors, tastes, and textures, enhancing the overall deliciousness by extending the shelf life of fermented products. These alterations are achieved through the utilization of various metabolites produced by diverse microorganisms such as LAB (Esayas, 2012). In the fermentation process of dairy products, LAB also play a vital role. Various fermentative LAB genera such as *Enterococcus, Lactobacillus, Leuconostoc*, *Pediococcus*, and *Streptococcus* contribute to this process. Generally, LAB imparts both preservative and detoxifying effects on fermented food (Beresford et al., 2001).

LAB is extensively employed as a starter culture in the industrial processing of fermented dairy, meat, vegetable, and cereal products. These bacteria play a crucial role in conferring primary preservation actions to fermented foods by lowering pH value and converting sugars into organic acids. Nevertheless, in the absence of starter cultures, distinct types of foods can still undergo fermentation through the activity of Indigenous LAB by imparting distinct properties to the products (Lelise *et al*., 2014). The conversion of carbohydrates (sugar) into lactic acid, the primary end-product of sugar fermentation by LAB leads to a pH reduction, which in turn assists bio-preservation of beverages and foods. The fermentation process carried out by LAB

in drinks and food generates diverse antimicrobial agents, including bacteriocin, organic acid, hydrogen peroxide, and diacetyl (Hemashenpagam, 2011; Jeevaratnam, 2005).

The primary focus of fermentation involves flora and fauna-based items, encompassing dairy products (such as yogurt and cheese), meat, fish, fruits, vegetables, and cereal products, all of which undergo fermentation facilitated by LAB (Adebo & Meza, 2020). Currently, a growing number of consumers prefer LAB fermentation for food preservation as it eliminates the need for chemical additives. The enzymes generated through LAB fermentation, including amylases, proteases, and lipases, play a crucial role in converting polysaccharides, proteins, and lipids into compounds that enhance flavors, aromas, and textures, thereby contributing to desirable product characteristics. LAB offers a significant advantage in enhancing the organoleptic properties, nutrient profile, and health benefits of food products (Gemechu, 2015).

LAB exhibit specific preferences for temperature, salinity, and p^H levels during their growth. They thrive within a temperature range of 10 to 45℃, tolerate a salinity of 6.5% NaCl, and prefer a p^H range between 4 and 9.6. The fermentation and biomass production processes facilitated by LAB occur rapidly within the temperature range of 26-42℃, with optimal bacterial growth observed between 35-37℃. LAB can be categorized into homofermentative (e.g., *Lactococcus*, *Streptococcus*) and heterofermentative (e.g., *Leuconostoc*, *Weissella*) based on their sugar fermentation capabilities. Homofermentative LAB utilizes the Embden-Meyerhof (EM) glucose degradation pathway to produce two lactate molecules from one glucose molecule, while heterofermentative LAB utilizes the 4-Phosphorylative pathway (4PP pathway) to generate lactate, ethanol, and carbon dioxide from a single glucose molecule (Gänzle, 2015).

2.4 The importance of LAB for properties of cereal-based beverages

2.4.1 Lipid transformation

LAB metabolic activity extends beyond the mere breakdown of carbohydrate compounds (Treimo *et al*., 2006). LAB contains an internal set of hydrolytic enzymes, specifically lipases and esterase, used for transforming lipids and fatty acids into tri acetyl glycerides (TAGs), released during the formation of various dairy products, like rennet-ripened cheese. These

enzymes, present within the cell, require an extended maturation period and eventual bacterial cell lysis to exhibit lipolytic activity. While this is observed in ripened cheese production, it is not typically seen in fermented beverages. Many *Lactobacillus* strains, particularly heterofermentative strains like *L. brevis*, have the potential to display lipolytic activity. If observed, the esters comprised of short and medium-chain fatty acids are typically limited (Pulido *et al*., 2007). The metabolic activity of these bacteria resulted in an almost threefold increase in the content of the fatty acid isomer 18:2 cis-9, trans-11 in the tested products. This metabolic activity observed in cereal and pseudo-cereal-based beverages can provide insights into the product (Akalin *et al*., 200).

2.4.2 Contents of vitamins

Several LAB strains cannot produce certain vitamins and rely on external sources for their nutritional requirements. However, some LAB strains exhibit the capacity to synthesize specific water-soluble vitamins, including various B vitamins like folic acid, vitamin B2, and vitamin B12. For instance, a strain of lactic acid bacteria isolated from sourdough, identified as *L. reuteri* CRL1098, has been recognized for its ability to produce cobalamin, a form of vitamin B12. Modern advancements, such as the genetic modification of LAB, have enabled the enhancement of vitamin production. An exemplary case is the genetically modified *L. lactis* sub-species *cremoris* NZ9000, which can produce vitamins. In the future, utilizing strains like *L. reuteri* CRL1098 as a starter and genetically modified strains like *L. lactis* sub-species *cremoris* NZ9000 could potentially increase the vitamin content in fermented plant-based beverages. Cereals and pseudo cereals naturally contain various nutrients, including most B vitamins (excluding B12). Genetic modification becomes particularly beneficial as the production process of beverages from plant-based sources may lead to the loss of bioactive substances. The impact of lactic acid fermentation on the vitamin B content of cereal-based beverages is subject to several factors, including the LAB strain's capability for vitamin B biosynthesis, incubation conditions, and the processing parameters applied to the plant-based materials. It is important to note that these findings apply to plant matrices beyond cereals or pseudo cereals (Champagne *et al*., 2011).

2.4.3 Enzymatic degradation of phytates

In cereal-based beverages, phytate is an enzyme that forms complexes with multivalent cations such as iron, zinc, calcium, and magnesium. Lactic acid fermentation can break down these complexes, leading to an increase in both the content and bioavailability of minerals through these enzymatic processes (Mohammadi *et al*., 2019). In certain flour-based and experimental cereal-based beverages, the enzymatic process has been illustrated. Microbial phytase, under the favorable conditions of low pH and fermentation temperature during lactic acid fermentation, can effectively break down phytic acid salts. This enzymatic activity contributes not only to the increased bioavailability of minerals but also enhances the digestibility of proteins and carbohydrates (Gotcheva *et al*., 2000).

2.4.4 β-glucosidase activity of LAB

LAB exhibits various activities, and one well-known function is the β-glucosidase activity, associated with certain carbohydrates and possessing antioxidant properties. These enzymes are utilized to hydrolyze a diverse range of substrates, including phenols, polyphenols, and flavonoids. They act by removing glucopyranosyl residues from the non-reducing end of β-D-glucosides through the hydrolysis of the glycosidic bond. Fermentation with LAB in plant materials has been demonstrated to elevate the concentrations of phytoestrogens, bioactive isoflavones, and phenolic compounds. This enhancement contributes to the nutritional qualities of fermented plant-based foods and cereal beverages (Michlmayr and Kneifel, 2014). The β-glucosidase activity plays a crucial role in unlocking appealing flavor compounds from glycosylated precursors in fermented products and improving the bioavailability of health-promoting plant metabolites. Most of the research in this domain has centered on fermented soybean products and fermented vegetables (Chien *et al*., 2006).

2.4.5 The digestibility of proteins

The lactic acid fermentation of cereal-based beverages can enhance protein digestibility by altering protein and amino acid levels, along with the degradation of phytates and polyphenols (Coda *et al*., 2012). The release of free amino acids is facilitated by the peptidase system of starter LAB, alongside the activation of endogenous proteinases in cereal flour due to

acidification. Studies have demonstrated that lactic acid fermentation can increase the availability of lysine, methionine, and tryptophan in cereals such as maize, millet, and sorghum. Nevertheless, these amino acid levels are influenced by the process parameters of lactic acid fermentation. While there are similarities and relationships observed in various fermented cereal-based beverages, this hypothesis lacks current support in the literature (Thiele *et al*., 2002).

2.5 Antimicrobial substances produced by Lactic acid bacteria

The inhibitions of foodborne pathogenic and spoilage microorganisms are significantly contributed by the antimicrobial metabolites produced by LAB. These metabolites encompass lactic acid, acetic acid, various organic acids, hydrogen peroxide, bacteriocins, and bacteriocin-like substances (Çadirci and Çitak, 2005).

2.5.1 Organic acids

The fermentation by LAB is marked by the buildup of organic acids and the consequent decrease in pH. The specific types and levels of organic acids produced during the fermentation process are influenced by the species of organisms, cultural composition, and growth conditions (Lindgren and Dobrogosz, 1990). Lactic acid demonstrates potent antagonistic activity against a variety of pathogenic microorganisms, including food spoilage organisms and other pathogens (Ten Brink *et al*., 1994).

Lactic acid serves as the primary metabolite in LAB fermentation, existing in equilibrium with its undissociated and dissociated forms, with the balance influenced by pH. At lower pH levels, a substantial portion of lactic acid remains undissociated, exhibiting toxicity to a range of bacteria, fungi, and yeasts. Nevertheless, the impact of lactic acid varies depending on the sensitivity of different microorganisms. While spore-forming bacteria are inhibited by lactic acid at a p^H of 5.0, this p^H level is ineffective against yeasts and molds (Woolford, 1975). LAB strains generate acetic and propionic acids via hetero fermentation, and these acids interact with cell membranes, leading to intracellular acidification and protein denaturation (Huang *et al*., 1986). Due to their elevated p^{Ka} values (lactic acid 3.08, acetic acid (4.75), and propionic acids (4.87), they exhibit greater antimicrobial effectiveness than lactic acid and, at a specific p^H, possess a higher percentage of undissociated acids compared to lactic acid (Earnshaw, 1992).

2.5.2 Hydrogen peroxide and carbon dioxide

As a result of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH), LAB generates hydrogen peroxide in the presence of oxygen. The antimicrobial impact of H_2O_2 arises from the denaturation of various enzymes and the peroxidation of membrane lipids. This occurs through the oxidation of sulfhydryl groups, leading to increased membrane permeability (Kong and Davison, 1980). H_2O_2 can serve as a precursor for the generation of bactericidal free radicals, including superoxide (O_2^-) and hydroxyl (OH^-) radicals. These radicals have the potential to cause damage to DNA (Byczkowski and Gessner, 1988). The antimicrobial mechanism of action of heterofermentative LAB, which produces carbon dioxide (CO_2), is not yet fully understood. CO_2 inhibits enzymatic decarboxylation, and its accumulation in the membrane lipid bilayer leads to membrane permeability dysfunction, creating an anaerobic environment, thereby playing a significant role(Eklund, 1984).

2.5.3 Bacteriocins and bacteriocin like substances

Bacteriocins, which are bacterial peptides, do not consistently inhibit or kill microorganisms, and their activity is typically limited to closely related strains of the producer bacterium. The antimicrobial activity of bacteriocins produced by LAB is under extensive investigation due to their effectiveness against foodborne bacteria. The competition between bacteriocin-producing LAB and other organisms in the intestine holds significant importance. Bacteriocins attach to specific cell receptors, featuring a biologically active protein moiety, and exhibit a bactericidal mechanism of action. Bacteriocins vary in molecular weight, biochemical properties, range of sensitive hosts, and mode of action, making them a diverse group of bacterial antagonists (Cladera *et al*., 2004).

According to Klaenhammer, (1993), the bactericidal activity of bacteriocins is specifically associated with the producer bacterium, and these substances can be defined as proteins or protein complexes. Both Gram-positive and Gram-negative bacteria produce bacteriocins. In the case of Gram-negative strains, *E. coli* produces bacteriocins known as colicins. (Braun *et al*., 1994). Bacteriocins produced by most Gram-positive bacteria enhance the permeability of their cytoplasmic membrane through their active membrane components(Jack *et al*., 1995). The bacteriocins produced by Gram-positive bacteria exhibit a much broader spectrum of bactericidal activity than those produced by Gram-negative bacteria. Naturally occurring inhibitors of pathogenic and food spoilage bacteria, such as LAB bacteriocins in various foods, are considered safe and effective. Nisin, a bacteriocin produced by LAB, offers several

advantages, including preventing *Clostridial* spoilage in processed and natural cheeses, inhibiting the growth of certain psychotropic bacteria in cottage cheeses, extending the shelf life of milk in warmer climates, preventing spoilage *lactobacilli* growth in beer and wine fermentations, and providing additional protection against *Bacillus* and *Clostridial* spores in canned foods. Nisin is a food additive under the trade name Nisaplin in over fifty countries, including the USA and Europe(Soomro *et al*., 2002).

2. 5.3.1 Classification of Bacteriocin

Over the years there have been various classification methods for sorting bacteriocins, while the bacteriocins for LAB have been classified alone in a different method (de Freire *et al*., 2015), resulting in two to four subcategories. Soltani and his colleagues suggest renewal bacteriocins classification for both Gram-negative and Gram-positive bacteria which are sorted into two huge parts, the Class I (modified) and Class II(unmodified) bacteriocins(Soltani *et al*., 2021).

Another study mentioned that Bacteriocins are now classified into major groups depending on their physicochemical and structural properties including Bacteriocins produced by Gram-Positive bacteria which are subdivided into the Class I of Bacteriocins (Lantibiotics)(Zacharof & Lovitt, 2012). Lantibiotics are tiny peptides heat stabile with molecular weight less than 5 kDa subject to modification after translation, as well as contain amino acids with polycyclic thioether like methyllanthionine and lanthionine, also contain unsaturated amino acids like like2-amino isobutyric acid, and dehydroalanine. According to the variety in charge, lantibiotics are also divided into two types. Lantibiotics Type A, like lacticin 3147 and nisin which are screw-shaped flexible molecules possess a positive charge and 2–4 kDa that cause pores in the target organism's cell membrane, leading to cytoplasmic membrane depolarization(Kaur & Kaur, 2015). Lantibiotics Type B are peptides with a molecular weight of 2–3 kDa without net charge or with a negative charge, these molecules are globular and affect cellular enzymatic activities, like the formation of cell walls. This category includes mersacidin, which is produced by *Bacillus* spp. (Sahl & Bierbaum, 1998).

Bacteriocins class II are tiny peptides with molecular weight less than 10 kDa without lanthionine and are heat stable as well this type is unmodified after translation, they also possess an amphiphilic structure with a helical shape that permits them to infix inside the membrane which in turn leads to depolarization as well as death to the target cell. Bacteriocins class III are

proteins that possess a high molecular mass of more than 30 kDa and heat-labile. several of the megacins secreted by *B. megaterium*, colicins, klebicin secreted by *K. pneumonia*, enterolysin secreted by *E. faecalis*, and Helvetica I secreted by *L. helveticus* consider members belong to this class(Kaur & Kaur, 2015), the summary of the classification of bacteriocin with several examples (Güllüce *et al*., 2013).

3. MATERIALS AND METHODS

3.1 Description of the study area

The study was conducted in Adama and Asella. Adama city is located at longitude and latitude 8.54 °N 39.27 °E at an elevation of 1712 meters, 99 km southeast of Addis Ababa, the capital city of Ethiopia. Adama city is in the East Shoa zone, Oromia Regional State (Figure 3), between the base of an escarpment to the west and the Great Rift Valley to the east, and the average annual temperature of the city is 20.7°C and about 371 mm of annual precipitation. The climate of this area is hot and arid climate type (Mekonen *et al*., 2019).

Asella is a town in central Ethiopia. It is located in the Arsi Zone of the Oromia Region (Figure 3) 126 km south of Addis Ababa. The Town has a latitude and longitude of 7°57′N 39°7′E, with an elevation of 2,430 meters above sea level.

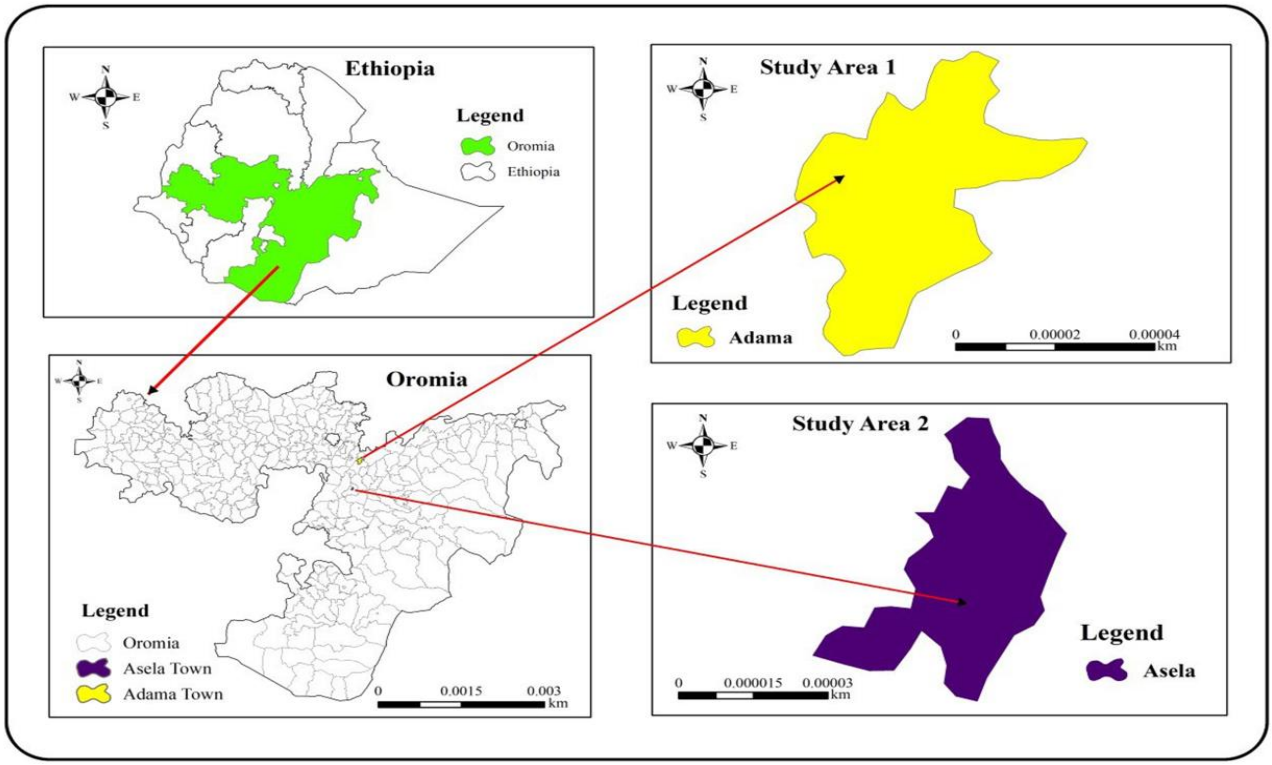

Figure 3: Map of Ethiopia showing the location of Adama City and Asella Town study areas. The orange and purple colors indicate Adama City and Asella Town respectively.

3.2 Sample Collection and Transportation

Thirty fermented *Tella* samples (300 mL each) were collected from local producers separately using 30 sterilized bottles. An equal number of samples (15 from each) were collected from both Adama city and Asella town, the study aims to ensure a balanced representation and comparative analysis of the probiotic properties of LAB isolated from *Tella* produced in these two regions. The areas were selected as popular and well-frequented to capture the diversity of *Tella* available for public consumption. The samples were prepared from different grains such as barley, and maize. Out of 15 Tella samples collected from Asella town 9 were prepared from barley and 6 samples were prepared from maize and barley in combination. Out of the 15 Tella samples collected from Adama city 12 were prepared from maize and barley but the 3 were prepared from maize only. The samples were labeled, properly sealed, and transported using an ice box to the Microbiology laboratory, Department of Applied Biology, Adama Science and Technology University within 6-8 h. The samples were kept in the refrigerator (4°C) (BINDER, GmbH, Germany) until the sample analysis was conducted within 24 h. The test organisms used for screening were standard strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. pyogenes* ATCC 13311) and the human pathogen (*S. aureus* ATCC 25923) obtained from Adama Public Health Research and Referral Laboratory (APHRRL).

3.3 Isolation of LAB

To isolate LAB from *Tella*, a series of dilutions were performed by adding 1 ml of fermented *Tella* into 9 ml of a standard saline solution (0.85% w/v) from 10^{-1} to 10^{-6}. The suspensions were thoroughly mixed using a vortex. Subsequently, 0.1 ml of the third and fifth dilutions were taken and spread onto the surface of the pre-dried de Man Rogosa Sharpe (MRS) agar (HiMedia Laboratories Pvt. Ltd.). The MRS medium consists of protease peptone 10 g, beef extract 10 g, yeast extract 5 g, dextrose 20 g, polysorbate 80 1 g, ammonium citrate 2 g, sodium acetate 5 g, magnesium sulfate 0.1 g, manganese sulfate 0.05 g, dipotassium phosphate 2 g, distilled water 1000 ml and agar 12 g. The inoculated plates were incubated in an anaerobic jar (Model No. HV-2-AJ, New Delhi, India) at 37°C for 24-48 h. The colonies that grew with distinct morphologies were sub-cultured using MRS agar and the purified isolates were maintained on slants at 4°C for further analysis (Lelise *et al*., 2014).

3.4 Morphological Tests

3.4.1 Gram Staining

Lactic acid bacteria were subjected to Gram's staining to distinguish between Gram- negative and Gram-positive species. On a clean glass slide, bacterial colonies were spread out using a drop of water, air-dried, and then heat-fixed gently. After a minute of flooding the slides with crystal violet dye (0.5-1%(w/v), the slides were rinsed under gently running tap water. After using Gram's iodine (1% w/v) was applied for one minute as a mordant, the slides were washed with tap water. Then, the slides were washed for 30 s with 70% (v/v) ethanol and immediately washed with tap water. Then, the slides were submerged in safranin (2.5% (w/v)) for a minute as a counterstain and washed with tap water. After allowing the slides to air-dry the stained slides are examined under a microscope (optechmicroscopes.co.uk) using an oil-immersion objective(x100) (Woodland, 2004).

3.4.2 Endospore Staining Procedures

Endospore staining procedures were performed to determine the presence of endospore. Bacterial colonies from slightly older cultures were smeared on a clean slide with a drop of water. Then the smears were heat-fixed. Blotting papers were cut to fit the size of the slides and placed on the slides. The slides were flooded with malachite green (0.5% w/v) solution and were subjected to a flame for 5 min with the addition of an ample amount of malachite green solution as needed. Then the blotting paper was removed and the slides were rinsed with tap water. Then the slides were again flooded with safranin (1% (w/v)) for 30 s and rinsed with tap water. After allowing the slides to air-dry the stained slides are examined under a microscope (optechmicroscopes.co.uk) using an oil-immersion objective(x100)(Harley & Prescott, 2002).

3.5 Biochemical Identification

3.5.1 Catalase Test

The catalase test involved placing a drop of a 3% hydrogen peroxide solution on a glass slide, onto which a colony of bacteria from a 24-h-old culture of each isolate was applied (or directly on the petri dish). Bacteria displaying a negative catalase reaction were taken for further analysis (Esayas, 2012).

3.5.2 Gas Production from Glucose

The identification of an isolate's homo-heterofermentative nature has relied on the generation of carbon dioxide gas from glucose. Pure cultures of LAB were introduced into a 10 ml MRS broth with an inverted Durham tube (Kimble-Chase, Pyrex, and Corning, US) and incubated at 37°C for 48 h (Goyal, 2012). For homofermentative LAB, gas production was absent, whereas, for heterofermentative LAB, gas was produced inside the Durham tube.

3.4.3 Motility test

A bacterial culture was poked into the center of semisolid nutritional agar medium tubes, and the growth of the culture in the medium allowed for the observation of motility (Bhagya *et al*., 2019).

3.6 Determination of probiotic properties

3.6.1 Acid tolerance

With minor adjustments, each LAB isolate's acid tolerance was determined using the techniques indicated by Vijayalakshmi *et al*., (2020). Five milliliters of MRS broth in test tubes were adjusted to pH 2.0, 2.5, and 3.0 before autoclaving. It was incubated for 0, 6, and 24 h at 37°C. To determine the survival rate of LAB isolates following exposure to low pH values, cultures were obtained at 0, 6, and 24 h and subsequently plated on MRS agar (protease peptone 10 g, beef extract 10 g, yeast extract 5 g, dextrose 20 g, polysorbate 80 1 g, ammonium citrate 2 g, sodium acetate 5 g, magnesium sulfate 0.1 g, manganese sulfate 0.05 g, dipotassium phosphate 2 g, and agar 12 g). The inoculated plates were incubated anaerobically at 37°C for 24 h. LAB isolate inoculated into pH 7.2 was used as a positive control. The survival rate was determined based on the following formula (Vijayalakshmi *et al*., 2020).

$$\text{Survival}(\%) = \frac{Number\ of\ surviving\ cells\ after\ 0,\ 6\ and\ 24\ h\ of\ incubation\left(\frac{\log CFU}{mL}\right)}{Ininital\ number\ of\ cells\ before\ incubation\left(\frac{\log CFU}{mL}\right)} \times 100 \qquad (3.1)$$

3.6.2 Bile salt tolerance

The bile salt tolerance of every LAB isolate was determined according to Mallappa *et* al., (2019). In short, 0.3, 0.75, and 1%(w/v) bile salt concentrations were added to MRS broth after 1 % LAB culture was inoculated, and the test tubes were incubated anaerobically for 3 and 6 h at 37°C. The survival rate of the LAB isolates was determined after exposing the cultures to bile salts, by taking a small amount of culture from 3 and 6 h incubated LAB culture. Thereafter,

the broth cultures were plated on MRS agar plates and incubated anaerobically at 37℃ for 24 h. LAB isolate cultured in MRS broth without bile salt was used as a negative control. The survival rate was determined using the following formula (Mallappa *et al*., 2019):

$$\text{Survival}(\%) = \frac{Number\ of\ surviving\ cells\ after\ 3\ and\ 6\ h\ of\ incubation\left(\frac{\log CFU}{mL}\right)}{Ininital\ number\ of\ cells\ before\ incubation\left(\frac{\log CFU}{mL}\right)} \times 100 \qquad (3.2)$$

3.6.3 Cell Auto-Aggregation

Using the procedure developed by Mallappa *et al*., (2019), the auto-aggregation potential of each culture was determined. The isolates were cultured in MRS broth for 18 h at 37°C and centrifuged for 10 min at 4°C using 8,000 × g. The cells were washed with phosphate buffer saline (PBS, pH 7) twice and resuspended in the buffer to bring the absorbance optical density (OD) to 600 nm (A_0). The cell suspensions were vortexed for 15 s and incubated for 0, 6, and 24 h at 37°C. A spectrophotometer (X-ma 6100/6300/6100S, South Korea) was used to measure the upper layer of this suspension (A_t) at a wavelength of 600 nm. The auto-aggregation percentage was determined:

$$\text{Cell auto aggregation}(\%) = \left[\frac{(\text{A0} - At)}{\text{A0}}\right] \times 100 \qquad (3.3)$$

3.6.4 Co-Aggregation

Co-aggregation characteristic of each LAB isolates with the pathogenic bacteria was examined using the techniques described by Mallappa *et al*. (2019). At 600 nm, the starting absorbances of each pathogenic bacterial culture (A_{path}) and LAB culture (A_{lac}) were adjusted to 8 log CFU/mL using saline salt solution (0.87 g/ml). Then, 10 s before incubation, equal quantities (1.5 mL) of each pathogenic bacteria and LAB cultures were vortexed in combination and incubated at 37°C for 0, 4, and 24 h. To determine the rate of co-aggregation of the mixtures absorbance (A_{mix}) was measured at 600nm.

$$\text{Co aggregation}\ (\%) = \frac{\left(\frac{Alac + Apath}{2}\right) - \text{Amix}}{\left(\frac{Alac + Apath}{2}\right)} \times 100 \qquad (3.4)$$

3.6.5 Cell Surface Hydrophobicity

Each isolate's hydrophobicity degree was assessed by measuring its capacity to adhere to organic solvents, using a modified version of the protocol outlined by Muñoz-Provencio *et al*., (2009). After adjusting each LAB culture to 8 log CFU/mL, the absorbance at 600 nm was determined

(A_0). 1 ml each of xylene (Loba Chemie Pvt. Ltd., jehangirJehangir Villa, Mumbai IndiaSamchun Pure Chemical Co., Ltd., Pyeongtaek, Republic of Korea) and chloroform (Duksan Pure Chemicals Co., Ltd., Ansan-si, Republic of Korea) were separately combined with 3 ml of each LAB culture grow in 10 ml of MRS broth. After 10 min of incubation at 37°C and vortexed, the mixtures were incubated for 0, 6, and 24 h at 37°C. The absorbance (A_t) at 600 nm was measured to determine the hydrophobicity of the cell surface.

$$\text{Cell surface hydrophobicity } (\%) = \left(\frac{1 - \text{At}}{\text{A0}}\right) \times 100 \qquad (3.5)$$

3.6.6 Antimicrobial Activity of Lactic Acid Bacteria against some human pathogenic MOs

Using the agar-well diffusion method, the antibacterial activity of the acid-bile-tolerant LAB strains was assessed against a few food-borne pathogens, according to Fontana et al., (2015). Adama Public Health Research and Referral Laboratory (APHRRL), Adama, Ethiopia, was provided the test organisms (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. pyogenes* ATCC 13311 and *S. aureus* ATCC 25923). The fresh and sterile 5ml of MRS broth containing 1% glucose was inoculated with the chosen acid-bile-tolerant LAB isolates from the slant cultures. The cultures were then incubated overnight at 37°C.

The cultures were centrifuged independently at 10,000 rpm for 10 min at 4°C. Each distinct culture's cell-free supernatant was used as a crude extract for the antagonistic investigation against the targeted food-borne pathogens. The pure cultures of food-borne pathogens were inoculated from slants to MRS broth. After 24 h incubation at 37°C, a volume of 100 µl (Log 10^8 CFU/ml) of inoculum of each strain was swabbed evenly over the surface of solidified Muller Hinton agar (Nissui Pharmaceutical Co., Ltd, Japan) plates with a sterile cotton swab. The plates were allowed to dry, and a sterile cork borer (diameter 5 mm) was used to cut uniform wells in the agar. Each well was filled with 50 µl culture-free supernatant (Log 10^8 CFU/ml) obtained from each of the acid-bile-tolerant LAB isolates. After incubation at 37°C for 24 h, the plates were observed for a zone of inhibition (ZOI) around the well. The diameter of the inhibition zone was measured by a caliper (Mitutoyo vernier caliper 500-196-20, Japan) in millimeters, and a clear zone of 1 mm or more was considered positive inhibition (Del Mar

Lleo' *et al*., 1998). The experiment was carried out in triplicates, and the activity was reported as the diameter of ZOI ± SD.

3.6.7 Antibiotics Susceptibility Test

The disc diffusion method reported by Zhang *et al*., (2016) was used to determine the antibiotic resistance of each acid-bile-tolerant isolate with an antagonistic effect. The antibiotics used to evaluate antibiotic susceptibility test included chloramphenicol (10µg/ml), tetracycline (30 µg/ml), streptomycin (10 µg/ml), erythromycin (15 µg/ml), ampicillin (10 µg/ml), and kanamycin (25 µg/ml). Using a sterile cotton swab, 100 µl (Log 10^8 CFU/ml) of actively growing cultures of acid-bile-tolerant lactic acid bacterial isolates were uniformly swabbed across the MRS plate surface. Following drying, the antibiotic-impregnated discs were placed on the solidified plate surface. The antibiotics were allowed to diffuse for 30 min at 4°C. The plates were then incubated anaerobically at 37°C for 24 h. The diameters of inhibition zones were measured using a caliper (TESA, TWIN-CAL IP67, Switzerland) (Vlková *et al*., 2006). Each antibiotic's zone of inhibition (measured in mm) was classified as susceptible (≥21 mm), intermediate (16–20 mm), and resistant (≤15 mm).

3.6.8 Antioxidant activity

The LAB strains' overnight culture with 7 ml MRS broth was subjected to centrifugation at 8000× g for 10 min at 4℃ to separate the cell-free culture supernatant (CFCS) from the cells. The CFCs were collected and filtered through a 0.2 µm Acro disc syringe filter. The CFCs were resuspended in PBS (pH 7) to achieve a concentration of 10^8 CFU/ml. The radical scavenging capabilities of the selected LAB strains were assessed using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical assay (Cao *et al*., 2019). This assay involved mixing 800 µL of a freshly prepared 0.2 mM DPPH solution in 80% methanol with 400 µL of CFCS, followed by vertexing for 30 s. The mixture was then left at room temperature in the dark room for 30 min. The scavenging ability was quantified by measuring the reduction in absorbance at 517 nm. Uninoculated MRS broth was employed as negative controls for the DPPH scavenging assessment of CFCS. The percentage of scavenging potential was determined using the following equation:

$$\text{DPPH scavenging capacity (\%)} = \left(\frac{\text{A control} - \text{A sample}}{\text{A control}}\right) \times 100 \qquad (3.6)$$

3.7 Growth of Isolates at Different Temperatures and NaCl Concentrations.

3.7.1 Growth at Different Temperature

Each of the 50 µl (log 10^{7-8}) LAB overnight cultures was divided into different tubes, each containing 5 ml of medium (modified MRS broth) with 0.12 g/l bromo cresol purple indicator. The inoculated test tubes were incubated at 15, 20, 25, 30, 40, 45, 50, and 55°C for 7 days, during this entire incubation period, if growth was observed at temperatures of 15, 20, 25, 30, 40, 45, 50, and 55°C the growth medium (cultures) turned yellow instead of purple (Yavuzdurmaz, 2007).

3.7.2 Growth at Different NaCl Concentrations

The ability of LAB isolates to withstand different NaCl concentrations was investigated and for this purpose, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 % NaCl concentrations were tested. Likewise, test tubes were filled to the correct capacity with 5 ml of modified MRS broth containing a bromo-cresol purple indicator. Following that, the test tubes containing 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 % NaCl were filled with 50 µL (log 10^{7-8}) of 1% of each overnight culture of the LAB isolate, and the tubes were then incubated at 37°C for 7 days. Color changing from purple to yellow was taken as evidence of cell development(Yavuzdurmaz, 2007).

3.8 Identification of Lactic Acid Bacteria from Fermented Tella Using MALDI–TOF MS

MALDI-TOF MS analysis of all isolates was performed according to the protocol specified in the manufacturer's user manual as follows. Samples were first inoculated in MRS agar and incubated at 37°C for 24 h. Every colony was moved to a 96-well microplate (Bruker®, Billerica, USA). One layer of lysis solution (70% formic acid; Sigma–Aldrich®) was applied to the bacterial sediment. A 1 L aliquot of the matrix solution (alpha-ciano-4-hidroxicinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma-Aldrich®) was added. Each sample's spectra were produced using a mass spectrometer (MALDI-TOF LT Microflex, Bruker®) was run under the Flex Control 3.3 programmed and had a linear path 337 nm nitrogen laser. The spectra were collected in a mass range between 2000 and 20,000 m/s and then analyzed with the MALDI Biotyper 2.0 program (Bruker®), using the standard bacterial

identification configuration, which compared the spectrum of the samples with the references in the database. The score values 1.7-1.99 provide genus level and >2 score values provide to species level.

3.9 Statistical Analysis

Statistical analysis was conducted utilizing Statistical Package for the Social Science software version 27 and Microsoft Excel spreadsheet. The results were presented with mean values ± standard deviation. Subsequently, the data underwent statistical analysis and comparison through one-way ANOVA, followed by the Tukey post hoc test. Data with a P-value < 0.05 was checked as statistically significant.

4. RESULTS AND DISCUSSION

4.1 Isolation of LAB

In this study, a total of 90 isolates (3 isolates from each of 30 *Tella* samples) were obtained from the traditionally fermented drink *Tella* (Appendix 4.4). Out of the 90 isolates, 87 were catalase-negative, 74 were Gram-positive, and 48 were endospores-negative. Forty-seven of the isolates were creamy, and the remaining 53 were white (Appendix 4.4). Gram-positive and catalase-negative bacteria are included in LAB according to the standards established by Amelia *et al*., (2021). Colonies that were creamy, white, cocci, spherical or ovoid and rod-shaped, Gram-positive, catalase-negative, and endospore-negative were screened and identified (Appendix 4.4).

4.2. Morphological, and Biochemical Characterization.

Out of the 48 Gram-positive, catalase-negative, and endospore-negative, cocci, spherical, or ovoid, and rod-shaped 6 of the isolates were acid-bile-tolerant LAB isolates identified using morphological and biochemical features (Appendix 4.4). Out of the 6 acid-bile-tolerant LAB isolates, the 4 LAB isolates (LABADI6, LABADI31, LABASI54, and LABASI83) produced gas from glucose and the remaining 2 LAB isolates (LABADI17 and LABASI49) were not produced gas (Table 6). All 6 LAB isolates were endospore-negative, non-motile, catalase-negative, and Gram-positive and the five LAB isolates were rod-shaped whereas isolates (LABADI31) were spherical or ovoid (Appendix 4.4 and Table 6). This result is inconsistent with the findings of Mulaw *et al*., (2019) who isolated *L. plantarum* strain CIP 103151, *L. paracasei subsp. tolerans strain* NBRC 15906, *L. paracasei strain* NBRC 15889, and *L. plantarum strain* JCM 1149 from traditional Ethiopian fermented *Teff, injera* dough, *Ergo*, and *Kocho* products and discovered that they could be divided into two groups: homofermentative and heterofermentative. However, the result of this study is consistent with the previous report by Negasi *et al*., (2017) who isolated probiotic LAB from *Shamita* and *Kocho* were recognized morphologically, biochemically, and physiologically, and they included both heterofermentative and homofermentative kinds.

4.3 Probiotic properties of isolates

4.3.1 Tolerance to Low pH and Bile Salts

The temporal viability counts of the chosen cell cultures were assessed for 0 h, 6 h, and 24 h at various pH values (2.0, 2.5, and 3.0) (Table 1). Out of the 48 of the 6 probiotic LAB isolates were subjected to pH (2.0, 2.5, and 3.0) for 0, 6, and 24 h in the current investigation (Table 1). Consequently, at pH 3, LABASI54 had the highest tolerance, with a survival rate of 87.33% followed by LABADI31 (85%) and LABADI6 (83%). At PH 2.5, LABADI31 had the highest tolerance, with a survival rate of 79.67% followed by LABASI54 (79%) and LABADI17 (78%). Likewise, at pH 2, LABADI31 had the highest tolerance with a survival rate of 78% followed by LABASI49 (70%) and LABASI54 (68%). After being exposed for 0 h under pH 2, pH 2.5, and pH 3, the 6 isolates' survival rates were 59 to 78, 62.33 to 79.67, and 76.67 to 80.33%, respectively. Nevertheless, after 6 h at pH 2, pH 2.5, and pH 3, the six isolates' survival rates ranged from 55 to 75, 55.33 to 72.6, and 71.33 to 79%, respectively (Table 2). Finally, after exposure for 24 h at pH 2, pH 2.5, and pH 3, the 6 isolates' survival rates ranged from 46 to 75, 52 to 66, and 63.67 to 77.33%, at 37℃ on MRS agar respectively. At pH 2, 2.5, and 3 for 0, 6, and 24 h, LABADI31 had the highest tolerance, with survival rates of 76, 66, 58, 77.6, 72.7, 66, 85, 79, and 77.3% respectively (Table 1). For all 6 isolates, the probiotic LAB was shown to have the greatest survival rate at pH 2, 2.5, and 3. However, the survival rate declined as the pH decreased to 2. The percentage of survivors varied significantly ($p < 0.05$) in their capacity to survive in acidic environments, ranging from 46 to 85% (Table 1). The result of this investigation was much like the previous research by Abushelaibi *et al*., (2017) and Song *et al*., (2021). Akbar *et al*., (2019), and Pan & Zhang, (2008) showed that most *L. lactis* strains have a survival rate from 65 to 70% following exposure to pH 2 for 3 h. The findings of this study are consistent with those reported by Sofyan *et al* (2013).

However, the viability of the LAB isolates at p^H 3 in this research had higher viability than the previous research by Torshizi *et al*., (2008) which had 55.89-67.76% of cell viability of *P. pentosaceus* TMU457, *L. rhamnosus* TMU094, and *L. fermentum* TMU121. The viability rate of this study was much greater than the results found by Castro *et al*., (2015), four *L. mesenteroides* isolates from *agave sap* were evaluated, and the results showed 40.9–49.12% tolerance to pH 2 for 3 h at 37°C. The survival rate of the current study was lower than the

previous research by Damayanti, *et al*., (2012) *P. pentosaceus* Db9 and *L. salivarius* I72 bacteria isolated from the duodenum of a broiler chicken and isolated from the ileum of a native chicken had the highest viability (75.66–81.76%) respectively. Notably, survival rates can differ based on the bacterial strain and the experimental setting, underscoring the need for additional study in this field (Clara, 2017). However, the examined strains' acid tolerance showed a varied response ($p < 0.002$), indicating that acid tolerance is a strain-specific characteristic. Since probiotics are subjected to stomach acidity following oral consumption, potential probiotic candidates must be able to withstand low pH (Giles-Gómez *et al*., 2016).

The survival of the six isolates of probiotic strains in MRS broth supplemented with 0.3, 0.75, and 1% bile salts are displayed in Appendix 10.3 and Figure 4. After a 3 and 6 h incubation period in an MRS medium enhanced with 0.3, 0.75, and 1% (w/v) bile salt concentration, the growth rates of each isolate were varied (Appendix 4.3 and Figure 4). At 0.3, 0.75, and 1% concentrations of bile salt for 3 and 6 h LABASI83 had the highest tolerance, with survival rates of 91.5 & 89.13%, 88.33, & 88.8%, 84.33, & 86.36% respectively (Appendix 4.3). In the current investigation, it was discovered that most of the chosen strains were tolerant to bile salt concentrations of 0.3 and 0.75% (w/v) after 3 h exposure and the viable counts of bacteria decreased as bile salt concentrations increased (Appendix 4.3 and Figure 4).

Nevertheless, the four isolates, including LABADI31, LABASI49, LABASI54, and LABASI 83, were able to withstand even the highest bile contents of 1.0% ox gall (Appendix 10.3 and Figure 4). These findings are consistent with other research that found some strains of *L. salivarius* can resist a 1% (w/v) concentration of bile salt (Bae *et al*., 2002) and some strains of *E. faecium* can survive in media containing 1% bile salt with a survival rate of up to 98 % (Strompfová, 2004) and Previous studies by Sukumar *et al*., (2010), *Pediococcus spp*. isolated from Indian fermented foods demonstrated that tolerant to bile salts. This investigation's result is consistent with Chowdhury *et al*., (2012), *Lactobacillus subsp*. isolated from yogurt was able to withstand 0.3% (w/v) bile salt. Isolates of *L. helveticus* exhibited tolerance as high as 0.3% and as low as 0.1%. This investigation coincided with observations made by Barua *et al*., (2015) and Rong *et al*., (2015) that *Lactobacillus spp*. had maximum growth at 0.1% and maximum tolerance at 0.3% concentration. Similar results were noted in a study by Baick & Kim, (2015).

The findings of this study suggest these isolates may be useful as prospective probiotic candidates (Appendix 4.3).

Tolerance to bile salts and acid is one of the most crucial screening criteria for isolates with probiotic potential. Acid tolerance is essential for the strain to withstand the severe conditions of the GIT and enables it to survive longer in higher-acidic beverages like *Tella* (Meng, 2010). For probiotics to successfully develop and colonize in the human GIT, they should ideally possess properties that enable them to withstand the acidity of the stomach and the exposure to bile in the upper section of the intestine (Mallappa *et al*., 2019; Sakoui *et al*., 2022). Finding bacteria that can withstand the extreme conditions of the gastrointestinal tract, which include low pH values between 2.0 to 3.0 and 0.3% bile concentrations, has been the focus of many in vitro investigations (Mallappa *et al*., 2019). The isolated LABASI83 and LABDSI31 have the greatest bile salt and acid tolerances, respectively.

Table 1: Acid tolerance of LAB strains (viability of cells after exposure—log CFU/mL).

	pH Tolerance								
Isolates	0 h			6 h			24 h		
	P^H 2	P^H 2.5	P^H 3	P^H 2	P^H 2.5	P^H 3	P^H 2	P^H 2.5	P^H 3
LABADI6	59±0.889^{e}	62.3±0.463^{d}	83±0.567^{c}	55±0.624^{e}	55.2±0.371^{e}	73±0.291^{c}	46±0.323^{f}	52±0.605^{e}	66±0.557^{c}
LABADI17	65.67±0.094^{d}	77±0.264^{a}	81.3±0.646^{b}	58.3±0.459^{d}	72.3±0.586^{a}	72±0.235^{d}	52±0.659^{d}	61.3±0.512^{c}	64±0.568^{d}
LABADI31	76± 0.360^{a}	77.6±0.533^{a}	85±0.567^{a}	66±0.656^{a}	72.7±0.611^{a}	79±0.634^{a}	59.3±0.426^{a}	66±0.529^{a}	77.3±0.028^{a}
LABADI49	70±0.12^{b}	74.7±0.129^{b}	76.6±0.094^{e}	65±0.810^{b}	70±0.854^{b}	72.1±0.326^{d}	59±0.267^{a}	65.3±0.509^{b}	63.6±0.371^{e}
LABASI54	68±0.148^{c}	73±0.624^{b}	80.3±0.055^{d}	66±0.816^{a}	67±0.535^{c}	76±0.211^{b}	55.7±0.611^{c}	59.6±0.507^{d}	66.3±0.023^{c}
LABASI83	64±0.557$^{\underline{d}}$	67.3±0.767^{c}	80.6±0.621^{d}	59±0.567^{c}	65±0.549^{d}	71.5±0.859^{e}	50.3±0.131^{e}	61.7±0.509^{c}	67.3±0.507^{b}

Data represents the mean (n=3) ± SD (standard deviation) of three independent experiments. Different superscript letters (a–e) represent significant differences ($p < 0.002$) among the means within the same column.

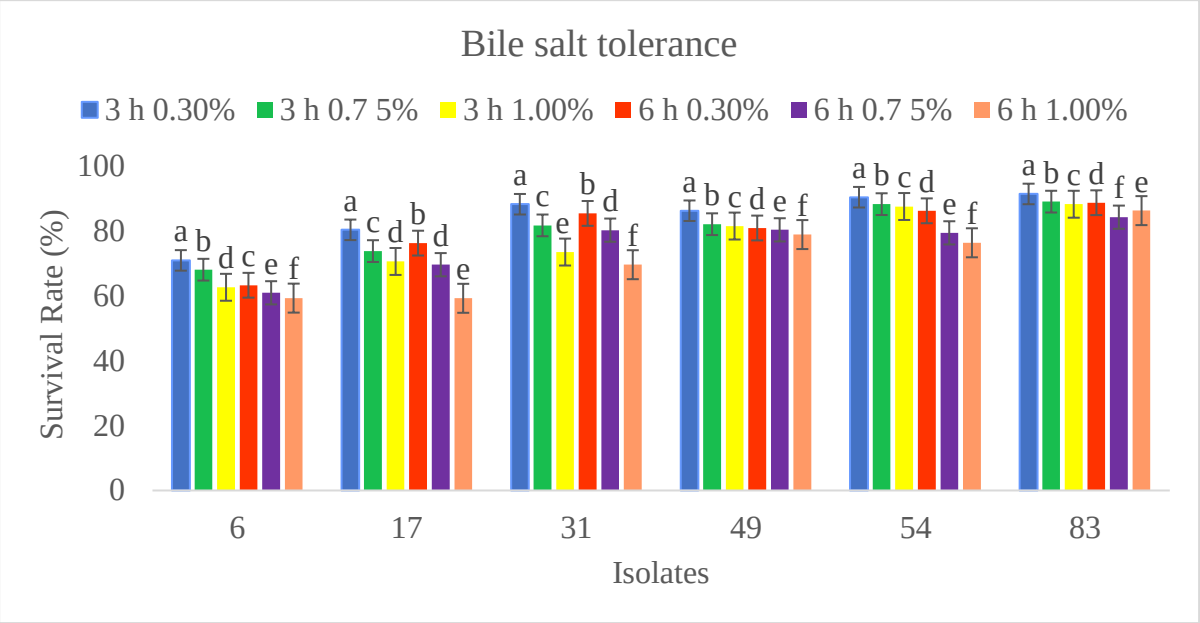

Figure 4: The percentage of LAB strains that survived after being isolated from *Tella* samples in bile salt conditions. Survival of the six isolates on MRS agar plates for 3 and 6 hours at 37°C in bile salt concentrations of 0.3, 0.7, and 1%. The data displayed are the mean ± SD of three independent experimental values. Different superscript letters (a–f) indicate a significant difference ($p < 0.03$).

4.3.2 Auto-aggregation and co-aggregation

Appendix 4.2 and Figure 5 showed that out of the six LAB isolates tested in this study, isolate LABADI31 had the highest auto-aggregation ability (74.2, 85.26, and 93.23%) at 0, 6, and 24 h, respectively but isolate LABADI6 showed the highest (96.4%) auto-aggregation ability at 4 h, whereas, isolate LABASI83 had the lowest auto-aggregation (28.6, 56.5, and 72.51%) at 0, 6, and 24 h, respectively. Strong adhesion is associated with high auto-aggregation ability (Al Seraih *et al*., 2018). The comparable results indicate that LABADI6 and LABADI31 isolates, possessed potent aggregation capabilities, suggesting a more reliable ability to attach to cells than other isolates. Aggregation elements that promote H-bonding between and among cell surfaces are thought to be correlated with enhanced general adhesion capacity (Dowdell *et al*., 2020). The auto-aggregation characteristics of the LAB isolates in this investigation showed greater values than those previously reported (26.3-38.75%) by Vijayalakshmi *et al*., (2020) and Sakoui *et al*., (2022). The isolate's auto-aggregation percentage varied significantly ($p < 0.003$) during the incubation periods since cell adhesion and clustering as a result of cell-to-cell contacts are the cause of this variation (Krausova *et al*., 2019).

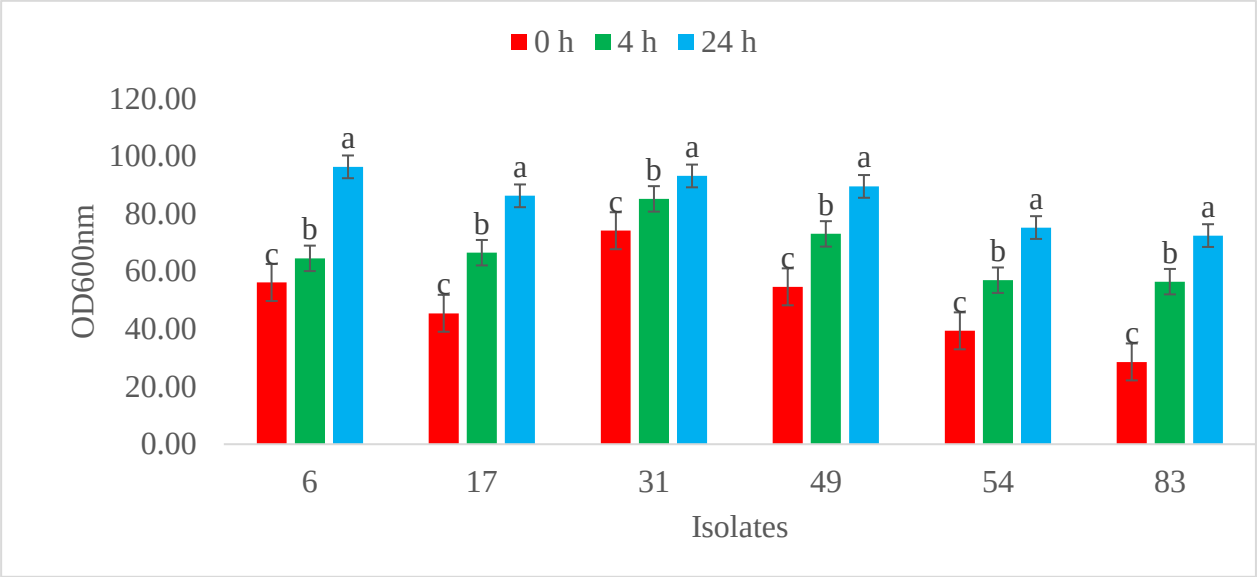

Figure 5: The OD600 values of all six isolates' auto aggregation percentages after 0, 6, and 24 h were incubated at 37°C. All the six LAB isolates in this study were maximum at 24 h but lower at 0 h. The data displayed are the mean ± SD of three independent experimental values. Different superscript letters (a–c) indicate a significant difference ($p < 0.003$).

Table 2 shows that the co-aggregation values of the LAB isolates ranged from 44.9% (isolate LABADI17 with *P. aeruginosa* ATCC 43895) at 0 h to 96.56% (isolate LABADI31 with *E. coli* ATCC 25923) at 24 h. There have been earlier reports of broader ranges (7.0–70.0%) of LAB co-aggregation qualities with other pathogens such as *E. Coli* ATCC8539, *L. monocytogenes* ATCC19115, *S. aureus* ATCC 114, and *S. Typhimurium* LT2 (Mallappa *et al*., 2019; Sakoui *et al*., 2022; Vidhyasagar & Jeevaratnam, 2013). The result of this study is in agreement with previous studies that found similar co-aggregation percentages of isolates from vinegar, camel milk, and sausages after 4 and 24 h of incubation with *P. acidilactici* and *S. Typhimurium* PTCC 1609 (Abushelaibi *et al*., 2017; Sui *et al*., 2021). The capacity of LAB attached to the surface of epithelial cells to co-aggregate with pathogens is strain-specific (Mallappa *et al*., 2019). The unique ability of isolates LABADI31 to *P. aeruginosa* ATCC 43895, LABASI54 to *E. coli* ATCC 13076, and LABADI17 to *S. aureus* ATCC 25923 was shown to be considerably distinct ($p < 0.001$) in their co-aggregation ability (Table 2). The study found that all isolates had varying co-aggregation percentages after 0, 4, and 24 h of incubation with pathogens. Moderate co-aggregation properties were observed in this investigation. Coaggregation refers to the intercellular adhesion that exists across distinct strains and is closely associated with their capacity to interact with pathogens.

As a result, aggregation could be one type of host defense mechanism against infection(Zhang *et al*., 2013). One reliable measure of gut colonization is the probiotics' capacity to both self- and co-

aggregate with pathogenic bacteria. Probiotics can live and endure in the GIT, which benefits their host, according to their aggregation ability, which is linked to their cell adherence capabilities(Cesena *et al*., 2001). Probiotics' capacity to survive and persist in the GIT and positively impact their host is indicated by their aggregation ability, which is linked to their cell adherence characteristics (Gupta *et al*., 2021). Several studies have found that cellular aggregation promotes probiotic bacteria cell colonization (Ayyash *et al*., 2021). The existence of certain chemicals on the surface of the LAB isolates that function as ligands for pathogen binding can account for this diversity (Campana *et al*., 2017). Additionally, the co-aggregation of pathogenic bacteria and probiotics may provide a barrier of defense that keeps infections from invading the gut (Vidhyasagar & Jeevaratnam, 2013). The selection of probiotic strains can be guided by the presence of both auto- and co-aggregation(Ferreira *et al*., 2011).

Table 2: Co-aggregation property of lactic acid bacteria isolates.

	Coaggregation								
	P. aeruginosa			*E. coli*			*S. aures*		
Isolates	0 h	4 h	24	0 h	4 h	24	0 h	4 h	24
LABADI6	66.2±0.721^{c}	77.2±0.655^{c}	78.07±0.281^{c}	79.3±0.2^{b}	83.47±0.450^{b}	87.83±0.318^{b}	59.07±0.750^{e}	62.06±0.145^{e}	67±0.8^{e}
LABADI17	44.9±0.8^{f}	51.86±0.950^{f}	56.06±1.096^{f}	59.27±0.568^{d}	66.09±0.089^{d}	78.2±0.654^{c}	90.4±0.808^{a}	95.12±0.351^{a}	97.2±0.552^{a}
LABADI31	78.97±0.585^{a}	85±0.818^{a}	89.4±0.5^{a}	90.6±0.26^{a}	93.2±0.6^{a}	96.56±0.802^{a}	66.83±0.829^{d}	74.17±0.450^{d}	77.3±0.850^{d}
LABADI49	74.16±0.305^{b}	79.81±0.812^{b}	82.96±0.575^{b}	53.53±0.321^{f}	59.5±0.360^{e}	66.4±0.258^{d}	70.87±1.021^{b}	80.07±0.405^{b}	83.03±0.802^{c}
LABASI54	61.16±0.305^{e}	69.97±0.513^{d}	73.1±0.702^{d}	55.34±0.305^{e}	52.63±0.055^{f}	54.17±0.045^{e}	56.56±0.251^{f}	59.4±0.264^{f}	64.65±0.776^{f}
LABASI83	63.37±0.493^{d}	66.5±0.4^{e}	69.31±0.321^{e}	69.4±0.360^{c}	72.23±0.416^{c}	78.4±0.458^{c}	68.6±0.050^{c}	78.1±0.4^{c}	88.37±0.305^{b}

1. Data represents the mean ± standard deviation of three independent experiments. Different superscript letters (a–f) represent significant differences ($p < 0.001$) among the means within the same column.
2. The ability of bacteria to co-aggregate is influenced by surface molecules such as adhesins, lectins, and polysaccharides. Variability in the expression or accessibility of these surface molecules among isolates can affect their ability to co-aggregate with one another.

4.3.3 Hydrophobicity

The hydrophobicity percentages of the isolates varied, as shown in Appendix 4.1 and Figure 6, from 46.01% in chloroform at 24 h for isolate LABASI83 to 99.73% in xylene at 0 h for isolate LABADI17. In general, LAB isolates in this study were more hydrophobic in xylene than the chloroform (Appendix 10.1 and Figure 6). LABASI17 exhibited the maximum hydrophobicity at 0 h towards xylene (99.73%) followed by LABASI54 towards chloroform (96.6%) (Appendix 10.1).

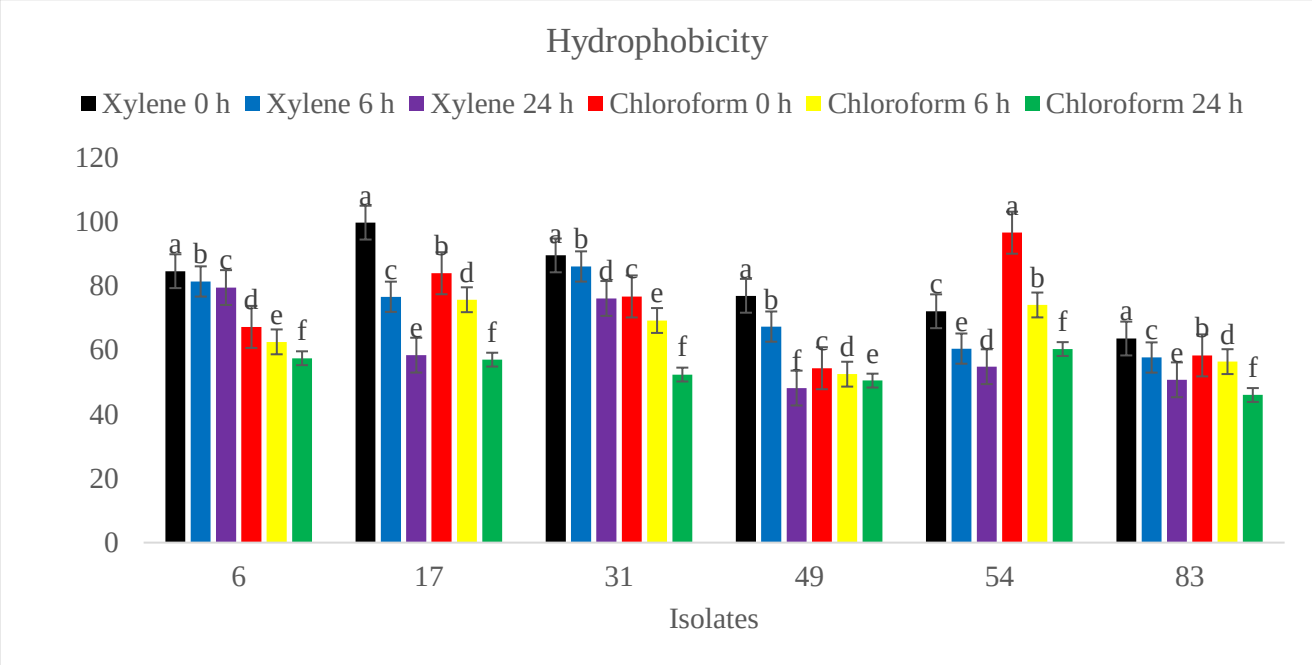

Figure 6: The percentage of hydrophobicity exhibits the cell adhesion ability of the six isolates in xylene and chloroform after 0, 6, and 24 h at 37°C. The data displayed are the mean ± SD of three independent experimental values. Different superscript letters (a–f) indicate a significant difference ($p < 0.01$).

In line with the present study, research by Claudia *et al*., (2007) indicates that a significant proportion of probiotic LAB exhibited cell surface hydrophobicity of over 40% against xylene. The hydrophobicity of the LAB isolates in this investigation showed greater values than those previously reported by Singh *et al*., (2016) who found strains of *L. reuteri* exhibit hydrophobicity levels ranging from 25.30 to 49.37% toward chloroform at 6 hours. Conversely, isolated LABASI49 showed the lowest percentages of xylene (48.17%) and chloroform (46.01%) at 24 h. For bacteria, the hydrophobicity test incubation duration ranged from 3 to 24 h. According to the present study, xylene, followed by chloroform, was

the most used hydrocarbon to perform the hydrophobicity test (Appendix 4.1 and Figure 6). Hydrophobicity has been observed between 2.0 to 88.0% for xylene (Gupta et al., 2021). The hydrophobicity of the LAB used in this investigation was higher than that of Mulaw *et al*., (2019), who reported that a LAB strain, *Lactobacillus* spp., isolated from traditionally fermented Ethiopian food products, had a hydrophobicity ability ranging from 32.75 to 36.30%. TokatlJ *et al*., (2015) found that the LAB strain, *L. plantarum*, isolated from traditional pickles, has an 82.41% hydrophobicity. Even though, the hydrophobicity of the LAB included in this investigation was lower than that of Harnentis *et al*., (2020), who stated that all the LAB isolates isolated from indigenous fermented foods in West Sumatera, Indonesia, had more than 84% hydrophobicity. The result of this study is lower than Amenu & Bacha, (2023) reports in which they stated that probiotic lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages have >69.08% cell surface hydrophobicity against xylene. Our LAB isolates demonstrated a moderate hydrophobicity in comparison to these investigations, suggesting their capacity to stably adhere to epithelial cells and encourage health (Schillinger *et al*., 2005).

The GIT's bacterial adherence and interactions with host cells are influenced by the crucial probiotic characteristic of cell surface hydrophobicity (Xu *et al*., 2009). Probiotic candidates are commonly tested for hydrophobicity in organic solvents like n-hexane, xylene, toluene, chloroform, and ethylene (Joghataei *et al*., 2019; Oliveira *et al*., 2019; Pino *et al*., 2019; Rao *et al*., 2019; Shivangi *et al*., 2020). The microbial adhesion to hydrocarbons test investigates a probiotic candidate's ability to stay in hydrocarbon solvents after mixing with phosphate buffer. According to studies, the hydrophobicity of a probiotic candidate correlates positively with its ability to adhere (Vinícius *et al*., 2018).

4.3.4 Antibacterial Activity

Table 3 and Figure 7 display the CFS's antibacterial activity levels against the four test pathogens, which include *E. Coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. pyogenes* ATCC 13311, and *S. aureus* ATCC 25923. Most of the pathogens were susceptible to antibacterial activity by the CFS from all LAB isolates. All six LAB isolates inhibited the growth of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. pyogenes* ATCC 13311, and *S. aureus* ATCC 25923. Isolate LABADI83 showed the maximum inhibition zone (37.45 mm) against *P. aeruginosa* ATCC 27853, whereas isolate LABASI17 exhibited the lowest inhibition zone (18.9 mm) against *S. aureus* ATCC 25923. Overall, the antibacterial activity

of isolates LABADI6, LABADI31, LABASI54, and LABASI83 was superior to that of the other two isolates.

Accordingly, Choi et al., (2018) have reported that *lactobacillus* strain entirely ceased the growth of food-borne pathogens that encompassed *S. enteritidis* KCCM 12021, *S. typhimurium* KCTC 1925, *E. coli* O157:H7 ATCC 35150, and *S. aureus*. The result of this study is much greater than that of the zone of inhibition by Tigu *et al*., (2016) shown that two *Lactobacillus* isolates, with inhibition zones ranging in diameter from 10.3 to 14.3 mm, inhibited the growth of *S. typhimurium* and *E. coli* out of the eleven probiotic LAB isolated from traditional Ethiopian fermented condiments, namely *Datta* and *Awaze* and the results reported by, Tadesse et al., (2015) have confirmed that all 118 LAB isolates from *Borde* and *Shamita*, belonging to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*, were found to have inhibition zones ranging in diameter from 15 to 17 mm that inhibited the growth of test strains, including *S. aureus*, *Salmonella spp*., and *E. coli* O157: H7. Numerous research has shown similar findings (Cesena *et al*., 2001; Sakoui *et al*., 2022).

The inhibitory impact on pathogens is caused by LAB's synthesis of bacteriocins, hydrogen peroxide, and organic acids (Holzapfel, 1997; Servin, 2004; Shah, 2007). In general, the results showed that the LAB isolates were more successful in their ability to inhibit the growth of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 than the *S. aureus* ATCC 25923 and *S. pyogenes* ATCC 1331. The selection of LAB as candidates for starter culture formation and probiotic application is contingent upon their antibacterial activity (Fei *et al*., 2018; Holzapfel, 1997; Servin, 2004).

CFS are widely used in food preservation due to their antimicrobial properties. CFS containing organic acids, bacteriocins, and other bioactive compounds can inhibit the growth of spoilage and pathogenic bacteria, extending the shelf life of foods. LAB-derived CFS can be used as a probiotic supplement or an alternative to live probiotic cultures. The bioactive compounds in the CFS may confer health benefits similar to those of live LAB cultures, such as supporting gut health and modulating the immune system. The CFSs of the LAB isolates were more active on gram-negative bacteria. Sometimes, the antimicrobial chemicals in the CFS perform better together to combat Gram-negative bacteria than they do separately. The combined effect of these two factors may increase the CFS's overall effectiveness against Gram-negative bacteria.

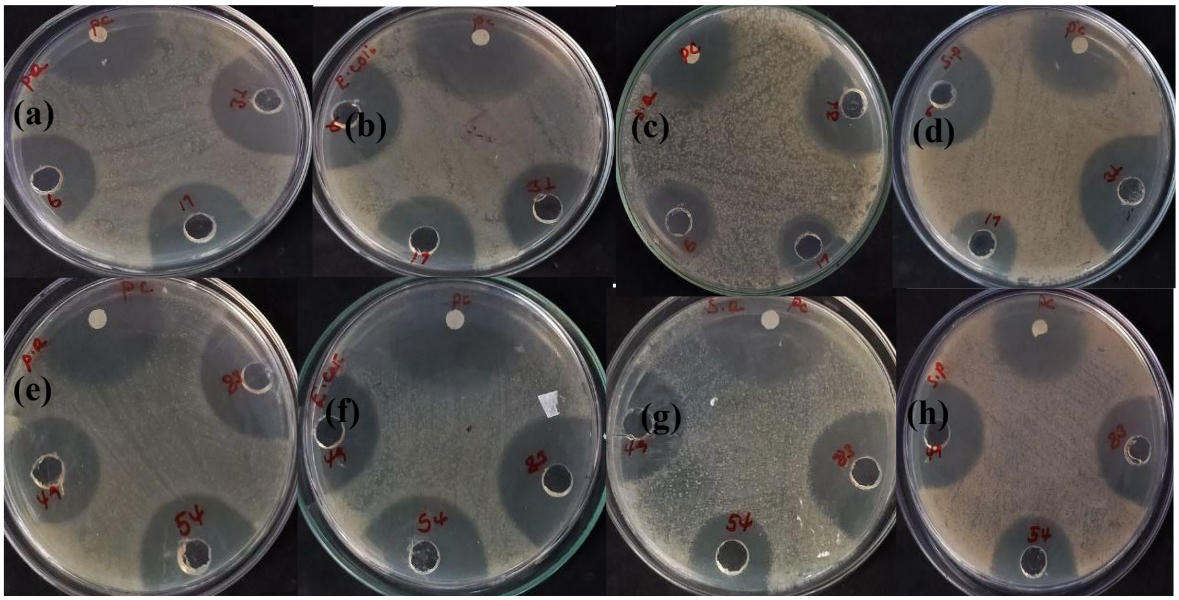

Figure 7: Antibacterial activity exhibited by the six LAB isolates (*P. pentosaceus* LABADI-6, *L. mesenteroides* LABADI-17, *L. lactis* LABADI-31, *L. paracasei* LABASI-49, *L. plantarum* LABASI-54, and *L. mesenteroides* LABASI-83) against the four pathogenic bacteria using the well diffusion method. (a) showed that the three LAB *P. pentosaceus* LABADI-6, *L. mesenteroides* LABADI-17, and *L. lactis* LABADI-31 against *P. aeruginosa* ATCC 27853 (b) showed that the three LAB *P. pentosaceus* LABADI-6, *L. mesenteroides* LABADI-17, and *L. lactis* LABADI-31 against *E. coli* ATCC 25922. (c) showed that the three LAB *P. pentosaceus* LABADI-6, *L. mesenteroides* LABADI-17, and *L. lactis* LABADI-31 against *S. aureus* ATCC 25923. (d) showed that the three LAB *P. pentosaceus* LABADI-6, *L. mesenteroides* LABADI-17, and *L. lactis* LABADI-31 against *S. pyogenes* ATCC 13311 (e) showed that the three LAB *L. paracasei* LABASI-49, *L. plantarum* LABASI-54, and *L. mesenteroides* LABASI-83 against *P. aeruginosa* ATCC 27853 (f) showed that the three *L. paracasei* LABASI-49, *L. plantarum* LABASI-54, and *L. mesenteroides* LABASI-83 against *E. coli* ATCC 25922. (g) showed that the three LAB *L. paracasei* LABASI-49, *L. plantarum* LABASI-54, and *L. mesenteroides* LABASI-83 against *S. aureus* ATCC 25923. (h) showed that the three *L. paracasei* LABASI-49, *L. plantarum* LABASI-54, and *L. mesenteroides* LABASI-83 against *S. pyogenes* ATCC 13311.

Table 3: Antibacterial activity of lactic acid bacteria (LAB) isolates against four foodborne pathogens.

	Antimicrobial activities			
	E. coli	*S. aureus*	*P. aeruginosa*	*S. pyogenes*
Isolates	ATCC25922	ATCC25923	ATCC27853	ATCC13311
6	34.67± 0.23^{bc}	20.13 ± 0.16^{f}	29.45 ± 0.30^{f}	35.76 ± 0.61^{b}
17	29.5± 0.67^{d}	18.9 ± 0.95^{g}	30.33 ± 0.61^{e}	21.37 ± 0.81^{f}
31	27.8± 0.31^{e}	33.5 ± 2.22^{c}	35.91 ± 0.31^{b}	37.25 ± 0.63^{a}
49	32.64 ± 0.6^{cd}	30.53 ± 0.28^{d}	31.35 ± 0.68^{d}	31.65 ± 0.71^{d}
54	35.36± 0.3^{abc}	23.46 ± 0.89^{e}	33.57 ± 0.75^{c}	27.76 ± 0.73^{e}
83	35.32± 0.84^{ab}	36.73 ± 0.39^{b}	37.45 ± 0.29^{ab}	33.68± 0.59^{c}
Pc	36.45±0.26^{a}	37.9±0.53^{a}	38.3±0.25^{a}	37.97±0.43^{a}

Data represent the mean ± standard deviation of three independent experiments. Different superscript letters (a–f) represent significant differences ($p < 0.04$) among the means within the same column.

4.3.5 Antibiotic Susceptibility

All the six LAB isolates in this investigation were intermediate to ampicillin and chloramphenicol, resistant to erythromycin and kanamycin, and sensitive to tetracycline and streptomycin (Table 4). This finding is similar to an earlier report in Ethiopia (Mulaw *et al*., 2020; Negasi *et al*., 2017) which found that LAB isolated from traditional fermented foods and beverages were more resistant to kanamycin but susceptible to tetracycline. Similarly, LAB isolated from traditional fermented milk in Spain (Niazi *et al*., 2014; Rajoka *et al*., 2019), were more responsive to tetracycline, but in inconsistency with the present investigation, these results were susceptibility to chloramphenicol and erythromycin. The present result is consistent with previous studies on the LAB isolated from different sources in western India where most LAB isolates exhibited remarkable resistance to Kanamycin but intermediate to streptomycin (Junnarkar *et al*., 2018). Antibiotics such as streptomycin and kanamycin typically target Gram-negative bacteria. Gram-positive LAB was resistant to these medicines because of their thick cell walls (Han *et al*., 2015). The observed resistance to tetracycline in this investigation is consistent with earlier studies against *Wesselia confusa* isolated from fermented sour rice, although the strain showed streptomycin resistance (Nath *et al*., 2021).

Furthermore, the *Lactobacillus* genus was naturally resistant to aminoglycosides like streptomycin and kanamycin, therefore its resistance doesn't present a risk to public health (Botta *et al*., 2014; Elkins & Mullis, 2004). The results are consistent with previous studies showing that medicines that block protein synthesis, like streptomycin and tetracycline, cause LAB isolates to become sensitive. On the other hand, resistance to aminoglycosides such as erythromycin and kanamycin was reported (Gupta *et al*., 2021; Zhou *et al*., 2005). The LAB isolates with varying susceptibilities to streptomycin and tetracycline show strain specificity (Danielsen *et al*., 2007). According to Borriello *et al*., (2003), probiotic isolates should be susceptible to at least two clinically relevant antibiotics; as a result, the isolates used in this investigation satisfy the safety standards for phenotypic resistance evaluation.

Furthermore, isolates may exhibit phenotypic resistance to several antimicrobial drugs without showing commonly studied resistance genes, or they may carry shortened antimicrobial resistance genes without displaying the phenotypic resistance pattern (Wang *et al*., 2012). Therefore, even in cases where an isolate does not show signs of phenotypic resistance, it is crucial to investigate the major resistance genes. It is imperative to evaluate the expression of

any resistance genes carried by isolates and the presence of mobile genetic elements that can spread these resistance determinants to other bacteria (Cruxen *et al*., 2019). By providing a favorable environment for the survival of resistant and multi-resistant bacteria, the food matrix can affect the spread of antimicrobial resistance (Bhargava & Zhang, 2012). Human health is seriously threatened by the spread of pathogens that are resistant to antibiotics(Chajecka *et al*., 2015). Since fermented foods can spread these resistant microbes, assessing antimicrobial resistance in native LAB isolates was an essential safety precaution. Furthermore, pathogenic bacteria can acquire antibiotic-resistant genes from probiotics through horizontal gene transfer; yet, LAB has been "generally recognized as safe."(Oh *et al*., 2018).

Table 4: Antibiotic susceptibility profile of potential probiotic LAB isolated from *Tella*.

	Antimicrobial susceptibility profile					
Isolates	TET	STR	KAN	AMP	ERY	CHL
LABADI6	S	S	R	R	I	I
LABADI17	S	S	R	R	I	I
LABADI31	S	S	R	R	I	I
LABADI49	S	S	R	R	I	I
LABASI54	S	S	R	R	I	I
LABASI83	S	S	R	R	I	I

The zone of inhibition (diameter in mm) for each antibiotic measured and expressed as susceptible, S (≥ 21 mm); intermediate, I (16–20 mm) and resistance R (≤ 15 mm). AMP, ampicillin (10 µg); KAN, kanamycin (30 µg); STR, streptomycin (10 µg); ERY, erythromycin (15 µg); TET, tetracycline (30 µg); CHL, chloramphenicol (30 µg).

4.3.6 Antioxidant Activity

The ability of the six different probiotic strains' cell-free extracts to scavenge free radicals in response to DPPH activities was evaluated (Table 5). All the isolates demonstrated antioxidant activity in the 33.8 to 52.2% range in the DPPH assay. Regarding the scavenging of DPPH, isolate LABADI6 (52.2%) showed the maximum antioxidant activity, while LABASI54 (33.8%) showed the lowest activity. Our results are significantly ($p<0.05$) lower, 33.8 to 52.2% than the findings reported by Abouloifa et al., (2020), which ranged from 43–93% using the same approach. The strains used in our investigation have lower DPPH scavenging activities than the strains produced by Liu *et al*., (2020), Zeng *et al*., (2021), and Zhao *et al*., (2021). Certain strains in this investigation exhibited better DPPH scavenging ability than LAB strains in the previous study (Czyżowska *et al*., 2017). Depending on the strain, Ayyash et al. (2018) have reported antioxidant values ranging from 5 to 20% on the initial day. Nevertheless, there was an increase up to 21 days of incubation with values varying between 10 and 50%. Antioxidant activity may therefore rise with the length of time the gut is occupied.

Table 5: Antioxidant activities of the six LAB isolates.

S. no.	Strain name	Radical Scavenging (%)
1	LABADI6	53.8 ± 0.015^{a}
2	LABADI17	41.2 ± 0.001^{c}
3	LABADI31	37.9 ± 0.0005^{d}
4	LABADI49	35.4 ± 0.001^{e}
5	LABASI54	33.8 ± 0.0015^{f}
6	LABASI83	52.1 ± 0.0015^{b}

Data represents the mean ± standard deviation of three independent experiments. Different superscript letters (a–f) represent significant differences ($p < 0.05$) among the means within the same row.

4. 5 Growth of Isolates at Different Temperatures and NaCl Concentrations.

Table 6 illustrates the salt concentration of isolates cultured at 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5% respectively. The results showed that all six strains were able to survive in 2.5, 3.5, and 4.5% NaCl, and the five isolates (LABADI6, LABADI31, LABASI49, LABASI54, and LABASI83) were able to survive in 5.5% NaCl, the four isolates (LABADI6, LABADI31, LABASI54, and LABASI83) were able to survive in 6.5% NaCl, the two isolates (LABADI6, and LABADI31) were able to survive in 7.5% NaCl, no growth was observed in 8.5% NaCl concentration. The growth was visualized by the dye's color change due to low pH from purple to yellow after 7 days of incubation, which indicates the growth of organisms at different NaCl concentrations. The current results showed that LAB isolates were able to tolerate tested concentrations of NaCl and good growth was observed at 2.5–4.5% NaCl with some differences as the growth was decreased when the concentration of NaCl was increased from 4.5 to 8.5%. The obtained results have similarities with the findings of Reale *et al*., (2015), they reported that lactobacilli isolated from the gastrointestinal tract of swine were tolerable to 4-8% NaCl. The result of this study is consistent with the result reported by Dhanvanti *et al*., (2023), who obtained both A2 and A5 strains were able to survive in 2 and 4% NaCl, and no growth was observed in 6.5% NaCl concentration. Mona *et al*., (2022) obtained similar results to the present study.

Table 6 illustrates the temperature of isolates cultured at 15, 20, 25, 30, 40, 45, 50, and 55℃ respectively. During incubation at different temperatures, growth was indicated by the change in color of the medium containing dye from purple to yellow due to a change in pH. The five LAB strains turned yellow at 15, 20, and 25℃. Except for isolate, LABASI54 at 40 and 45℃, and LABADI31 at 45℃ the isolated growth was indicated by the color change of medium containing dye from purple to yellow due to a change in pH but in the five isolates (LABADI6, LABADI17, LABADI31, LABASI49, and LABASI54) was not shown color change ≥50℃. Isolate LABASI83 showed positive results at 50℃ but not at 55℃. Our findings concur with earlier research reported by Reuben *et al*., (2019) which found that all strains tested grew best at 37°C. the result of this study is consistent with the results obtained by Dhanvanti *et al*., (2023)who found A2 and A5 strains turned yellow at 10, 30, and 37ºC. Maximum growth was observed at 37ºC and no growth was reported at 50ºC.

Table 6: Some biochemical and growth of isolates at temperature and NaCl concentrations.

Isolates	Gas Production	Motility test	Temperature (℃)								Salt Concentration (%)						
			15℃	20℃	25℃	30℃	40℃	45℃	50℃	55℃	2.5%	3.5%	4.5%	5.5%	6.5%	7.5%	8.5%
LABADI6	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
LABADI17	-	-	-	-	-	+	+	+	-	-	+	+	+	-	-	-	-
LABADI31	+	-	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-
LABASI49	-	-	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-
LABASI54	+	-	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-
LABASI83	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-

Keynote: LABADI= Lactic acid bacteria isolated from Adama city

LABASI=Lactic acid bacteria isolated from Asella town

"+" indicates positive "-" indicates negative

4.6 Proteomic identification (MALDI–TOF MS)

The six LAB isolates were subjected to MALDI-TOF MS analysis (LABADI6, LABADI17, LABADI31, LABADI49, LABASI54, and LABASI83 (Table 7). The strains were identified as *P. pentosaceus*, *L. mesenteroides*, *L. lactis*, *L. paracasei*, *L. plantarum*, and *L. mesenteroides* according to the identification list suggested by the MALDI BioTyper 3.0 software (Bruker). The strains' respective identification scores were 2.21, 2.06, 1.95, 1.88, 2.23, and 2.34 for strains LABADI6, LABADI17, LABADI31, LABADI49, LABASI54, and LABASI83 respectively (Table 7).

As per the manufacturer's criteria, this result indicates a high probability of identification and good reliability: a log (score) between 1.70 and 1.99 allowed a probable identification of the probable genus with low confidence; a log (score) between 2.00 and 2.29 indicates secure genus identification; and a log (score) between 2.30 and 3 indicates high probable species identification with high confidence. These results show good reliability and a high probability of identification. The result of this study agrees with the study reported by Gantzias *et al*., (2020), who identified four species containing several subspecies, *La. plantarum*, *La. paracasei*, *L. mesenteroides*, and *L. Lactis* from Algerian raw cow's milk. The score values of the strains of this study were higher than the score values of the strains reported by Abdeslam *et al*., (2019) who isolated from some artisanal dairy products in Algeria. They reported that only four strains out of the twenty isolates identified as *L. plantarum* had scores higher than 2.

Run info:

Run identifier: 211101-1557-1011027865

Comment: Bacteria ID – samples from Adama (any bacteria)

Msganaw Mola

Operator: tof-user@MBT-WIN10

Run creation date/Time: 2024-05-8.

Number of tests: 6

Type: Standard

BTS-QC passed

BTS-QC Position D8:3

Instrument ID: 8604832.05385

Table 7: Rate classification results as determined by Bruker MALDI-MS Biotyper.

Sample Name	Sample ID	Organisms (best match)	Score Value	Organisms (second best match)	Score value
A1 (++) (C)	31859 (Standard)	*Pediococcus pentosaceus*	2.21	*Pediococcus*	1.87
A2 (++) (A)	31687 (Standard)	*Leuconostoc mesenteroides*	2.06	*Leuconostoc*	1.83
A3 (+) (B)	31657 (Standard)	*Lactococcus lactis*	1.95	*Lactococcus*	1.91
A4 (+) (B)	31732 (Standard)	*Lactobacillus paracasei*	1.88	*Lactobacillus*	1.79
A5 (-) (C)	31663 (Standard)	*Lactobacillus plantarum*	2.23	*Lactobacillus pentosus*	2.10
A6 (+) A)	31769 (Standard)	*Leuconostoc mesenteroides*	2.34	*Leuconostoc mesenteroides*	2.01

5. CONCLUSION AND RECOMMENDATION

Unquestionably, probiotic microorganisms are crucial to human health and welfare. Particularly LABADI6, LABADI31, LABADI49, LABASI54, and LABASI83 of the LAB isolated from *Tella* showed growth at 6.5% salt concentration. Additionally, all the LAB isolates showed moderate survivability in GIT-like environments. Furthermore, every single isolated strain exhibited moderate to high adhesion qualities, with LABADI31 being the most proficient in terms of hydrophobicity, auto-aggregation, and co-aggregation. All strains showed evidence of antibiotic susceptibility and antibiotic resistance on two antibiotics, but they also showed antimicrobial activities on four pathogenic bacteria. Nevertheless, more investigation is required to assess their probiotics and possible in vivo effects, as well as complete genome sequencing to identify and examine their antimicrobial resistance genes.

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APPENDICES

Appendix 1: Representative Pictures of samples of *Tella* collected from Adama City and Asella town.

Figure -A_1: Samples of the study collected from Adama City and Asella town Oromia Ethiopia, which are prepared from maize and barley.

Appendix 2: Photos of LAB culture on the anaerobic jar.

Figure -A_2: An anaerobic jar is an instrument used in the production of an aerobic environment. This method of anaerobiosis, as others is used to culture bacteria that die or fail to grow in the presence of oxygen (anaerobes).

Appendix 3: Graphs of the probiotic property tests of LAB isolates.

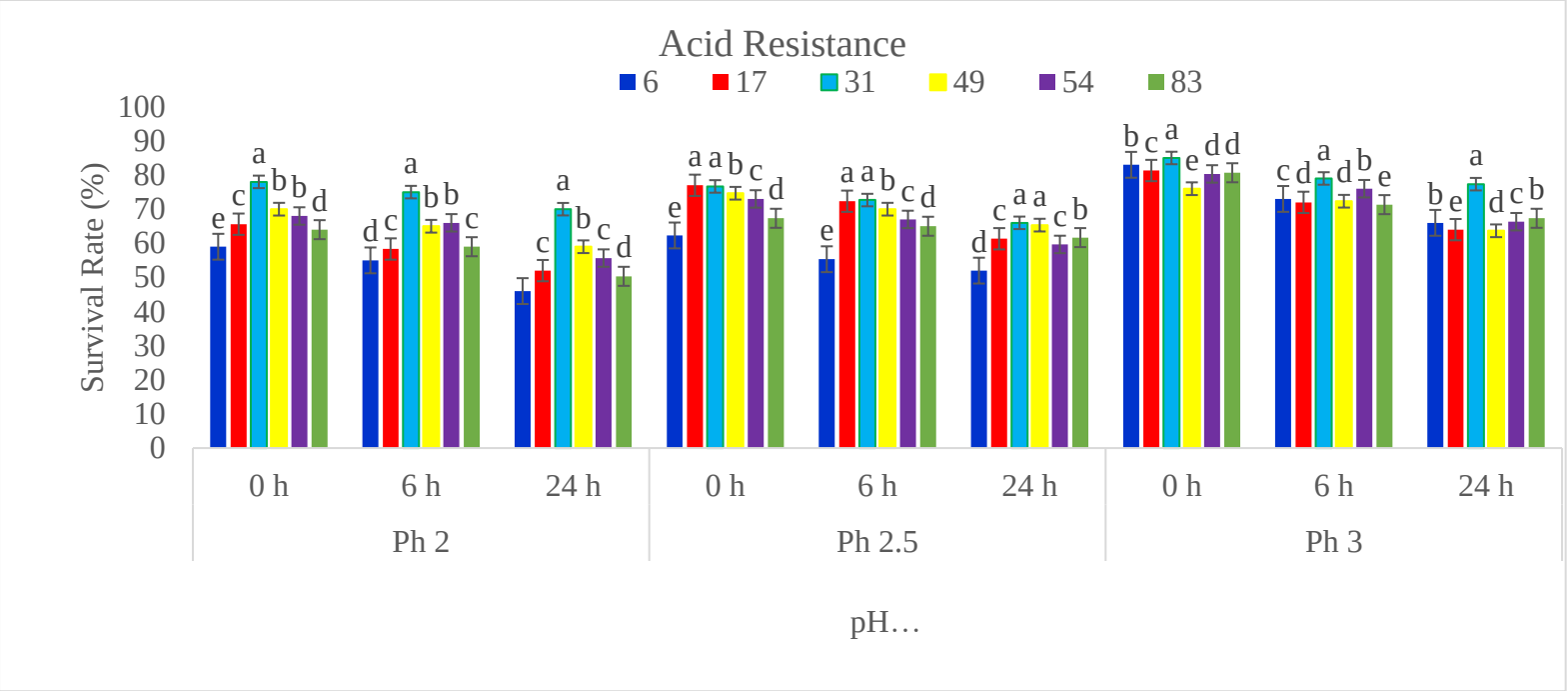

Figure A$_3$: The survival rate of LAB isolates obtained from traditionally fermented Tella samples which are produced in Adama city and Asella town Oromia Ethiopia. Survival of the six isolates in MRS agar plates for 0, 6, and 24 h at 37°C under pH 2, 2.5, and 3 conditions. The data displayed are the mean ± SD of three independent experimental values. Isolate 31 showed the highest survival rate at all three pH levels under these time durations. Different superscript letters (a–d) indicate a significant difference ($p < 0.002$).

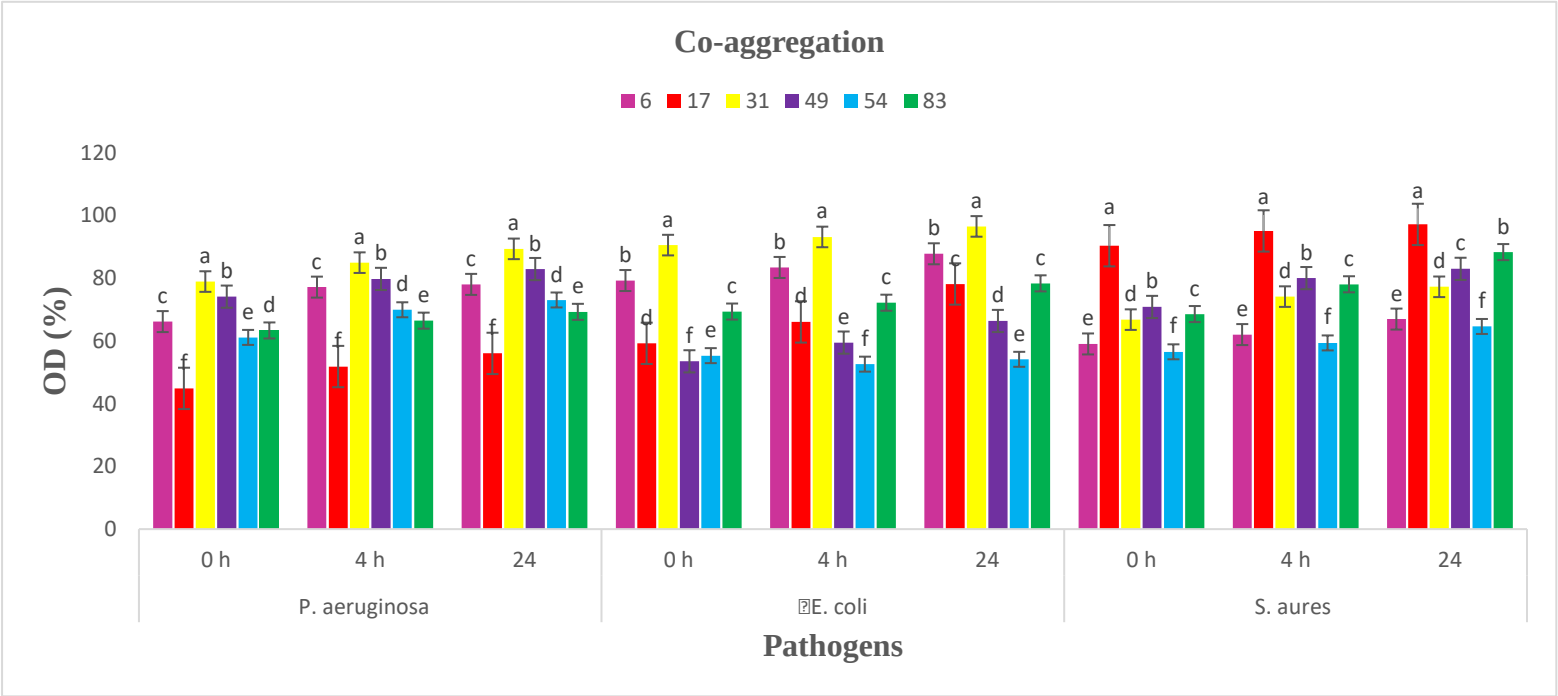

Figure-A$_4$: Percentage of the isolates' co-aggregation against the three pathogens for 0, 4, and 24 hours of incubation at 37°C. P < 0.05 indicated that the mean ± SD values were significant. Different superscript letters (a–f) indicate a significant difference (p < 0.001).

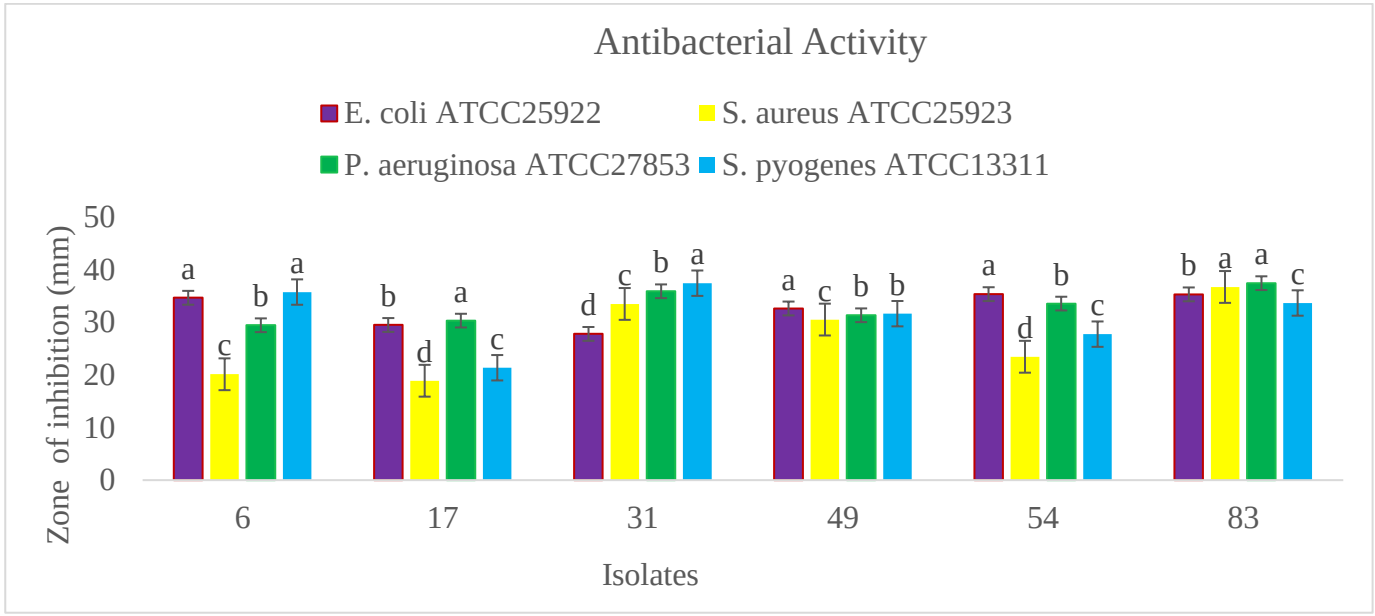

Figure-A_5: The antibacterial activity of LAB strains' cell-free supernatants obtained from *Tella* samples was tested against four pathogenic strains obtained from Adama Public Health Research and Referral Laboratory (APHRRL), which were represented as the millimeter-scale zone of inhibition against four pathogenic bacteria: *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes* after 24 h incubation at 37°C. All the LAB isolated in this study showed a maximum inhibition zone on *P. aeruginosa* than the three pathogenic bacteria. The mean ± SD values were significant at p <0.04.

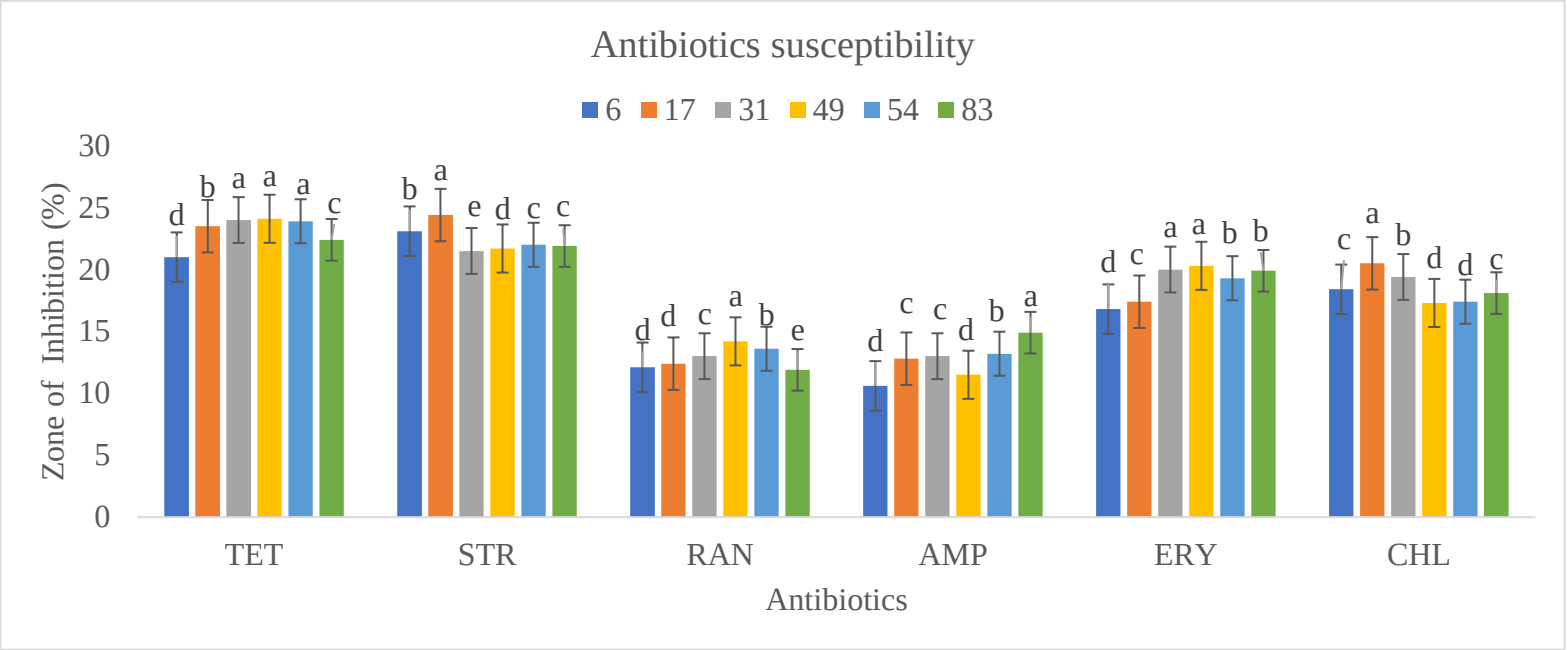

Figure-A_6: Antibiotic susceptibility test for lactic acid bacteria isolated from Ethiopian traditionally fermented *Tella* samples against the six antibiotics such as ampicillin, chloramphenicol, erythromycin, kanamycin, tetracycline, and streptomycin. The result showed that all isolates were resistant, susceptible, and intermediate to two antibiotics. The data displayed are the mean ± SD of three independent experimental values.

Appendix 4: Tables of probiotic properties of LAB isolated from *Tella* samples.

Appendix 4.1 Hydrophobicity properties of lactic acid bacteria (LAB) isolate.

	Hydrophobicity					
	Xylene			Chloroform		
Isolates	0 h	6 h	24 h	0 h	6 h	24 h
LABADI6	84.6±0.3^{c}	81.4±0.365^{b}	79.5±0.4^{a}	67.23±0.230^{d}	62.57±0.204^{d}	57.47±0.35^{b}
LABADI17	99.73±0.199^{a}	76.63±0.025^{c}	58.43±0.477^{c}	83.96±0.627^{b}	75.7±0.428^{a}	57.03±1.15^{b}
LABADI31	89.52±0.145^{b}	86.07±0.850^{a}	76.1±0.264^{b}	76.73±0.288^{c}	69.23±0.404^{c}	52.38±0.135^{c}
LABADI49	76.93±1.295^{d}	67.33±0.404^{d}	48.17±0.282^{e}	54.36±0.230^{f}	52.5±0.4^{f}	50.5±0.4^{d}
LABASI54	72.11±0.06^{e}	60.47±0.151^{e}	54.83±0.141^{d}	96.6±0.655^{a}	74.070.925^{b}	60.33±0.148^{a}
LABASI83	63.61±0.413^{f}	57.7±0.2^{f}	50.75±0.193^{e}	58.35±0.152^{e}	56.41±0.208^{e}	46.01±0.787^{e}

Data represent the mean ± standard deviation of three independent experiments. Different superscript letters (a–f) represent significant differences ($p < 0.01$) among the means within the same column.

The surface characteristics of microbial cells, such as the presence of hydrophobic substances (e.g. lipids and proteins) on the cell surface, have an impact on hydrophobicity. Variations in the hydrophobicity of isolates can result from variations in the location and content of these molecules. Measurements of hydrophobicity frequently entail interactions between microorganisms and solvents or substrates that are hydrophobic. The results of the hydrophobicity test may differ depending on changes in the test substrate's characteristics or composition.

Appendix 4.2 Auto-aggregation properties of lactic acid bacteria (LAB) isolate.

	Auto aggregation		
Isolates	0 h	6 h	24 h
LABADI6	56.2 ± 0.2^{b}	64.6 ± 0.360^{d}	96.4 ± 0.264^{a}
LABADI17	45.5 ± 0.1^{d}	66.56 ± 0.416^{c}	86.33 ± 0.564^{d}
LABADI31	74.2 ± 0.305^{a}	85.26 ± 0.493^{a}	93.23 ± 0.493^{b}
LABADI49	54.7 ± 0.251^{c}	73.1 ± 0.644^{b}	89.6 ± 0.3^{c}
LABASI54	39.4 ± 0.351^{e}	57.0 ± 0.230^{e}	75.3 ± 0.360^{e}
LABASI83	28.6 ± 0.550^{f}	56.5 ± 1.001^{f}	72.51 ± 0.378^{f}

Data represent the mean ± standard deviation of three independent experiments. Different superscript letters (a–f) represent significant differences ($p < 0.003$) among the means within the same column.

Appendix 4.3 Bile salt tolerance (0.3%, 0.75%, and 1% bile salt concentration) of LAB strains (viability of cells after exposure–log CFU/mL)

	Bile salt tolerance					
	3 h			6 h		
Isolates	0.3%	0.75%	1%	0.3%	0.75%	1%
LABADI6	71±0.156 ^{e}	68.13±0.026^{d}	62.7±0.126 ^{e}	63.33±0.154^{e}	61±0.178^{e}	59.36±0.553^{e}
LABADI17	80.43±0.750^{d}	73.87±0.808^{c}	70.67±0.527 ^{d}	76.33±0.081^{d}	69.67±0.516^{d}	59.3±0.608^{e}
LABADI31	88.33±0.081 ^{b}	81.8±0.928^{b}	73.57±0.227 ^{c}	85.5±0.802^{b}	80.33±0.309^{b}	69.67±0.055^{d}
LABADI49	86.33±0.577^{c}	82.17±0.288^{b}	81.6±0.608 ^{b}	81±0.708^{c}	80.43±0.665^{b}	78.97±0.080^{c}
LABASI54	90.5±0.435^{a}	88.36±0.547^{a}	87.63±0.025 ^{a}	86.3±0.360^{b}	79.5±0.458^{c}	76.43±0.404^{b}
LABASI83	91.5±0.040^{a}	89.13±0.115^{a}	88.33±0.351 ^{a}	88.8±0.582^{a}	84.33±0.378^{a}	86.36±0.665^{a}

Data represent the mean ± standard deviation of three independent experiments. Different superscript letters (a–e) represent significant differences ($p < 0.03$) among the means within the same column.

Appendix 4.4 LAB bacterial isolate sample source, code, catalase test, microscopic shape, gram staining, and endospore staining.

S. N	Sample sources	Code	Colony color	Catalase test	Gram staining	Endospore staining	Microscopic shape
1	Adama	LABADI1	white	-	+	-	Rod
2	Adama	LABADI2	creamy	-	+	+	Cocci
3	Adama	LABADI3	creamy	-	+	+	Rod
4	Adama	LABADI4	white	-	+	+	Rod
5	Adama	LABADI5	white	-	+	+	Rod
6	Adama	LABADI6	white	-	+	-	Spherical cocci
7	Adama	LABADI7	creamy	-	-	+	Rod
8	Adama	LABADI8	creamy	-	+	-	Cocci
9	Adama	LABADI9	white	-	+	-	Cocci
10	Adama	LABADI10	creamy	-	+	+	Rod
11	Adama	LABADI11	creamy	-	+	+	Cocci
12	Adama	LABADI12	white	-	+	+	Rod
13	Adama	LABADI13	white	-	+	-	Rod
14	Adama	LABADI14	creamy	-	+	-	Cocci
15	Adama	LABADI15	white	-	+	+	Cocci
16	Adama	LABADI16	creamy	-	+	-	Rod
17	Adama	LABADI17	white	-	+	+	Rod
18	Adama	LABADI18	white	-	+	-	Rod
19	Adama	LABADI19	white	-	+	+	Cocci
20	Adama	LABADI20	white	-	+	-	Cocci
21	Adama	LABADI21	creamy	-	+	+	Rod
22	Adama	LABADI22	white	-	-	+	Cocci
23	Adama	LABADI23	creamy	-	+	-	Cocci
24	Adama	LABADI24	creamy	-	+	+	Rod
25	Adama	LABADI25	white	-	+	-	Cocci
26	Adama	LABADI26	creamy	-	+	-	Cocci
27	Adama	LABADI27	creamy	-	+	+	Cocci
28	Adama	LABADI28	white	-	-	+	Rod
29	Adama	LABADI29	creamy	-	+	+	Rod
30	Adama	LABADI30	white	-	+	+	Cocci
31	Adama	LABADI31	white	-	+	-	spherical or ovoid
32	Adama	LABADI32	white	-	+	-	Cocci
33	Adama	LABADI33	creamy	-	+	+	Rod
34	Adama	LABADI34	creamy	-	+	-	Cocci
35	Adama	LABADI35	white	-	+	+	Rod
36	Adama	LABADI36	creamy	-	-	+	Rod
37	Adama	LABADI37	white	-	+	+	Cocci
38	Adama	LABADI38	white	-	+	-	Rod

39	Adama	LABADI39	white	-	+	+	Rod
40	Adama	LABADI40	white	-	+	+	Rod
41	Adama	LABADI41	creamy	-	+	-	Cocci
42	Adama	LABADI42	white	-	+	-	Cocci
43	Adama	LABADI43	creamy	-	+	+	Rod
44	Adama	LABADI44	white	-	+	+	Cocci
45	Adama	LABADI45	creamy	-	+	-	Cocci
46	Assela	LABASI46	white	-	+	-	Cocci
47	Assela	LABASI47	creamy	-	+	+	Rod
48	Assela	LABASI48	white	-	+	-	Cocci
49	Assela	LABASI49	creamy	-	+	-	Rod
50	Assela	LABASI50	white	+	+	+	Rod
51	Assela	LABASI51	creamy	-	-	-	Rod
52	Assela	LABASI52	creamy	-	+	+	Cocci
53	Assela	LABASI53	white	-	+	-	Rod
54	Assela	LABASI54	creamy	-	+	+	Rod
55	Assela	LABASI55	creamy	-	+	-	Cocci
56	Assela	LABASI56	white	-	+	+	Rod
57	Assela	LABASI57	white	-	+	+	Rod
58	Assela	LABASI58	white	-	+	+	Cocci
59	Assela	LABASI59	creamy	-	+	+	Rod
60	Assela	LABASI60	white	-	+	-	Rod
61	Assela	LABASI61	creamy	-	+	+	Cocci
62	Assela	LABASI62	white	-	+	-	Rod
63	Assela	LABASI63	creamy	-	+	-	Cocci
64	Assela	LABASI64	white	-	+	+	Rod
65	Assela	LABASI65	creamy	-	+	+	Cocci
66	Assela	LABASI66	white	-	+	+	Cocci
67	Assela	LABASI67	creamy	-	-	-	Rod
68	Assela	LABASI68	creamy	-	+	+	Cocci
69	Assela	LABASI69	white	-	+	+	Rod
70	Assela	LABASI70	white	-	+	-	Cocci
71	Assela	LABASI71	white	-	+	+	Cocci
72	Assela	LABASI72	white	-	+	-	Rod
73	Assela	LABASI73	creamy	-	+	+	Cocci
74	Assela	LABASI74	white	-	+	+	Cocci
75	Assela	LABASI75	creamy	-	+	-	Cocci
76	Assela	LABASI76	white	-	+	-	Rod
77	Assela	LABASI77	white	-	+	+	Cocci
78	Assela	LABASI78	creamy	-	+	+	Cocci
79	Assela	LABASI79	creamy	+	+	+	Rod
80	Assela	LABASI80	white	-	+	-	Rod
81	Assela	LABASI81	white	-	+	+	Cocci
82	Assela	LABASI82	creamy	-	+	-	Cocci
83	Assela	LABASI83	creamy	-	+	-	Rod
84	Assela	LABASI84	white	-	+	+	Cocci

85	Assela	LABASI85	white	-	+	-	Rod
86	Assela	LABASI86	creamy	-	+	+	Cocci
87	Assela	LABASI87	white	-	+	-	Rod
88	Assela	LABASI88	white	-	+	+	Rod
89	Assela	LABASI89	creamy	-	+	+	Rod
90	Assela	LABASI90	creamy	-	+	+	Cocci

Keynote: LABADI= Lactic acid bacteria isolated from Adama city

LABASI=Lactic acid bacteria isolated from Asella town

"+" indicates positive "-" indicates negative