



**PROBIOTIC AND BACTERIOGINOGENIC POTENTIAL OF
LACTIC ACID BACTERIA OF FERMENTED FOODS AND
BEVERAGES ORIGIN**

Ph.D. DISSERTATION

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BACTERIA OF FERMENTED FOODS AND BEVERAGES ORIGIN**

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LIST OF ACRONOMYS/ABBREVIATIONS

AAB	Acetic Acid bacteria
ATCC	American Type Culture Collection
BSH	Bile Salt Hydrolase
CFS	Cell Free Supernatant
Cfu	Colony Forming units
CHCA	Cyno-4-hydroxycinnamic acid
DNase	Deoxyribonuclease
DGGF	Denaturing Gradient Gel Electrophoresis
DHB	2, 5-dihydroxybenzoic acid
EPHI	Ethiopian public Health Institute
ETFFB	Ethiopian traditional fermented food and beverages
GC-MS	Gas chromatography–mass spectrometry
GIT	Gastro-intestinal Tract
GRAS	Generally Recognized as Safe
HPLC MS	High Performance Liquid Chromatography–Mass Spectrometry
JU	Jimma University
LAB	Lactic acid bacteria
MALDI-TOF	Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry
Mg	Microgram
MHA	Muller Hintor Agar
MRS	De Man, Rogosa and Sharpe agar
MS	Mass Spectrometry
NGS	Next Generation Sequencing
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PGA	Polyglutamic Acid
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
Rpm	Revolution per minutes
SGJI	Simulated Gastro-intestinal juice

SIJ	Simulated intestinal juice
SPSS	Statistical Package for the Social Sciences
SR	Survival rate
SSDP	Simulated stomach Deudnum passage
TRFLP	Restriction Fragment Length Polymorphism
WGS	Whole Genomic Sequance

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Probiotic and Bacteriocinogenic Potential of Lactic Acid Bacteria of Fermented Foods and Beverages Origin

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GENERAL ABSTRACT

*Food quality, safety, flavor, and aroma are improved by metabolites produced by lactic acid bacteria (LAB) that include organic acids, bacteriocins, and hydrogen peroxides. The metabolites could inhibit the growth of food borne pathogens and spoilage microorganisms in foods. Furthermore, LAB play a crucial role in the fermentation process by giving unique flavors and textures to food products, prolonging its shelf life, emhancing safety and quality. LAB also have probiotic roles that effectively combat foodborne pathogens. LAB have gained attention for bacteriocin production and their biopreservative properties. Thus, the objective of this study was to isolate and characterize LAB retrieved from Ethiopian traditional fermented foods and beverages for their probiotic, bacteriocin production, and preservative activities. In addition, the study was conducted to assess the bio-preservative potential of LAB in milk, fruits, and vegetables. Traditional fermented foods and beverages were collected from East Wollega Zone and Jimma Town. About, 180 samples, 30 each (kotcho, bulla, borde, shamita, bukuri, and ergo) were collected and transported to Jimma University's Microbiology Laboratory for isolation of LAB following standard methods. LAB isolates were characterized using **morphological, biochemical, physiological, and MALDI ToF/Ms techniques**. Concurrently, the effects of acid, temperature, enzymes, and food preservatives on the antagonistic activities of LAB strains were evaluated. The study further evaluated potential probiotic LAB based on their tolerance to bile salt and acid, gastrointestinal simulation, auto- and co-aggregation, hydrophobicity, antibiotic resistance, bacteriocin production, and bio-preservation activity. The bacteriocin assay was evaluated at various temperatures, incubation times, and growth medium to determine the optimal conditions for bacteriocin production. Solvent extraction methods for concentrating of bacteriocins were developed, and various extraction procedures were tested for successful bacteriocin recovery. The ability of the LAB isolates to prolong the shelf life of test foods was evaluated. About, 956 LAB isolates were recovered from 180 traditional fermented food and beverage samples. As revealed from phenotypic and morphological characteristics, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, and *Enterococcus* species dominated all the six traditional fermented foods and beverages. *Lactobacillus* was the most abundant genus with even distribution in all the traditional fermented foods and beverages. Bulla harbored the highest number of LAB communities, followed by Ergo. Over 580 of the 956 isolates showed strong inhibitory activity against selected food borne pathogens. Furthermore, 340 LAB had antagonistic effects against all the selected food borne pathogens. Using MALDI-ToF/MS analysis, the majority of the isolates were identified as *Pediococcus* species (*P. acidilactici* and *P. pentosaceus*) followed by *Enterococcus* species mainly *E.**

faecium (n = 6), *Lactococcus lactis* (n = 2), *Bacillus* species (*B. infantis*), and *Rothia dentocariosa* (n=1)). *Pediococcus pentosaceus* (23.33%) dominated the microbial ecology of all the selected Ethiopian traditional fermented foods and beverages followed by *Enterococcus faecium* (20%), and the least common species was *Lactococcus lactis* (6.67%). All of the isolates, 17 of them showed potential capacity to produce antimicrobial activity, putative bacteriocins, survive in simulated gastrointestinal tracts, and inhibitor substances were all studied in vitro and in artificially simulated environments. Out of 340 that demonstrated potential antagonistic effects, 44 (13%) of isolates survived at pH 2 and 3 for 3 and 6 h, respectively, with survival rates ranging from 33.825% to 100%. Because they were acid- and bile-tolerant isolates, 125 of the 340 LAB isolates were screened as potential probiotic candidates. Among 125 isolates, 39 (31.20 %) were identified as the best acid and bile salt-tolerant, with the potential capacity to produce bacteriocin. All the thirty-nine promising bacteriocin producers were susceptible to tetracycline (30 g/ml) and chloramphenicol (15 g/ml), but highly resistant to kanamycin (20 g/ml), with some being resistant or susceptible to streptomycin (10 g/ml) and ampicillin (10 g/ml). Additionally, the isolates showed nearly 75%–95% survival rates at low pH (2, 2.5, and 3), bile salt (0.3%), intestinal inhibitor substances, and simulated gastro-intestinal conditions, respectively. The potent isolates also showed strong potency for aggregation (50-120 %) and cell surface hydrophobicity (70-116%). *P. pentosaceus* JULABB16 was identified as the most effective isolate in controlling the spoilage microorganisms and extended the shelf life of tested fruits and vegetables by at least three weeks. Likewise, *Lactococcus lactis* was found to be the potential isolate to maintain the quality of milk. The entire potent and selected LAB demonstrated potential preservative efficiency because they did not cause browning or off-odor on fruits and vegetables for three to four weeks. In conclusion, this study demonstrates the significant potential of lactic acid bacteria (LAB) to extend the shelf life of fruits, milk, and vegetables. The research has established a foundation for the selection of specific antimicrobial peptides and the further development and application of LAB for antibacterial and probiotic activities. The findings underscore the efficacy of certain LAB isolates in enhancing the longevity and safety of various food products. Moving forward, further research should focus on the practical applications of these LAB isolates in food preservation strategies and probiotic formulations, aiming to leverage their full potential in extending shelf life and promoting health benefits.

Key words: Antimicrobial activity, Probiotic, Bacteriocinogenic Potential, Lactic Acid Bacteria, Fermented Foods, Fermented Beverages.

List of Publications and Manuscripts Extracted from the study

The following articles and manuscripts are extracted from the current study

- 1) **Amenu, D., Bacha, K. (2024).** Antagonistic Effects of Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Foods and Beverages Against Foodborne Pathogens. *Probiotics & Antimicro. Prot.* (2024). <https://doi.org/10.1007/s12602-024-10231-5>. Published
- 2) **Amenu, D., Bacha, K. (2023).** Probiotic potential and safety analysis of lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages. *Ann Microbiol* 73, 37. <https://doi.org/10.1186/s13213-023-01740-9>. **Published**
- 3) **Amenu, D., Bacha, K. (2024).** Bio-Preservation Potential and Antimicrobial Activity of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Dairy Products. *Probiotics & Antimicro. Prot.* (2024). <https://doi.org/10.1007/s12602-024-10272-w>. **Published**
- 4) **Amenu, D., & Bacha, K. (2024).** Isolation and Characterization of Bacteriocin Producing *Enterococcus* species from Ethiopian Traditional Fermented Food and Beverages. Submitted to *BMC Research Notes* (**Under review**).
- 5) **Amenu, D., & Bacha, K. (2024).** Shelf-Life Enhancement of Fruits and Vegetables Using Lactic Acid Bacteria isolated Ethiopian traditional fermented foods and beverages. **Submitted to *Probiotics and Antimicrobial Proteins* (Under review)**

1. ● GENERAL INTRODUCTION

1.1. Background of the study

Fermentation is a natural phenomenon that occurs due to the natural activity of microbes in spontaneous fermented products. Among the most important groups of microorganisms involved in the natural fermentation of foods and beverages are basically lactic acid bacteria (LAB) and yeasts (Ashenafi 2006; Karssa *et al.*, 2014; Wambugu 2015). Furthermore, traditional fermentation, has long been used and performed in rural communities as a food production and preservation strategy(Ashenafi, 2008).

African traditional fermented foods and beverages contain also live microbes that can produce beneficial substances for our health such as antimicrobial peptides, essential minerals, or molecules with antioxidant activity as well as probiotic strains (Diaz et al .,2019; Franz et al.,2014). These fermentation process are mainly carried out by LAB that are designated as "generally recognized as safe" (GRAS) (Mokoena et al., 2016). They metabolize carbohydrates into lactic acid, resulting in a lowering of pH, which in turn reduces the number of food-borne pathogens and spoilage microbes. Foodborne diseases are serious and costly issues in the food industry; and calls for the designing of strategies for prevention and control methods such as the use of probiotic LAB and their metabolites as bio-preservatives and biocontrol agents. These approaches have shown promising results in inhibiting the growth of pathogenic/spoilage bacteria and can extend the shelf life of food products (Moore et al., 2018).

Antibiotic applications are popular clinical treatments for the control of many foodborne pathogen infections. However, extensive use of antibiotics encourage the development of antibiotic resistance among microbial pathogens (Castillo et al., 2012; Nair et al., 2008). Accordingly, a proper food safety management system should be developed in order to estimate the risks to human health from food

consumption and to identify, select, and implement mitigation techniques to control and reduce these risks (Lee., 2009; Wray et al., 2018). To this end, different strains of microorganisms with potential probiotic properties have been discovered and reported (Dowarah et al., 2018; Tadesse et al., 2005; Tesfaye et al., 2011). Besides their application as promising food preservatives, the consumption of a sufficient number of these live beneficial microorganisms together with food and beverages fundamentally enhance consumer health while also lowering the risks of foodborne diseases/spoilage microbes (Mills et al., 2011). The search for more efficient and stable probiotic microbes could continue using improved and state-of-the art techniques for their proper identification.

LAB are among the frequently used probiotic bacteria. They are non-pathogenic, tolerant to high acid and bile salt, produce several antimicrobial compounds (Mokoena *et al.*, 2016) and designated as GRAS. Probiotic LAB may prevent food spoilage and pathogenic microorganisms through their production of antimicrobial compounds such as acids, hydrogen peroxide, and bacteriocins with bio-preservatives potential (Oelschlaeger, 2010). Furthermore, probiotic LAB contribute to the flavor, aroma, and texture of traditional fermented foods and beverages (O'Bryan et al., 2015; Ricci et al., 2019).

In Ethiopian, probiotic LAB isolated from a few traditional fermented foods and beverages were reported to antagonize several foodborne pathogens and spoilage microorganisms (Dowarah et al., 2018; Mulaw et al., 2019; Negasi et al., 2017; Tadesse et al., 2005; Tesfaye et al., 2011; Tigu et al., 2016). The antagonistic action of LAB strains, whether pure or defined mixed cultures could be more efficient against some foodborne pathogens during fermentation and storage.

Several studies have been conducted to identify possible antibacterial-producing probiotic LAB as well as the microbial diversity of Ethiopian fermented foods and beverages (Berhe et al., 2019; Mulaw et al., 2019; Negasi et al., 2017; Tesfaye et al., 2011; Tigu et al., 2016). However, there is a few research studies made to evaluate the microbial ecology of traditional fermented products to identify potential probiotic LAB species for starter culture development and biopreservative potential. Understanding genetic diversity and molecular characterization of potential probiotic LAB species is crucial for identification of suitable and safe starter cultures for application in food and beverage industries.

The majority of Ethiopia's traditionally fermented food/beverage products are produced without further heat processing, making them a perfect vehicle for delivering of probiotic LAB into the human gastrointestinal tract. Probiotic LAB strains found in traditionally fermented foods and beverages may be useful as a starter culture for large-scale production of the traditionally fermented products as well as having a desirable functional characteristic for their use as probiotics against food-borne infections. To this end, the main objective of this study was to explore the potential of microbes isolated from Ethiopian traditional fermented foods and beverages to study their probiotic potential and other other bio-preservative efficiency towards shelf life extension of some preshible products.

1.2. Statement of the problem

Foodborne pathogens can persist and proliferate at low temperatures, creating challenges for low-temperature food preservation by increasing microbial risks. Chemical preservatives, while useful, have limitations and can pose health risks if their concentrations are not carefully controlled. Concerns over side effects such as cancer, cardiovascular diseases, and aging have led modern consumers to favor natural preservatives with less processed foods/beverages (Sahoo et al., 2022). Many foodborne outbreaks have been caused by *Listeria monocytogenes*, *Salmonella Typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, and molds like *Aspergillus* spp and *Botrytis* spp (Moghadam & Larsen, 2019). Hence, resistance of these pathogens to widely used chemical preservatives has created challenges to food and beverage industries and calls for safe management strategies using beneficial microbes (Bintsis, 2018).

Due to the aforementioned concerns about chemical preservatives, there has been a renewed interest in using beneficial LAB and their key metabolites as natural food preservatives (Kuniyoshi et al., 2021). These natural preservatives can reduce food-borne illnesses without posing health risks. Probiotic LAB and their antimicrobial metabolites such as bacteriocins inhibit spoilage and pathogenic bacteria, while enhancing food quality (De La Fuente et al., 2015). This study aims to isolate, identify, and characterize beneficial LAB from Ethiopian traditional fermented foods and beverages to explore their probiotic properties and biopreservative potential of some selected preshible food staff.

1.3. Objectives

1.3.1. General objective

The general objective of this study was to evaluate the probiotic properties, bacteriocin producing and biopreservative potential of **Lactic Acid Bacteria** from Ethiopian traditional fermented foods and beverages for their industrial application.

1.3.2. Specific objectives

The specific objectives of the current study were to

- 1) **Isolate and characterize** Lactic Acid Bacteria retrieved from some selected Ethiopian traditional fermented foods and beverages,
- 2) **Evaluate the antimicrobial activity** of Lactic Acid Bacteria isolated from some selected Ethiopian traditional fermented foods and beverages,
- 3) **Assess the probiotic potential** of Lactic acid bacteria isolated from some Ethiopian traditional fermented foods and beverages
- 4) **Screen bacteriocin producing** capacity of Lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages
- 5) **Evaluate Shelf life extension of fruits and vegetables** using Lactic Acid Bacteria isolated from selected Ethiopian traditional fermented foods and beverages,
- 6) **Evaluating** the antimicrobial activity and biopreservative potential of LAB isolated from Ethiopian traditional fermented dairy product (*ergo*)-on raw milk collected from Jimma Town.

2. ● GENERAL LITERATURE REVIEW

2.1. Overview of fermented foods and beverages

Fermentation, an ancient practice across cultures worldwide, enriches diets by enhancing food's nutritional value through microorganisms-driven synthesis of proteins, vitamins, and amino acids (Zhang et al. 2016). This process historically provided sustenance for rural populations and remains crucial for food security, especially for marginalized communities (Marshall and Mejia, 2012). Overall, fermentation stands as one of the oldest and cost-effective ways to preserve food safety and quality.

Traditional fermentation of foods and beverages is a crucial economic activity globally, especially in the informal sector and supports livelihoods for many families and commercial endeavors (Thairu et al., 2014). This indigenous knowledge is often passed down orally, limiting its widespread documentation. Consequently, traditional fermentation practices are primarily common in rural and tribal cultures due to factors related to cost, accessibility, preferences, and social ties to these products (Paulos et al. 2010).

Traditional fermented foods and beverages exhibit unique characteristics as influenced by environmental factors, culture, tastes, raw material availability, and technological advancements (Nair et al., 2008). The diversity of microorganisms involved in fermentation shapes these products' qualities (Vogel et al., 2011). These microbes can be native to the food or added intentionally as starter cultures in industrial processes (Stevens et al., 2009). Some of these beneficial organisms produce compounds with food additive potential or enhance fermentation processes naturally (Longo, 2006).

Traditional fermented foods and beverages undergo *ergo* spontaneous fermentation, leading to variability in their microbiological and chemical composition (Bacha et al., 2010). The process relies on

microorganisms present in substrates, utensils, or from previous batches, influencing the final product and its quality (Steinkraus, 2002). These microbes biochemically transform the substrates into culturally accepted edible products. Key components of the fermentation ecosystem include organic matter, microbiota (bacteria, yeasts, and molds), the fermentation solution, and the fermenting container (Stolier et al., 2008).

2.2. Types of fermentation

Fermentation remains an energy-efficient and cost-effective method crucial for food preservation and safety, extending shelf life (Sadat Kamal Amit†, Md. Mezbah Uddin†, Rizwanur Rahman, 2017). Despite modern technologies, fermentation remains fundamental in food production. Different types such as lactic acid, acetic acid, alkaline, and alcoholic fermentation are commonly used, depending on the desired food products (Anal, 2019) (Figure 2.1).

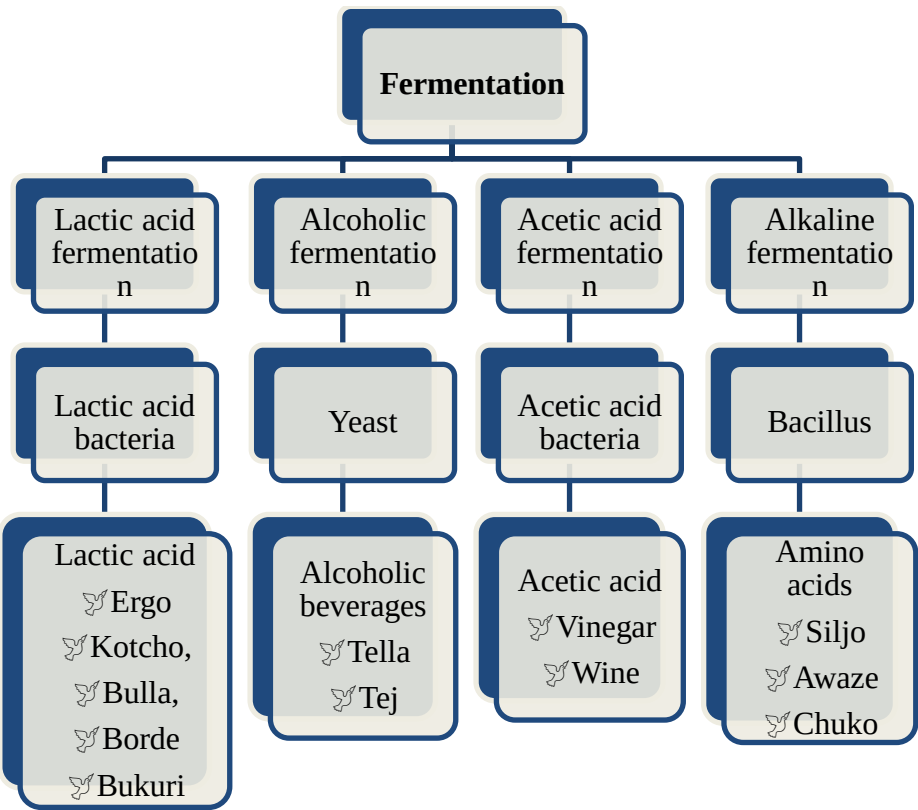


Figure 2. 1. Illustrats the schematic representation of types of fermentation, the microorganisms involved, and the resulting end products (Anal, 2019).

2.2.1. Lactic acid fermentation

Lactic acid bacteria are crucial for lactic acid fermentation, playing key roles in food preservation and production without requiring much heat, making them cost-effective. The quality and characteristics of these fermented foods depend on microbial actions influenced by factors like water activity, pH, salt, temperature, and food composition (Blandino et al., 2003). While various microorganisms participate in fermentation, specific types significantly impact product quality. The development of microbial communities in food is guided by ecological factors, affecting the physiological behavior of LAB (Park et al., 2018). Although the number of microorganisms in food are small, their impact on flavor and other sensory aspects is significant (Okafor et al., 2009).

Fermented foods are a significant part of diets worldwide, constituting 25% in developed nations and 60% in many developing countries (Ogunbanwo et al., 2003). Ethiopia, with its diverse cultural backgrounds, has a rich variety of traditional fermented foods and beverages like *ergo*, *ayib*, *qibe*, *arrera* (Gonfa et al., 2001), *kotcho*, *siljo* (Mehari and Ashenafi, 1995), *awaze*, *datta* (Idris et al., 2001) and *tella*, (Derbew et al., 2013), *borde* and *shamita* (Bacha et al., 2011), *tej*, *borde* and *tella* ((Nemo & Bacha, 2020), *cabbage-shamita*, *bukuri* and *kocho* (Amenu & Bacha, 2024).

Lactic acid bacteria play a crucial role in food fermentation by rapidly acidifying the environment through the production of lactic acid. Additionally, they generate various metabolites such as acetic acid, ethanol, aromatic compounds, bacteriocins, exopolysaccharides, and enzymes. These compounds not only enhance the texture and sensory attributes of fermented products but also contribute to their extended shelf life and microbiological safety. A wide range of fermented foods and beverages, including dairy products, meat and fish products, fresh vegetables, cereals, and alcoholic/non-alcoholic beverages, are primarily the result of lactic acid fermentation (Rhee et al., 2011). Different species of *Lactobacillus* are commonly utilized in traditional fermentation practices for items like meat, vegetables, and dairy due to their ability to produce lactic acid. The adoption of these probiotic strains in industrial food technology is justified by their role in improving the conversion process and enhancing the flavor and texture properties of fermented foods (Anal, 2019). Figure 2.2 illustrates the metabolic pathways carried out by lactic acid bacteria.

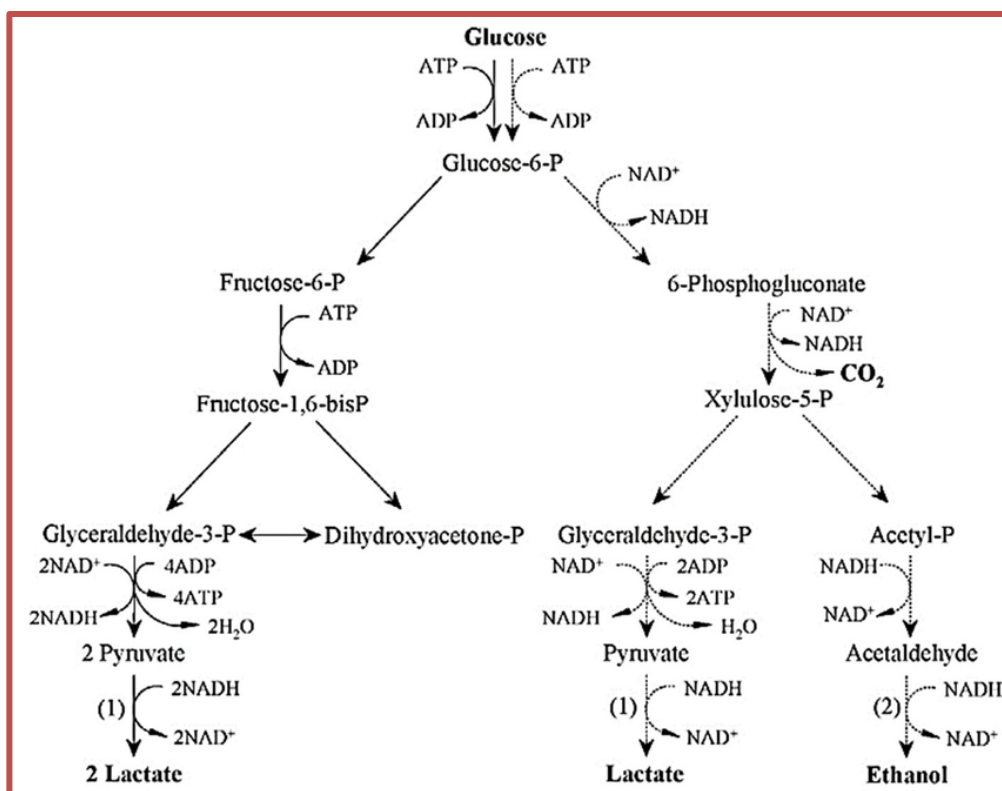


Figure 2. 2. Metabolic pathways of homofermentative and heterofermentative LAB (Basso et al. 2014).

Metabolic pathways of homofermentative (solid line) and heterofermentative (dotted line) LAB: P, phosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; NAD⁺, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); (1), lactate dehydrogenase; (2), alcohol dehydrogenase.

2.2.2. Alcoholic Fermentation

Alcoholic fermentation, an ancient and crucial food processing method, has been used since ancient times to produce various alcohol-based beverages like beers, wines, and spirits. This process involves microorganisms such as yeast, like *Endomycopsis*, and bacteria like *Zymomonas mobilis* fermenting sugars or starch hydrolysates to produce ethanol and carbon dioxide (Anal, 2019). In Africa, sorghum-based beers are extensively produced, dolo in Burkina Faso (Sawadogo-Lingani et al., 2021). Ethiopia is known for traditional fermented beverages like *tella* (Abegaz, 2014), *borde* (Abegaz et al., 2007) and *shamita* (Bacha et al., 1999).

2.2.3. Acetic Acid Fermentation

Fermented foods and beverages often contain acetic acid bacteria (AAB), like *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter*. They oxidize carbohydrates, sugars, alcohols, and ethanol in food substrates to acetic acid. Despite their significance, AAB are not as extensively studied as other industrial bacteria and can spoil alcohol production (De et al., 2018). According to Awulachew (2021), the dominant species in acetic acid fermentation are *S. cerevisiae* and *L. pastorianum*. *Tella*, with a pH of 4.5-4.8 and 2.8-5.0% (v/v) ethanol, is considered the best for consumption. However, excessive growth of *Acetobacter* spp. during aerobic fermentation can make *?tella?* too sour to drink (Awulachew, 2021).

2.2.3. Alkanie Fermentation

Alkaline fermentation involves *Bacillus* species and other bacteria to break down proteins into peptides and amino acids, leading to a pH increase to 8-10. This process is crucial in regions where starchy foods are staple but lack diversity in nutrients (Samedi & Charles, 2019). The common *Bacillus* species found in legume-based fermented foods include *B. amyloliquefaciens*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis*, *B. subtilis* var. *natto*, and *B. thuringiensis*, while *B. cereus* strains are associated with *Prosopis* fermentation. Some *B. subtilis* strains produce polyglutamic acid (PGA), contributing to the sticky texture of Asian fermented soybean dishes (Elshaghabe et al., 2017; Owusu et al., 2022).

2.3. Functions of fermented foods and beverages

Further research is essential to ensure the safety and nutritional value of traditional fermented foods and beverages, which are increasingly vital due to population growth and food insecurity. The wide array of fermented foods available globally highlights their significance not just for preservation and safety but also for their valued sensory properties (Holzapfel, 2002). In developing countries, millions rely on fermentation for affordable food preservation (Kalui et al., 2010). Because, the fermentation improves digestibility, flavor, stability, and nutritional value, along with reduced food toxicity, cooking time, energy requirements, and extended shelf life (Parkouda et al., 2009).

2.3.1. Nutritional Quality

Fermented products, typically maintained at 22-25°C, encourage microorganisms and metabolic activity due to their acidic nature (Mokoena et al.,2016). LAB fermentation not only boosts enzyme activity but also reduces anti-nutrients like phytic acid and tannins in these foods, enhancing the availability of minerals like iron, protein, and simple sugars(Chelule et al., 2010).Compared to unfermented foods, fermented ones are generally more nutritious (Oladeji et al.,2018). Many fermentative microorganisms involved in traditional fermentation processes break down complex compounds and produce complex vitamins and growth factors, elevating the nutritional value of the foods and beverages Mahlia et al., 2014).

2.3.2. Enhancement of Sensory Quality

Fermentation enhances food and beverage organoleptic features such as flavors, smells, and textures, adding new sensory properties to the diet and making the food more palatable and, ultimately, more popular than unfermented food (Blandino et al.,2003; Chelule et al.,2010). These organoleptic properties made fermented products more popular than unfermented ones in terms of consumer acceptance (Blandino et al., 2003). Thus, fermentation is unique in that it modifies the unfermented food in diverse ways, resulting in new sensory properties in the fermented product(De Vuyst & Leroy, 2007).

2.3.3. Bio-preservation potential

The preservative effect of fermented food and beverages is due to several antimicrobial compounds produced by LAB during fermentation process (Santos et al., 2018). These bacteria also produce antimicrobial metabolites, including organic acids, hydrogen peroxides and bacteriocin (Chelule et al.,2010; Santos et al. 2018) which can increase the bio-preservative potential of LAB. In general, many fermented foods and beverages have good keeping qualities and can be kept without refrigeration for longer than fresh, unfermented foods. Fermentation increases the shelf life and decreases the need for refrigeration or other methods of food preservation (Ahn et al., 2002; Aidoo et al., 2011).

2.3.4. Health Benefits

Most species of LAB used for the production of fermented foods and beverages are generally recognized as safe (GRAS), thus eliminating any health risk (Endeshaw et al., 2018). Those LAB isolates, largely isolated from traditional fermented foods and beverages are the normal residents of the complex ecosystem of the gastrointestinal tract (GIT) (Tamang, 2003). The probiotic effects and lower levels of pathogenic bacteria found in fermented foods and beverages are especially important in developing countries, where fermented foods and beverages have been shown to reduce the severity, duration, and morbidity of diarrhea. According to a study conducted in the Netherlands and Sweden, regular consumption of fermented dairy products has the potential capacity to lower the incidence of bladder cancer, cholesterol level, (Keszei et al., 2009) and cardiovascular disease (Sonestedt et al., 2011).

2.4. LAB associated with Foods and beverages fermentation

Fermented foods have been part of human diets for over 6,000 years, offering benefits like improved shelf life, safety, digestibility, and nutrition (Ther, 2013). Lactic acid fermentation, where microorganisms convert sugars into lactic acid, is widely used in households and the food industry for preservation and creating various food items (Dorrington & Bowdish, 2013). This natural preservation method is cost-effective and economically vital in developing countries (Moyane et al., 2013). Fermentation of cereals enhances taste, flavor, acidity, and digestibility. In addition, fermented meats and fish, can be sources of probiotic bacteria if processed correctly (Anihouvi et al., 2012). The authors further revealed that, fermentation is also a key fish preservation method in coastal African regions, though processing methods and hygiene levels can affect the safety and quality of fermented fish products. There's a lack of research on the health benefits of fermented foods, the specific bacterial strains utilized, and the potential probiotic microbes in African fermented foods for applications in the food and pharmaceutical sectors.

2.5. Ethiopian traditional fermented foods and beverages

Ethiopian traditional fermented foods and beverages, crafted with rural skills and indigenous knowledge, are vital in global diets, particularly in Ethiopia's culturally diverse landscapes. Ethiopian traditional fermented foods and beverages that have received attention include *ergo* (sour milk), *ayib*

(cottage cheese), *qibe* (traditional butter), *arrera* (defatted buttermilk) (Gonfa et al., 2001), injera, *kocho*, *siljo* (Mehari & Ashenafi, 1995), (Idris et al., 2020). Despite their local-scale production, they play a significant role in sustaining communities (Ashenafi, 2006). Extensive research has investigated potential LAB in Ethiopian fermented foods, revealing their ability to combat foodborne pathogens and enhance food shelf life through antimicrobial (Birhanu Abegaz, 2014; Mamo Nigatu et al., 2015). Furthermore, because of their ability to produce antimicrobial metabolites, they can control food spoilage and improve food shelf life by extending the food and controlling the proliferation and establishment of pathogens in food.

2.5.1. Bukuri

Bukuri is one of Ethiopian traditional fermented nonalcoholic beverage, which is the most popular among all people of East Wollega zone and its surroundings area, which is consumed by large communities of the rural areas. The ingredients for the preparation of *Bukuri* are cereals like barely (*Hordeum vulgare*), maize (*Zea mays*), sorghum (*Sorghum bicolor*), wheat (*Triticum sativum*), finger millet (*Eleusine coracana*) or teff (*Eragrostis tef*) and it is without gesho. The communities use *Bukuri* as meal replacement, on holidays, wedding ceremony, and social works and as sources of income of many households. The popularity of traditionally fermented *Bukuri* is more common among the religious groups because it is considered as low alcoholic beverages. *Bukuri* is a cereal based Ethiopian traditional fermented beverages prepared from locally available cereals (maize, barley, sorghum, millet, wheat, teff and malt flours)(Chali et al., 2024). It is considered as alcohol free drink although the preparation follows a lot of fermentation process. The picture depicts the traditional *Bukuri* fermenting process, which is widely practiced in regions consumed in and around East Wollega Zone. This beverage is frequently used in the communities, particularly during holidays, social work, and wedding ceremonies (Figure 2.4).

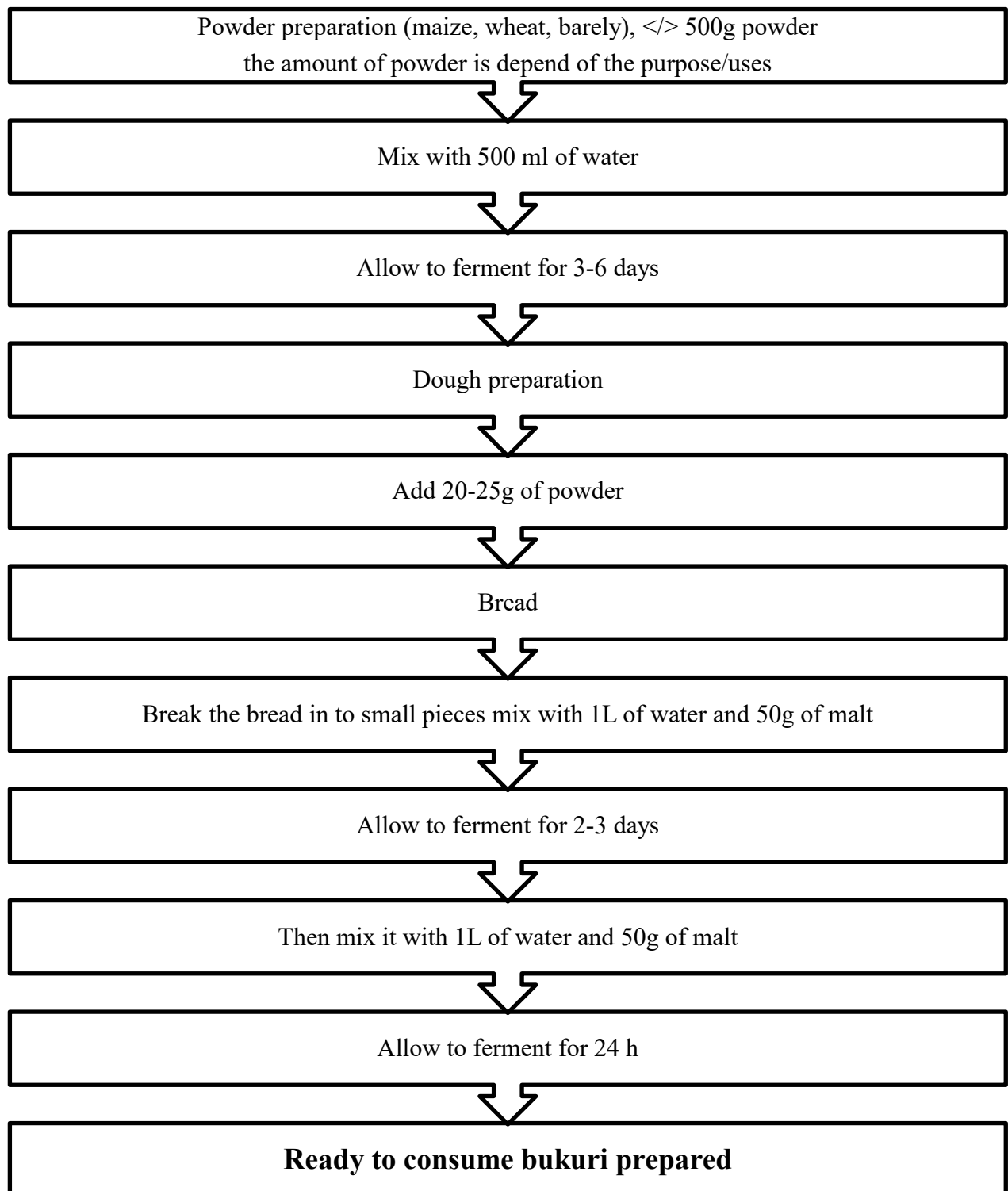


Figure 2. 3. Ethiopian traditional fermented beverage, *bukuri* preparation (Chali et al., 2024)

2.5.2. *Cabbage-shamita*

Cabbage-shamita is a thick, traditionally fermented non-alcoholic beverage mostly consumed by women in rural and urban parts of East Wollega zone and its environments. Cereals such as barley, maize, cabbage seeds, and spices are used in cabbage-shameta fermentation (Figure 2.5). Because *cabbage-shamita* fermentation takes time and energy, it is not for sale, meal replacement, vacations, or social work. Cabbage seed is crushed with a native crusher and pestle and immersed in water for 7-14 days in *cabbage-shamita* fermentation. The filtered liquid half is boiled with barely or maize flour, chilled, and spices added before being preserved in a pot after 7-14 days (Chali et al., 2024).

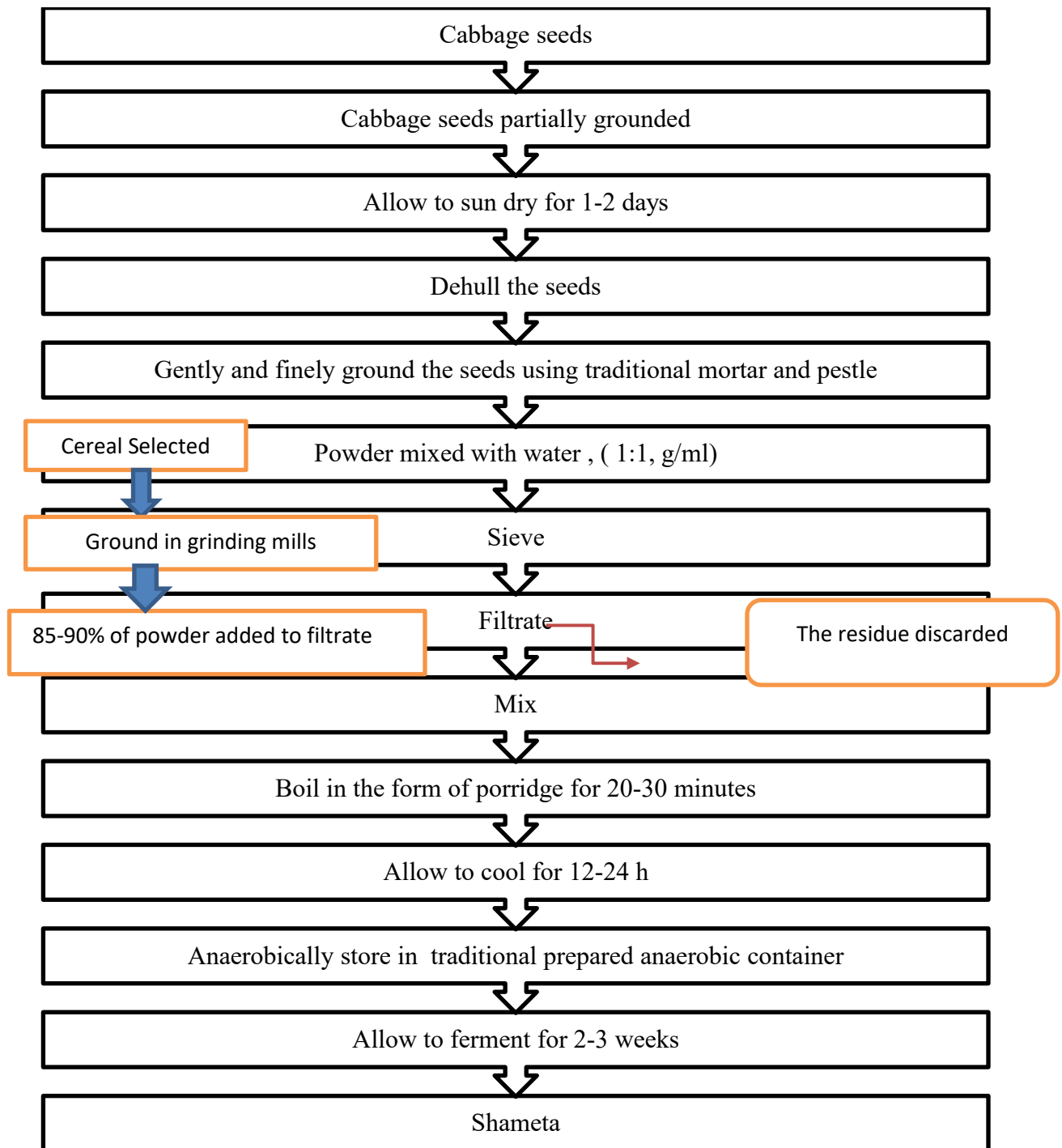


Figure 2. 4 Flow chart of *Cabbage-shamita* preparation (Chali et al., 2024).

2.5.3. *Borde*

Borde is one of the most commonly prepared and consumed traditional fermented beverages in many parts of the county, Nekemte city and its surrounding communities are using *borde* specially during ceremony, wedding and on holly days' occasion. Even though, many ingredients such as maize, wheat, teff, sorghum, and finger millet are mostly used for *borde* preparation, at the study area, mostly *borde* has been prepared from barley (*Hordeum vulgar L*), as the information gathered from the traditional practioners. Therefore, it is a popular meal substitute in several regions of the country. *Borde* is a low-alcohol beverage, and most people believe it helps during lactation. After giving delivery, mothers are recommended to consume large amounts of it. The components for *Borde* fermentation appear to differ between *Borde*-producing villages. The most often utilized indigenous raw materials for *borde* preparation are maize, wheat, teff, sorghum, and finger millet (Hotessa & Robe, 2020; Lemi, 2020; Nemo & Bacha, 2020; Tilahun et al., 2018). Barley is necessary for the production of malt *borde*. Barley is washed to remove dirt and unwanted impurities before steeping in water for roughly a day to make malt. Excess water is drained, and the soaked barley is wrapped in insete eaves for 2-5 days in order to germinate. Germination barley can afterward be sun-dried and crushed finely, result Malt.

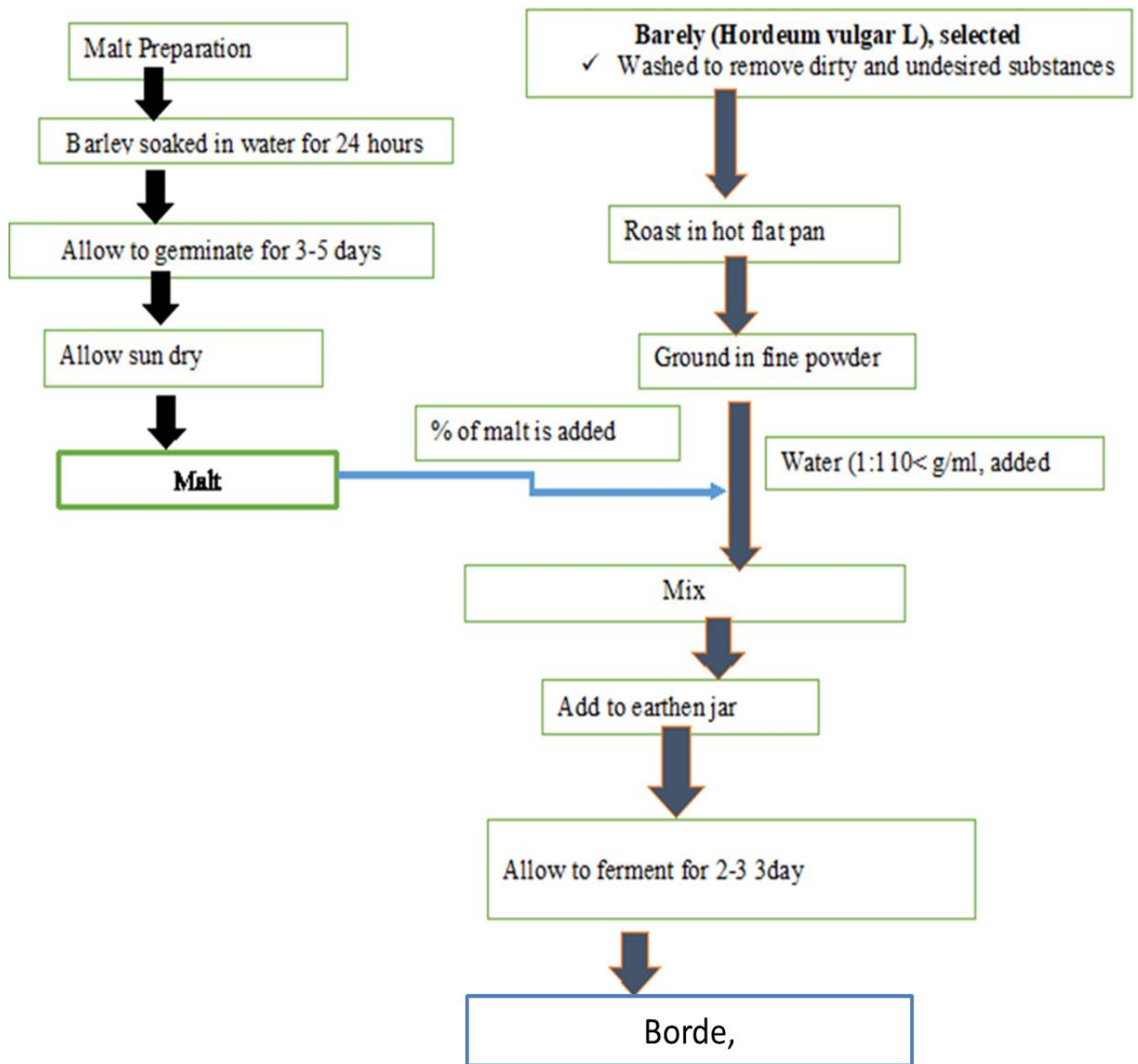


Figure 2. 5. Flow chart of *borde* preparation (source: local practioners)

2.5.4. Kocho

Kocho, along with injera is one of the most popular fermented dishes. It has a high starch content due to the fermentation of pseudostem, pulverized corm, and decorticated pulp (Bosha et al., 2016; Dibaba et al., 2018). The fermentation duration of kocho varied from location to location, depending on their incubation temperature. The period of kocho fermentation is one year (Dibaba et al., 2018; Yemata, 2020). However, fermentation times of traditional fermented kocho take place in a pit for two to three weeks (Dibaba et al. 2018; Karssa et al. 2014) and its fermentation time is varied from region to region based on their indigenous knowledge of people (Karssa et al., 2014; Tsegaye et al., 2015; Weldemichael et al., 2019).



Figure 2. 6. Ethiopian Traditional fermented food (Kocho), prepared at home and ready for market, collected from Jimma Town

2.5.5. Bulla

Bulla is one of the principal enset products, "the best part," acquired during enset-to-kocho transformation process. To separate the starch from the fiber, the inside part of the pseudostem is scraped using a piece of split bamboo. This decantation yields the *bulla*, which is next fermented for many weeks until it is highly starchy and of the highest quality. However, the nutritive value of starchy foods is determined primarily by their nutrient content, starch physicochemical properties, and the presence of nutritional factors and toxic substances (Atlabachew and Chandravanshi, 2008).

Bulla is a little amount of water-insoluble starchy substance that can be isolated from kocho by squeezing and decanting the liquid during processing. After decanting, the *Bulla* is let to dry or ferment in the same manner as Kocho. However, instead of being fermented, it is usually dehydrated to make flour that can be stored for long periods or cooked directly without fermentation. It is the highest-quality enset meal, derived primarily from fully matured enset plants (Dibaba et al., 2018; Yemata, 2020).



Figure 2. 7. Ethiopian Traditional fermented food (*Bulla*), prepared at home and send to market, collected from Jimma Town

2.5.6. *Ergo*

Ergo is a popular Ethiopian fermented milk similar to yogurt. It's thick, smooth, and white, with a pleasant taste and smell. It can last up to 15-20 days when stored correctly. Widely enjoyed across the country, it's made without specific starter cultures by relying on natural milk flora for fermentation. Urban and rural households alike use various methods for fermentation, with temperatures and inoculation methods affecting the final product's consistency (Gonfa et al., 2001). LAB colonize the inside walls of the container and act as starting cultures. Incubation temperature rarely varies much, and the taste of the fermented product may be more or less homogeneous in general (Assefa et al., 2008; Temesgen et al., 2020).

2.6. Classification and physiology of lactic acid bacteria

Lactic acid bacteria (LAB) are classified based on their cultural characteristics, which provide valuable insights into their growth preferences and metabolic activities. Firstly, LAB exhibit specific requirements for growth, such as optimal pH levels, temperature ranges, and nutrient compositions of their culture media. These factors dictate whether a particular LAB species thrives in acidic environments typical of fermented foods or prefers neutral pH conditions. Additionally, LAB can vary in their temperature preferences, with psychotrophic LAB thriving in cooler environments and mesophilic LAB preferring moderate temperatures (Wang et al., 2021).

Cultural characterization also involves observing LAB growth patterns. This includes assessing colonial morphology, such as colony size, shape (e.g., round, irregular), texture (smooth, rough), and color variations (white, cream, yellow). The rate of growth is another distinguishing factor, where some LAB species grow rapidly, forming large colonies or producing turbid cultures, while others exhibit slower growth rates. Furthermore, LAB can be classified based on their oxygen requirements, categorized as aerobic (requiring oxygen), anaerobic (growing in the absence of oxygen), or facultatively anaerobic (able to grow in both conditions) (Mokoena, 2017).

Metabolic characteristics play a crucial role in cultural classification as well. LAB are known for their ability to ferment specific carbohydrates like glucose, lactose, maltose, and sucrose into lactic acid, which is fundamental to their metabolic pathways. Other metabolic traits evaluated include enzyme production (e.g., proteases, lipases), acid and gas production from sugars, and utilization of various nitrogen sources (Khushboo et al., 2023).

Lactic acid bacteria (LAB) are characterized biochemically by their ability to ferment various carbohydrates into lactic acid, a key trait that sets them apart from other bacteria and underpins their metabolic diversity and adaptability to diverse environments. Additionally, LAB demonstrate versatile nitrogen metabolism, utilizing amino acids and peptides for growth, which enhances their resilience in environments ranging from food products to the gastrointestinal tract. These bacteria may possess additional characteristics such as antimicrobial compound production, tolerance to environmental stresses, and the ability to utilize alternative carbon sources, highlighting their versatility in applications such as food fermentation, probiotics, and biotechnology. Biochemical characterization is crucial for

understanding LAB's functional roles, facilitating the selection and engineering of strains tailored for specific purposes in enhancing human health and optimizing industrial processes(Wang et al., 2021).

2.6.1. MALDI-TOF MS bacterial identification system

MALDI-ToF, or matrix-assisted laser desorption ionization time-of-flight analysis, is a mass spectrometry method used for identifying microorganisms like bacteria, yeast, and molds. A laser breaks down bacteria into protein fragments, which travel through a detection tube. The "time of flight" measures how long these peptides take to reach the tube. By comparing, the mass spectrometry profile with a MALDI-TOF database, bacterial genera and species can be identified. Updating the database can be done by laboratory personnel or through purchasing an updated version. It relies on detecting the mass-to-charge ratio of ribosomal proteins by providing a unique mass spectrum for bacteria in a short time, and identification is based on the closest match with reference strains' spectra (Ashfaq et al., 2022).

2.6.1.1. Sample preparation

Two main sample preparation methods for MALDI-ToF analysis are direct transfer and protein extraction. Direct transfer involves quickly recovering bacterial cells from growth plates or liquid media by providing reliable and accurate results. Protein extraction using solvents like ethanol (avoiding toxic ones like formic acid), is recommended for higher spectral quality (Diaz et al., 2019). After preparation, bacterial cells or extracted proteins are placed on a MALDI biotarget plate, covered with a matrix solution and dried. This plate is then inserted into the MALDI-TOF instrument to generate a spectrum for comparison with the database (Gonzalo et al., 2021). An overview of the MALDI-ToF mass analysis process is presented in Figure 2.13.

2.6.1.2. Bacterial identification

Online databases as if the MALDI Biotyper Library (MBL) compares mass spectra of unknown bacteria to known ones for identification, using software like Biotyper. Other search engines and fingerprint libraries are also available. MALDI-ToF can achieve over 90% accuracy in identifying organisms to the species level, but reproducibility depends on factors like sample culture, preparation, storage, and matrix type (Clark et al., 2013). In MALDI-TOF bacterial identification, scores below 1.7

suggest uncertainty; between 1.7 and 2.0 indicate a probable genus-level identification and scores above 2.0 are reliable for species-level identification.

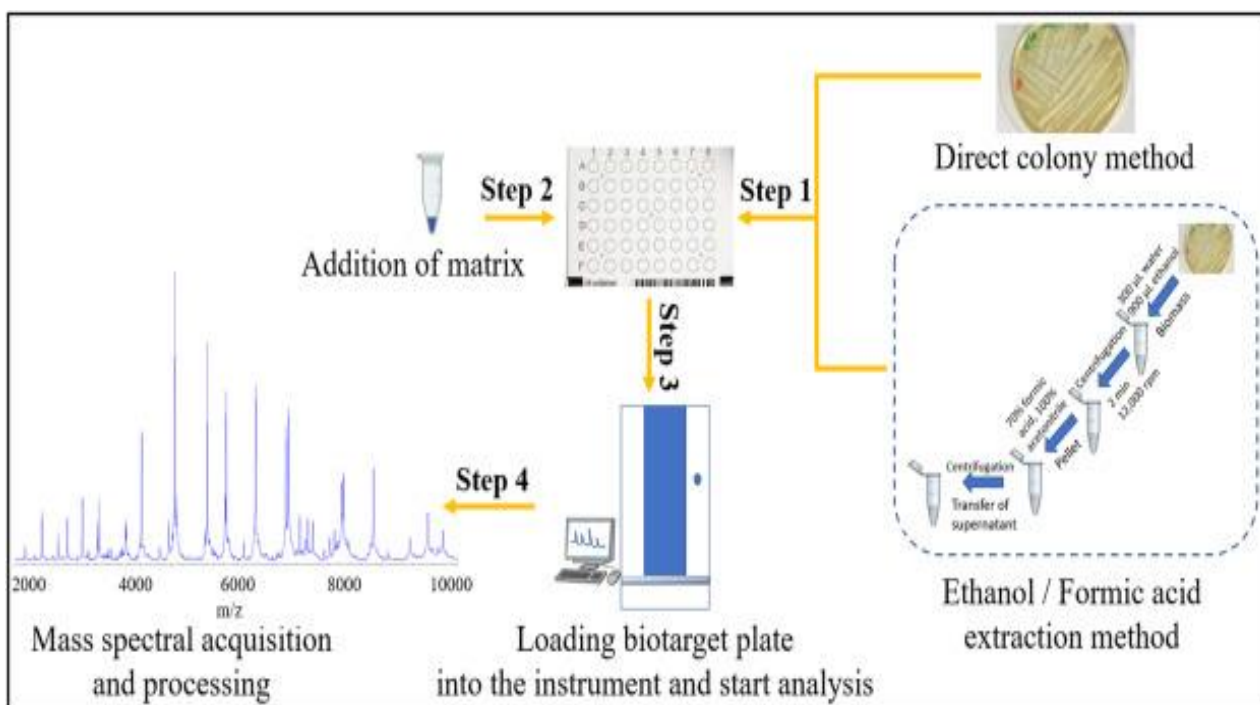


Figure 2. 8. Steps and procedures for bacterial identification using MALDI-TOF MS (Gonzalo et al., 2021; Šedo et al., 2011; Toh-Boyo et al., 2012).

2.6.1.3. Microorganism typing

The MALDI-TOF method is widely used for fast and accurate bacterial identification, including subtyping and serotyping. While it is excellent for genus and species identification, its use for subspecies identification is still being explored due to similarities within species. However, some success has been reported with bacteria like *Yersinia enterocolitica* for subspecies-level typing using MALDI-TOF MS (Sandrin et al., 2013). Different methods exist for identifying strains using MALDI-TOF MS. Comparing mass spectra visually helps distinguish species by specific peaks. The gel view enhances this comparison by highlighting peak presence or absence. Certain peaks, like those around 2000 and 5000 m/z, are often considered biomarkers across many bacteria (Alsayegh et al., 2021).

The LAB group is currently classified in the phylum **Firmicutes** and **Actinobacter**, class **Bacilli**, and order **Atopobium**, **Bafidobacterium** and **Latobacillales**. LAB are classified based on cellular morphology, mode of glucose fermentation, range of growth temperature, and sugar utilization patterns and sequencing of 16S rRNA (Mokoena, 2017; Yang et al. 2014). *Lactobacillus* species are often found in human and animal guts, as well as in vegetables and their fermented versions. *Leuconostoc* species come from meats, clinical sources, plants, fermented dairy, and wines (Goldstein et al., 2015). *Pediococcus* species are associated with beer spoilage, while *Lactococcus* is more common in dairy (Parada & Aguilera, 2007). *Lactobacillus* members are gram-positive, non-motile, non-sporulating, acid-tolerant, facultative anaerobes, and can ferment in different ways (Alkema et al., 2016). Lactic acid bacteria (LAB) play a crucial role in food fermentation by enhancing sensory qualities and ensuring microbiological safety. Their antimicrobial activity, achieved through organic acids, hydrogen peroxide, ethanol, and bacteriocins that have garnered interest for pathogen control in foods. Utilizing LAB and natural components for food preservation may reduce economic losses from microbial degradation and lower foodborne illness occurrences (Dalié et al., 2010; Delesa, 2017).

2.7. Phylogenetic characterization of Lactic acid bacteria

The 16S rRNA gene is widely used in bacterial taxonomy due to its ubiquitous presence, stable function, and sufficient size for informatics (Janda, J. Michael, Abbott, 2007). Yet, to propose a new bacterial taxon, additional data like isolation source, phenotypic traits, biochemical profiles, and antibiotic susceptibility are essential. Other genes like 23S rRNA, 16S-23S rRNA ITS, *rpoB*, and *groEL* contribute to characterization (Drancourt & Raoult, 2005).

For Lactic acid bacteria (LAB), genomic studies are crucial for understanding their metabolism, evolution, and classification (Callan and Westblade 2020). Whole-genome sequences aid in functional genomics, providing permanent scientific resources (Nowrousian et al., 2010). Comparative genomics show LAB genomes are small but diverse, with gene losses reflecting adaptation to nutrient-rich environments (Makarova & Koonin, 2007). Advancements like next-generation sequencing (NGS) and metagenomics offer deeper insights, although pyrosequencing's error rates can affect rare phylotype estimations (Nowrousian et al., 2010). Metagenomics has explored various environments, showcasing its utility in diverse scenarios (Brugger et al., 2012).

2.8. Probiotics property of Lactic acid bacteria

Probiotics are live microorganisms and substances that promote gut balance and may benefit health when consumed adequately (Vilander & Dean, 2019). LAB are the most common probiotics, found in nutrient-rich environments and various body parts (Didari et al., 2014). They are considered safe and therapeutic for treating gastrointestinal issues. To qualify as probiotics, LAB must be safe, survive stomach acids, adhere to intestinal walls, combat harmful pathogens, and maintain viability during production and storage (Feng *et al.*, 2017).

2.9. Criteria for Selection of Probiotic Lactic acid bacteria

For probiotics, strains must meet safety, functionality, and storage stability criteria. They should survive processing and storage while maintaining their properties. LAB strains for human use must originate from humans; ideally, probiotic strains for human use should originate from human sources. This enhances their compatibility with the human microbiota and improves colonization potential in the gastrointestinal tract. have a non-pathogenic history, and lack transmissible antibiotic resistance genes (Lim & Im, 2009) (Table 2.1).

2.9.1. Acid and bile tolerance

A critical step in probiotic selection is ensuring LAB can survive the acidic stomach environment with pH as low as 1.5 (Gotcheva et al., 2002). Studies show probiotic LAB can endure gastric transit at pH 2.0 for short periods (1-2 h) (Dowarah et al., 2018). Resistance to bile salts is also key, with recommended concentrations of 0.15-0.3% for human usage, matching physiological bile levels of 0.1-0.3% and occasionally up to 0.5% (Mulaw et al., 2020).

2.9.2. Antimicrobial activity

Antimicrobial activity is a key factor in selecting probiotic LAB strains. These strains must produce antimicrobial compounds like organic acids, hydrogen peroxide, and bacteriocin to be effective probiotics. Their ability to create these compounds is crucial for developing probiotic supplements and diets (Fugaban et al., 2021).

2.9.3. Adhesion Properties of Probiotics

The ability of probiotic strains to adhere to the intestinal surface is crucial for their effectiveness. Strains that adhere well are more likely to persist in the gut, leading to better metabolic and immunomodulatory effects. Adhesion is a critical characteristic as it competitively excludes harmful bacteria from the intestinal epithelium, making it a key selection criterion for probiotic production (Bour *et al.*, 2015).

Table 2. 1. Selection criteria for potential probiotic lactic acid bacteria

The selection criteria of the potential probiotic Lactic acid bacteria
1) Human or animal origin, these criteria is important for species dependent health effects.
2) Isolated from the gastrointestinal tract of healthy individuals
3) History of safe use, safe for food and clinical use; no invasion and no degradation of intestinal mucus.
4) Absence of data regarding an association with infective disease, no adverse effect on human
5) Important for oral consumption even if it may not be for other applications for survival though the intestine, maintaining adhesiveness and metabolic activity.
6) Lack of genes liable for antibiotic resistance localized in non-stable elements
7) Much enough to compete with pathogens, this expresses their ability to survive and maintain the metabolic activity, and to grow in the target site
8) Should resist to low pH in the stomach, bile salts and enzymes
9) Should have to show potential antagonistic activity towards pathogens
10) Resistance to bacteriocin and acids produced by the endogenic intestinal microbiota
11) Adherence and ability to colonize some particular sites within the host organism
12) Easy production of high biomass amounts and high productivity of cultures.
13) Viability and stability of the desired properties of probiotic bacteria during the fixing process, preparation, and distribution of probiotic products
14) High storage survival rate in finished products
15) Guarantee of desired sensory properties of finished products
16) Genetic stability
17) Important to improve immune system, competition with pathogens, maintain metabolic activity, prevent pathogens to adhesion and colonization

(Paulina Markowiak and Katarzyna Sli ´ zewska-, 2017).

2.9.4. Antibiotic susceptibility

Antibiotic susceptibility is a critical attribute of *Lactobacillus acidophilus* strains, ensuring their safety and efficacy as probiotics in therapeutic applications. These bacteria typically exhibit susceptibility to antibiotics commonly prescribed in clinical settings. This susceptibility is essential because it prevents *Lactobacillus acidophilus* from harboring antibiotic resistance genes or transferring resistance to other bacteria in the gut microbiota (Mayrhofer et al., 2010). By remaining susceptible to antibiotics, *Lactobacillus acidophilus* maintains its probiotic integrity and reduces the risk of contributing to antibiotic resistance development. This characteristic is particularly significant in clinical contexts where probiotics are used alongside antibiotic treatments. It allows *Lactobacillus acidophilus* to support gut health without compromising the effectiveness of antibiotic therapies intended to treat bacterial infections (Shahali & Soltani, 2023). Moreover, the susceptibility of *Lactobacillus acidophilus* to antibiotics underscores its safety profile for human consumption. Probiotic products containing *Lactobacillus acidophilus* can be confidently utilized in various therapeutic and preventive applications, including managing gastrointestinal disorders, enhancing immune function, and promoting overall well-being. This characteristic highlights the importance of selecting probiotic strains with demonstrated antibiotic susceptibility to ensure their beneficial impact on human health while minimizing potential risks associated with antibiotic resistance (Milner et al., 2021).

2.9.5. Exopolysaccharide production

Exopolysaccharides (EPS) production in *Lactobacillus acidophilus* is a significant feature that can affect its functionality and health-promoting properties. EPS are complex polysaccharides synthesized and secreted by certain strains of *Lactobacillus acidophilus* during their growth. These EPS play diverse roles in gastrointestinal health and probiotic efficacy. EPS produced by *Lactobacillus acidophilus* can enhance the probiotic's ability to adhere to intestinal epithelial cells, which is crucial for colonization and persistence in the gut environment (Werning et al., 2023). Adherence to intestinal cells allows probiotics to establish a stable population, interact closely with the host epithelium, and exert their beneficial effects effectively.

Furthermore, EPS contribute to strengthening the gut barrier function by forming a protective layer on the intestinal mucosa. This barrier enhancement potentially reduces intestinal permeability, preventing the passage of harmful substances across the gut epithelium and maintaining gut health (Ruas-Madiedo et al., 2010). These polysaccharides can interact with the host immune system, modulating immune responses and promoting a balanced immune function. Overall, EPS production in *Lactobacillus acidophilus* underscores its potential as a beneficial probiotic strain for enhancing gastrointestinal health. The ability of EPS to facilitate adherence, strengthen gut barrier function, and potentially modulate immune responses highlights their importance in probiotic functionality and therapeutic applications.

2.9.6. Cell auto-aggregation

Cell auto-aggregation in *Lactobacillus acidophilus* is a notable characteristic that enhances its effectiveness as a probiotic in the gastrointestinal tract. Recent research has highlighted that auto-aggregation plays a pivotal role in the persistence and functionality of *Lactobacillus acidophilus* in the gut environment. The formation of clusters aids in the development of biofilms, protective communities of microorganisms adhering to surfaces, including the intestinal mucosa (Idrees et al., 2022). Biofilms contribute to the probiotic's ability to adhere firmly to the intestinal epithelium, thereby enhancing colonization and persistence in the gut. This adherence is crucial for *Lactobacillus acidophilus* to exert its health-promoting effects, such as modulating the immune system and improving gut barrier function. Moreover, auto-aggregation can facilitate interactions with other gut microbes and host cells, potentially influencing the gut microbiota composition and metabolic activities (Mahmoudi et al., 2019). In summary, the ability of *Lactobacillus acidophilus* to auto-aggregate and form clusters underscores its adaptability and efficacy as a probiotic. This property not only supports its colonization and persistence in the gut but also enhances its potential to promote gastrointestinal health and overall well-being.

2.9.7. Co-aggregation

Co-aggregation is a phenomenon where probiotic bacteria aggregate with other microorganisms or food particles, which has implications for food preservation and microbial interactions. Co-aggregation plays a significant role in the ecology and functionality of probiotic bacteria. Probiotics, such as certain

strains of *Lactobacillus* and *Bifidobacterium*, are known to co-aggregate with pathogens and spoilage organisms, influencing the microbial balance and competitive exclusion in food systems (Mahmoudi et al., 2019; Nallala et al., 2017). This interaction involves specific adhesion mechanisms between probiotics and other bacteria or food particles, mediated by surface proteins and polysaccharides. Moreover, co-aggregation with food particles or components can affect the sensory properties and texture of fermented foods. For instance, in yogurt production, co-aggregation of probiotic strains with milk proteins or fat globules can influence product consistency and mouthfeel (Jrad et al., 2021). Understanding the mechanisms and factors influencing co-aggregation is crucial for optimizing probiotic functionality in food applications. It allows for the development of probiotic-containing foods with enhanced microbial stability, safety, and sensory quality. In summary, co-aggregation of probiotic bacteria with other microorganisms or food components is a multifaceted phenomenon that affects microbial interactions, food preservation, and product characteristics in fermented foods.

2.9.8. Cell surface hydrophobicity

Cell surface hydrophobicity is a critical characteristic of microorganisms that influences their interactions in various environments, including food matrices and host tissues. It is determined by the composition and arrangement of surface molecules such as proteins, lipids, and polysaccharides, which can either attract or repel water molecules. Recent research underscores the significance of cell surface hydrophobicity in microbial adhesion and biofilm formation. For instance, hydrophobic interactions play a crucial role in the initial attachment of pathogens to surfaces and subsequent biofilm development, impacting food safety and product shelf life (Tang et al., 2023). Moreover, in probiotics, moderate hydrophobicity can enhance their adhesion to intestinal epithelial cells, facilitating colonization and potential health benefits in the gut (Latif et al., 2023). Understanding and manipulating cell surface hydrophobicity is essential for applications in food microbiology and biotechnology. Techniques to measure and modulate hydrophobicity contribute to optimizing the performance of probiotics and starter cultures in fermented foods, improving texture, stability, and sensory attributes while controlling microbial spoilage (Fugaban et al., 2021). In summary, recent studies highlight the multifaceted role of cell surface hydrophobicity in microbial behavior and its implications for food safety, probiotic functionality, and product quality in modern food systems.

2.9.9. Anti-oxidant activity

Antioxidant activity refers to the ability of substances to inhibit oxidation, thereby protecting cells from oxidative damage caused by free radicals and reactive oxygen species (ROS). Here is an expanded explanation with recent citations: Antioxidants play a crucial role in food preservation and human health by neutralizing free radicals and preventing oxidative stress-related diseases. Natural antioxidants derived from plants, fruits, and microbial sources have garnered significant attention due to their potential health benefits and applications in food industries. Recent research has highlighted various compounds with antioxidant properties, including phenolic compounds, flavonoids, vitamins (e.g., vitamin C and E), and enzymes (e.g., superoxide dismutase). These antioxidants scavenge free radicals and inhibit lipid peroxidation, protein oxidation, and DNA damage, thereby mitigating cellular oxidative stress (Kagkli et al., 2016).

In food preservation, antioxidants help maintain the quality and shelf life of products by preventing rancidity, color changes, and nutrient degradation caused by oxidation processes. For example, natural antioxidants extracted from plants or added as food additives can delay lipid oxidation in oils, extend the freshness of packaged foods, and enhance stability during storage (Song et al., 2021). Moreover, antioxidants contribute to human health by protecting cells from oxidative damage associated with aging, cardiovascular diseases, cancer, and neurodegenerative disorders. They also exhibit anti-inflammatory properties and support immune function, highlighting their therapeutic potential beyond food preservation (Boya et al., 2022; Li et al., 2023). Understanding the antioxidant activity of compounds and their mechanisms of action is crucial for optimizing their use in both food technology and health applications. Advances in antioxidant research continue to explore novel sources and formulations to enhance efficacy and safety in various applications. In summary, antioxidants serve as key players in both food preservation and human health, offering protective effects against oxidative stress and contributing to overall well-being.

2.10. Mechanisms of Probiotic Action

Probiotic LAB employ various mechanisms to defend against pathogens and intestinal diseases. Primarily, they modulate the host's immune response, crucial for preventing infections and addressing gut inflammation. LAB also compete with pathogens by blocking their attachment to intestinal cells, aiding infection prevention and microbial balance restoration (Figure 2.12). Additionally, probiotics can influence microbial and host products, contributing to their overall beneficial effects (Anees et al., 2020; Plaza-Díaz et al., 2018).

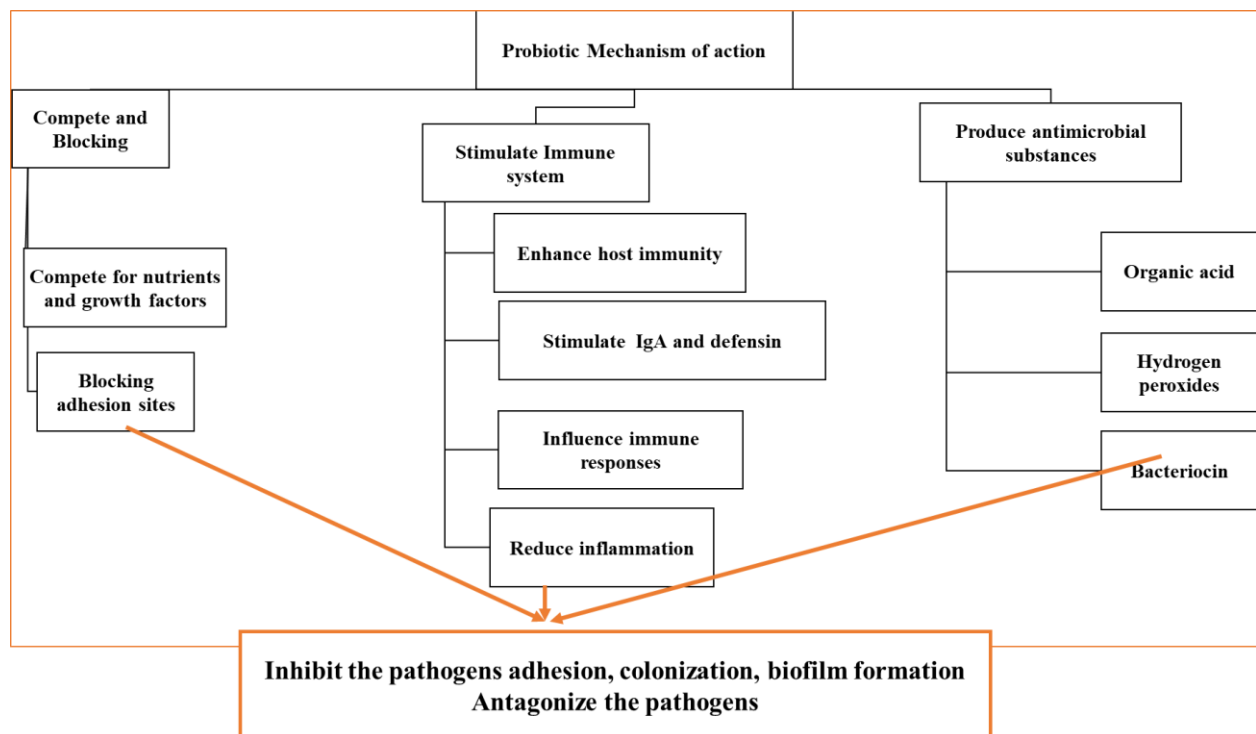


Figure 2. 9. Mechanisms of probiotic action by LAB (Latif et al., 2023).

2.11. Health Benefits of Probiotics

Research shows that probiotic bacteria, when consumed with traditional fermented foods and beverages, significantly contribute to maintaining good health, preventing intestinal infections, and improving overall well-being. These benefits include reducing antibiotic-related diarrhea, allergies, lactose intolerance, and colon cancer risk, as well as preventing eczema in children, lowering cholesterol, supporting immune system development, and protecting against gut pathogens (Hong et

al. 2010; Rajoka et al. 2019). In addition, probiotic bacteria can enhance immunity by influencing the immune system's function, particularly in boosting natural killer (NK) cell activity. They also regulate inflammatory cytokines, contributing to overall health improvement (Gardiner, 2002).

Probiotic LAB exhibit strong antimicrobial activity against various food and human pathogens. Studies by George Kerry et al. (2018) and Tejero-Sariñena et al. (2012) showed that probiotic LAB can inhibit the survival of pathogens like *S. Typhimurium* and *C. difficile*. Additionally, research by Zapašnik et al. (2022), revealed that probiotics produce antimicrobial compounds such as bacteriocin, ethanol, organic acids, and peptides. These probiotics not only directly target infections but also enhance host defense mechanisms against pathogens (Figueroa et al., 2011).

Many people experience lactose maldigestion, leading to symptoms like loose stools and bloating. Interestingly, individuals often tolerate lactose in yogurt better than in raw milk. This is due to lactase released from probiotic LAB and yogurt, which breaks down lactose, making it easier to digest and reducing discomfort (Marteau, Seksik, et al., 2002). Lactic acid bacteria can reduce serum cholesterol, lowering the risk of heart disease. They achieve this by producing bile salt hydrolase (BSH) enzyme, which breaks down bile salts. This process increases bile salt excretion, synthesizes more bile salts from serum cholesterol, and reduces cholesterol uptake from the gut, as shown in studies of Nguyen et al. (2013) and Nguyen et al. (2007).

In addition, Probiotics are recognized for their ability to promote human health when consumed in adequate quantities. They play a crucial role in supporting digestive health by fostering a balanced gut microbiome, essential for effective digestion and nutrient absorption. This balance can mitigate symptoms associated with gastrointestinal disorders such as diarrhea, irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD) (Hill et al., 2014). Moreover, probiotics have demonstrated potential in enhancing immune function, potentially decreasing the susceptibility to and severity of infections such as respiratory and urinary tract infections (Gao & Xu, 2020). Recent research has also highlighted their influence on mental health through the gut-brain axis, suggesting benefits in managing conditions like anxiety, depression, and stress (Slykerman et al., 2020).

Certain strains of probiotics have been studied for their ability to manage allergies, eczema, and urogenital health conditions such as bacterial vaginosis (West et al., 2014). Additionally, probiotics may contribute to cardiovascular health by modestly lowering blood pressure and cholesterol levels (Khalesi et al., 2014). Their role in improving nutrient absorption and potentially influencing weight management and metabolic health has also been explored (Wang et al., 2016).

2.11.1. Selection criteria of probiotic microbes

Safety is one the most important criteria and issue should be considered to select potential and good probiotic candidate. To be considered a potential probiotic bacterium, an organism must demonstrate non-pathogenicity and non-toxicity profiles while exhibiting tolerance to harsh conditions along the digestive tract. Additionally, it should adhere effectively to mucosal surfaces and have documented health effects validated through scientific research (Marteau et al., 2002). Moreover, they should not carry transmissible antibiotic resistance genes (Saarela et al., 2002). This is because antibiotic resistant genes, especially those encoded by plasmids could be transferred between microorganisms. The potential strains need to be assayed for their antibiotic resistance to prevent the undesirable transfer of resistance to other endogenous bacteria. Furthermore, the presence of an antibiotic may facilitate the growth of antibiotic resistant mutants (Marteau et al., 2002).

2.12. Lactic Acid Bacteria as Biological food preservatives

Bio-preservation involves using living LAB and their metabolites (Organic acid, Hydrogen peroxides, Carbon dioxdes, bacteriocins, and bacteriocins) to enhance food safety and prolong shelf life. LAB and their antimicrobial compounds are employed to increase food safety without compromising sensory qualities (Ghafur et al., 2019). These bacteria produce various compounds like organic acids, bacteriocins, hydrogen peroxide, and antifungal agents, which suppress spoilage organisms and food-borne diseases. Their long history in food fermentation and GRAS status make them ideal alternatives to chemical preservatives. LAB's preservative effect is due to the production of active metabolites such as organic acids, ethanol, fatty acids, and antifungal compounds, which lower pH and inhibit microbial growth (Al-Yami et al., 2022; Zapašnik et al., 2022).

2.10.1. Organic acids

Lactic acid and acetic acid, both produced by lactic acid bacteria (LAB) during fermentation, play essential roles as antimicrobial agents in food preservation. Lactic acid primarily functions by acidifying the environment, lowering pH levels, and creating conditions unfavorable for the growth of spoilage and pathogenic microorganisms. This acidity disrupts microbial metabolism and inhibits their ability to proliferate, thereby extending the shelf life of food products (Reis et al., 2012). The solubility characteristics of lactic acid are crucial in its antimicrobial action. While it remains soluble in water, lactic acid can penetrate microbial cells, disrupting their internal pH balance and metabolic processes. This disruption impairs essential cellular functions and reduces the energy available for microbial growth, contributing significantly to the preservation of fermented foods (Sabry Othman, 2014).

Acetic acid, a component found in vinegar, exhibits broad-spectrum antimicrobial activity. It is particularly effective against bacteria, yeasts, and fungi, functioning optimally under acidic conditions. This acid disrupts microbial cell membranes and enzymatic activities, leading to cell death and inhibition of growth, further enhancing the safety and stability of food products (Menteş et al., 2007). In conjunction with other preservation methods such as temperature control and heat treatment, lactic acid and acetic acid provide robust protection against foodborne pathogens. These acids not only inhibit microbial growth but also help maintain the sensory attributes and nutritional quality of fermented foods. Their combined effects contribute to the overall safety and longevity of food products, making them integral components of traditional and modern food preservation practices. In summary, lactic acid and acetic acid produced by LAB are pivotal in food preservation due to their antimicrobial properties, ability to alter pH environments, and effectiveness against a wide range of microorganisms. Understanding their roles and interactions with food matrices is crucial for optimizing preservation strategies and ensuring the quality and safety of fermented foods.

2.12.1. Diacetyl, acetaldehyde and acetoin

Heterofermentative lactic acid bacteria (LAB) are capable of producing acetaldehyde as a key metabolite during fermentation. Acetaldehyde interacts with pyruvate to form α -acetolactate, which is subsequently converted to diacetyl through enzymatic processes. Diacetyl is well-known for imparting a buttery aroma to fermented dairy products. However, its strong aroma and high concentrations often

restrict its use as a preservative in food products. Despite its limitations as a preservative, acetaldehyde plays a crucial role in inhibiting microbial growth in fermented foods. Although present in lower amounts compared to diacetyl, acetaldehyde, along with other LAB metabolites, contributes to creating an environment hostile to spoilage and pathogenic microorganisms. This antimicrobial activity is part of the broader preservation mechanism employed by LAB, which includes acidification and the production of various metabolites during fermentation(Jyoti et al., 2003).

Acetaldehyde and diacetyl, products of heterofermentative LAB metabolism in fermentation, play pivotal roles in enhancing the sensory attributes and ensuring microbial safety of fermented dairy products. Diacetyl is well-known for its buttery aroma, which significantly enriches the flavor profile of dairy products but can limit its use as a preservative due to its strong aroma at high concentrations. In contrast, acetaldehyde, despite being present in smaller amounts, exhibits antimicrobial properties that contribute to inhibiting the growth of spoilage and pathogenic microorganisms in fermented foods(Sharma et al., 2020). These metabolites, along with acidification and other microbial byproducts, form a comprehensive preservation mechanism employed by LAB. Managing the production and concentrations of acetaldehyde and diacetyl during fermentation is critical for achieving product quality and extending shelf life naturally. This involves optimizing fermentation conditions such as temperature control and culture management to maintain a balance between sensory attributes and microbial safety(Xu et al., 2021)

2.12.2. Carbon dioxide

Recent studies have affirmed the multifaceted preservative effects of carbon dioxide (CO₂) in food preservation. CO₂ exhibits potent antimicrobial and antifungal activities by disrupting cellular processes such as enzymatic activity and membrane integrity, effectively extending the shelf life of perishable foods(Pawlowska et al., 2012). In Modified Atmosphere Packaging (MAP), CO₂ plays a pivotal role in maintaining food quality by creating an anaerobic environment that controls microbial growth and enzymatic reactions, thereby preserving the freshness and nutritional value of products (Lee et al., 2022).

Firstly, it creates an anaerobic environment by displacing molecular oxygen, which is crucial because many spoilage organisms require oxygen for growth and metabolism. This anaerobic condition helps

inhibit the growth of aerobic microorganisms, thereby extending the shelf life of products. Secondly, CO₂ exhibits direct antimicrobial and antifungal activities (Sanlibaba et al., 2015). Its antimicrobial properties include inhibiting enzymatic decarboxylation processes within microbial cells. CO₂ also affects microbial membranes by accumulating in the lipid bilayer, which disrupts membrane integrity and leads to defective permeability. This disruption further compromises the ability of microorganisms to maintain cellular homeostasis and function properly (Mabrouk et al., 2023).

2.12.3. Hydrogen peroxide (H₂O₂)

According to many research reports, the production of H₂O₂ by LAB can prevent the growth of foodborne pathogens and can also be act as food preservation. At refrigeration temperatures, LAB that produce H₂O₂ have been demonstrated to limit the growth of psychotrophic and pathogenic microbes. However, it increases the rancidity and colors the final product and might interfere with the organoleptic qualities of fermented meat products (Jaroni & Brashears, 2000; Németh et al., 2005).

According to several research reports, the production of hydrogen peroxide (H₂O₂) by lactic acid bacteria (LAB) serves as a dual mechanism for food preservation. LAB that produce H₂O₂ have been shown to effectively inhibit the growth of foodborne pathogens, especially at refrigeration temperatures where psychotrophic and pathogenic microbes are prevalent. This property makes H₂O₂ a valuable natural preservative in various food products (Jaroni & Brashears, 2000). However, despite its antimicrobial benefits, the presence of H₂O₂ can also have some drawbacks. It has been observed that H₂O₂ production by LAB can contribute to increased rancidity and discoloration of the final product. These effects are particularly notable in fermented meat products, where maintaining desirable organoleptic qualities is crucial (Németh et al., 2005). Thus, while H₂O₂ produced by LAB offers significant potential for extending shelf life by inhibiting pathogens, its impact on product quality, particularly in terms of taste, color, and aroma, requires careful consideration in food preservation strategies.

2.12.4. Bacteriocins

Bacteriocins are antibacterial compounds produced by bacteria, known for their amphiphilic, membrane-permeabilizing nature. They can reduce the need for chemical preservatives and intense heat treatment in food processing, leading to naturally preserved foods with enhanced sensory and

nutritional qualities (Yang et al. 2012). To maximize their efficacy, careful study of bacteriocin-producing LAB strains is essential, including optimization of production techniques and use of inducible production systems. Heterologous expression of bacteriocin genes can further enhance their properties and production systems (Zhou et al.,2006).

2.12.5. Classification of Bacteriocin

Bacteriocin classification depends on various factors like molecular weight, producer strains, structure, and mode of action, biosynthesis, and biological activity. Recent studies categorize LAB bacteriocins into Class I, Class II, and Class III based on aforementioned criteria (Dicks et al., 2018; Soltani et al., 2021). Class I bacteriocins are characterized by unique amino acids and structures resulting from posttranslational modifications, making them small peptides. These modifications include rare amino acids like lanthionine, heterocycles, head-to-tail cyclization, and glycosylation. They also feature a leader peptide crucial for enzyme recognition, transport, and peptide inactivation (Arnison et al., 2012). Genes responsible for lantibiotic biosynthesis are organized in operons, with genes for bacteriocin maturation typically found within the same gene structure (Bacteriocins of Lactic Acid Bacteria: Extending the Family, 2016).

Class II bacteriocins are characterized by a flexible hinge that separates the peptide tract into variable and conserved portions. They feature an N-terminal leader with a C-terminal attached. The cationic N-terminal leader contains two cysteine residues linked by a conserved disulfide bridge, crucial for target engagement and a lethal mechanism of action. In contrast, the C-terminus is less conserved, resulting in reduced specificity in target contact (Johnsen et al., 2005). Class III bacteriocins are larger, heat-labile proteins with unique peptide domains. They encompass non-lytic bacteriocins with diverse modes of action, including inhibition without cell lysis, binding to sugars like mannose or glucose to affect target organisms' sugar absorption, and causing membrane leakage, leading to essential molecule loss (Choyam et a.,2021). Table 2.2, describes the major types of bacteriocins produced by Lactcia acid bacteria.

The use of LAB bacteriocin as a unique natural food preservative is based on its health benefits. To be considered a safe and effective natural preservative, it must meet these criteria: (1) not harm consumers' gastrointestinal health; (2) have broad-spectrum activity against various food spoilage microorganisms;

(3) be resistant to food enzymes; and (4) show thermal stability across different pH levels, high salt concentrations, and other factors. Failure to meet these criteria would limit bacteriocin's potential as a safe natural food preservative (Ananou et al., 2010).

LAB bacteriocin has been explored as a novel natural food preservation method using various approaches, including LAB starter cultures, protective LAB strains, reintroduction of previously fermented foods with bacteriocin-producing LAB during processing, and direct application of purified bacteriocin as a preservative (Ananou et al., 2010). These strategies are adaptable to different food types and specific LAB targets for preservation. Given LAB bacteriocin's "GRAS" status, it's suitable for industrial use, meeting the rising demand for fresh, safe, naturally preserved, and nutritionally enhanced food products with improved sensory qualities (Dhundale et al., 2018). Bacteriocins are commonly used as biopreservatives in foods through three methods: 1) Immunizing food with LAB that produce bacteriocins, crucial for their efficacy; 2) Directly using pure or semi-purified bacteriocins as preservatives; and 3) Incorporating a bacteriocin-producing strain's fermented product into food processing (Valledor et al., 2022).

Table 2. 2. Bacteriocins of LAB and their main characteristics(Yang *et al.*, 2014).

Classification/features	Bacteriocins	Mwt (Da)	Producing strain
CLASSI			
The bacteriocins are post-transnationally modified, linear or globular peptides containing lanthionine, β -methyl lanthionine and dehydrated amino acids	Nisin A	3352	<i>Lactococcus lactis</i> subsp. <i>lactic</i>
	Nisin U	3029	<i>Streptococcus uberis</i>
	Nisin Z	3493	<i>Lactococcus lactis</i> subsp. <i>lactic</i>
	Mersacidin	1824	<i>Bacillus</i> sp.Y85,54728
	Labyrinthopeptin A	21922	<i>Actinomadura</i> sp.
	subtilisin A	3399	<i>Bacillus subtilis</i> 168
CLASSII			
Heat stable, unmodified, bacteriocins, heterogeneous class of small peptides			
Class IIa (pediocinPA-1like bacteriocins)	Pediocin PA-1	4629	<i>Pediococcus acidilactici</i> i PAC-1.0
Class IIb (composed of two peptides)	Carnobacteriocin X	3602	<i>Carnobacterium maltaromaticum</i> C2
	lactacinF	4755	<i>Lactobacillus</i> spp.
	ABP-11	84096	<i>Lactobacillus salivariussubsp. salivarius</i> UCC118
Class IIc (circular peptide)	carnocyclinA	5862	<i>Carnobacterium maltaromaticum</i> UAL307
Class IId (linear, non-pediocinlike, single-peptide)	enterocin AS-4	87149	<i>Enterococcus faecalis</i>
	epidermicin NI01	6074	<i>Staphylococcusepidermidis</i>
	lactococcin A	5778	<i>Lactococcus lactis</i> subsp. <i>Cremoris</i>
CLASSIII			
Large, heat unstableproteins	Caseicin 80	~42000	<i>Lactobacillus casei</i> B80
	Enterolisin A	34501	<i>Enterococcus faecalis</i> LMG2333
	Helveticin J	37511	<i>Lactobacillus helveticus</i> 481

2.12.6. Separation, purification and identification of bacteriocins

The process of isolating bacteriocins from lactic acid bacteria (LAB) typically begins with screening LAB strains sourced from various environments such as plant materials, food products, and isolates from humans or animals. For instance, Ogawa et al. (2019), identified LAB strains in dairy and cheese products, highlighting the diversity of potential sources for bacteriocin-producing strains. Conversely, Rajaram et al. (2020), employed the well diffusion method on agar plates to screen for antagonistic activity among LAB strains, emphasizing a common approach in bacteriocin research.

To mitigate interference from hydrogen peroxide, which can affect bacteriocin activity, researchers often supplement the culture medium with catalase. Catalase breaks down hydrogen peroxide into water and oxygen, thereby neutralizing its inhibitory effects. Additionally, using phosphate buffer in the solid growth medium helps maintain optimal pH and prevents organic acids from inhibiting bacteriocin production. In summary, the isolation and purification of bacteriocins involve careful selection of LAB strains, screening for antagonistic activity, and employing specialized techniques to ensure optimal conditions for bacteriocin production and purification. These efforts contribute to advancing our understanding of bacteriocins and their practical applications in various fields

2.12.7. Chromatographic separation and purification

The purity of antibacterial compounds from LAB is crucial for structural analysis. Purification typically involves chromatographic techniques or a combination of methods based on different separation principles. For instance, reuterin was successfully separated and purified using reversed-phase high-performance liquid chromatography (RP-HPLC) with two C18 columns in series and water as the mobile phase (Pauter et al., 2020).

2.12.8. Ammonium sulfate precipitation

Ammonium precipitation is a widely used method for bacteriocin purification, typically as a first step. After culturing bacteriocin producers on MRS agar and recovering the cell-free supernatant through centrifugation, crude bacteriocin is obtained via protein precipitation with ammonium salt saturation. This crude bacteriocin is then further purified through steps like centrifugation, dissolution in water or buffer, and additional purification methods like membrane filtering and gel filtration. While this

process, known as partial purification, yields bacteriocin suitable for use as a food preservative but it's not the final purification step (Fugaban et al., 2021).

2.12.9. Identification of antimicrobial compounds

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) can be used to identify antimicrobial substances. The latter is frequently employed to determine molecular mass. Reuterin was identified primarily using proton (^1H) and carbon (^{13}C). HPLC-MS and GC-MS analysis of NMR data in deuterium oxide (D_2O) and deuterated methanol revealed that the molecular mass of the cyclic dimer form of reuterin was 148 (Ibrahim et al. 2021; Perez et al., 2014; Ye et al. 2021).

2.12.10. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis is used to estimate the molecular weight of bacteriocin after purification, and purified bacteriocin is treated with tricine sodium dodecyl sulfate polyacrylamide electrophoresis. Bacteriocin standard solutions are commonly used as a standard to measure the molecular weight of bacteriocin using gels (stacking solution and separating gel) (Struck et al., 2016; Xu et al., 2021; Yson et al., 2013; Zhu et al., 2015). Here, the steps following for bacteriocins extraction and purification is depicted in figure 2.13.

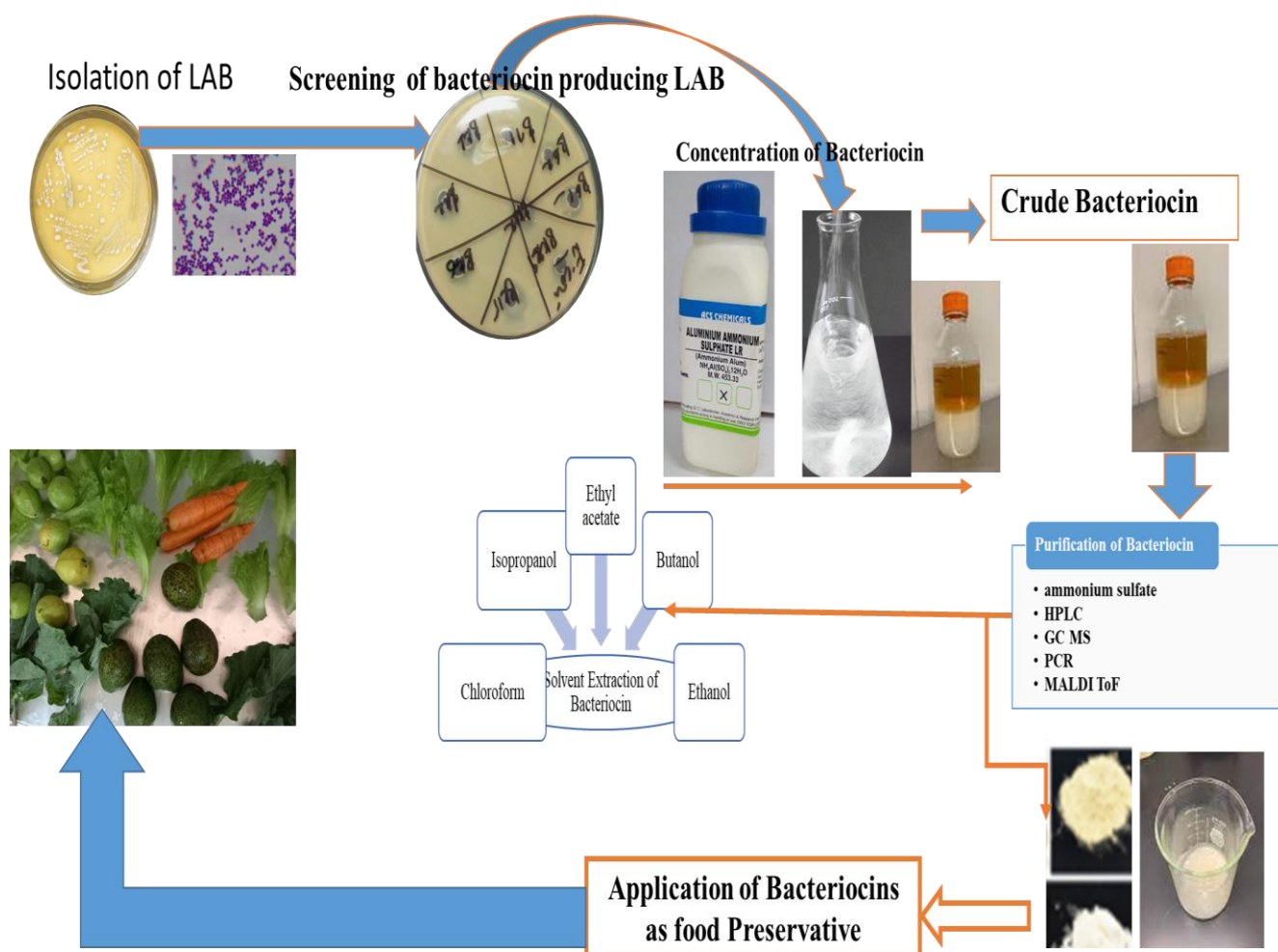


Figure 2. 10. Steps for bacteriocin screening, production and purification.

2.13. Therapeutic properties of probiotics and their safety

Probiotics, defined as live microorganisms that confer health benefits when administered in adequate amounts, have garnered significant attention for their therapeutic properties and safety profile. Probiotics have been extensively studied for their beneficial effects on gastrointestinal health, including the management of conditions such as diarrhea, irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD). They are also investigated for their potential in improving immune function, preventing allergies, and even influencing mental health through the gut-brain axis (Hill et al., 2014). Generally, probiotics are considered safe for most individuals, including infants, children, and adults.

Clinical trials and systematic reviews have shown that adverse effects are rare and typically mild, such as gastrointestinal symptoms that resolve without intervention (Hempel et al., 2012; Sanders et al., 2019). However, safety considerations include potential risks in immunocompromised individuals and those with serious underlying health conditions, highlighting the importance of individualized assessment and medical supervision in such cases (Didari et al., 2014). Regulatory bodies such as the FDA and EFSA provide guidelines and safety assessments for probiotic products, ensuring their quality, efficacy, and safety before they reach the market (Hill et al., 2014). In conclusion, probiotics offer promising therapeutic benefits across a spectrum of health conditions, supported by a generally favorable safety profile. Continued research and regulatory oversight are essential to further elucidate their mechanisms of action and optimize their clinical applications.

2.14. Delivery system of probiotics

The delivery system of probiotics plays a critical role in preserving their viability, stability, and effectiveness as they navigate the gastrointestinal tract. Effective delivery systems are essential to protect probiotics from the acidic stomach environment and bile salts in the small intestine, which can compromise their survival and therapeutic potential. Encapsulation and microencapsulation techniques are widely employed to enhance probiotic survival during transit through the digestive system (W. Xu et al., 2021). Microencapsulation represents a further advancement, wherein probiotic cells or clusters are encapsulated within small particles. This approach provides controlled release and protection from environmental stresses, thereby enhancing probiotic stability and prolonging shelf life in food and pharmaceutical applications (Tanhaeian et al., 2020). Ongoing research endeavors to optimize delivery systems continue to explore innovative techniques such as nanotechnology and genetically engineered carriers.

2.15. Commercially available probiotics and their global markets

The global probiotics market is experiencing substantial growth, with its size estimated at \$73.65 billion in 2023 and projected to reach \$80.48 billion in 2024. By 2029, the market is expected to hit \$115.07 billion, growing at a compound annual growth rate (CAGR) of 8.3%. Key drivers of this growth include increasing consumer awareness of the health benefits of probiotics, a shift towards preventive healthcare, and rising demand for functional foods and beverages. Probiotic foods and beverages represented 83.06% of the market share in 2023 and are expected to grow at a CAGR of

8.62% during the forecast period. Leading companies in the probiotics market include Nestle, Danone, Ch. Hansen, Yakult Honsha, and Probi. These companies are focusing on strategic acquisitions and collaborations to expand their product offerings. For example, Nestle has launched new probiotic products in China and the UK, targeting both infant foods and children's respiratory health. The market is also segmented by ingredient type, with bacteria accounting for 88.07% of the market in 2023. This segment is expected to grow due to its broad applications in digestive wellness. Distribution channels for probiotics include supermarkets, hypermarkets, pharmacies, and online stores, with supermarkets and hypermarkets holding the largest share. Overall, the rising interest in natural and functional foods, the growth of e-commerce platforms, and increasing awareness of gut health are significant trends driving the expansion of the probiotics market.

2.16. Conceptual Frame Work/Work plan

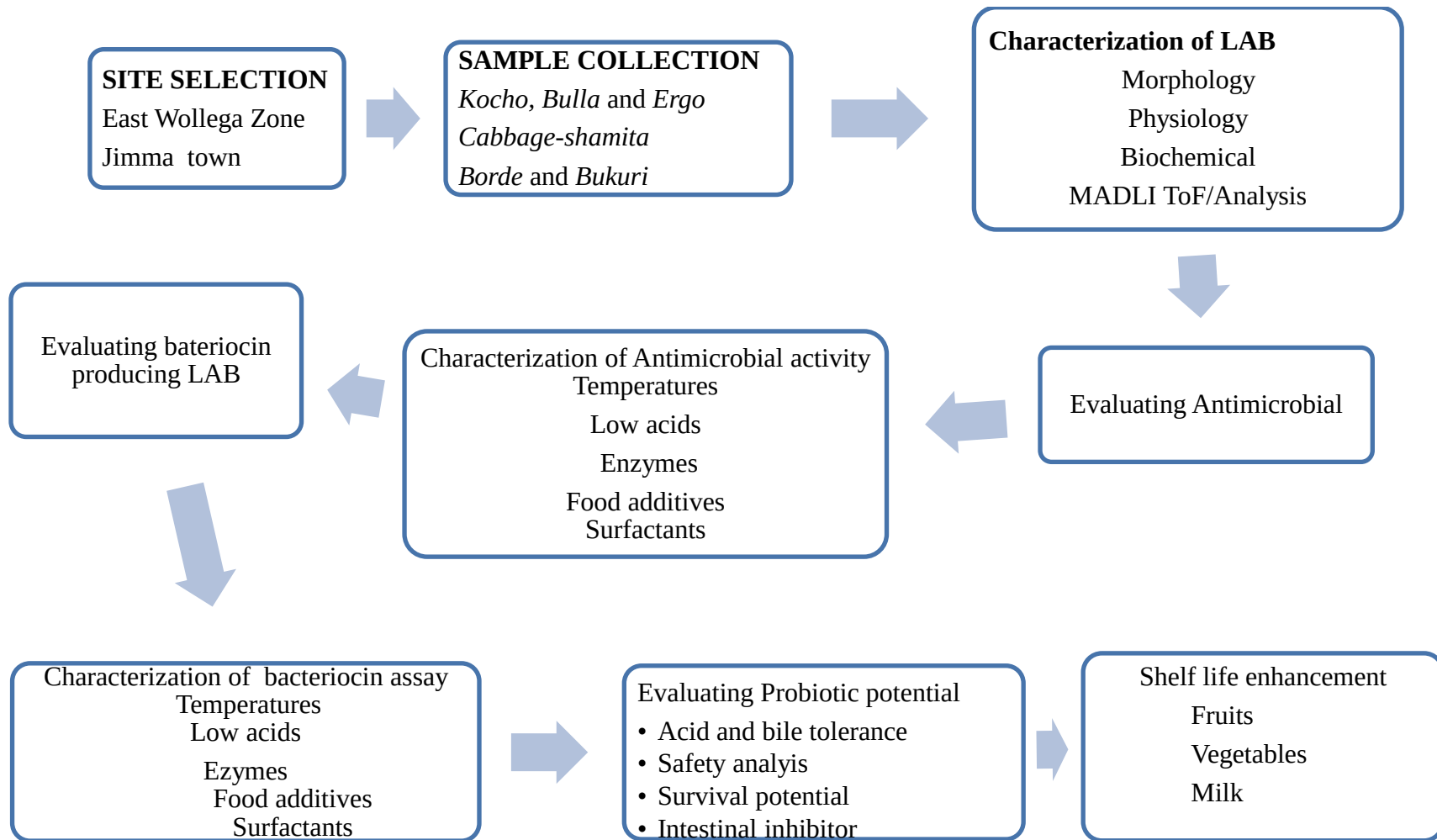


Figure 2. 11. Flow chart for conceptual framework of the study

3.

CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM ETHIOPIAN TRADITIONAL FERMENTED FOODS AND BEVERAGES

**The finding of this paper is published in *Probiotics and
Antimicrobial Proteins***

Amenu, D., & Bacha, K. (2024). Antagonistic Effects of Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Foods and Beverages Against Foodborne Pathogens. *Probiotics and Antimicrobial Proteins*, 0123456789. <https://doi.org/10.1007/s12602-024-10231-5>

The cover page with the abstract is annexed in the Appendix section

ABSTRACT

In Ethiopia, numerous communities have long been producing and using a variety of traditional fermented foods and beverages including Kocho, Bulla, Ergo, Cabbage-Shamita, Borde, and Bukuri. These products are made using traditional knowledge and locally available resources. The main microorganisms involved in fermenting these foods and beverages are lactic acid bacteria (LAB), yeast, molds, and some *Bacillus* species. LAB in Ethiopian fermented foods have shown potential in preventing food-borne pathogens through the production of antimicrobial compounds like hydrogen peroxides, carbon dioxides and bacteriocins. However, there are no detailed reports on the characterization of antimicrobial substances produced by LAB isolated from Ethiopian traditional fermented foods and beverages. Therefore, this study was carried out to evaluate the antagonistic activity of LAB against food-borne pathogens and to characterize their antimicrobial substances. The activities of LAB were tested against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella Typhimurium* ATCC 13311, and *Candida albicans* ATCC 14053. Additionally, the impact of pH, temperature, enzymes, and food additives on the antagonistic effects of LAB-produced cell free supernatants was examined. LAB were identified and characterized using a combination of phenotypic methods and MALDI TOF MS spectrum analysis. Data were analyzed using one-way ANOVA and Tukey post hoc analysis using SPSS 25 statistical software. A total of 956 LAB were isolated, of which seventeen (17 LAB) isolates, including *Pediococcus pentosaceus* (n = 7), *Enterococcus faecium* (n = 6), *Lactococcus lactis* (n = 2), and *Pediococcus acidilactici* (n = 2) were screened for antagonistic activity based on their ability to produce bacteriocins, probiotic activities, and preservative potential. . *Pediococcus pentosaceus* JULABB16, *P. pentosaceus* JULABB01, and *Enterococcus faecium* JULABBr39 demonstrated strong antagonistic activity against all the selected pathogens, with mean inhibition zone diameters that ranged from 23.50 to 35.50 mm. In addition, the bioactive metabolites produced by LAB were sensitive to proteolytic enzymes but capable of withstanding high temperatures (80–100°C) and different acid concentrations (pH 2–10). Furthermore, the antimicrobial activity of cell free supernatant (CFS) was effective over a wide range of pH (pH 2 to 10). Accordingly, CFS produced by *Lactococcus lactis*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, and *Enterococcus faecium* showed the most promising antagonistic activity against all the selected food borne pathogens. Hence, these isolates are candidates for application as natural food preservatives to enhance the shelf life of foods for long storage. What about implication for human health improvement?

Key words: Antagonistic activity, Ethiopian, food borne pathogens, traditional fermented product, Lactic acid bacteria.

3.1. INTRODUCTION

Traditional fermented foods and beverages are deeply ingrained in local cultures, often made from locally available plant and animal sources based on indigenous knowledge. These preparations are a common and essential part of the diet in rural communities globally, providing significant nutrition and sometimes even serving as meal replacements or refreshments (Ashenafi, 2008; Birhanu Abegaz, 2014). In developing nations, they form a substantial portion of dietary intake, including in Ethiopia, where they are consumed widely among different ethnic groups (Ashenafi, 2008). These traditional products harbor potentially beneficial Lactic acid bacteria (LAB), known for their antimicrobial properties against various food-borne pathogens, making them valuable additions to the diet (Berhe et al., 2019; Negasi et al., 2017).

LAB are considered GRAS (generally recognized as safe) due to their non-toxic nature, stability in various conditions including the presence of preservatives, and effectiveness at low concentrations (Angmo et al., 2016). They are extensively used to combat foodborne pathogens, extend shelf life, and offer potential probiotic benefits (Negasi et al., 2017). Among LAB genera like *Pediococcus*, *Enterococcus*, and *Lactococcus*, notable antimicrobial activity against food spoilage and pathogens has been observed, particularly in strains like *P. pentosaceus* and *P. acidilactici*, which effectively combat *Listeria monocytogenes* even at low temperatures (2–4°C) (Darbandi et al., 2022; Fugaban et al., 2021). *Lactococcus lactis*, known for bacteriocin production, is utilized as a food additive against pathogens like *L. monocytogenes* and *Staphylococcus aureus* (Loh et al., 2017). Additionally, strains of *Enterococcus faecium*, isolated from traditional fermented foods and camel milk, respectively, exhibit antagonistic actions through bacteriocin-like substances, showcasing potential probiotic and preservative properties (Farias et al., 2021).

Ethiopia has a deep-rooted habit of consuming traditional fermented foods and beverages. However, there is a limited documentation on the potential use of isolates as antimicrobial agents for preserving food and beverages, especially from products like *Bukuri* and *Cabbage-shamita*. Therefore, this study aimed to isolate, characterize, and assess the antagonistic activity of LAB obtained from Ethiopian traditional fermented foods (*Bulla*, *Kocho*, and *Ergo*) and beverages (*cabbage-shamita*, *borde*, and *bukuri*) against food spoilage and food-borne pathogens, while also examining their antimicrobial substances.

3.2. MATERIALS AND METHODS

3.2.1. Description of the study area

The study was conducted at Jimma University in Jimma Town and Wollega University in Nekemte Town by focusing on traditionally fermented foods and beverages from specific areas in Southwestern and Western Ethiopia (Fig. 3.2). The selected locations included Sasiga d, Sibu Sire, Guto Gidda districts, and Nekemte town in the East Wollega Zone as well as Jimma town in the Jimma Zone (Figure 3.2). The research spanned from September 2018 to September 2022. Nekemte is situated in the Oromia Regional State, East Wallaga Zonewith coordinates of 9°5' N latitude, 36°33' E longitude, and an elevation of 2,088 meters above sea level. Traditional fermented beverages were gathered from Nekemte and its environs. The samples were transported under aseptic conditions to the Microbiology Laboratory at Wollega University for LAB isolation and characterization. Meanwhile, traditional fermented foods were collected from Jimma town and analyzed at the Microbiological Laboratory of Jimma University, Jimma town.

3.2.2. Samples collection

A total of 180 fermented food and beverage samples were collected from East Wollega Zone and Jimma town, then transported to Jimma and Wollega Universities' Microbiology Laboratories for LAB isolation and screening. The samples included Cabbage- *shamita*, *bukuri*, and *borde* from East Wollega, and *kocho*, *ergo*, and *bullla* from Jimma Zone. Each sample (500 g/mL) was collected aseptically in sterilized containers and transported in iceboxes to the laboratories, where they were refrigerated at +4°C until further analysis.

The study sites were chosen though purposive sampling based on the availability and popularity of the aforementioned traditional fermented foods and beverages. The sites included East Wollega Zone (Guto district (Nekemte town), Sibu Sire, and Sasiga districts) and Jimma Zone (Jimma town). Similarly, a specific location was identified based on the number of individuals practicing the production and consumption of fermented beverages. The sample size was determined using the minimum sample size ($n = 30$). In total, 180 samples were collected for the current investigation, including 30 each of *kocho*, *bullla*, cabbage *shamita*, *bukuri*, *borde*, and *ergo*.

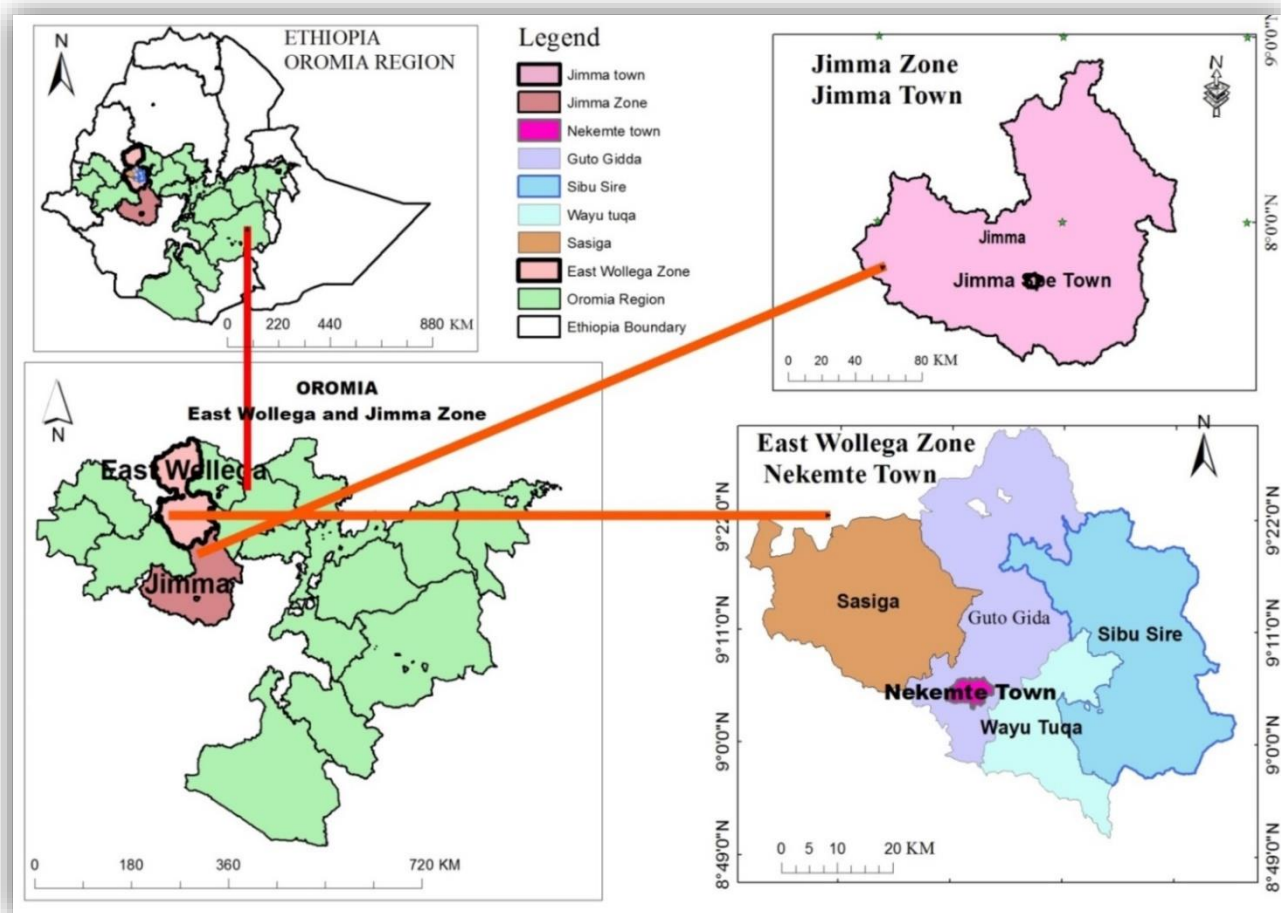


Figure 3. 1. Map of the study areas (Jimma Zone and East Wollega Zone)

3.2.3. Isolation and Characterization of Lactic Acid Bacteria

About 500 mL (500 g) each of the selected Ethiopian traditional fermented foods and beverages were collected in sterile bottles, and approximately 25 g (25 ml) of each food or beverage sample was first homogenized well and later mixed with 225 mL of sterile buffered peptone water and further homogenized for 30 seconds with a blender (homogenizer, Edmund Buhler, GmbH, Germany) followed by serial dilution in 9 ml peptone water. Appropriate dilutions from the diluted samples were transferred onto MRS agar (M6411-500, Hemedia; *Lactobacillus* MRS Agar, New Dell, India) and incubated in an anaerobic jar (MODEL NO. HV-2-AJ, New Dell, India) at 37°C for 48 h. Snow white colonies were subcultured in MRS broth and later transferred to MRS agar after right incubation period to ensure purity of the isolates (Lobo et al., 2010).

Characteristic LAB colonies: gram positive, catalase- negative, oxidase negative and that showed a clear zone around the colonies in the MRS media supplemented with 0.3% CaCO₃ were selected as presumptive LAB (Shyh-Chang et al., 2013). Then, distinct bacterial colonies were streaked on MRS agar plates to purify the isolates. The resulting pure colonies were subcultured in MRS broths [(dextrose (20 g), peptone (10 g), beef extract (10 g), yeast extract (0.5 g), sodium acetate (0.5 g), ammonium citrate (0.2 g), disodium phosphate (0.2 g), tween 80 (0.1 ml), MgSO₄ (0.05 g), agar (20 g), distilled water (1000 ml), pH (6.5)], and subsequently maintained at -32°C with 15% glycerol until used for further characterization (Patil et al., 2010). Give space between numeral and unit! Correct; 3 g but wrong 3g!

3.2.4. Confirmation of Lactic Acid Bacteria isolates

To confirm the LAB, biochemical tests like catalase, oxidase, citrate utilization and spore producing ability of LAB isolates were performed. The confirmed LAB isolates were designated with initials derived from the source of the isolates: as JULABK, JULABB, JULABBk, JULABBr, JULABE and JULABS. Where, 'JU' stands for Jimma University, 'LAB' for Lactic acid bacteria, 'B' for *Bulla*, 'K' for Kocho, 'Bk' for *Bukuri*, 'Br' for *Borde*, 'S' for *Cabbage-shamita* and 'E' for *Ergo*, while the numbers indicated the consecutive samples number.

3.2.5. Characterization of LAB isolated from traditional fermented foods and beverages

3.2.5.1. Cell morphology

Gram staining

Pure isolates were smeared on a clean slide, air-dried, and heat-fixed. The smear was stained with crystal violet for 1 minute, rinsed with water, then covered with iodine for another minute, and rinsed again. After decolorizing with ethanol for 10-15 seconds and rinsing, the smear was counterstained with safranin for 1 minute and dried. Gram status was observed under a microscope (Brand and country of origin!!) at 100x magnification (Thairu et al., 2014).

Motility and Endospore test

LAB motility was assessed by stabbing a loopful of LAB colonies into a test tube with sulfide indole motility (SIM) medium and incubating for 48 h at 37°C. A negative result is when the colony grows

along the stab line, while a positive result is when the bacteria spread across the medium surface or throughout it (Cappuccino & Sherman, 2005). The isolates were prepared on a glass slide, air-dried, heat-fixed, flooded with 0.5% malachite green solution, and steamed for 5 minutes. Safranin was used for counterstaining. The slide was cleaned with tap water, air-dried, and examined under an oil immersion objective after cooling.

3.2.5.2. Biochemical tests

3.2.5.2.1. KOH test

Visible cells from fresh cultures were mixed with 3% KOH on a clean slide using a sterile loop. Only isolates not producing a viscous product were selected, as LAB are typically gram-positive cells.

Catalase Test

The experiment involved adding two drops of 3% hydrogen peroxide to 24-hour-old LAB cultures on a glass slide. Only isolates without gas bubble production were selected as presumptive LAB isolates.

3.2.5.2.2. Oxidase test

A filter paper soaked in oxidase reagent was dried, then a well-isolated colony from a 24-hour pure culture was rubbed onto it using an inoculating loop. The observation of a purple or blue color change indicated oxidase positivity.

3.2.5.2.3. Citrate utilization tests

Citrate utilization was tested using citrate reagent. A slant was streaked with a wire loop for inoculation. Positive results showed a color change from green to blue, while negative results remained green.

3.2.5.3. Physiological characterization of Lactic Acid Bacteria

The isolates were tested for their physiological resilience under varying conditions. They were exposed to temperatures of 25°C and 45°C to assess their thermal tolerance and growth potential. Additionally, their ability to withstand different salt concentrations (4% and 6.5%) was examined by inoculating them into MRS broth with varying salt levels and bromocresol purple indicator. The isolates' fermentation characteristics, including homo-fermentation, hetero-fermentation, and CO₂ production from glucose, were evaluated in modified MRS broth with 1% glucose using Durham tubes. Furthermore, their capacity for arginine hydrolysis was assessed.

3.2.6. Antimicrobial activity of Lactic acid bacteria

3.2.6.1. Bacteria strains

The standard strains used in this study were *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Listeria monocytogens* ATCC7644, *Pseudomonas auroginoasa* ATCC27853, *Salmonella Typhimurium* ATCC13311, and *Candida albicans* ATCC 14053. The strains were kindly obtained from Ethiopian Public Health Institute (EPHI).

3.2.6.2. Primary screening

Out of 956 LAB isolates, 583 displayed robust antimicrobial effects, and 340 showed notable antagonistic effects against chosen foodborne pathogens. Consequently, 125 isolates were chosen based on their antagonistic activity, bacteriocin production, probiotic traits, and preservative potential for primary screening. The isolates were streaked horizontally on Muller Hinton Agar alongside the tested pathogens, which were streaked vertically on the same medium. Incubation at 37°C for 24 h was followed by examination for zones of inhibition around the streaked lines or points to assess antimicrobial activity (Avaiyarasi et al., 2016).

3.2.6.3. Extraction of Antibacterial Compound from LAB

Out of the initial 125 LAB isolates, 54 were chosen for assessing their antimicrobial effects. These isolates were grown in 100 mL MRS broth in a 250 mL Erlenmeyer flask and kept at 30°C for 48 h. The active cells were then separated by centrifugation at 10,000 rpm for 15 minutes at 4°C to obtain cell-free culture supernatant (CFS). This CFS was treated with 80% ammonium sulfate to cause the precipitation of bioactive compounds, which were subsequently isolated using a separating funnel and concentrated under vacuum using a rotary evaporator at 44°C (Hassan et al., 2020).

3.2.6.4. Secondary screening

After neutralizing the cell-free culture supernatant (CFS) to pH 7 with 1 M NaOH to eliminate organic acid inhibition, 17 LAB isolates were chosen for secondary screening. These selections were based on their tolerance to acid and bile, potential antagonistic effects, preservative efficacy, and bacteriocin production, aiming to characterize potential antimicrobial compounds (Arena et al., 2016).

3.2.6.5.Characterization of the antimicrobial substances

The bioactive compounds from LAB isolates were tested for enzyme sensitivity using pepsin, trypsin, proteinase K, and lysozyme at 5 mg/mL concentration (Goa et al., 2022). The treated cell-free culture supernatant (CFS) was tested for antimicrobial activity against foodborne pathogens using the agar well diffusion method. Heat treatment was applied to CFS at temperatures of 45°C, 65°C, 85°C, and 100°C for 30 minutes to assess its antimicrobial effectiveness against food borne pathogens. The pH variations (2, 4, 6, 8, and 10) were induced in CFS using 1 N HCl and 1 N NaOH, with pH readings taken using a pH meter to study their antibacterial effects. Additionally, 10% methanol and ethanol were added to CFS to observe their influence on bioactivity against the test pathogens. The stability and antagonistic potential of CFS were further evaluated by introducing 1% (w/t) sodium citrate and potassium sorbate as food preservatives by examining their impact on antibacterial activity. The treated CFS was then incubated for 24 h at 37°C using the disc diffusion method, and residual inhibitory activity was gauged by observing the zone of inhibition (Arena et al., 2016).

3.2.6.6.MALDI –TOF/ MS, characterization of Lactic acid bacteria

For sample preparation, 2-5 single colonies of actively growing LAB cultures were used. In a 1.5 mL centrifuge tube, 1-5 mg of colonies were mixed with 300 µL of ultra-pure water and 900 µL of 70% ethanol, then centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded, and the pellet dried at room temperature. To remove any remaining supernatant, the pellet was centrifuged again and dried for 5 minutes. Next, 20 µL of 70% formic acid was added and the mixture left for 5 minutes, followed by 20 µL of acetonitrile (ACN). The mixture was centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred to a centrifuge tube, and 1 µL was placed on a sample target plate, covered with a matrix solution, and dried. MALDI-TOF MS EXS3000 (Zybio, China) was used for LAB identification. MS spectra were obtained in linear mode in the 2,000-20,000 Da range. *E. coli* ATCC 25922 was used for calibration. Log scores >2.0 were used for species identification, scores between 2.0 and 1.7 for genus or probable species, and scores <1.7 were considered unreliable (Yu et al., 2021). Figure 3.3 shows the details steps for MALDI ToF/MS analysis of LAB isolates.

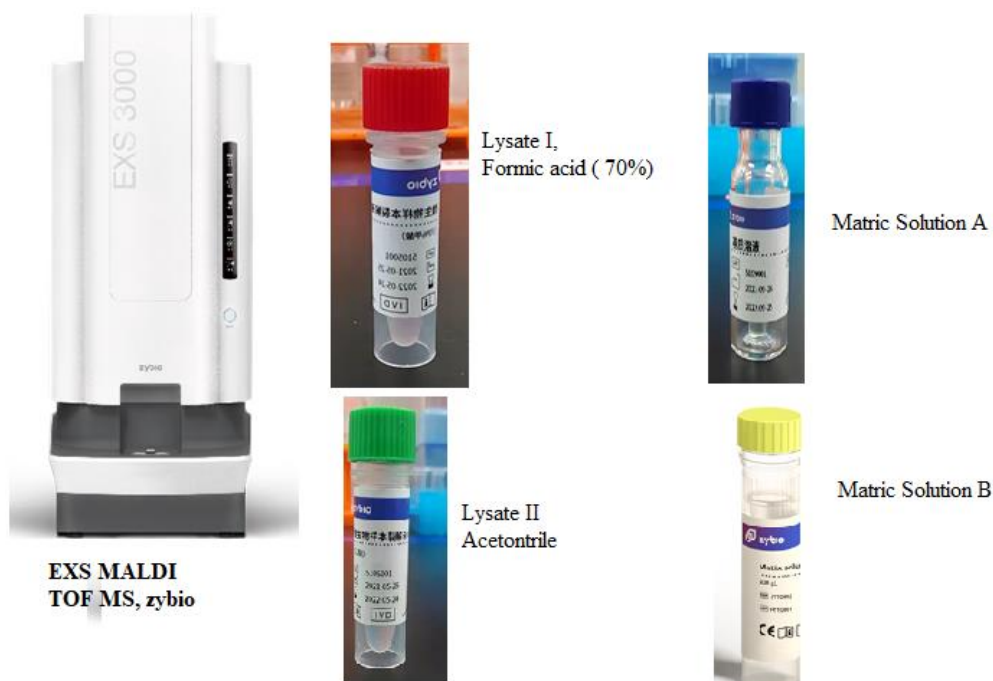


Figure 3. 2. Sample preparation, analysis and identification of LAB using MALDI TOF-MS analysis

3.2.6.7.Data Analysis

The data analysis for this study utilized the SPSS Statistics software package (version 26 IBM Corp., Armonk, NY, USA). To compare the mean differences in antimicrobial activities (zone of inhibition) analysis of variance (ANOVA) was conducted. Additionally, post hoc multiple comparison analysis was performed to further examine specific differences between groups. Statistical significance was determined at a significance level of $p < 0.05$, indicating that any observed differences were unlikely to have occurred by chance.

3.3. RESULTS

3.3.1. Isolation and distribution of LAB in fermented foods and beverages

A total of **1800** isolates were identified from six different samples (*cabbage-shamita*, *b*, *Bukuri*, *bull*, *kocho*, and *ergo*). Out of the total **1800** isolates, **956 (53.23 %)** were confirmed presumptive LAB isolates. Out of these presumptive LAB isolates, 340 (32.23%), 173 (18.09%), 128 (14%), 122 (12.72%), 112 (11.71%), and 81 (8.48%) were isolated from *bull*, *kocho*, *ergo*, *cabbage-shamita*, *borde*, and *bukuri*, respectively (Table 3. 1).

Table 3. 1. Distribution and diversity of LAB in Ethiopian traditional fermented foods and beverages (n=956).

Samples	No of Isolates	Tentative LAB genera identified in this study				
		<i>Lactobacillus</i>	<i>Lactococcus</i>	<i>Pediococcus</i>	<i>Enterococcus</i>	<i>Streptococcus</i>
<i>Bulla</i>	340(35.56%)	120	70	60	60	30
<i>Kotcho</i>	173(18.09%)	66	30	30	30	17
<i>Ergo</i>	128(13.40%)	50	27	23	15	13
<i>Cabbage-shamita</i>	122(12.72%)	52	24	20	14	12
<i>Borde</i>	112(11.71%)	45	15	22	17	13
<i>Bukuri</i>	81(8.48%)	35	12	12	12	10
Total	956	368(38.5%)	178(18.62%)	167(17.5%)	148(16%)	95(9.94%)

The antimicrobial activity of LAB isolates indicated that, 583 (61%) of 956 presumptive LAB isolates had significant inhibitory action against selected foodborne pathogens, out of which 147 (25.00%) and 202 (34.70%) showed strong antagonistic activity against *S. aureus* and *E. coli*, respectively. Furthermore, 340 (36% of them) demonstrated significant antagonistic action against all the tested food-borne pathogens (Table 3.2).

Table 3. 2. Antimicrobial activity of LAB isolated from Ethiopian traditional fermented foods and beverages (n=583)

Samples	No of isolates	<i>Escherchia coli</i> ATCC®25922	<i>Salmonella</i> <i>Typhimirium</i> ATCC®13311	<i>Pseudomonas</i> <i>auroginosa</i> ATCC® 27853	<i>Staphylococcus</i> <i>aureurs</i> ATCC®25923	<i>Listeria</i> <i>monocytogenes</i> ATCC® 7644)	<i>Candida albicans</i> ATCC® 14053
<i>Bulla</i>	187(32.07%)	53(9.09%)	20 (3.43%)	17 (2.92%)	47 (8.1%)	23 (3.95%)	27 (4.63%)
<i>Kotcho</i>	100(17.15%)	39(6.69%)	5 (0.86%)	5 (0.86%)	25 (4.3%)	12 (2.06%)	14 (2.40%)
<i>Ergo</i>	81(13.90%)	29(4.97%)	5 (0.86%)	4 (0.69%)	21 (3.6%)	10 (1.72%)	12 (2.06%)
<i>Cabbage shamita</i>	80(13.72%)	31(5.32%)	4 (0.69%)	4(0.69%)	20 (3.4%)	9 (1.54%0	12 (2.06%)
<i>Borde</i>	75(12.86%)	29(4.97%)	4 (0.69%)	3 (0.51%)	18 (3.1%)	10 (1.72%)	11 (1.89%)
<i>Bukuri</i>	60(10.24%)	21(3.60)	3 (0.51%)	3(0.51%)	16 (2.75)	8 (1.37%)	9 (1.54%)
Total	583	202(34.70%)	40 (7.03%)	36 (6.18%)	147 (25%)	72 (12.40%)	85 (15%)

3.3.2. Morphological, Biochemical and Physiological Characterization

The majority of the isolates (over 40%) were rod-shaped *Lactobacillus* species, while the rest were cocci-shaped *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus* species. In terms of acid-bile tolerance, 85% survived bile salt concentrations between 4% and 6.5%. Additionally, 340 LAB isolates grew rapidly at 4% salt concentration. All the 340 isolates grew at 25°C, but only 55 (16.17%) could grow at 45°C, with 285 (84%) failed to grow at this temperature (Table 3.3). Only 54 (16%) of the 340 LAB isolates produced gas during glucose fermentation, while 286 (84%) did not. For arginine hydrolysis, 142 (42%) tested positive, but 198 (58%) did not hydrolyze arginine (Table 3.3).

3.3.3. Carbohydrate utilization (Fermentation) profile of lactic acid bacteria

All the *E. faecium* species utilized the majority of the tested carbohydrates (lactose, maltose, fructose, sucrose, xylose, sorbitol, dextrose, galactose, starch, and glucose). However, none of the seventeen LAB isolates (*P. pentosaceus*, *P. acidilactici*, *E. faecium*, and *L. lactis*) produced gas during glucose fermentation but produced only acids (Table 3.4).

Table 3. 3. Morphological, biochemical and physiological characterization of Lactic acid bacteria isolates (n=340)

Characteristics	Group of Lactic Acid Bacteria isolated from Ethiopian traditional fermented foods and beverages				
Morphology	Rod	Coccus			
Cell arrangement	Short or long Chains	Spherical or ovoid single or chains	Coccus in pairs and tetrads	Coccus in chain	Pairs, or short chain
Gram reaction	+	+	+	+	+
Catalase	-	-	-	-	-
Oxidase	-	-	-	-	-
Motility	-	-	-	-	-
Endospore	-	-	-	-	-
Gas production	+/-	+/-	+/-	+/-	+/-
Arginine production	+/-	+/-	+/-	+/-	+/-
Fermentation type	Homo/Hetero	Homo/Hetero	Homo/Hetero	Homo/Hetero	Homo/Hetero
Growth (T°C)					
25	+	+	+	+	+
45	+/-	+/-	+/-	+/-	+/-
Salt tolerance					
4%	+	+	+	+	+
6.5%	+	+	+	+	+
pH tolerance					
4.7	+	+	+	+	+
9.6	+/-	+/-	+/-	+/-	+/-
Likey LAB Genera	<i>Lactobacillus</i>	<i>Lactococcus</i>	<i>Pediococcus</i>	<i>Streptococcus</i>	<i>Enterococcus</i>
Number of isolates	138 (40.58%)	70 (20.58%)	51 (15.00%)	38 (11.18%)	43 (12.64%)

Where, “+” = Positive/Presence/ Growth; “-” = Negative/Absence/ No growth

Table 3. 4. Carbohydrate utilization pattern of LAB isolated from Ethiopian traditional fermented foods and beverages.

Carbohydrates	Group of Lactic Acid Bacteria isolated from Ethiopian traditional fermented foods and beverages				
	Rod		Coccus		
	Short or long Chains	Spherical or ovoid single or chains	Coccus in pairs and tetrads	Coccus in chain	Pairs, or short chain
Lactose	35 (10.29%)	129 (37.94%)	66 (19.41 %)	50 (14.71%)	40 (11.76 %)
Xylose	35 (10.29%)	123 (36.18%)	66 (19.41%)	50 (14.71%)	40 (11.76 %)
Maltose	36 (10.59%)	127 (37.35 %)	64 (18.82 %)	51 (15.00%)	36 (10.59%)
Fructose	42 (12.35%)	116 (34.12 %)	65 (19.12 %)	51 (15.00%)	35 (10.29 %)
Dextrose	35 (10.29%)	115 (33.82 %)	65 (19.12 %)	51 (15.00%)	36 (10.59%)
Sucrose	38 (11.18%)	121 (35.59 %)	68 (20.00%)	51 (15.00%)	37 (10.88%)
Glucose	42 (12.35%)	122 (35.88 %)	69 (20.29 %)	51 (15.00%)	40 (11.76 %)
Galactose	38 (11.18%)	122 (35.88 %)	67 (19.71 %)	51 (15.00%)	40 (11.76 %)
Citrate	38 (11.18%)	124 (36.47 %)	70 (20.59 %)	50 (14.71%)	40 (11.76 %)
Sorbitol	42 (12.35%)	130 (38.24 %)	70 (20.59 %)	51 (15.00%)	40 (11.76 %)
Likey LAB Genera	<i>Lactobacillus</i>	<i>Lactococcus</i>	<i>Pediococcus</i>	<i>Streptococcus</i>	<i>Enterococcus</i>

Note: (+) = Positive activity/ growth, (-) = no growth / negative activity

3.3.4. Antimicrobial Activities of Lactic acid bacteria (LAB)

The antimicrobial activity of LAB isolates was evaluated against selected food borne pathogens (Table 3.5). The average zone of inhibition of LAB isolates against food borne pathogens ranged from 13.33 mm to 35.94 mm, of which 17 isolates had the largest diameter of inhibition zone (> 15 mm) and these were screened for probiotic evaluation. Isolates *Pediococcus pentosaceus* JULAB-B01, *Pediococcus pentosaceus* JULAB-B16, *Pediococcus pentosaceus* JULAB-Br35 and *Enterococcus faecium* JULAB-Br39 showed the highest inhibition zone against *E. coli* ATCC 5922, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, and *Pseudomonas aeruginosa* ATCC 27853, with the minimum inhibition zone being 25.00 mm and 30.58 mm against *Pseudomonas aeruginosa* and *Candida albicans* ATCC 14053, respectively. The diameter of zone of inhibitions recorded for different isolates were significantly different at $p < 0.05$ (Table 3.5).

Out of all the LAB isolates selected as potential probiotic candidates, JULAB-Br39 against *E. coli* ATCC@25922 (33.50 mm), and JULABB16 against *S. aureus* ATCC@25923 (32.50 mm), *L. monocytogenes* ATCC@7644 (35.50 mm), *P. aeruginosa* ATCC@27853 (30.50 mm), and *C. albicans* ATCC@14053 (30.50 mm) had relatively the largest diameter of inhibition zones. Finally, *P. pentosaceus* JULAB-B16, *P. pentosaceus* JULAB-B01, and *P. pentosaceus* JULAB-E31 demonstrated the highest and most efficient antagonistic activity against fungal infections, with inhibition zone diameters against *Candida albicans* of 30.50, 29.67, and 24.00 mm, respectively (Table 3.5).

In light of this, 340 of the 583 isolates showed a strong inhibitory zone against all the selected foodborne pathogens, among which 125 isolates exhibited probiotic properties. Finally, based on their acid and bile tolerance, potential antagonistic effects, preservative efficiency, and bacteriocin production, seventeen isolates (17) were subjected to MALDI ToF/Mass analysis and identified as *Pediococcus pentosacues* (n = 7), *Pediococcus acidilactici* (n = 2), *Enterococcus faecium* (n = 6), and *Lactococcus lactis* (n = 2) (Table 3.7).

Table 3. 5. Inhibition zone diameters (mm) of LAB isolates against some foodborne pathogens (n=125)

S/N	LAB isolates	<i>E. coli</i> ATCC® 25922	<i>S. aureus</i> ATCC® 25923	<i>L.monocytogenes</i> ATCC® 7644	<i>P. auroginosa</i> ATCC® 27853	<i>S. Tphimurium</i> ATCC® 13311	<i>C.albicans</i> ATCC® 14053
1)	JUB01	30.13±0.18 ^{ab}	28.30±0.40 ^b	25.40±0.53 ^c	29.67±0.33 ^a	20.50±0.71 ^{abc}	29.67±0.33 ^a
2)	JUB02	19.28±1.45 ^{cd}	16.28±0.11 ^{ef}	16.95±0.59 ^{ef}	17.95±0.96 ^{def}	18.28±0.43 ^{bcd}	16.62±0.16 ^d
3)	JUB04	18.95±2.56 ^{cde}	16.95±0.56 ^{ef}	15.95±0.54 ^e	16.95±0.45 ^f	18.28±0.01 ^{bcd}	14.62±0.66 ^d
4)	JUB05	18.28±0.43 ^{cdef}	14.95±0.03 ^f	16.28±0.63 ^{ef}	17.28±0.36 ^f	17.62±0.29 ^c	17.62±0.18 ^c
5)	JUB06	16.95±0.54 ^{def}	16.28±0.8 ^{ef}	16.62±0.70 ^{ef}	17.62±0.07 ^f	20.62±0.53 ^{ab}	17.95±0.08 ^c
6)	JUB07	20.95±0.32 ^c	16.95±003 [?]	15.28±0.43 ^e	16.28±0.35 ^{???}	21.28±0.08 ^{ab}	17.95±0.62 ^c
7)	JUB08	18.78±0.06 ^{cde}	15.78±0.58 ^e	14.78±0.93 ^f	15.78±0.25 ^{fg}	17.12±0.31 ^c	15.78±0.42 ^d
8)	JUB10	27.79±0.30 ^b	18.40±0.53 ^{cde}	24.50±0.71 ^c	18.33±0.33 ^{def}	19.67±0.33 ^b	15.33±0.34 ^d
9)	JUB11	18.12±0.67 ^{cde}	16.45±0.24 ^{ef}	14.12±0.24 ^f	15.12±0.25 ^f	14.45±0.96 ^c	15.78±0.26 ^d
10)	JUB12	16.12±0.07 ^f	13.78±0.18 ^f	13.78±0.45 ^g	14.78±0.36 ^f	15.45±0.77 ^c	17.45±0.85 ^c
11)	JUB13	16.45±0.37 ^{ef}	12.45±0.45 ^{fg}	16.12±0.66 ^{ef}	17.12±0.25 ^f	20.12±0.50 ^{ab}	19.12±0.27 ^c
12)	JUB14	20.78±0.83 ^c	13.78±0.13 ^f	14.78±0.86 ^f	15.78±0.12 ^{fg}	19.78±0.19 ^b	18.12±0.33 ^d
13)	JUB15	19.17±.076 ^{cde}	11.17±0.26 ^{gh}	14.50±0.78 ^f	15.5±0.12 ^{fg}	18.50±0.50 ^{bcd}	17.50±0.80 ^c
14)	JUB16	26.00±0.57 ^b	32.50±0.71 ^a	35.50±0.71 ^a	30.50±0.71 ^a	20.50±0.71 ^{abc}	30.50±0.71 ^a
15)	JUB17	18.17±0.29 ^{cde}	10.83±0.75 ^{gh}	14.50±0.04 ^e	15.5±0.5 ^{fg}	14.83±0.75 ^c	17.50±0.18 ^c
16)	JUB18	17.50±0.18 ^{def}	10.50±0.50 ^{gh}	13.83±0.69 ^g	14.83±0.14 ^f	14.50±0.50 ^c	18.83±0.59 ^c
17)	JUB19	18.83±0.26 ^{cde}	8.83±0.04 ^{ij}	18.17±0.02 ^{ef}	19.17±0.25 ^{cde}	17.50±0.87 ^c	17.17±0.65 ^c
18)	JUB20	21.83±0.48 ^c	10.50±0.50 ^{gh}	16.17±0.48 ^{ef}	15.17±0.6 ^{fg}	17.50±0.97 ^{bcd}	16.50±0.77 ^d
19)	JUB23	20.17±0.04 ^c	11.17±0.61 ^{gh}	16.50±0.09 ^{ef}	15.5±0.2 ^{fg}	17.17±0.45 ^c	17.50±0.32 ^c
20)	JUB26	20.50±0.04 ^c	10.83±0.84 ^{gh}	17.17±0.69 ^{ef}	16.17±0.3 ^{fg}	14.17±0.25 ^c	19.17±0.58 ^c
21)	JUB27	25.63±0.88 ^{bc}	18.00±0.00 ^{cde}	17.50±0.71 ^{def}	18.33±0.34 ^{cdef}	25.34±0.34 ^a	24.34±0.34 ^{bcd}
22)	JUB29	19.83±0.65 ^{cd}	12.50±0.50 ^{fg}	17.17±0.69 ^{ef}	16.17±0.1 ^{fg}	16.50±0.50 ^{de}	20.17±0.33 ^c
23)	JUB30	20.67±0.66 ^c	20.30±0.36 ^{cd}	21.30±0.81 ^{cde}	20.50±0.71 ^{cde}	22.50±0.71 ^{ab}	17.75±0.35 ^c
24)	JUB31	17.50±0.28 ^{cdef}	10.83±0.62 ^{gh}	18.83±0.76 ^{de}	17.83±0.5 ^f	17.50±0.76 ^c	18.83±0.25 ^d
25)	JUB32	17.83±0.75 ^{cdef}	10.50±0.50 ^{gh}	17.17±0.63 ^{ef}	16.17±0.58 ^f	17.50±0.77 ^c	18.17±0.30 ^d
26)	JUB35	30.25±0.35 ^{ab}	30.00±0.00 ^{ab}	30.40±0.53 ^b	30.00±1.41 ^a	25.50±0.71 ^a	30.58±1.14 ^a
27)	JUB36	17.50±0.5 ^{cdef}	10.17±0.04 ^h	17.50±0.28 ^{ef}	16.5±0.36 ^f	17.17±0.29 ^c	18.50±0.50 ^d
28)	JUB39	19.75±±0.35 ^{cd}	15.70±0.93 ^e	15.50±0.71 ^e	20.00±1.41 ^{cde}	18.50±0.71 ^{bcd}	18.50±0.71 ^c
29)	JUB44	19.17±0.25 ^{cde}	10.50±0.15 ^{gh}	19.50±0.87 ^{de}	18.5±0.36 ^{def}	16.50±0.22 ^{de}	20.50±0.50 ^{cde}
30)	JUB45	21.50±0.5 ^c	11.17±0.96 ^g	19.50±0.87 ^{de}	18.5±0.25 ^{cdef}	17.17±0.36 ^c	20.50±0.50 ^{cde}
31)	JUB47	25.63±0.88 ^{bc}	18.00±0.00 ^{cde}	17.50±0.71 ^{def}	18.33±0.34 ^{cdef}	25.34±0.34 ^a	24.34±0.34 ^{bcd}
32)	JUB50	19.42±0.58 ^{cd}	11.42±0.36 ^{gh}	18.75±0.98 ^{de}	17.75±0.25 ^f	16.75±0.17 ^{de}	18.75±0.16 ^d

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33)	JUB51	18.08±0.88 ^{cde}	11.08±0.88 ^g	16.75±0.38 ^{ef}	15.75±0.14 ^{fg}	17.42±0.09 ^c	18.08±0.13 ^c
34)	JUB52	17.08±0.2 ^{ef}	10.42±0.01 ^{gh}	18.42±0.64 ^{de}	17.42±0.25 ^f	16.42±0.77 ^{de}	18.08±0.01 ^c
35)	JUB53	19.17±0.59 ^{cd}	12.50±0.39 ^{fg}	20.50±0.09 ^d	21±0.1 ^c	15.50±0.52 ^c	19.83±0.66 ^c
36)	JUB54	21.83±0.88 ^c	13.50±0.82 ^f	20.50±0.09 ^d	21±0.2 ^c	19.17±0.13 ^b	21.17±0.64 ^{cde}
37)	JUB56	18.50±0.71 ^{cde}	18.33±0.33 ^{cde}	20.40±0.53 ^d	20.60±0.85 ^c	19.50±0.71 ^b	15.50±0.71 ^d
38)	JUB60	18.00±0.57 ^{cde}	22.10±0.15 ^c	19.00±0.41 ^d	20.00±0.00 ^{cde}	15.00±0.00 ^c	25.00±0.00 ^b
39)	JUB62	18.17±0.88 ^{cde}	12.50±0.39 ^{fg}	18.17±0.45 ^{de}	18.6±70.15 ^{de}	23.17±0.94 ^{ab}	22.83±0.13 ^{cde}
40)	JUB71	17.50±0.58 ^{cdfe}	10.50±0.66 ^{gh}	19.83±0.39 ^{de}	20.33±0.12 ^{cde}	24.83±0.82 ^a	22.17±0.65 ^{cde}
41)	JUBK01	17.83±0.4 ^{cdef}	11.83±0.36 ^g	22.17±0.50 ^{de}	22.67±0.16	16.17±0.36 ^{de}	19.17±0.81 ^c
42)	JUBK02	20.93±0.11 ^c	18.30±0.71 ^{cde}	15.30±0.35 ^e	18.25±0.35 ^{cdef}	18.34±0.34 ^{bcd}	23.67±0.88 ^{bcd}
43)	JUBK06	14.50±2.12 ^f	15.50±0.71 ^e	18.33±0.33 ^{de}	14.50±0.71 ^f	18.45±0.63 ^{bcd}	18.50±0.71 ^c
44)	JUBK15	24.00±0.00 ^{bcd}	15.00±0.00 ^e	14.00±0.00 ^e	24.50±0.71 ^c	15.50±0.71 ^c	19.00±1.41 ^c
45)	JUBK21	22.33±0.33 ^c	18.00±0.00 ^{de}	17.67±0.33 ^{def}	22.33±0.34 ^c	19.67±0.34 ^b	19.67±0.34 ^c
46)	JUBK29	18.63±0.88 ^{cde}	15.50±0.71 ^e	18.33±0.34 ^{de}	20.50±0.71 ^{cde}	20.45±0.63 ^{abc}	18.00±0.00 ^c
47)	JUBK32	16.50±2.12 ^{cdef}	18.50±0.71 ^{cde}	23.00±1.40 ^c	20.50±0.71 ^{cde}	20.45±0.63 ^{abc}	20.50±0.71 ^c
48)	JUBK33	16.50±2.12 ^d	18.50±0.71 ^{cde}	23.00±1.40 ^c	20.50±0.71 ^{cde}	20.45±0.63 ^{abc}	20.50±0.71 ^c
49)	JUBK34	18.67±0.66 ^{cde}	15.00±0.00 ^e	15.60±0.87 ^e	23.50±0.71 ^c	20.45±0.63 ^{abc}	21.00±1.14 ^{cde}
50)	JUBK38	21.00±1.41 ^c	20.30±0.40 ^{cd}	17.67±0.34 ^{def}	15.50±0.85 ^c	23.45±0.63 ^{ab}	26.00±1.41 ^b
51)	JUBK39	20.25±0.35 ^c	15.50±0.71 ^e	18.90±0.31 ^{de}	21.50±0.71 ^c	20.50±0.63 ^{abc}	19.00±1.41 ^c
52)	JUBK39	20.25±0.35 ^c	15.50±0.71 ^e	18.90±0.31 ^{de}	21.50±0.71 ^c	20.50±0.63 ^{abc}	19.00±1.41 ^c
53)	JUBK40	21.00±1.41 ^c	20.30±0.40 ^{cd}	17.67±0.34 ^{def}	15.50±0.85 ^e	23.45±0.63 ^{ab}	26.00±1.41 ^b
54)	JUBR03	19.00±1.41 ^{cd}	20.10±0.10 ^{cd}	16.00±0.41 ^e	19.50±0.71 ^d	17.66±0.33 ^{cde}	18.25±0.35 ^c
55)	JUBR04	15.00±0.25 ^f	18.50±0.58 ^{cde}	18.50±0.71 ^{de}	14.33±0.33 ^f	18.45±0.63 ^{bcd}	18.00±0.00 ^c
56)	JUBR05	18.25±0.35 ^{cde}	15.30±0.37 ^e	24.23±0.71 ^c	13.00±1.41 ^f	18.45±0.63 ^{bcd}	19.50±0.71 ^c
57)	JUBR12	18.67±0.66 ^{cde}	15.00±0.00 ^e	14.50±0.71 ^e	16.50±0.71 ^e	24.00 ±1.00 ^a	19.33±0.33 ^c
58)	JUBR15	20.83±0.50 ^c	10.83±0.22 ^{gh}	19.83±0.70 ^{de}	20.33±0.25 ^{cde}	22.50±0.13 ^{ab}	21.50±0.13 ^c
59)	JUBR17	18.88±1.24 ^{cd}	15.60±0.87 ^e	19.67±0.33 ^d	16.67±0.66 ^e	13.45±0.63 ^d	20.50±0.71 ^c
60)	JUBR20	21.17±0.23 ^c	12.50±0.13 ^{fg}	20.17±0.13 ^d	20.67±0.31 ^{cde}	21.17±0.17 ^{ab}	22.83±0.02 ^{cde}
61)	JUBR21	20.28±0.40 ^c	17.90±0.16 ^d	21.00±1.42 ^{cde}	18.75±1.06 ^{cdef}	15.45±0.63 ^c	22.50±0.71 ^{bcd}
62)	JUBR24	20.83±0.13 ^c	10.17±0.64 ^{gh}	21.17±0.18 ^{de}	21.67±0.52 ^c	24.83±0.95 ^a	24.50±0.66 ^{bcd}
63)	JUBR29	19.17±0.69 ^{cd}	11.17±0.54 ^{gh}	21.50±0.30 ^{de}	22±0.30 ^{cd}	15.50±0.13 ^c	18.50±0.25
64)	JUBR30	22.83±0.66 ^c	12.17±0.09 ^{fg}	18.83±0.96 ^{de}	19.3±0.30 ^{cde}	18.17±0.22 ^{bcd}	19.83±0.62 ^c
65)	JUBR31	19.50±0.18 ^{cd}	10.83±0.96 ^g	19.83±0.96 ^{de}	20.33±0.12 ^{cde}	22.17±0.04 ^{ab}	21.83±0.14 ^c
66)	JUBR33	33.50±0.71 ^a	23.50±0.71 ^c	26.00±1.41 ^{bcd}	18.63±0.88 ^{cdef}	20.50±0.71 ^{abc}	21.00±1.41 ^{cde}

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67)	JUBR34	20.08±0.11 ^c	15.50±0.49 ^e	29.67±0.33 ^{bc}	29.50±0.71 ^a	19.00±1.41 ^b	30.50±0.71 ^a
68)	JUBR38	21.83±0.18 ^c	11.83±0.51 ^g	20.83±0.12 ^d	21.33±0.41 ^c	18.50±0.19 ^{bcd}	25.17±0.22 ^b
69)	JUBR39	20.08±0.11 ^c	15.50±0.49 ^e	29.67±0.33 ^{bc}	29.50±0.71 ^a	19.00±1.41 ^b	30.50±0.71 ^a
70)	JUBR40	18.50±0.71 ^{cde}	19.80±0.35 ^{cde}	15.80±0.06 ^e	17.00±0.00 ^e	17.66±0.34 ^{cde}	16.33±0.33 ^d
71)	JUE01	20.00±1.15 ^c	14.00±0.00 ^{efg}	15.10±0.18 ^e	19.33±0.33 ^{cde}	20.50±1.41 ^{abc}	14.50±0.71 ^d
72)	JUE02	21.17±0.18 ^c	9.83±0.13 ^h	20.50±0.77 ^{de}	21±0.67 ^c	22.50±0.82 ^{ab}	23.83±0.22 ^{bcd}
73)	JUE05	20.28±0.40 ^c	20.30±0.37 ^{cd}	29.33±0.66 ^{bc}	17.50±0.71 ^e	19.25±0.47 ^b	15.75±1.43 ^d
74)	JUE07	15.45±0.63 ^f	19.60±0.52 ^{cde}	20.50±0.70 ^d	17.25±0.25 ^e	19.67±0.33 ^b	20.33±0.33 ^{cde}
75)	JUE08	18.67±0.33 ^{cde}	21.00±0.00 ^c	17.50±0.71 ^{def}	19.33±0.33 ^{cde}	15.50±0.71 ^c	18.00±0.00 ^c
76)	JUE12	18.13±0.18 ^{cde}	20.50±0.71 ^{cd}	18.33±0.33 ^{de}	19.50±0.71 ^d	17.50±0.50 ^{cde}	19.00±0.00 ^c
77)	JUE16	19.17±0.17 ^{cde}	9.17±0.75 ^h	18.83±0.25 ^{ef}	19.3±30.32 ^{de}	16.50±0.80 ^{de}	17.50±0.25 ^c
78)	JUE20	21.67±0.26 ^c	10.00±0.95 ^{gh}	18.00±0.63 ^{de}	18.5±0.06 ^{cdef}	18.33±0.27 ^{bcd}	20.00±0.77 ^{cde}
79)	JUE23	21.75±0.62 ^c	20.25±0.25 ^{cd}	22.50±0.71 ^{cde}	17.50±0.50 ^e	15.50±1.41 ^c	18.50±0.71 ^c
80)	JUE27	18.67±0.66 ^{cde}	9.67±0.65 ^h	19.33±0.63 ^{de}	19.83±0.16 ^{cde}	18.33±0.90 ^{bcd}	21.67±0.62 ^c
81)	JUE29	20.00±0.88 ^c	10.67±0.91 ^{gh}	20.33±0.20 ^d	20.83±0.22 ^{cde}	14.67±0.08 ^c	23.33±0.10 ^c
82)	JUE30	18.67±0.65 ^{cde}	10.00±0.05 ^h	17.33±0.68 ^{ef}	17.83±0.46 ^f	18.33±0.25 ^{bcd}	20.00±0.02 ^c
83)	JUE31	18.67±0.33 ^{cde}	21.00±0.00 ^c	17.50±0.71 ^{def}	19.33±0.33 ^{cde}	20.00±0.40 ^b	24.33±0.43 ^{bcd}
84)	JUE33	21.33±1.76 ^c	18.00±0.00 ^{cde}	23.50±0.28 ^{cd}	17.00±0.00 ^e	17.00±1.41 ^{cde}	20.50±0.71 ^c
85)	JUE34	21.33±1.76 ^c	18.00±0.00 ^{cde}	23.50±0.28 ^{cd}	17.00±0.00 ^e	17.00±1.41 ^{cde}	20.50±0.71 ^c
86)	JUE35	21.28±1.02 ^c	20.30±0.40 ^{cd}	19.33±0.66 ^d	17.00±0.00 ^e	24.00±1.41 ^a	20.50±0.71 ^c
87)	JUE36	18.50±0.50 ^{cde}	21.00±0.0 ^c	18.50±0.71 ^{de}	19.00±0.00 ^{cde}	20.00±1.41 ^{abc}	23.00±0.00 ^{bcd}
88)	JUE40	18.50±0.50 ^{cde}	21.00±0.0 ^c	18.50±0.71 ^{de}	19.00±0.00 ^{cde}	20.00±1.41 ^{abc}	23.00±0.00 ^{bcd}
89)	JUK01	16.33±0.63 ^{ef}	13.00±0.34 ^{fg}	17.33±0.74 ^{ef}	19.33±0.57 ^{cde}	19.33±0.38 ^b	23.67±0.88 ^{bcd}
90)	JUK02	16.00±0.66 ^{ef}	16.00±0.13 ^{ef}	18.67±0.40 ^{de}	20.67±0.44 ^c	17.00±0.85 ^c	23.33±0.82 ^{bcd}
91)	JUK03	19.08±1.52 ^c	22.50±0.71 ^c	24.50±0.50 ^c	19.50±0.71 ^d	18.50±0.71 ^{bcd}	14.50±0.71 ^d
92)	JUK04	17.92±0.32 ^{cdef}	18.58±0.81	20.25±0.63 ^d	22.25±0.16 ^{cd}	19.92±0.56 ^{ab}	18.58±0.21 ^d
93)	JUK07	22.28±0.40 ^c	20.50±0.71 ^{cd}	23.50±0.71 ^c	20.50±0.71 ^{cde}	15.17±0.71 ^c	15.50±0.71 ^d
94)	JUK14	18.92±0.57 ^{cde}	17.25±0.50 ^{de}	19.25±0.30 ^{de}	21.25±0.13 ^c	20.25±0.81 ^{ab}	21.25±0.58 ^{cde}
95)	JUK20	15.28±0.66 ^f	14.62±0.43	15.62±0.66 ^e	17.62±0.66 ^f	19.95±0.38 ^b	20.28±0.28 ^{cde}
96)	JUK21	19.50±0.71 ^{cd}	18.80±1.12 ^{cde}	17.50±0.71 ^{def}	21.50±0.71 ^c	18.83±0.71 ^{bcd}	18.00±0.00 ^c
97)	JUK23	17.95±0.85 ^{cdef}	15.95±0.96 ^e	17.62±0.86 ^{def}	19.62±0.96 ^{cdef}	16.95±0.08 ^{de}	23.28±0.73 ^c
98)	JUK27	15.45±0.63 ^f	15.80±1.12 ^e	17.50±0.71 ^{def}	19.50±0.71 ^d	17.83±0.71 ^{cde}	15.50±0.71 ^d
99)	JUK29	24.68±0.96 ^{bcd}	15.50±0.71 ^e	20.40±0.53 ^d	17.50±0.71 ^e	19.67±0.34 ^b	18.33±0.34 ^c
100)	JUK30	17.28±0.37 ^{def}	16.95±0.5 ^{ef}	25.28±0.86 ^c	27.28±0.58 ^a	20.62±0.70 ^{ab}	18.28±0.51 ^c

S/N	LAB isolates	<i>E. coli</i> ATCC® 25922	<i>S. aureus</i> ATCC® 25923	<i>L.monocytogenes</i> ATCC® 7644	<i>P. aeruginosa</i> ATCC® 27853	<i>S. Typhimurium</i> ATCC® 13311	<i>C.albicans</i> ATCC® 14053
101)	JUK31	18.95±0.17 ^{cde}	18.62±0.15	23.28±0.53 ^c	25.28±0.85 ^b	21.62±0.20 ^{ab}	18.28±0.97 ^c
102)	JUK33	17.62±0.14 ^{cdef}	17.95±0.1 ^d	22.28±0.86 ^c	24.28±0.58 ^c	19.95±0.45 ^b	15.28±0.82 ^d
103)	JUK37	21.68±0.45 ^c	17.80±0.30 ^d	21.40±0.53 ^{cde}	20.33±0.33 ^{cde}	26.83±0.71 ^a	18.50±0.71 ^c
104)	JUK40	20.28±0.25 ^c	16.62±0.73 ^{ef}	22.62±0.16 ^c	24.62±0.71 ^c	20.95±0.93 ^{abc}	23.28±0.58 ^{bcd}
105)	JUK41	24.85±1.20 ^{bcd}	17.50±0.71 ^d	20.40±0.53 ^d	21.33±0.33 ^c	23.78±0.11 ^{ab}	15.33±0.33 ^d
106)	JUK46	19.00±0.57 ^{cd}	18.00±0.00 ^{de}	20.33±0.33 ^d	13.33±0.33 ^e	24.5 6±0.22 ^a	14.33±0.33 ^d
107)	JUK49	18.33±0.66 ^{cde}	20.00±0.00 ^{cd}	25.33±0.33 ^c	17.33±0.33 ^e	20.34±0.33 ^{bcd}	18.33±0.33 ^c
108)	JUK50	24.68±0.96 ^{bcd}	15.50±0.71 ^e	20.40±0.53 ^d	17.50±0.71 ^e	19.67±0.34 ^b	18.33±0.34 ^c
109)	JUS01	23.00±0.57 ^c	15.00±0.00 ^e	27.67±0.33 ^{bc}	20.50±0.50 ^{cde}	19.67±0.00 ^b	15.50±0.50 ^d
110)	JUS06	15.62±0.80 ^f	16.95±0.94 ^{ef}	24.28±0.72 ^{cd}	26.28±0.67 ^b	22.95±0.95 ^{ab}	16.62±0.17 ^d
111)	JUS07	17.28±.97 ^{cdef}	17.62±0.64	23.95±0.61 ^c	21.95±0.10 ^c	21.62±0.62 ^{ab}	16.95±0.52 ^d
112)	JUS08	16.95±0.99 ^{cdef}	16.95±0.12 ^{ef}	21.62±0.12 ^{cde}	19.62±0.60 ^{cde}	22.28±0.28 ^{ab}	15.95±0.95
113)	JUS09	18.95±0.54 ^{cde}	14.95±0.38	22.62±0.06 ^{cd}	20.62±0.08 ^{cde}	22.95±0.95 ^{ab}	16.62±0.30 ^c
114)	JUS12	18.50±0.50 ^{cde}	18.00±0.00 ^{de}	19.50±0.50 ^d	24.50±0.71 ^c	24.83±0.71 ^a	21.50±0.71 ^{cde}
115)	JUS13	17.79±0.29 ^{cdef}	17.50±0.71 ^d	18.40±0.53 ^{de}	19.00±0.00 ^{cde}	17.83±0.71 ^{cde}	16.50±0.71 ^d
116)	JUS15	19.62±0.73 ^{cd}	15.28±0.93 ^{ef}	21.95±0.27 ^{cde}	19.95±0.72 ^{cde}	22.28±0.28 ^{ab}	19.28±0.63 ^c
117)	JUS16	18.28±0.61 ^{cde}	15.62±0.05	23.95±0.02 ^{cd}	21.95±0.06 ^c	23.95±0.95 ^{ab}	17.62±0.45 ^c
118)	JUS18	18.95±0.73 ^{cde}	18.95±0.51	23.28±0.12 ^c	21.28±0.62 ^c	23.95±0.95 ^{ab}	17.95±0.13 ^c
119)	JUS30	18.78±0.3 ^{cde}	17.50±0.71 ^d	28.40±0.53 ^{bc}	12.50±0.71 ^f	20.33±0.94 ^{abc}	20.50±0.71 ^c
120)	JUS31	19.00±0.00 ^{cd}	18.00±0.00 ^{de}	28.33±0.33 ^{bc}	13.67±0.66 ^f	19.11±0.56 ^b	21.33±0.33 ^{cde}
121)	JUS33	18.67±0.1 ^{cde}	13.30±0.73 ^f	19.01±0.71 ^d	17.01±0.67 ^f	19.12±0.12 ^b	19.45±0.93 ^c
122)	JUS36	21.67±0.82 ^c	20.00±0.00 ^{cd}	28.33±0.33 ^{bc}	13.33±0.33 ^f	20.34±0.07 ^{abc}	26.33±0.33 ^b
123)	JUS38	17.95±0.08 ^{cdef}	22.50±0.71 ^c	20.40±0.53 ^d	16.00±1.41 ^e	19.50±0.71 ^b	20.50±0.71 ^c
124)	JUS41	18.38±0.53 ^{cde}	20.50±0.71 ^{cd}	17.50±0.71 ^{def}	19.50±0.71 ^d	24.00±1.41 ^a	21.00±0.00 ^{cde}
125)	JUS43	17.00±1.41 ^{cdef}	18.50±0.71 ^{cde}	20.50±0.71 ^d	22.00±0.00 ^c	18.50±0.71 ^{bcd}	21.50±0.71 ^{cde}
	Tetracycline	20.75±0.35^c	20.50±0.71^{cd}	21.40±0.53^{cde}	19.25±0.35^{cde}	21.67±0.94^{ab}	18.00±0.00^c

Mean values denoted by the same letter within a column are not significantly different (p< 0.05). JU= Jimma University, B=Bulla, S=Cabbage Shamita, Bk=Bukuri, Br= Borde, E=Ergo, *Escherichia coli* (*E. coli*), *Salmonella Typhimurium* (*S. Typhimurium*), *Klebsella pneumoneae* (*K. pneumoneae*), *Pseudomonas aeruginosa* (*P. aeruginosa*) *Staphylococcus aureus* (*S. aureus*), *Listeria monocytogenes* (*L. monocytogenes*), *Streptococcus pyogens* (*S. pyogens*) and *Candida albicans* (*C. albicans*), *Pediococcus pentosacues* (*P. pentosacues*), *Enterococcus faecium* (*E. faecium*) and *Lactococcus lactis* (*L.lactis*).

3.3.5. Characterization of antimicrobial substances produced by LAB

The antibacterial compound produced by LAB showed thermal stability with sustained antagonistic activity against *S. aureus* within temperatures ranging from 60°C to 100°C (Figure 3.3.) However, its efficacy diminished at 120°C (Figure 3.3). Regarding pH effects on antimicrobial activity, the CFS of LAB displayed increased activity across pH 2-10, but its effectiveness declined notably at pH 10 and higher (Figure 3.4), with acidic conditions (pH 2-6) being more conducive to its inhibitory effects. The CFS retained its antimicrobial activity even after exposure to 10% methanol and ethanol (Table 3.6), and it remained effective when treated with food preservatives like sodium citrate, ascorbic acid, and potassium sorbate. However, proteolytic enzymes completely neutralized the inhibitory substance, while lysozymes had no effect on its antagonistic activity (Table 3.6).

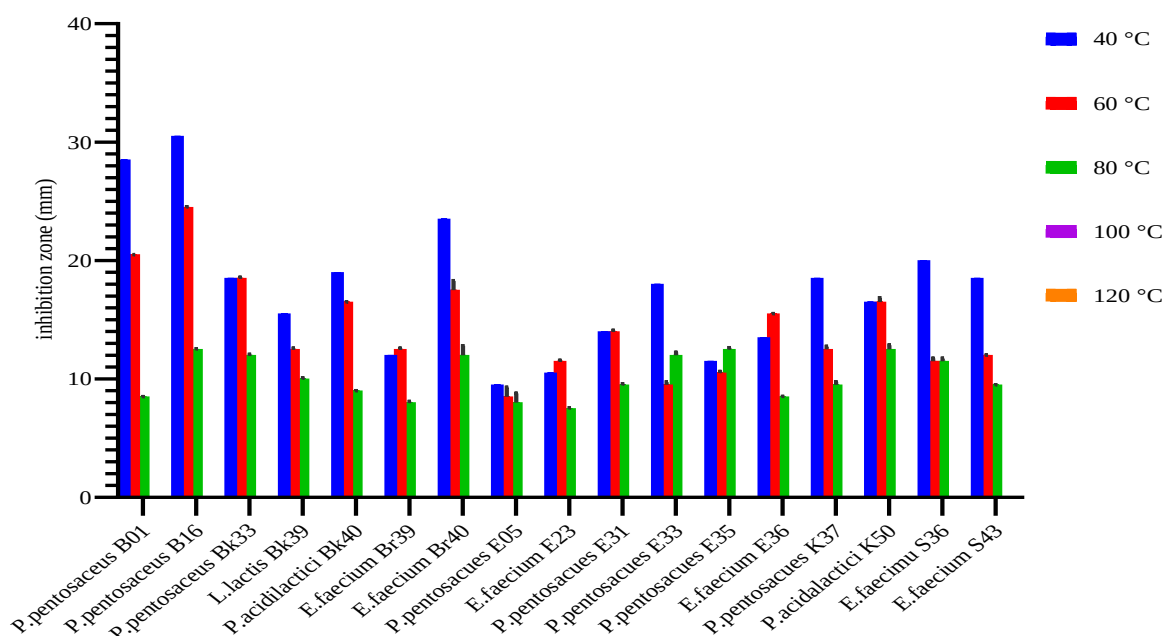


Figure 3. 3. Effects of temperatures on inhibitory activity of CFS produced by LAB isolated from Ethiopian tradition fermented foods and beverages against food-borne pathogen (*S. aureus*).

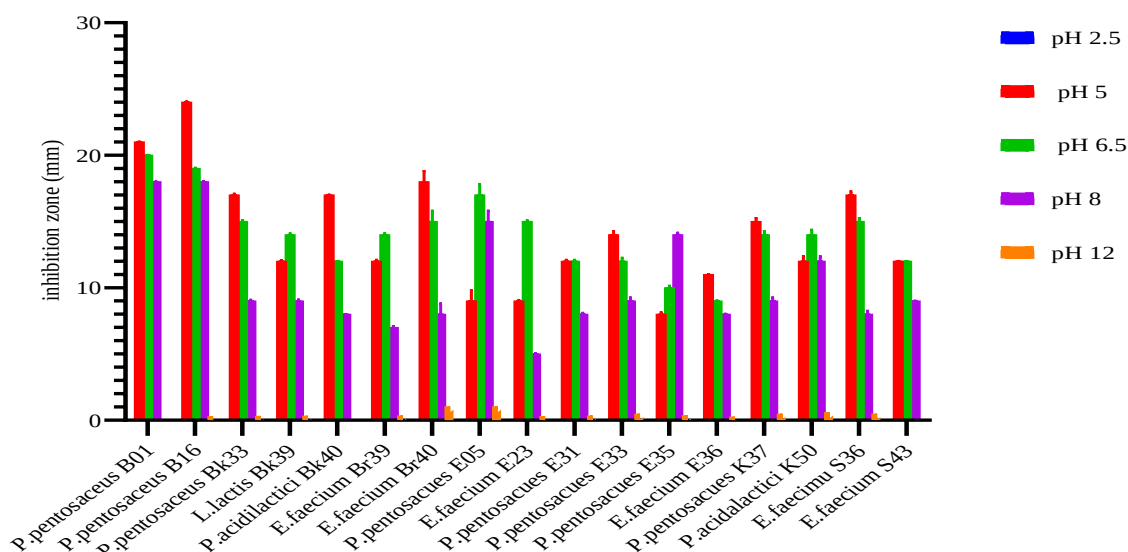


Figure 3. 4. Effects of pH on inhibitory activity of CFS produced by LAB isolated from Ethiopian tradition fermented foods and beverages against food borne pathogen (*S. aureus*).

Table 3. 6. Effects of enzymes, preservatives and alcohols on inhibitory activity of CFS produced by LAB isolated from Ethiopian tradition fermented foods and beverages against food borne pathogen (*S. aureus*).

LAB isolates	Alcohols (10%)		Preservatives (1%)		Ascorbic acid	Enzymes (5 mg/mL)		
	Methanol	Ethanol	Potassium sorbate	Sodium citrate		Pepsin	Trypsin	Proteinase K
1) JULABB01	+++	++	++	++	++	-	-	-
2) JULABB16	+++	++	++	++	++	-	-	-
3) JULABBk33	++	++	++	++	++	-	-	-
4) JULABBk39	++	++	++	++	++	-	-	-
5) JULABBk40	++	++	++	++	++	-	-	-
6) JULABBr39	++	++	++	++	++	-	-	-
7) JULABBr40	++	++	++	++	++	-	-	-
8) JULABE05	++	++	++	++	++	-	-	-
9) JULABE23	+++	++	++	++	++	-	-	-
10) JULABE31	++	++	++	++	++	-	-	-
11) JULABE33	++	++	++	++	++	-	-	-
12) JULABE35	++	++	++	++	++	-	-	-
13) JULABE36	++	++	++	++	++	-	-	-
14) JULABK37	++	++	++	++	++	-	-	-
15) JULABK50	+++	++	++	++	++	-	-	-
16) JULABS36	++	++	++	++	++	-	-	-
17) JULABS43	++	++	++	++	++	-	-	-

Note: +++= inhibition zone>20; ++=inhibition zone>15; and -= no inhibition zone. JU= Jimma University, B=*Bulla*, S=Cabbage *Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*.

3.3.6. Identification of LAB using MALDI TOF Mass Spectrum analysis

In this study, representative LAB species which displayed tolerance to certain stresses and found to display antimicrobial activities were identified using MALDI-TOF/MS identification (Figure 3.5). A total of seventeen LAB species were identified to species level, including *Pediococcus pentosaceus* was the most dominant and frequently identified LAB, followed by *Enterococcus faecium*, *Lactococcus lactis* and *Pediococcus acidilactici* (Table 3.7).

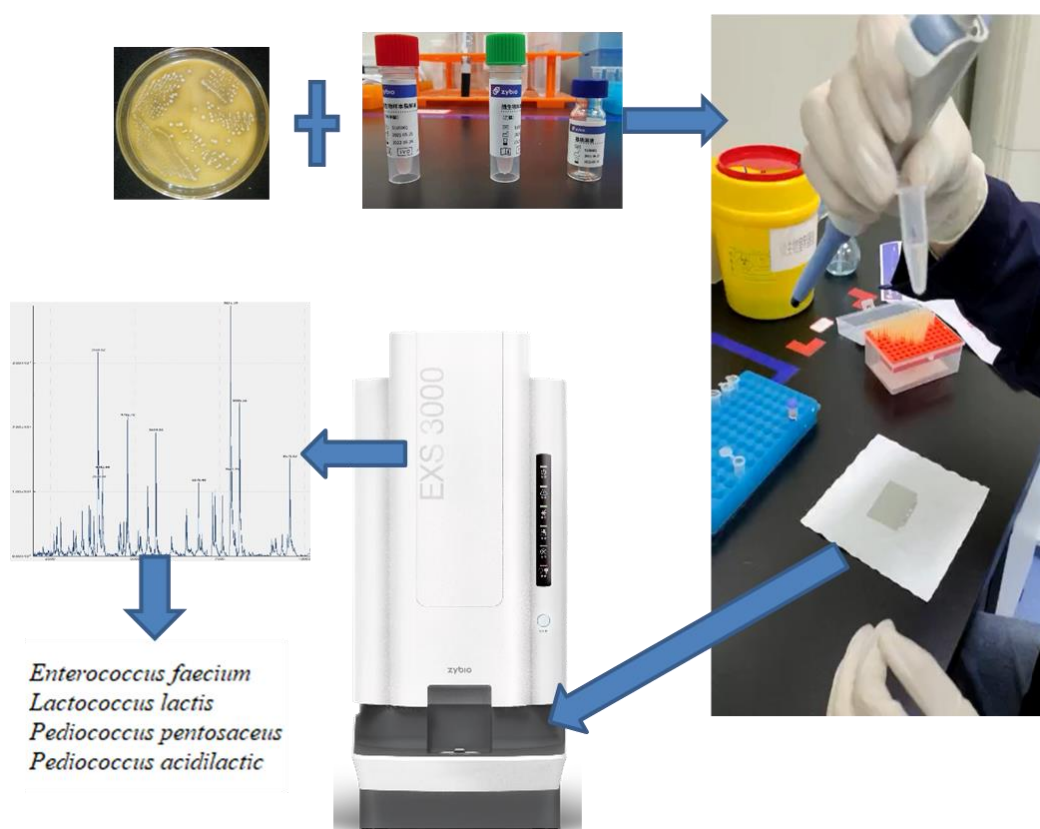


Figure 3. 5. MALDI ToF/MS spectrum aanlyis of LAB isolated from Ethiopian traditional fermented foods and beverages

Table 3.7. Microbial ecology of some Ethiopian traditional fermented foods and beverages identified by MALI TOF MS spectrum analysis

S/N	Sources/Samples	LAB isolates	LAB Species	Score
1)	<i>Borde</i>	JULAB-Br40	<i>Enterococcus faecium</i>	2.07
		JULAB-Br39	<i>Enterococcus faecium</i>	2.17
2)	<i>Bukuri</i>	JULAB-Bk39	<i>Lactococcus lactis</i>	1.77
		JULAB-Bk40	<i>Pediococcus acidilactici</i>	2.17
		JULAB-Bk33	<i>Pediococcus pentosaceus</i>	2.17
3)	<i>Bulla</i>	JULAB-B01	<i>Pediococcus pentosaceus</i>	1.83
		JUALAB-B16	<i>Pediococcus pentosaceus</i>	1.76
4)	<i>Ergo</i>	JUALAB-E23	<i>Enterococcus faecium</i>	2.06
		JULAB-E36	<i>Enterococcus faecium</i>	1.78
		JULAB-E35	<i>Lactococcus lactis</i>	1.76
		JULAB-E31	<i>Pediococcus pentosaceus</i>	1.72
		JULAB-E05	<i>Pediococcus pentosaceus</i>	1.84
		JULAB-E33	<i>Pediococcus pentosaceus</i>	1.87
5)	<i>Kocho</i>	JULAB-K50	<i>Pediococcus acidilactici i</i>	2.12
		JULAB-K37	<i>Pediococcus pentosaceus</i>	1.89
6)	<i>Shamita</i>	JULAB-S43	<i>Enterococcus faecium</i>	2.19
		JULAB-S36	<i>Enterococcus faecium</i>	1.83

Note= JU= Jimma University, B=*Bulla*, S=Cabbage *Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*, SGIJ

3.4. DISCUSSION

The rise of human pathogens in food systems is a significant public health concern, leading to risks and fatalities in communities (Girma & Aemiro, 2021). Hence, there's a pressing need to explore novel and effective natural food preservatives to mitigate foodborne illnesses. LAB, recognized as GRAS, are crucial in food fermentation and preservation due to their production of beneficial metabolites such as organic acids, H₂O₂, CO₂, and bacteriocins, which inhibit the growth of human pathogens in food (Dejene et al., 2021; Mulaw et al., 2019).

LAB isolated from Ethiopian traditional fermented foods and beverages displayed significant antagonistic activity against various food-borne pathogens, (Dejene et al., 2021; Mulaw et al., 2020; Tesfaye et al., 2011). Thi study identified potential isolates belonging to the genera *Lactococcus*, *Pediococcus*, and *Enterococcus*, with *Pediococcus* being the most prevalent, followed by *Enterococcus* and *Lactococcus*, consistent with other studies (Dejene et al., 2021;Taye et al., 2021).

During primary screening, *P. pentosaceus* JULABB16 exhibited the highest antagonistic effects against various pathogens, including *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 25923, and *P. auroginosa* ATCC 27853, with inhibition zone diameters of 35.50±0.50, 32.50±0.50 and 30.50±0.71 mm, respectively. Similarly, *E. faecium* JULABBr39 showed potent inhibition against *E. coli* ATCC 25922 and *L. monocytogenes* ATCC 7644, with the inhibition zone diameter of 33.50 and 29.33 mm, respectively. These findings align with other studies reporting LAB isolates from Ethiopian fermented foods with significant antagonistic effects against pathogens (Dejene et al., 2021; Girma & Aemiro, 2021; Goa et al., 2022), with inhibition zone diameters ranging from 12.00 to 20.00 mm.

The study observed significantly higher antimicrobial effects from LAB compared to previous reports, possibly due to rigorous screening and identification criteria involving bacteriocin production, probiotic potential, preservative effects, and antimicrobial substance characterization (Assefa et al., 2008; Darbandi et al., 2022). Moreover, the study screened only 17 LAB isolates from 956 characterized isolates, which could also contribute to the observed differences. Assefa et al.(2008) have reported that *Pediococcus acidilactici* isolated from *ergo* (an Ethiopian dairy product), and *Lactococcus lactis* isolates (Fn-555, Ad-411, and Ad-211) demonstrated potential antimicrobial activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, respectively as verified in in this study.

The heat-treated cell-free supernatant (CFS) of LAB showed increased antagonistic activity as temperatures rose, especially in isolates like *P. pentosaceus* JULABB16, *E. faecium* JULABBr39, *E. faecium* JULABBr40, and *P. pentosaceus* JULABB01. These LAB-derived antimicrobial substances exhibited resilience across a wide temperature range and effective even at 80°C as a result of their low molecular weight and diverse chemical structures (Dejene et al., 2021; Girma & Aemiro, 2021; Goa et al., 2022; Tesfaye et al., 2011). This resilience suggests that LAB can potentially be used as a novel and promising food preservative at high temperatures.

The 17 LAB isolates displayed a heightened antimicrobial activity within acidic environments with a pH range of 2.5-6.5. Notably, strains *P. pentosaceus* JULABB01, *P. pentosaceus* JULABB16, *E. faecium* JULABBr39, and *E. faecium* JULABBr40 showed promising antagonistic effects even at pH 8. However, as the pH rose above 8 towards 12, the overall antimicrobial activities of the LAB isolates diminished. This decline in activity suggests that the antimicrobial effect was primarily due to the presence and production of organic acids except for a few isolates. These findings align with previous studies (Goa et al., 2022; Ren et al., 2018), which highlight the effectiveness and stability of LAB antimicrobial activity in acidic environments, typically between pH 2-6.

The antimicrobial activity of LAB isolates remained effective and showed strong antagonistic potential even after exposure to alcohols, as the cell-free supernatant (CFS) that did not dissolve in the alcohols and maintained its structure. This finding aligns with studies by Ren et al. (2018), but differs from reports by Avaiyarasi et al. (2016) and Girma & Aemiro (2021). However, discrepancies in outcomes across studies by Avaiyarasi et al. (2016) and Girma & Aemiro (2021) could stem from differences in LAB strains, experimental methods, or alcohol concentrations used.

Besides potent efficacy under different pH conditions, the antimicrobial activity of LAB isolates was found effective and displayed good antagonistic potential even after treated with alcohols since the CFS retained its structure in the test alcohols. This result was similar with the research reported elsewhere (Ren et al., 2018), but contradicted the study reported by others (Avaiyarasi et al., 2016; Girma & Aemiro, 2021). Furthermore, the effects of enzymes on antimicrobial activity of LAB isolates were also evaluated, and the activities of some LAB isolates were partially inactivated after treated with pepsin, trypsin and proteinase K. The current observation might be due to the fact that antimicrobial substances of some of the isolates may be associated with the production of organic acids rather than other bioactive compounds like bacteriocins and bacteriocins like substances. This finding

is in line with the study conducted by Ren et al.(2018) in which the activity of metabolites of LAB was reduced or totally inactivated after treated with enzymes like pepsin, trypsin, papain, and proteinase K. Similarly, other research reports (Dejene et al., 2021; Gebreselassie et al., 2016; Goa et al., 2022) showed that the antimicrobial activity of LAB isolated from Ethiopian traditional fermented foods and beverages was partially unstable after treated with α -amylase, lysozyme enzymes and proteinase K.

To evaluate the effects of food preservatives on potential antagonistic effects of LAB, all CFS were treated with sodium citrate, potassium, to verify the effect of food additives on the antibacterial components of LAB. These findings suggest that the use of sodium citrate and potassium sorbate as food preservatives may be compatible with the growth and survival of LAB in food products. Nevertheless, it is important to continue monitoring their effects on LAB to ensure the safety and quality of food products. Therefore, it is crucial to consider the potential interactions between food preservatives and other additives in food products to maintain the efficacy of antimicrobial agents. Similarly, some reports (Heredia-Castro et al., 2015; Ren et al., 2018) revealed that most LAB isolates showed potential antagonistic activity after CFS was treated with sodium citrate and potassium sorbate. This suggests that these additives can be safely used in food preservation without compromising the antibacterial properties of LAB and their metabolites.

3.5. CONCLUSION

Lactobacillus species were the most prevalent among the characterized isolates. Using MALDI TOF mass spectrum analysis, *Pediococcus pentosaceus* emerged as the most frequently identified species, followed by *E. faecium*, *L. lactis*, and *P. acidilactici*. All 17 LAB isolates exhibited strong antagonistic effects against selected foodborne pathogens, demonstrating sensitivity across a wide range of pH and temperature, even in the presence of food additives. Further research incorporating whole genome sequencing is recommended to explore resistance and cluster genes responsible for bioactive peptide production. Molecular analysis of promising LAB is crucial for identifying probiotic candidates for human health and starter culture development. Given the rise of multidrug-resistant pathogens, LAB with preservative properties could serve as an alternative to antibiotics, supporting gut microbiota growth while suppressing harmful microorganisms.

4.

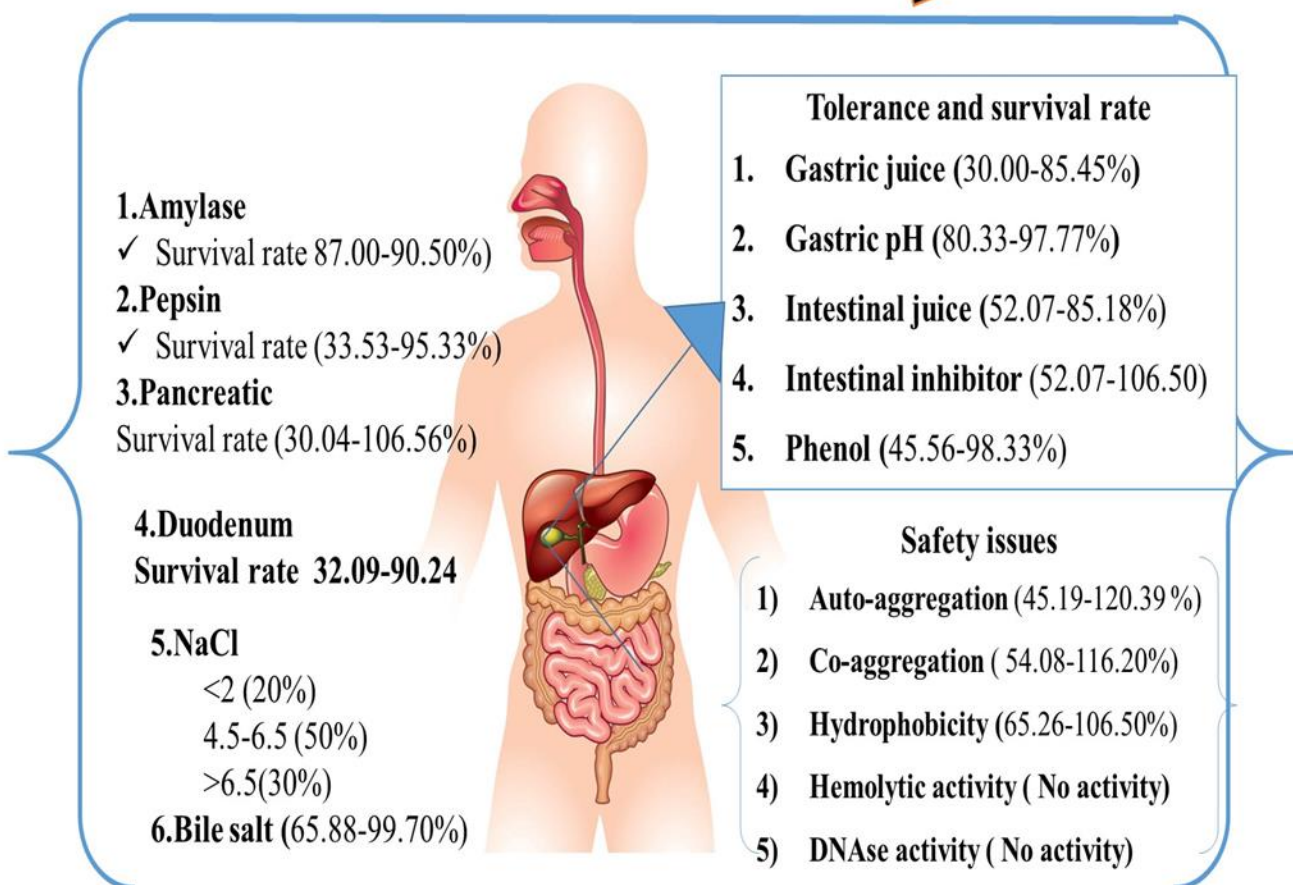
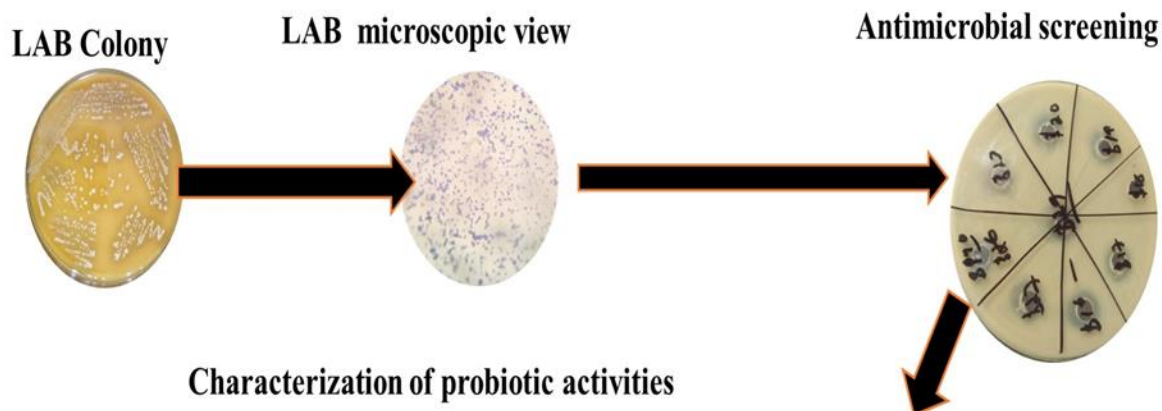
PROBIOTIC POTENTIAL AND SAFETY ANALYSIS OF LACTIC ACID BACTERIA ISOLATED FROM ETHIOPIAN TRADITIONAL FERMENTED FOODS AND BEVERAGES

The finding of this paper is published in *Annals of Microbiology* as:

Amenu, D. and Bacha, K. 2023. Probiotic potential and safety analysis of lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages. *Ann Microbiol* 73 (37): <https://doi.org/10.1186/s13213-023-01740-9>.

The cover page with the abstract is attached in appendix section

Graphic Abstract



ABSTRACT

*Probiotics are live microorganisms that effectively combat foodborne pathogens, promoting intestinal health when consumed in sufficient amounts. This study evaluated the probiotic potential and safety analysis of lactic acid bacteria (LAB) from Ethiopian traditional fermented foods and beverages, including Kotcho, Bulla, Ergo, Cabbage-Shamita, Borde, and Bukuri. The bacteria were characterized through morphological, physiological, biochemical, and MALDI ToF/Mass analysis. To assess their probiotic activity, the tolerance and survival rate of LAB to a variety of stress conditions, including low pH, intestinal inhibitor substances, salt concentration, bile salt, simulated gastric/intestinal juice, and low pH, were studied. LAB isolates were also tested for antagonistic activity against common bacterial pathogens such as *S. aureus*, *S. Typhimurium*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *C. albicans*. Furthermore, the safety analysis (auto-aggregation, co-aggregation, cell surface hydrophobicity, hemolytic and DNase activities as well as antibiotic susceptibility were evaluated to determine the safety analysis of LAB isolates. A total of 125 probiotic LAB were identified in this study. Out of 125 isolates, 54 LAB isolates survived low pH (2, 2.5, and 3), bile salt (0.3%), intestinal inhibitor chemicals (phenol, bile, low acidity, pepsin, and pancreas juice), and simulated gastro-intestinal settings with a near 60–94% survival rate. The isolates' ability to withstand acids, intestinal inhibitor chemicals, simulated intestinal circumstances, and bile salt tolerance as well as effective antagonistic activities could contribute to good and more effective antagonistic activity against all the selected foodborne pathogens. All the 54 LAB isolates were resistant to Ampicillin, Vancomycin, Gentamycin, Kanamycin, Clindamycin, and Chloramphenicol, while they were susceptible to Streptomycin and Tetracycline. The seventeen efficiency probiotic LAB isolates were identified using MALDI TOF Mass spectrometry as belonging to *Pediococcus pentosaceus*, *P. acidilactic*, *Enterococcus faecium*, and *Lactococcus lactis*. . Hence, all the 54 LAB strains were found to be the most potentially lactic acid bacteria, and were selected as likely probiotic LAB that can be applied as the best starter culture and bio-preservative for the enhancement of foods shelf life. This study established the groundwork for the selection of excellent probiotic candidates as well as the development and application of LAB for possible antibacterial action, starter culture production, and biopreservatives.*

Key words: Antimicrobial activity, Ethiopian, Lactic acid bacteria, Probiotic potential, Traditional fermented products.

4.1. INTRODUCTION

Probiotics are living microorganisms that have beneficial aspects for human health if administered in adequate amounts (Mulaw et al., 2020). LAB are a group of bacteria that are generally recognized as safe (GRAS) and are widely used in fermented foods. They produce antimicrobial substances such as acids, hydrogen peroxide, and bacteriocins and have great potential as food biopreservatives (Paulos Mokoena et al., 2010). In addition to their preservative properties, LAB have also been shown to have immunomodulatory effects and may improve gut health. Studies have suggested that consuming probiotics containing LAB can alleviate symptoms of gastrointestinal disorders such as irritable bowel syndrome and inflammatory bowel disease (Mulaw et al., 2020; Mokoena et al., 2010).

Foodborne illness outbreaks caused by pathogens are a major concern for food safety agencies (Akbar et al., 2019). Probiotic LAB, including *Lactobacillus*, *Bifidobacteria*, and others, have shown probiotic potential (Birri et al., 2010). Notably, *Pediococcus* species like *P. pentosaceus* and *P. acidilactici* demonstrate significant probiotic properties (Altermann et al., 2005; Federici et al., 2014). Probiotic LAB protect against enteric pathogens through mechanisms such as producing antimicrobial substances, competing for resources, and anti-adhesive effects (Oelschlaeger, 2010). In traditional fermented foods and beverages, they enhance flavor and texture, while also potentially improving digestion and boosting the immune system (Ricci et al., 2019). These benefits extend to various fermented products in Ethiopia such as *Ergo*, *Kocho*, and teff flour (Mulaw et al., 2019) and *Borde* (Negasi et al., 2017), *Bukuri* (Chali et al., 2024) and injera (Tilahun et al., 2018). These products not only provide unique and delicious flavors but also offer potential health benefits to those who consume them regularly.

Consequently, investigating the roles and potential adaptations of microbial communities can deepen our understanding of their interactions and environments by offering practical benefits for large-scale production. Identifying strains with specific qualities such as probiotic potential and safety is crucial for developing effective starter cultures for industrial applications. Furthermore, comprehending the metabolic processes involved in fermentation can enhance efficiency and minimize environmental impact. Thus, this study aimed to evaluate LAB from Ethiopian fermented foods and beverages for their probiotic attributes, safety profile, and their ability to inhibit pathogens like *S. aureus*, *S. Typhimurium*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *C. albicans*.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains

The LAB used in this study were isolated from Ethiopian fermented foods and beverages (Section 3.2.4). Standard strains of *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Listeria monocytogens* ATCC7644, *Pseudomonas aeruginosa* ATCC27853, *Salmonella Typhimurium* ATCC13311, and *Candida albicans* ATCC 14053 were provided by the Ethiopian Public Health Institute (EPHI) (Section 3.2.7).

4.2.2. Characterization of probiotic Lactic acid bacteria

4.2.2.1. Resistant to Low pH

LAB isolates were grown in MRS broth at various pH levels, and their optical density was measured using UV-spectrophotometry after 0 h, 3 h, and 6 h of anaerobic incubation at 37°C. The survival rate was computed as $SR(\%) = \frac{OD_{03hr/6hr}}{OD_{600nm}}$. Where OD600 nm= the bacterial cell density at 0 h, OD3/6 h= bacterial turbidity or cell density at 3 h or 6 h (Grosu-Tudor & Zamfir, 2012).

4.2.2.2. Tolerance to Bile Salts

To estimate bile tolerance probiotic LAB isolates (those only survived at pH 2.0, 2.5, and/or 3.0) were separately grown overnight in MRS broth at 37°C under anaerobic conditions (Grosu-Tudor & Zamfir, 2012). The bacterial cell or cultures were inoculated into sterile MRS broth supplemented with 0.3% (w/v) bile salt (Oxgall, USA) after the bacterial cell density was adjusted to a log of 7 CFU/ml at 600 nm. Samples were taken at 24 h from the onset of incubation to determine the survivability rate of the isolates as described previously (Grosu-Tudor & Zamfir, 2012). The survival rate was calculated as $:SR(\%) = \frac{OD_{024h}}{OD_{600nm}} * 100$. Where OD600 nm= the bacterial cell density at 0 h, O24 h= bacterial turbidity or cell density after 24 h incubation, with the bacterial density ($\log 10^{6-8}$ CFU/ml).

4.2.3. Production of Bacteriocins

The isolates' bacteriocin-producing potential was tested using the agar-well diffusion assay against specific pathogens (Yang et al., 2014). To neutralize acids and hydrogen peroxides, 1 NaOH was used

to adjust the pH of the cell-free supernatant (CFS) to 7.00, and catalase was added at 5 g/ml. Confirming bacteriocin production involved treating the CFS with acid neutralization and heat, then applying proteinase K (Sigma, St. Louis, USA) and incubating for 2 h at 37°C. Antimicrobial activity was assessed using the agar well diffusion method (Saad et al., 2015).

4.2.4. Tolerance to intestinal inhibitory substances

The probiotic qualities of LAB isolates were assessed by testing their tolerance to intestinal inhibitors. The selected LAB Isolates were cultured in MRS broth overnight at +37°C under anaerobic conditions. Survival rate was tested under adverse conditions like phenol (0.3-0.5 v/v %), bile salt (0.3-0.8 w/v %), low pH (2-2.5), and enzymes (pancreatic and pepsin). Cultures were incubated for 24 h at 37°C in static conditions, and growth was monitored. The survival rate of the isolates was calculated as: % tolerance (survival)rate = $\frac{OD_{0h} - OD_{24h}}{OD_{0h}} * 100$. Where, OD₀ and OD₂₄ refer to bacterial density at 0 h and bacterial density after 24 h incubation period, respectively (Yasmin et al., 2020).

4.2.5. Survival of LAB in simulated gastrointestinal environment

Simulated stomach and intestinal fluids were prepared by mixing 13.3 mg/L pepsin with 0.5% sterile saline at pH 2.5 for stomach fluid, and 250 mg/L pancreatic with 0.5% sterile saline at pH 7.5 for small intestinal juice (Kondrashina et al., 2023). LAB cells at 10⁶ CFU/mL were added to 5 mL of the test solution and incubated for 3 h under anaerobic conditions at 37°C. After exposure, one percent v/v of each sample was transferred to sterile MRS broth and incubated for 24 h at 37°C under anaerobic conditions. In all cases, growth was measured at 600 nm at zero h, three h, and twenty-four h, and the percentage was calculated as $SR(\%) = \frac{(OD_{0h} - OD_{24h})}{OD_{0h}} * 100$ Where OD 0 h and OD 3 h/24 h, bacterial optical density at zero hour and at three or twenty-four h, respectively (Kondrashina et al., 2023).

4.2.6. Survival of LAB to Simulated Stomach Duodenum Passage

The screened and selected LAB isolates were cultured in MRS broth (pH 3.0), synthetic duodenum juice (Na₂HCO₃, KCl, NaCl (0.85%) in distilled water, pH 7.5), and bile salt solution (0.3%) After inoculation (10⁶ CFU/mL), samples were collected at zero, 3, and 24 h by measuring optical density with a spectrophotometer. Bile salt and duodenal juice were added after 1 hour, followed by incubation

at 37°C for 2-3 h before optical density assessment to estimate percent survival after 3 h. Finally, the percent survival rate was calculated using the following formula: $SR(\%) = \frac{(OD_{0h} - 24h)}{OD_{0h}} * 100$ Where OD 0 h and OD 3 h/24 h, bacterial optical density at zero hour and at three or twenty-four h, respectively (Yadav et al., 2016).

4.2.7. Safety Evaluation of LAB Strains

4.2.7.1. Antibiotic susceptibility

The antibiotic susceptibility of LAB was tested using an antibiotic disc diffusion method (B. Zhang et al., 2016) on MRS agar plates. LAB broth cultures prepared with MRS were adjusted to 0.5 McFarland standards. A 100 µl suspensions of these cultures were spread on MRS agar plates, and antibiotic discs were placed on the agar surface. The plates were then incubated at 37°C for 48 h. The antibiotic susceptibility pattern of the isolates was assessed using ampicillin (10 µg/disc), vancomycin (30 µg/disc), gentamicin (10 µg/disc), kanamycin (30 µg/disc), streptomycin (10 µg/disc), chloramphenicol (30 µg/disc), erythromycin (15 µg/disc), clindamycin (2 µg/disc), and tetracycline (30 µg/disc) (Zhang et al., 2016). The zone of inhibition diameter was measured using the clinical and laboratory standards institute (CLSI) scale for antibiotics (CLSI, 2020). The results were categorized as susceptible, moderately susceptible, or resistant based on interpretative zone diameters outlined in the Performance Standards for Antimicrobial Disc Susceptibility Test (Callan and Westblade, 2020).

4.2.7.2. Hemolytic Activity

To determine the hemolytic activity of the LAB, the isolates were streaked onto blood agar plates containing 5% (w/v) sheep blood and incubated at 37°C for 48 h. After incubation, the plates were examined for β-hemolysis, α-hemolysis, and non-hemolytic activities (Yadav et al., 2016).

4.2.7.3. DNase Activity

The LAB isolates were streaked onto a deoxyribonuclease (DNase) agar medium to test for the production of the DNase enzyme. The plates were then incubated at 37°C for 48 h and observed for the zone of DNase activity. A clear pinkish zone around the colonies was considered as positive DNase activity (Shuhadha et al., 2017).

4.2.7.4.Auto Aggregation

To evaluate the auto-aggregation potential of LAB, isolates the overnight culture was harvested by centrifugation (at 8,000 rpm at 4°C for 10 min) and washed with phosphate buffer solution twice and re-suspended in PBS buffer. The sample was allowed to stand awhile, incubating anaerobically at 37°C, and the upper suspension was checked for absorbance at 600 nm at time intervals of 0, 1, 2, 3, 4, and 5 h. The auto aggregation was measured (in percentage) using the formula auto aggregation % = $[1 - (A_{\text{time}}/A_0) \times 100]$, where, A time represents the absorbance at a particular time and A0 represents the absorbance at time 0 h (Zommiti et al., 2017).

4.2.7.5.Co-aggregation

For evaluation of co-aggregation of LAB, the selected strains were grown in 10 mL of MRS and with some selected indicator bacteria pathogens (*E. coli* and *S. aureus*) in brain heart infusion, respectively, at 37 °C. Co-aggregation was calculated using the following equation:

$$\text{Co - aggregation}(\%) = \frac{\frac{AODx + AODy}{2} - A(ODx + ODy)}{AODx + \frac{AODy}{2}} * 100$$

OD0 refers to the initial OD, taken immediately after the relevant strains were paired. ODt refers to the OD of the supernatant at 0 h, 1 h, 5 h and 24 h (Zommiti et al., 2017). Where x and y represent each of the two strains in the control tubes and in the (x + y) mixture

4.2.7.6.Cell Surface Hydrophobicity

The *in vitro* cell surface hydrophobicity of the LAB isolates was evaluated by measuring the microbial cell adhesion to hydrocarbons according to the method described by Rokana et al. (2018). The overnight cultures in MRS broth were harvested by centrifugation (at 8,000 rpm at 4°C for 10 min), washed twice with PBS, and re-suspended in PBS buffer followed by absorbance (A0) measurement at 600 nm. A cell suspension of about 3 ml was blended with 1 ml of hydrocarbon (xylene) and incubated at 37°C without shaking for 1 h for separation of the aqueous and organic phases. The aqueous phase (1 ml) was removed carefully and the absorbance (A1) was measured at 600 nm. The

percent hydrophobicity was measured by a decrease in absorbance and calculated using the following formula: % cell surface hydrophobicity = $(1 - A1/A0) \times 100$).

4.2.7.7.Data analysis

The data were analyzed statistically using the SPSS Statistics software package (version 26, IBM Corp., Armonk, NY, USA). The statistical significance of the differences between bacteria different samples was analyzed using analysis of variance (ANOVA) post hoc, multiple variation comparison, statistical significance was determined at $p < 0.05$.

4.3. RESULTS

4.3.1. Characterization of Probiotic Lactic acid bacteria (LAB)

4.3.1.1. Resistant low pH.

The study identified 125 potential probiotic LAB isolates that tolerated acid conditions and bile salts (Table 4.1). Ultimately, 54 LAB were identified as promising probiotic candidates. Of these, 17 isolates (13.60%) were selected as potential probiotics based on criteria such as high inhibition zone, acid and bile tolerances, and a survival rate under simulated gastrointestinal conditions (Table 4.5). These isolates showed survival rates ranging from 33.82% to 98.82% at pH levels of 2, 2.5, and 3 for over 3- and 6-hour periods. Survival rates varied ($p>0.05$) significantly among the isolates at different pH levels and exposure times. Notably, survival rates were higher at higher pH levels and shorter durations. For example, JULABB16 demonstrated the highest tolerance with a 98.82% survival rate after 3 h, while JULABS30 had the lowest (33.82%) survival rate (Table 4.5). JULABB35 showed the highest tolerance at pH 2 for 6 h with a 90.07% survival rate. Survival rates at pH 2.5 for 6 h ranged from 68.27% to 90.07%, and at pH 3 for 6 h, rates varied from 32.32% to 90.07%. JULABB16 displayed exceptional tolerance with a 98.82% survival rate at pH 3 for 6 h.

Table 4. 1. Acid and bile tolerance of potential probiotic LAB from Ethiopian traditional fermented foods and beverages

S/N	Isolates	pH tolerance rate (Percentage of survival rate)						Bile salt
		3 h			6 h			
		pH2	pH2.5	pH3	pH2	pH2.5	pH3	
1.	JULABB01	74.23±0.69 ^{bcd}	89.80±0.69 ^a	95.42±0.72 ^a	63.26±0.65 ^{efg}	85.33±0.58 ^b	97.67±0.58 ^{ab}	98.00±1.00 ^a
2.	JULABB10	78.28±0.26 ^{abcd}	84.00±0.50 ^b	89.74±0.65 ^{bcde}	80.23±0.69 ^b	85.33±0.58 ^b	93.33±0.58 ^{bce}	85.52±0.89 ^f
3.	JULABB15	68.03±0.05 ^{def}	81.83±0.24 ^{bcd}	96.03±0.05 ^a	69.53±0.75 ^d	76.53±0.66 ^d	97.53±0.75 ^{ab}	98.56±0.62 ^a
4.	JULABB16	75.35±0.56 ^c	80.33±0.58 ^{bcd}	84.67±0.58 ^c	73.00±2.65 ^c	83.00±6.24 ^b	98.82±1.00 ^a	99.20±0.72 ^a
5.	JULABB30	66.78±0.32 ^{defg}	83.53±0.66 ^b	94.78±0.32 ^{abc}	68.78±0.32 ^{de}	74.78±0.32 ^d	95.78±1.10 ^b	100.53±0.67 ^a
6.	JULABB35	78.47±0.50 ^{abcd}	80.40±0.53 ^{bcd}	85.50±0.87 ^c	85.61±0.53 ^a	90.07±0.12 ^a	97.67±0.58 ^{ab}	96.67±0.58 ^b
7.	JULABB39	53.84±0.22 ^{ghij}	81.78±0.32 ^{bcd}	81.84±0.22 ^{def}	55.84±0.22 ^g	61.84±0.22 ^g	82.84±1.19 ^{def}	83±.50±0.71 ^{fg}
8.	JULABB45	65.84±0.23 ^{defg}	80.84±0.23 ^{bcd}	93.84±0.23 ^{abc}	67.84±0.23 ^{de}	73.84±0.23 ^d	94.84±1.19 ^b	93.51±0.69 ^c
9.	JULABB56	66.83±0.24 ^{defg}	72.90±0.15 ^{efg}	94.83±0.24 ^{abc}	68.83±0.24 ^{de}	74.83±0.24 ^d	95.83±1.18 ^b	100.76±0.35 ^a
10.	JULABBK02	68.75±0.35 ^{def}	83.75±0.35 ^b	96.75±0.35 ^a	70.00±0.35 ^d	76.75±0.35 ^d	97.75±1.07 ^{ab}	84.93±0.10 ^f
11.	JULABBK06	53.58±0.60 ^{ghij}	79.89±0.15 ^c	81.08±0.11 ^{def}	55.58±0.60 ^g	61.58±0.60 ^g	82.58±0.82 ^{def}	76.53±0.66 ^g
12.	JULABBK15	52.70±0.43 ^g	79.56±0.63 ^c	80.20±0.28 ^{def}	54.70±0.43 ^{ghi}	60.70±0.43 ^g	81.70±0.99 ^{def}	91.51±0.69 ^d
13.	JULABBK21	72.16±0.77 ^{cde}	81.00±1.00 ^{bcd}	88.00±1.00 ^{bcdef}	73.33±0.58 ^c	84.67±1.53 ^{bc}	94.67±0.58 ^{bce}	86.09±0.87 ^{efg}
14.	JULABBK29	51.74±0.37 ^{gh}	79.00±0.00 ^c	79.74±0.37 ^{def}	53.74±0.37 ^{ghi}	59.74±0.37 ^{gh}	80.74±1.05 ^{defg}	81.93±0.11 ^{fgh}
15.	JULABBK32	50.53±0.66 ^{gh}	75.56±0.62 ^d	78.03±0.05 ^d	52.53±0.66 ^{ghi}	58.53±0.66 ^{gh}	79.53±0.76 ^e	100.95±0.36 ^a
16.	JULABBK33	50.80±0.28 ^{gh}	78.91±0.13 ^{cde}	78.80±0.28 ^d	52.80±0.28 ^{ghi}	58.80±0.28 ^{gh}	79.80±1.13 ^e	88.93±0.11 ^e
17.	JULABBK39	47.97±0.04 ^{hij}	74.62±0.53 ^{ef}	75.97±0.04 ^d	49.97±0.04 ^h	55.97±0.04 ^h	76.97±1.37 ^e	71.77±0.33 ^g
18.	JULABBK40	46.12±1.02 ⁱ	69.00±1.00 ^f	84.67±0.58 ^c	65.29±0.61 ^e	78.33±0.58 ^{cd}	85.67±0.58 ^d	81.33±1.53 ^{fgh}
19.	JULABBR03	60.14±0.58 ^{defgh}	84.67±0.58 ^b	89.33±0.58 ^{bcde}	76.87±0.22 ^{bcd}	86.33±1.15 ^b	94.00±1.00 ^{bce}	85.73±0.64 ^f
20.	JULABBR04	47.76±0.34 ^{hij}	73.71±0.41 ^e	75.76±0.34 ^d	49.76±0.34 ^h	55.76±0.34 ^h	76.76±1.07 ^e	60.80±0.29 ^j
21.	JULABBR05	47.66±0.48 ^{hij}	73.67±0.46 ^e	75.16±0.22 ^d	49.66±0.48 ^h	55.66±0.48 ^h	76.66±0.93 ^e	81.86±0.19 ^{fgh}
22.	JULABBR12	70.0±80.88 ^d	75.67±1.53 ^d	87.33±1.53 ^b	77.37±0.55 ^{bcd}	82.00±6.08 ^b	97.00±2.00 ^{ab}	94.00±1.00 ^c
23.	JULABBR17	47.50±0.71 ^{hij}	73.59±0.59 ^e	75.00±0.00 ^d	49.50±0.71 ^h	55.50±0.71 ^h	76.50±0.71 ^e	88.76±0.33 ^e
24.	JULABBR21	46.84±0.22 ⁱ	72.94±0.08 ^{efg}	74.84±0.22 ^{efg}	48.84±0.22 ^h	54.84±0.22 ^h	75.84±1.19 ^{efg}	73.83±0.24 ^g
25.	JULABBR33	85.39±0.35 ^a	87.40±0.53 ^{ab}	89.63±0.64 ^{bcde}	83.20±0.72 ^{ab}	88.50±0.87 ^{abc}	96.33±0.58 ^{abc}	92.07±0.90 ^{cd}
26.	JULABBR39	46.82±0.26 ⁱ	70.74±0.37 ^{efg}	74.82±0.26 ^{efg}	48.82±0.26 ^h	54.82±0.26 ^h	75.82±1.16 ^{efg}	51.79±0.30 ^{klm}
27.	JULABBR40	68.63±0.55 ^{def}	89.33±0.58 ^a	96.00±1.00 ^a	55.47±0.50 ^{ghi}	71.00±3.46 ^e	86.00±0.00 ^{cdef}	82.20±0.72 ^{fgh}

S/N	Isolates	pH tolerance rate (Percentage of survival rate)						Bile salt
		3 h			6 h			
		pH2	pH2.5	pH3	pH2	pH2.5	pH3	
28.	JULABE01	61.62±0.53 ^{uergr}	70.53±0.67 ^{efg}	89.12±0.18 ^{bcue}	63.62±0.53 ^{erg}	69.62±0.53 ^{erg}	90.62±0.88 ^c	71.83±0.24 ^g
29.	JULABE05	71.20±0.72 ^{cde}	74.87±0.81 ^{def}	89.08±0.14 ^{bcde}	49.32±0.59 ^h	78.33±0.58 ^{cd}	88.00±1.00 ^{cde}	84.21±0.71 ^{fg}
30.	JULABE07	69.82±0.26 ^{def}	84.82±0.26 ^b	97.82±0.26 ^a	72.00±0.45 ^c	77.82±0.26 ^{cd}	98.00±1.16 ^a	81.78±0.31 ^{fgh}
31.	JULABE12	80.62±0.54 ^b	84.67±2.08 ^b	89.33±0.58 ^{bcde}	54.82±0.31 ^{ghi}	85.00±1.00 ^b	88.00±1.00 ^{cde}	83.31±0.60 ^{fg}
32.	JULABE23	45.94±0.09 ^{hijk}	69.91±0.13 ^f	67.94±0.09 ^f	41.94±0.09 ^{ikl}	47.94±0.09 ^j	68.94±1.33 ^{fg}	77.88±0.17 ^g
33.	JULABE31	46.85±0.21 ⁱ	69.93±0.10 ^f	71.85±0.21 ^{efg}	45.85±0.21 ⁱ	51.85±0.21 ^{hij}	72.85±1.20 ^f	51.84±0.23 ^{klm}
34.	JULABE33	46.70±0.42 ⁱ	69.64±0.51 ^f	67.20±0.29 ^f	41.70±0.42 ^{jkl}	47.70±0.42 ^j	68.70±1.00 ^{fg}	73.63±0.53 ^g
35.	JULABE35	43.88±0.17 ^j	69.60±0.57 ^f	61.88±0.17 ^{fgh}	35.88±0.17 ^k	41.88±0.17 ^k	62.88±1.25 ^h	74.71±0.41 ^g
36.	JULABE36	42.92±0.12 ^j	68.84±0.22 ^f	60.92±0.12 ^{fgh}	34.92±0.12 ^k	40.92±0.12 ^{kl}	61.92±1.29 ^h	70.79±0.30 ^{gh}
37.	JULABK07	60.56±0.62 ^{defgh}	67.70±0.43 ^{fgh}	88.06±0.08 ^b	62.56±0.62 ^{fgh}	68.56±0.62 ^{efg}	89.56±0.79 ^c	52.57±0.61 ^{klm}
38.	JULABK29	59.62±0.53 ^{defgh}	65.80±0.28 ^h	87.12±0.17 ^b	61.62±0.53 ^{fgh}	67.62±0.53 ^{efg}	88.62±0.88 ^{cde}	87.72±0.39 ^{efg}
39.	JULABK30	39.67±0.47 ^{klm}	66.74±0.37 ^h	57.17±0.24 ^g	33.67±0.47 ^{kl}	37.67±0.47 ^{klm}	58.67±0.94 ^{hi}	54.59±0.06 ^{klm}
40.	JULABK37	59.17±1.17 ^{defgh}	65.53±0.66 ^h	86.17±0.25 ^{cde}	60.67±0.46 ^f	66.67±0.46 ^{efg}	87.67±0.95 ^{cde}	57.56±0.62 ^{kl}
41.	JULABK39	42.64±0.51 ^j	68.58±0.60 ^f	60.14±0.20 ^{fgh}	34.64±0.51 ^k	40.64±0.51 ^{kl}	61.64±0.91 ^h	75.72±0.39 ^g
42.	JULABK41	55.33±0.58 ^{ghij}	79.00±1.00 ^c	84.67±0.58 ^c	65.00±1.00 ^e	77.72±0.31 ^{cd}	87.96±0.06 ^{cde}	83.26±0.65 ^{fg}
43.	JULABK46	53.10±0.86 ^{ghij}	80.50±0.50 ^{bcd}	85.83±0.76 ^c	69.21±1.07 ^{de}	81.00±1.00 ^{cde}	90.83±0.76 ^c	85.22±0.69 ^f
44.	JULABK49	63.25±1.09 ^{defgh}	75.00±1.00 ^d	84.83±1.04 ^c	44.40±0.53 ^{ijk}	68.27±0.64 ^{efg}	87.86±0.24 ^{cde}	82.12±0.82 ^{fgh}
45.	JULABK50	58.71±0.41 ^{efgh}	65.70±0.43 ^h	86.21±0.30 ^{cde}	60.71±0.41 ^f	66.71±0.41 ^{efg}	87.71±1.01 ^{cde}	80.90±0.13 ^{fgh}
46.	JULABS01	66.67±1.53 ^{defg}	79.00±1.00 ^c	82.67±0.58 ^{def}	63.40±0.53 ^{efg}	77.00±1.00 ^{cd}	86.67±0.58 ^{cde}	82.83±1.04 ^{fgh}
47.	JULABS12	41.61±0.56 ^j	67.59±0.58 ^{fgh}	59.11±0.15 ^g	33.61±0.56 ^{kl}	39.61±0.56 ^{kl}	60.61±.86 ^h	94.60±0.56 ^c
48.	JULABS13	58.59±0.59 ^{efgh}	62.91±0.13 ^{hij}	86.09±0.12 ^{cde}	60.59±0.59 ^f	66.59±0.59 ^{efg}	87.59±0.83 ^{cde}	76.81±0.41 ^g
49.	JULABS30	33.82±0.26 ^{lkm}	62.76±0.34 ^{hij}	51.82±0.26 ^h	33.82±0.26 ^{kl}	32.32±0.96 ^l	52.82±1.16 ^{ij}	54.81±0.27 ^{klm}
50.	JULABS31	71.13±0.81 ^{cde}	74.67±0.58 ^{d^{ef}}	79.33±0.58 ^{defg}	59.45±0.51 ^f	69.33±0.58 ^{efg}	85.67±0.58 ^d	81.36±0.91 ^{fgh}
51.	JULABS36	46.50±0.00 ^{hijk}	59.67±0.58 ^{hij}	63.00±1.00 ^{fgh}	66.23±0.69 ^{defg}	77.00±1.00 ^{cd}	89.67±0.58 ^{cde}	85.53±0.50 ^f
52.	JULABS38	54.91±0.13 ^{ghij}	62.66±0.48 ^{hij}	82.91±0.13 ^{def}	56.91±0.13 ^g	62.91±0.13 ^g	83.91±0.28 ^{g^{def}}	68.57±0.60 ^h
53.	JULABS41	35.67±0.46 ^k	62.97±0.04 ^{hij}	53.170.24 ^h	33.67±0.46 ^{kl}	33.67±0.46 ^l	54.67±0.95 ⁱ	83.76±0.34 ^{fg}
54.	JULABS43	50.70±0.43 ^{gh}	76.62±0.53 ^{def}	78.20±0.28 ^d	52.70±0.43 ^{ghi}	58.70±0.43 ^{gh}	79.70±0.99 ^e	100.85±2.62 ^a

^{a-z} Means not connected by the same letter within a column are significantly different at $p < 0.05$. Results expressed as average ($n = 3$) $\pm$ SD (standard deviation), JU= Jimma University, B=*Bulla*, S=*Cabbage Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*.

4.3.1.2.Tolerance to Bile Salts

All the 54 LAB isolates demonstrated tolerance against 0.3% bile salt concentration, with survival rates ranging from 51.84% to 100.82% (Table 4.1). These survival rates significantly varied ($p < 0.05$) (Table 4.1). Notably, JULABS43 and JULABB30 exhibited the highest survival rates at 100.85% and 100.53%, respectively. After incubation periods of 3 and 6 h at a 0.3% bile salt concentration, all the isolates displayed high resistance to low pH levels (2.00, 2.5, and 3.00), with minimal mortality rates. Consequently, these isolates were selected as promising probiotic candidates for further characterization.

4.3.1.3.Antimicrobial Activities of Lactic acid bacteria (LAB)

The antimicrobial activity of acid-bile-tolerant LAB against the selected foodborne pathogens was examined, and the average zone of inhibition ranged from 13.55 mm to 35.50 mm (Table 4.2). The mean of the antagonizing activity's inhibition zone displayed a significant variation at $p < 0.05$ (Table 4.2). Isolates JULABBR33, JULABBB16, JULABBB35, and JULABBR33 had the strongest antagonistic activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella Typhimurium*, and *Pseudomonas aeruginosa*, with inhibition zones that ranged from 25.40 mm to 35.50 mm. Isolates JULABK06, JULABBE01, JULABBR12, and JULABK46 had the smallest inhibition zone against all of the tested foodborne pathogens. Isolate JULAB01 had the highest inhibition zone against *Pseudomonas aeruginosa* with 29.67 mm, while isolates JULABK31 and JULABBB35 had the highest inhibition zones against *Salmonella Typhimurium* with 26.83 (Table 4.2). Finally, JULABB35, JULABBR33, and JULABB16 demonstrated the greatest and most effective antagonistic activity against *Candida albicans* with inhibition zone diameters of 30.58, 30.50, and 30.50 mm, respectively (Table 4.2).

Table 4. 2. Antimicrobial activities of LAB (traditional fermented foods and beverages) against some foodborne pathogens

S/N	LAB isolates	<i>E. coli</i> ATCC® 25922	<i>S. aureus</i> ATCC® 25923	<i>L. monocytogenes</i> ATCC® 7644	<i>P. aeruginosa</i> ATCC® 27853	<i>S. Tphimurium</i> ATCC® 13311	<i>C. albicans</i> ATCC® 14053
1)	JULABB01	30.13±0.18 ^{ab}	28.30±0.40 ^b	25.40±0.53 ^c	29.67±0.33 ^a	20.50±0.71 ^{abc}	29.67±0.33 ^a
2)	JULABB10	27.79±0.30 ^b	18.40±0.53 ^{cde}	24.50±0.71 ^c	18.33±0.33 ^{def}	19.67±0.33 ^b	15.33±0.34 ^d
3)	JULABB15	25.63±0.88 ^{bc}	18.00±0.00 ^{cde}	17.50±0.71 ^{def}	18.33±0.34 ^{cdef}	25.34±0.34 ^a	24.34±0.34 ^{bcd}
4)	JULABB16	26.00±0.57 ^b	32.50±0.71 ^a	35.50±0.71 ^a	30.50±0.71 ^a	20.50±0.71 ^{abc}	30.50±0.71 ^a
5)	JULABB30	20.67±0.66 ^c	20.30±0.36 ^{cd}	21.30±0.81 ^{cde}	20.50±0.71 ^{cde}	22.50±0.71 ^{ab}	17.75±0.35 ^c
6)	JULABB35	30.25±0.35 ^{ab}	30.00±0.00 ^{ab}	30.40±0.53 ^b	30.00±1.41 ^a	25.50±0.71 ^a	30.58±1.14 ^a
7)	JULABB39	19.75±0.35 ^{cd}	15.70±0.93 ^e	15.50±0.71 ^e	20.00±1.41 ^{cde}	18.50±0.71 ^{bcd}	18.50±0.71 ^c
8)	JULABB45	18.50±0.71 ^{cde}	18.33±0.33 ^{cde}	20.40±0.53 ^d	20.60±0.85 ^c	19.50±0.71 ^b	15.50±0.71 ^d
9)	JULABB56	18.00±0.57 ^{cde}	22.10±0.15 ^c	19.00±0.41 ^d	20.00±0.00 ^{cde}	15.00±0.00 ^c	25.00±0.00 ^b
10)	JULABBk02	20.93±0.11 ^c	18.30±0.71 ^{cde}	15.30±0.35 ^e	18.25±0.35 ^{cdef}	18.34±0.34 ^{bcd}	23.67±0.88 ^{bcd}
11)	JULABBk06	17.83±0.4 ^{cdef}	11.83±0.36 ^g	22.17±0.50 ^{de}	22.67±0.16	16.17±0.36 ^{de}	19.17±0.81 ^c
12)	JULABBk15	20.93±0.11 ^c	18.30±0.71 ^{cde}	15.30±0.35 ^e	18.25±0.35 ^{cdef}	18.34±0.34 ^{bcd}	23.67±0.88 ^{bcd}
13)	JULABBK21	22.33±0.33 ^c	18.00±0.00 ^{de}	17.67±0.33 ^{def}	22.33±0.34 ^c	19.67±0.34 ^b	19.67±0.34 ^c
14)	JULABBK29	18.63±0.88 ^{cde}	15.50±0.71 ^e	18.33±0.34 ^{de}	20.50±0.71 ^{cde}	20.45±0.63 ^{abc}	18.00±0.00 ^c
15)	JULABBK32	16.50±2.12 ^d	18.50±0.71 ^{cde}	23.00±1.40 ^c	20.50±0.71 ^{cde}	20.45±0.63 ^{abc}	20.50±0.71 ^c
16)	JULABBk33	20.25±0.35 ^c	15.50±0.71 ^e	18.90±0.31 ^{de}	21.50±0.71 ^c	20.50±0.63 ^{abc}	19.00±1.41 ^c
17)	JULABBk39	16.50±2.12 ^{cdef}	18.50±0.71 ^{cde}	23.00±1.40 ^c	20.50±0.71 ^{cde}	20.45±0.63 ^{abc}	20.50±0.71 ^c
18)	JULABBk40	18.67±0.66 ^{cde}	15.00±0.00 ^e	15.60±0.87 ^e	23.50±0.71 ^c	20.45±0.63 ^{abc}	21.00±1.14 ^{cde}
19)	JULABBBr03	20.25±0.35 ^c	15.50±0.71 ^e	18.90±0.31 ^{de}	21.50±0.71 ^c	20.50±0.63 ^{abc}	19.00±1.41 ^c
20)	JULABBBr04	15.00±0.25 ^f	18.50±0.58 ^{cde}	18.50±0.71 ^{de}	14.33±0.33 ^f	18.45±0.63 ^{bcd}	18.00±0.00 ^c
21)	JULABBBr05	19.00±1.41 ^{cd}	20.10±0.10 ^{cd}	16.00±0.41 ^e	19.50±0.71 ^d	17.66±0.33 ^{cde}	18.25±0.35 ^c
22)	JULABBBr12	15.00±0.25 ^f	18.50±0.58 ^{cde}	18.50±0.71 ^{de}	14.33±0.33 ^f	18.45±0.63 ^{bcd}	18.00±0.00 ^c
23)	JULABBBr17	18.67±0.66 ^{cde}	15.00±0.00 ^e	14.50±0.71 ^e	16.50±0.71 ^e	24.00 ±1.00 ^a	19.33±0.33 ^c
24)	JULABBBr21	20.28±0.40 ^c	17.90±0.16 ^d	21.00±1.42 ^{cde}	18.75±1.06 ^{cdef}	15.45±0.63 ^c	22.50±0.71 ^{bcd}
25)	JULABBBr33	33.50±0.71 ^a	23.50±0.71 ^c	26.00±1.41 ^{bcd}	18.63±0.88 ^{cdef}	20.50±0.71 ^{abc}	21.00±1.41 ^{cde}
26)	JULABBBr39	20.08±0.11 ^c	15.50±0.49 ^e	29.67±0.33 ^{bc}	29.50±0.71 ^a	19.00±1.41 ^b	30.50±0.71 ^a
27)	JULABBBr40	33.50±0.71 ^a	23.50±0.71 ^c	26.00±1.41 ^{bcd}	18.63±0.88 ^{cdef}	20.50±0.71 ^{abc}	21.00±1.41 ^{cde}
28)	JULABE01	18.13±0.18 ^{cde}	20.50±0.71 ^{cd}	18.33±0.33 ^{de}	19.50±0.71 ^d	17.50±0.50 ^{cde}	19.00±0.00 ^c

S/N	LAB isolates	<i>E. coli</i> ATCC®25922	<i>S. aureus</i> ATCC®25923	<i>L. monocytogenes</i> ATCC®7644	<i>P. aeruginosa</i> ATCC®27853	<i>S. Typhimurium</i> ATCC®13311	<i>C. albicans</i> ATCC®14053
29)	JULABE05	21.17±0.18 ^c	19.83±0.13 ^{cde}	20.50±0.77 ^{de}	21.00±0.67 ^c	22.50±0.82 ^{ab}	23.83±0.22 ^{bcd}
30)	JULABE07	18.67±0.33 ^{cde}	21.00±0.00 ^c	17.50±0.71 ^{def}	19.33±0.33 ^{cde}	15.50±0.71 ^c	18.00±0.00 ^c
31)	JULABE12	18.13±0.18 ^{cde}	20.50±0.71 ^{cd}	18.33±0.33 ^{de}	19.50±0.71 ^d	17.50±0.50 ^{cde}	19.00±0.00 ^c
32)	JULABE23	18.67±0.66 ^{cde}	19.67±0.65 ^{cde}	19.33±0.63 ^{de}	19.83±0.16 ^{cde}	18.33±0.90 ^{bcd}	21.67±0.62 ^c
33)	JULABE31	16.67±0.38 ^{ef}	14.00±0.42 ^f	18.67±0.28 ^{de}	20.67±0.32 ^{cde}	19.33±0.63 ^b	18.33±0.76 ^d
34)	JULABE33	21.33±1.76 ^c	18.00±0.00 ^{cde}	23.50±0.28 ^{cd}	17.00±0.00 ^e	17.00±1.41 ^{cde}	20.50±0.71 ^c
35)	JULABE35	21.28±1.02 ^c	20.30±0.40 ^{cd}	19.33±0.66 ^d	17.00±0.00 ^e	24.00±1.41 ^a	20.50±0.71 ^c
36)	JULABE36	18.50±0.50 ^{cde}	21.00±0.0 ^c	18.50±0.71 ^{de}	19.00±0.00 ^{cde}	20.00±1.41 ^{abc}	23.00±0.00 ^{bcd}
37)	JULABK07	22.28±0.40 ^c	20.50±0.71 ^{cd}	23.50±0.71 ^c	20.50±0.71 ^{cde}	15.17±0.71 ^c	15.50±0.71 ^d
38)	JULABK29	21.68±0.45 ^c	17.80±0.30 ^d	21.40±0.53 ^{cde}	20.33±0.33 ^{cde}	26.83±0.71 ^a	18.50±0.71 ^c
39)	JULABK30	24.68±0.96 ^{bcd}	15.50±0.71 ^e	20.40±0.53 ^d	17.50±0.71 ^e	19.67±0.34 ^b	18.33±0.34 ^c
40)	JULABK37	19.00±0.57 ^{cd}	18.00±0.00 ^{cde}	20.33±0.33 ^d	13.33±0.33 ^e	24.5 6±0.22 ^a	14.33±0.33 ^d
41)	JULABK39	19.08±1.52 ^c	22.50±0.71 ^c	24.50±0.50 ^c	19.50±0.71 ^d	18.50±0.71 ^{bcd}	14.50±0.71 ^d
42)	JULABK41	24.85±1.20 ^{bcd}	17.50±0.71 ^d	20.40±0.53 ^d	21.33±0.33 ^c	23.78±0.11 ^{ab}	15.33±0.33 ^d
43)	JULABK46	19.00±0.57 ^{cd}	18.00±0.00 ^{cde}	20.33±0.33 ^d	13.33±0.33 ^e	24.5 6±0.22 ^a	14.33±0.33 ^d
44)	JULABK49	18.33±0.66 ^{cde}	20.00±0.00 ^{cd}	25.33±0.33 ^c	17.33±0.33 ^e	20.34±0.33 ^{bcd}	18.33±0.33 ^c
45)	JULABK50	15.62±0.80 ^f	16.95±0.94	24.28±0.72 ^{cd}	26.28±0.67 ^b	22.95±0.95 ^{ab}	16.62±0.17 ^d
46)	JULABS01	23.00±0.57 ^c	15.00±0.00 ^e	27.67±0.33 ^{bc}	20.50±0.50 ^{cde}	19.67±0.00 ^b	15.50±0.50 ^d
47)	JULABS12	18.50±0.50 ^{cde}	18.00±0.00 ^{cde}	19.50±0.50 ^d	24.50±0.71 ^c	24.83±0.71 ^a	21.50±0.71 ^{cde}
48)	JULABS13	17.79±0.29 ^{cdef}	17.50±0.71 ^d	18.40±0.53 ^{de}	19.00±0.00 ^{cde}	17.83±0.71 ^{cde}	16.50±0.71 ^d
49)	JULABS30	19.00±0.00 ^{cd}	18.00±0.00 ^{cde}	28.33±0.33 ^{bc}	13.67±0.66 ^f	19.11±0.56 ^b	21.33±0.33 ^{cde}
50)	JULABS31	19.00±0.00 ^{cd}	18.00±0.00 ^{cde}	28.33±0.33 ^{bc}	13.67±0.66 ^f	19.11±0.56 ^b	21.33±0.33 ^{cde}
51)	JULABS36	21.67±0.82 ^c	20.00±0.00 ^{cd}	28.33±0.33 ^{bc}	13.33±0.33 ^f	20.34±0.07 ^{abc}	26.33±0.33 ^b
52)	JULABS38	17.95±0.08 ^{cdef}	22.50±0.71 ^c	20.40±0.53 ^d	16.00±1.41 ^e	19.50±0.71 ^b	20.50±0.71 ^c
53)	JULABS41	18.38±0.53 ^{cde}	20.50±0.71 ^{cd}	17.50±0.71 ^{def}	19.50±0.71 ^d	24.00±1.41 ^a	21.00±0.00 ^{cde}
54)	JULABS43	17.00±1.41 ^{cdef}	18.50±0.71 ^{cde}	20.50±0.71 ^d	22.00±0.00 ^c	18.50±0.71 ^{bcd}	21.50±0.71 ^{cde}
	Gentamycin(30 g/ml)	20.75±0.35^c	20.50±0.71^{cd}	21.40±0.53^{cde}	19.25±0.35^{cde}	21.67±0.94^{ab}	18.00±0.00^c

Note: JU= Jimma University, B=Bulla, S=Cabbage Shamita, Bk=Bukuri, Br= Borde, E=Ergo. Mean values denoted by the same letter within a column are not significantly different at $p < 0.05$. , *Escherichia coli* (*E. coli*), *Salmonella Typhimurium* (*S. Typhimurium*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Listeria monocytogenes* (*L. monocytogenes*), and *Candida albicans* (*C. albicans*).

4.3.1.4.Evaluating bacteriocins producing potential

Among the 54 isolates, 39 showed potential as bacteriocin producers with inhibition zones spanning 11.33 to 35.94 mm against the test foodborne pathogens (Table 4.3). The potent isolates included JULABB01, JULABB10, JULABB35, JULABB16, JULABBK02, JULABBK21, JULABBK39, JULABE12, JULABK30, JULABK3, and JULABS31 with JULABBB01 showing the highest inhibition zone (35.94 mm) against *Listeria monocytogenes*. JULABBK02 and JULABB45 showed strong inhibition zones (28.25 and 30.26 mm) against *Escherichia coli* and *Candida albicans*, respectively (Table 4.3). The results demonstrate that several isolates have the capability to produce potent bacteriocins, which were confirmed to be protein-based through enzyme sensitivity tests. This suggests their promising potential in combating foodborne pathogens, pending additional characterization studies.

4.3.1.5.Antibiotic Susceptibility Test

Regarding the subset of isolates, some were susceptible to streptomycin (10 g/ml), ampicillin (10 g/ml), and erythromycin (15 g/ml). The varying percentages of susceptibility among these antibiotics, as shown in Table 4.4, suggest a complex resistance pattern within this subset, indicating the need for further investigation to understand the underlying mechanisms behind these different responses to antibiotics at specific concentrations.

4.3.1.6.Survival of potential of LAB under Simulated Stomach Duodenum Passage and Gastro-intestinal juice

After a 3-hour incubation, potential probiotic LAB were subjected to simulated stomach duodenum passage (SSDP) to assess their survival rates (Table 4.5). The LAB isolates displayed survival rates ranging from 32.09% to 90.24%. JULABB01 and JULAB10 were the most resilient, with survival rates of 90.24% and 86.44%, respectively. Conversely, JULABBr40 exhibited the lowest (32.09%) survival rate under simulated SSDP conditions. Approximately 43 (80%) of the isolates had survival rates exceeding 50%, with a statistically significant ($p < 0.05$) variation in mean survival rates (Table 4.5). Out of 125 LAB isolates, 54 exhibited the highest survival rates under simulated gastrointestinal conditions, ranging from 30.07% to 85.45% after 3 and 24 h of incubation (Table 4.5). JULABB01 stood out with the highest survival rate at 85.45%. Conversely, JULABBBk40 showed the lowest survival rate among the isolates.

Table 4. 3. Antimicrobial activity of putative bacteriocins producing LAB against foodborne pathogens

S/N	LAB isolates	<i>E. coli</i> ATCC®25922	<i>S. aureus</i> ATCC®25923	<i>L. monocytogenes</i> ATCC®7644	<i>P. aeruginosa</i> ATCC®27853	<i>S. Typhimurium</i> ATCC®13311	<i>C. albicans</i> ATCC®14053
1.	JULABB01	17.75±0.35 ^c	30.29±0.41 ^a	35.94±1.32 ^a	34.13±0.18 ^a	33.49±0.69 ^a	18.50±0.71 ^{de}
2.	JULABB10	19.50±0.71 ^{abc}	15.50±0.71 ^d	15.33±0.46 ^{de}	15.50±0.71 ^{ef}	24.88±0.18 ^b	18.13±0.18 ^{de}
3.	JULABB16	18.25±0.35 ^c	30.00±0.00 ^a	30.13±0.18 ^b	13.25±0.35 ^e	24.82±0.69 ^b	14.50±0.71 ^e
4.	JULABB15	15.00±0.57 ^{cde}	12.00±0.00 ^{efg}	15.33±0.34 ^e	15.33±0.34 ^{ef}	23.34±0.34 ^b	21.34±0.34 ^c
5.	JULABB30	23.75±0.35 ^b	17.50±0.71 ^{def}	20.07±0.10 ^{de}	17.13±0.18 ^d	18.82±0.69 ^{bcd}	15.38±0.53 ^{ef}
6.	JULABB35	23.88±0.18 ^b	18.40±0.57 ^{de}	17.95±0.07 ^d	18.50±0.71 ^d	19.88±0.18 ^{bc}	15.13±0.18 ^{ef}
7.	JULABB45	14.75±0.35 ^{cde}	18.00±0.00 ^{de}	23.75±0.35 ^{cd}	19.13±0.18 ^{de}	20.13±0.18 ^{bc}	30.26±0.37 ^a
8.	JULABB56	23.75±0.35 ^b	14.75±0.35 ^d	20.29±0.41 ^{de}	12.13±0.18 ^{fg}	16.16±0.69 ^c	15.38±0.53 ^{ef}
9.	JULABBK02	28.25±0.35 ^a	15.00±0.00 ^d	18.25±0.35 ^d	22.13±0.18 ^c	20.88±0.18 ^{bc}	18.26±0.37 ^{de}
10.	JULABBK21	19.75±0.35 ^{abc}	20.75±1.06 ^c	20.29±0.41 ^{de}	17.13±0.18 ^d	19.49±0.69 ^{bc}	25.38±0.53 ^{cd}
11.	JULABBK33	15.67±0.66 ^{cde}	13.00±0.00 ^e	14.67±0.34 ^e	19.33±0.34 ^{bd}	16.67±0.34 ^c	19.67±0.34 ^{de}
12.	JULABBK39	19.67±0.82 ^{abc}	17.00±0.00 ^{def}	25.33±0.33 ^c	11.33±0.33 ^{fg}	17.34±0.07 ^{bcd}	23.33±0.33 ^c
13.	JULABBK40	20.02±0.58 ^{ab}	18.50±0.58 ^{de}	23.050±0.87	15.26±0.89 ^{ef}	17.00±0.05	22.30±0.36
14.	JULABBR03	18.60±0.85 ^c	20.50±0.71 ^c	17.15±0.44 ^d	17.13±0.18 ^d	18.13±0.18 ^{bcd}	19.38±0.53 ^{de}
15.	JULABBR12	21.33±1.76 ^{ab}	15.00±0.00 ^d	16.33±0.66 ^{de}	16.33±0.33 ^{ef}	16.67±0.33 ^c	17.33±0.33 ^{de}
16.	JULABBR33	13.50±0.71 ^d	16.00±1.41 ^{def}	35.31±0.44 ^a	20.50±0.71 ^c	18.88±0.18 ^{bcd}	16.13±0.18 ^d
17.	JULABBR39	21.75±0.62 ^{abc}	17.25±0.25 ^{def}	20.0±0.28 ^{de}	14.25±0.25 ^e	16.25±0.47 ^c	13.75±1.43 ^e
18.	JULABBR40	17.75±0.35 ^c	17.50±0.71 ^{def}	28.32±0.45 ^{bc}	16.13±0.18 ^{ef}	15.13±0.18 ^c	24.38±0.53 ^{cd}
19.	JULABE01	17.75±0.35 ^c	20.50±0.71 ^c	23.25±0.35 ^{cd}	17.13±0.18 ^d	20.13±0.18 ^{bc}	14.26±0.37 ^e
20.	JULABE05	17.75±0.35 ^c	17.50±0.71 ^{def}	20.26±0.37 ^{de}	19.13±0.18 ^{de}	20.13±0.18 ^{bc}	20.38±0.53 ^c
21.	JULABE07	17.25±0.35 ^c	20.50±0.71 ^c	18.43±0.60 ^d	19.13±0.18 ^{de}	18.13±0.18 ^{bcd}	16.26±0.37 ^d
22.	JULABE23	18.50±0.21 ^c	22.20±0.50 ^c	18.50±0.41 ^d	18.50±0.33 ^d	22.50±0.33 ^{bc}	18.36±0.36 ^{de}
23.	JULABE31	19.20±0.35	22.50±0.25 ^{abcd}	17.36±0.41 ^d	19.60±0.69 ^{de}	21.50±0.36 ^{bd}	23.00±0.00 ^c

S/N	LAB isolates	<i>E. coli</i> ATCC® 25922	<i>S. aureus</i> ATCC® 25923	<i>L. monocytogenes</i> ATCC® 7644	<i>P. aeruginosa</i> ATCC® 27853	<i>S. Typhimurium</i> ATCC® 13311	<i>C. albicans</i> ATCC® 14053
24.	JULABE33	20.12±0.36 ^{ab}	19.25±0.58 ^{de}	18.63±0.50 ^d	20.36±0.34 ^{abc}	19.50±0.25 ^{bcd}	19.38±0.76 ^{de}
25.	JULABE35	20.00±0.00 ^{abc}	13.00±0.0 ^e	12.00±0.00 ^f	17.00±0.00 ^{cd}	13.00±0.00 ^d	23.00±0.00 ^c
26.	JULABE36	22.50±1.76 ^b	17.50±0.25 ^{def}	15.69±0.25 ^e	18.90±0.33 ^d	20.58±0.25 ^{bc}	18.26±0.37 ^{de}
27.	JULABE12	19.93±0.11 ^{abc}	14.50±0.71 ^d	15.18±0.25 ^e	20.50±0.71 ^c	20.88±0.18 ^{bc}	18.13±0.18 ^{de}
28.	JULABK07	18.75±1.06 ^c	14.50±0.71 ^d	18.38±0.53 ^d	22.13±0.18 ^c	19.88±0.18 ^{bc}	20.26±0.37 ^c
29.	JULABK30	24.50±0.71 ^b	20.50±0.71 ^c	25.00±0.00 ^c	30.25±0.35 ^a	25.83±0.71 ^b	19.50±0.71 ^{de}
30.	JULABK37	20.87±0.18 ^{abc}	14.75±0.35 ^d	14.13±0.18 ^e	18.50±0.71 ^d	19.88±0.18 ^{bc}	18.13±0.18 ^{de}
31.	JULABK46	18.50±0.71 ^c	18.00±0.00 ^{de}	28.25±0.35 ^{bc}	13.25±0.35 ^e	20.17±0.71 ^{bc}	21.50±0.71 ^c
32.	JULABK49	16.00±0.57 ^{cd}	15.00±0.00 ^d	17.33±0.33 ^d	17.33±0.33 ^d	16.67±0.34 ^c	15.33±0.34 ^{ef}
33.	JULABK50	20.87±0.18 ^{abc}	20.25±0.35 ^{abc}	20.20±0.35 ^{abc}	19.26±0.37 ^{bd}	20.25±0.35 ^{abc}	20.87±0.18 ^{abc}
34.	JULABS01	17.93±0.11 ^c	15.50±0.71 ^d	20.13±0.18 ^{de}	14.13±0.18 ^e	18.13±0.18 ^{bcd}	30.26±0.37 ^a
35.	JULABS12	18.25±0.35 ^c	19.75±0.35 ^c	14.75±0.35 ^e	18.50±0.71 ^d	19.88±0.18 ^{bc}	18.13±0.18 ^{de}
36.	JULABS13	18.93±1.31 ^{abc}	17.50±0.71 ^{def}	17.75±0.35 ^d	19.25±0.35 ^{bd}	18.83±0.71 ^{bcd}	16.50±0.71 ^d
37.	JULABS31	20.50±0.71 ^{abc}	17.50±0.71 ^{def}	20.26±0.37 ^{de}	21.25±0.35 ^c	20.17±0.71 ^{bc}	15.50±0.71 ^{ef}
38.	JULABS36	20.25±0.35 ^{abc}	17.50±0.71 ^{def}	25.25±0.35 ^c	16.13±0.18 ^{ef}	19.13±0.18 ^{bcd}	19.26±0.37 ^{de}
39.	JULABS43	20.25±0.35 ^{abc}	19.26±0.37 ^{de}	17.33±0.33 ^d	20.87±0.18 ^{abc}	19.26±0.37 ^{de}	20.25±0.35 ^{abc}
	Gentamycin (30mg/ml)	18.75±1.06 ^c	17.50±0.71 ^{def}	18.38±0.53 ^d	14.13±0.18 ^e	20.13±0.18 ^{bc}	25.26±0.37 ^{cd}

Note: As mentioned in Table 4.2.

Table 4. 4.Antibiotic susceptibility profile of potential probiotic LAB isolated from Ethiopian traditional fermented foods and beverages

S/N	Isolates	Antimicrobial susceptibility profile					
		Tetracycline (30µg/ml)	Streptomycin (10µg/ml)	Kanamycin(25µg/ml)	Amplicin (10µg/ml)	Erythromycin (15µg/ml)	Chloramphenicol (15µg/ml)
1.	JULABB01	S	S	R	S	S	S
2.	JULABB10	S	S	R	S	R	S
3.	JULABB16	S	S	R	R	R	S
4.	JULABB15	S	R	R	S	S	S
5.	JULABB30	S	R	R	S	S	S
6.	JULABB35	S	R	R	S	S	S
7.	JULABB39	S	R	R	S	S	S
8.	JULABB45	S	R	R	S	S	S
9.	JULABB56	S	S	R	R	S	S
10.	JULABBK02	S	S	R	S	R	S
11.	JULABBK06	S	S	R	S	S	S
12.	JULABBK15	S	R	R	S	S	S
13.	JULABBK21	S	R	R	S	S	S
14.	JULABBK29	S	R	R	S	S	S
15.	JULABBK32	S	S	R	S	S	S
16.	JULABBK33	S	S	R	S	S	S
17.	JULABBK39	S	R	R	S	S	S
18.	JULABBK40	S	R	R	S	S	S
19.	JULABBR03	S	S	R	S	S	S
20.	JULABBR04	S	S	R	S	R	S
21.	JULABBR05	S	S	R	R	R	S
22.	JULABBR12	S	R	R	S	S	S
23.	JULABBR17	S	R	R	S	S	S
24.	JULABBR21	S	R	R	S	S	S
25.	JULABBR33	S	R	R	S	S	S
26.	JULABBR39	S	R	R	S	S	S
27.	JULABBR40	S	S	R	R	S	S
28.	JULABE01	S	S	R	S	R	S
29.	JULABE05	S	S	R	S	S	S
30.	JULABE07	S	R	R	S	S	S
31.	JULABE12	S	R	R	S	S	S
32.	JULABE23	S	R	R	S	S	S
33.	JULABE31	S	S	R	S	S	S
34.	JULABE33	S	S	R	S	S	S
35.	JULABE35	S	R	R	S	S	S
36.	JULABE36	S	R	R	S	S	S
37.	JULABK07	S	S	R	S	S	S

S/N	Isolates	Antimicrobial susceptibility profile					
		Tetracycline (30µg/ml)	Streptomycin (10µg/ml)	Kanamycin(25µg/ml)	Amplicin (10µg/ml)	Erythromycin (15µg/ml)	Chloramphenicol (15µg/ml)
38.	JULABK29	S	S	R	S	R	S
39.	JULABK30	S	S	R	R	R	S
40.	JULABK37	S	R	R	S	S	S
41.	JULABK39	S	R	R	S	S	S
42.	JULABK41	S	R	R	S	S	S
43.	JULABK46	S	R	R	S	S	S
44.	JULABK49	S	R	R	S	S	S
45.	JULABK50	S	S	R	R	S	S
46.	JULABS01	S	S	R	S	R	S
47.	JULABS12	S	S	R	S	S	S
48.	JULABS13	S	R	R	S	S	S
49.	JULABS30	S	R	R	S	S	S
50.	JULABS31	S	R	R	S	S	S
51.	JULABS36	S	S	R	S	S	S
52.	JULABS38	S	S	R	S	S	S
53.	JULABS41	S	R	R	S	S	S
54.	JULABS43	S	R	R	S	S	S

The zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥ 21 mm); intermediate, I (16-20 mm) and resistance R (≤ 15 mm).

4.3.1.7.Simulated intestinal juice tolerance

Totally, 125 LAB isolates were selected as prospective LAB and tested for their capacity to survive in simulated intestinal juice. Accordingly, as a result, 54 LAB isolates had the highest survival rate ranging from 41.04% to 85.18 for their ability to tolerate and survive under simulated intestinal juice (Table 4.5). Hence, 54 LAB had high survival rates that ranged from 41.04% (the lowest) to 85.18% (highest). The isolates JULABB01 had the greatest survival rate followed by JULABS31 and JULABS36, which had survival rates of 83.27% and 82.24%, respectively. The mean values of the isolates survival rates demonstrated a significant ($p < 0.05$) difference (Table 4.5). In general, approximately 36 (65.16%) of the total isolates exhibited a survival rates more than 50%.

Table 4. 5. Survival rate of potential of probiotic LAB in simulated gastro-intestinal conditions

S/N	LAB isolates	Survival rate (percentage of the survival potential)					
		SGIJ	SIJ	SSDP	Intestinal inhibitory substances		
					Phenol	Pepsin	Pancreatic
1.	JULABB01	85.45±0.64 ^a	85.18±0.26 ^a	90.24±0.34 ^a	79.82±0.25 ^e	88.21±0.30 ^a	106.50±0.71 ^a
2.	JULABB10	83.06±0.08 ^{ab}	78.35±0.50 ^b	86.44±0.63 ^{abc}	77.16±0.22 ^e	88.09±0.13 ^a	98.06±0.09 ^b
3.	JULABB15	82.07±0.10 ^{ab}	75.40±0.56 ^b	74.71±0.40 ^{cd}	67.89±0.15 ^g	85.27±0.38 ^{ba}	87.02±0.03 ^d
4.	JULABB16	82.21±0.29 ^{ab}	76.17±0.24 ^b	81.41±0.58 ^b	67.45±0.64 ^g	86.07±0.09 ^{ba}	91.00±0.00 ^c
5.	JULABB30	80.06±0.08 ^{abc}	70.01±0.01 ^c	73.61±0.55 ^{cde}	66.96±0.06 ^{gh}	81.14±0.20 ^b	82.11±0.15 ^{fg}
6.	JULABB35	76.20±0.29 ^b	64.29±0.41 ^e	70.29±0.41 ^d	63.91±0.12 ^h	77.35±0.50 ^{bc}	81.09±0.12 ^{fgh}
7.	JULABB39	76.17±0.24 ^b	64.23±0.33 ^e	70.26±0.37 ^d	63.70±0.42 ^h	77.22±0.31 ^{bc}	78.25±0.35 ^e
8.	JULABB45	40.02±0.56 ^{ijk}	60.49±0.29 ^{efg}	77.10±0.10 ^c	80.59±0.30 ^{def}	83.83±0.17 ^b	84.16±0.16 ^{fg}
9.	JULABB56	75.22±0.31 ^b	63.03±0.04 ^e	68.31±0.44 ^{de}	62.67±0.47 ^h	71.39±0.55 ^{cde}	76.16±0.22 ^e
10.	JULABBK02	74.41±0.58 ^b	62.36±0.51 ^{efg}	66.13±0.18 ^{def}	58.80±0.29 ⁱ	71.26±0.37 ^{cde}	73.20±0.28 ^{gh}
11.	JULABBK06	71.22±0.31 ^{bc}	61.29±0.41 ^{efg}	66.03±0.04 ^{def}	58.75±0.36 ⁱ	71.11±0.16 ^{cde}	72.36±0.51 ^{gh}
12.	JULABBK15	70.07±0.09 ^c	60.27±0.38 ^{efg}	64.26±0.36 ^e	58.10±0.14 ⁱ	70.30±0.42 ^{cde}	72.15±0.22 ^{gh}
13.	JULABBK21	69.92±0.11 ^c	59.46±0.65 ^f	64.15±0.21 ^e	56.31±0.44 ^{ij}	70.20±0.28 ^{cde}	71.23±0.32 ^f
14.	JULABBK29	68.45±0.64 ^{cd}	59.23±0.33 ^f	63.26±0.36 ^{efg}	56.19±0.26 ^{ij}	70.02±0.03 ^{cde}	71.10±0.14 ^f
15.	JULABBK32	68.33±0.47 ^{cd}	58.37±0.52 ^f	62.34±0.48 ^{efg}	55.30±0.43 ^j	69.44±0.63 ^{cde}	68.34±0.48 ^g
16.	JULABBK33	68.01±0.02 ^{cd}	57.36±0.51 ^{fg}	62.23±0.32 ^{efg}	55.02±0.03 ^j	69.29±0.41 ^{de}	66.48±0.68 ^{gh}
17.	JULABBK39	67.40±0.57 ^{cd}	57.30±0.43 ^{fg}	61.37±0.52 ^{efg}	54.44±0.62 ^j	69.04±0.05 ^{de}	66.09±0.13 ^{gh}
18.	JULABBK40	30.70±0.35 ^k	48.82±0.43 ^h	62.21±0.21 ^{efg}	97.32±0.34 ^{ab}	84.82±0.18 ^b	85.49±0.29 ^{fg}
19.	JULABBR03	67.25±0.35 ^{cd}	57.02±0.03 ^{fgh}	60.40±0.57 ^{efg}	54.25±0.35 ^j	66.26±0.37 ^d	64.48±0.67 ^{gh}
20.	JULABBR04	67.21±0.30 ^{cd}	57.02±0.02 ^{fgh}	60.39±0.55 ^{efg}	54.16±0.23 ^{jk}	66.24±0.33 ^d	64.07±0.10 ^{ghi}
21.	JULABBR05	53.63±0.32 ^f	48.31±0.34 ^h	75.56±0.29 ^{cd}	91.85±0.15 ^{bcd}	56.00±0.00 ^{ghi}	57.33±0.33 ^{hg}
22.	JULABBR12	66.39±0.55 ^{cde}	55.10±0.13 ^g	59.97±0.05 ^f	53.32±0.46 ^{jk}	65.38±0.53 ^d	63.44±0.62 ^{ghi}
23.	JULABBR17	66.36±0.51 ^{cde}	54.31±0.44 ^g	59.77±0.33 ^f	52.21±0.29 ^{jk}	65.22±0.31 ^d	63.41±0.57 ^{ghi}
24.	JULABBR21	66.18±0.26 ^{cde}	52.48±0.68 ^g	59.62±0.54 ^f	52.07±0.10 ^{jkl}	65.02±0.03 ^d	63.18±0.25 ^{ghi}
25.	JULABBR33	66.13±0.19 ^{cde}	52.30±0.42 ^g	58.13±0.18 ^{fg}	52.03±0.05 ^{kl}	64.26±0.36 ^{ef}	63.16±0.23 ^{ghi}
26.	JULABBR39	53.03±0.03 ^f	85.00±0.00 ^a	32.09±0.09 ^{lnm}	85.63±0.32 ^d	79.73±0.27 ^{bc}	80.73±0.27 ^{fgh}
27.	JULABBR40	65.34±0.48 ^d	52.03±0.04 ^g	58.08±0.11 ^{fg}	51.33±0.47 ^{jkl}	62.06±0.08 ^{ef}	62.49±0.69 ^{ghi}
28.	JULABE01	43.42±0.71 ^{ij}	49.39±0.31 ^h	59.54±0.29 ^f	98.67±0.33 ^a	69.03±0.55 ^{de}	70.03±0.55 ^f

S/N	LAB isolates	Survival rate (percentage of the survival potential)					
		SGIJ	SIJ	SSDP	Intestinal inhibitory substances		
					Phenol	Pepsin	Pancreatic
29.	JULABE05	51.97±0.55 ^{fg}	44.10±0.10 ⁱ	65.08±0.08 ^e	85.07±0.07 ^d	84.67±0.33 ^b	64.44±0.30 ^{gh}
30.	JULABE07	63.11±0.15 ^d	49.21±0.29 ^h	57.36±0.51 ^{fg}	51.21±0.30 ^{ijkl}	60.20±0.28 ^{ef}	62.22±0.31 ^{ghi}
31.	JULABE12	53.00±0.00 ^f	60.33±0.33 ^{efg}	42.09±0.09 ^k	90.33±0.33 ^{bcd}	71.00±0.00 ^{cde}	72.00±0.00 ^{gh}
32.	JULABE23	62.10±0.14 ^{de}	48.17±0.24 ^h	57.22±0.31 ^{fg}	51.05±0.07 ^{ijkl}	58.20±0.28 ^e	60.07±0.09 ^{hik}
33.	JULABE31	61.49±0.70 ^{def}	48.15±0.22 ^h	56.38±0.54 ^{fgh}	51.02±0.03 ^k	58.02±0.03 ^e	59.76±0.34 ^h
34.	JULABE33	61.28±0.39 ^{def}	48.06±0.08 ^h	56.34±0.49 ^{fgh}	50.45±0.63 ^k	58.02±0.03 ^e	58.37±0.52 ^h
35.	JULABE35	61.21±0.29 ^{def}	48.00±0.00 ^{hi}	54.45±0.63 ^g	50.95±0.07 ^k	58.01±0.01 ^e	58.15±0.22 ^h
36.	JULABE36	39.15±0.15 ^{jk}	68.31±0.31 ^{cde}	69.56±0.30 ^{de}	83.11±0.49 ^{def}	83.74±0.26 ^b	84.41±0.30 ^{fg}
37.	JULABK07	60.28±0.40 ^{def}	47.26±0.37 ^{hi}	54.28±0.40 ^g	50.44±0.62 ^k	57.43±0.60 ^e	58.10±0.14 ^h
38.	JULABK29	60.02±0.03 ^{def}	47.22±0.31 ^{hi}	53.03±0.05 ^g	50.19±0.27 ^k	57.32±0.46 ^e	57.41±0.58 ^{hg}
39.	JULABK30	59.36±0.52 ^e	46.26±0.36 ^{hij}	52.17±0.24 ^g	49.83±0.24 ^k	57.16±0.23 ^{fg}	57.35±0.50 ^{hg}
40.	JULABK37	59.01±0.01 ^e	46.06±0.09 ^{hij}	52.13±0.18 ^{gh}	49.52±0.68 ^k	56.42±0.59 ^{fg}	56.17±0.24 ^{hi}
41.	JULABK39	59.00±0.00 ^e	46.05±0.06 ^{hij}	51.34±0.48 ^{gh}	48.93±0.10 ^k	56.37±0.52 ^{fg}	56.14±0.20 ^{hi}
42.	JULABK41	63.95±0.53 ^d	64.34±0.33 ^e	49.76±0.24 ^h	83.85±0.15 ^{def}	51.66±0.33 ^f	53.32±0.34 ^{hi}
43.	JULABK46	41.18±0.18 ^{ijk}	57.42±0.30 ^{fgh}	81.21±0.21 ^b	81.95±0.05 ^{def}	35.38±0.31 ^{ijk}	36.04±0.04 ^{klm}
44.	JULABK49	42.60±0.31 ^{ij}	54.07±0.07 ^g	76.17±0.17 ^c	91.00±0.58 ^{bcd}	72.17±0.17 ^{cde}	73.84±0.16 ^{gh}
45.	JULABK50	58.07±0.07 ^{ef}	70.19±0.19 ^c	46.15±0.15 ⁱ	89.35±0.33 ^c	69.78±0.22 ^{de}	70.44±0.29 ^f
46.	JULABS01	56.33±0.33 ^{efg}	45.02±0.02 ⁱ	71.05±0.05 ^d	98.33±0.33 ^a	75.33±0.33 ^c	76.99±0.01 ^e
47.	JULABS12	57.45±0.64 ^{ef}	43.19±0.27 ⁱ	49.97±0.04 ^h	47.12±0.17 ^{kl}	55.33±0.46 ^{fgh}	55.04±0.06 ^{hi}
48.	JULABS13	57.36±0.52 ^{ef}	42.10±0.14 ^{ij}	49.39±0.55 ^h	46.80±0.28 ^{klm}	55.26±0.37 ^{fgh}	54.49±0.69 ^{hi}
49.	JULABS30	56.35±0.49 ^{efg}	42.09±0.13 ^{ij}	49.32±0.45 ^h	45.78±1.10 ^l	54.47±0.66 ^f	54.26±0.36 ^{hi}
50.	JULABS31	77.14±0.14 ^b	83.27±0.27 ^{ab}	67.52±0.29 ^{def}	80.44±0.29 ^{def}	75.00±0.00 ^c	75.66±0.33 ^e
51.	JULABS36	76.05±1.02 ^b	82.24±0.24 ^{abc}	58.10±0.10 ^{fg}	90.95±0.53 ^{bcd}	63.98±0.02 ^{ef}	64.98±0.02 ^{gh}
52.	JULABS38	54.49±0.69 ^f	41.16±0.22 ^{ij}	48.23±0.33 ^h	45.29±0.4 ^l	53.46±0.65 ^f	52.45±0.63 ^{hij}
53.	JULABS41	54.43±0.61 ^f	41.11±0.16 ^{ij}	48.18±0.25 ^{hi}	45.14±0.19 ^l	53.45±0.64 ^f	51.34±0.48 ^{hij}
54.	JULABS43	54.13±0.19 ^f	41.04±0.06 ^{ij}	48.07±0.10 ^{hi}	45.06±0.08 ^l	53.43±0.60 ^f	51.31±0.44 ^{hijk}

JU= Jimma University, B=*Bulla*, S=*Cabbage Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*, SGIJ=simulated gastro-intestinal juice SIJ=Simulated intestinal juice, SSDP=Simulated stomach duodenum passage. Mean values denoted by the same letter within a column are not significantly different at p< 0.05.

4.3.1.7.1. Phenol tolerance

After demonstrating acid-bile tolerance, 54 LAB isolates were assessed for their ability to withstand 0.3% phenol concentration (Table 4.5). All the isolates had over 45.05% survival rates by displaying significant ($p < 0.05$) variations in mean survival rates. Survival rates ranged from 45.06% to 98.67%, with JULABE01 (98.67%) and JULABS01 (98.33%) showing the highest rates (Table 4.5). The high tolerance to phenol concentration by the potent isolates indicates the untapped potential for selecting probiotic candidates for future applications (Table 4.5). However, JULABS43 had the lowest (45.06%) survival rate

4.3.1.7.2. Determining Pancreatic and pepsin tolerance

Probiotic LAB were tested for survival under simulated gastrointestinal conditions, pancreatin, and pepsin, with survival rates ranging from 36.84% to 106.50% (Table 4.5). JULABB01, JULABB10, and JULABB16 showed high survival rates of 106.50%, 98.06%, and 96.99%, respectively with JULABB01 demonstrating the highest pancreatin tolerance. Tolerance to these conditions is crucial for screening potential probiotic LAB. All the isolates were resistant to pepsin, with survival rates ranging from 35.38% to 88.21%. Notably, JULABB01, JULABB10, and JULABB16 showed survival rates of 88.21%, 88.09%, and 86.07%, respectively (Table 4.5).

4.3.1.8. Safety Evaluation of LAB isolates

All the 54 identified putative probiotic LAB were assessed for hemolytic and DNase activities, confirming their non-pathogenic status as they showed no hemolysis or DNase production (Table 4.6). Moreover, their aggregation potential, critical for biofilm formation and gastrointestinal survival, was evaluated. The isolates displayed auto-aggregation rates ranging from 45.19% to 120.39%, with JULABB01, JULABB10, and JULABB16 exhibiting the highest rates. Co-aggregation potential with *E. coli* and *S. aureus* ranged from 54.08% to 116.20%, with JULABB01 and JULABB10 displaying the strongest co-aggregation. All the isolates had cell-surface hydrophobicity percentages above 54.08%, indicating a good adherence capacity, particularly JULABB01, JULABB10, and JULABB16 with the highest rates. These findings underscore the safety and potential of these LAB for probiotic applications (Table 4.6).

Table 4. 6.Aggregation, hydrophobicity and safety analysis of potential probiotic LAB isolates

S/N	Isolates	Hemolytic activity	DNase Activity	Auto-aggregation	Co-aggregation	Hydrophobicity
1)	JULABB01	γ-haemolytic	-	120.39±0.55 ^a	116.20±0.29 ^a	106.50±0.14 ^a
2)	JULABB10	γ-haemolytic	-	105.23±0.33 ^b	108.04±0.05 ^b	82.23±0.33 ^d
3)	JULABB15	γ-haemolytic	-	83.84±0.45 ^{fg}	91.06±0.09 ^{def}	97.19±0.80 ^b
4)	JULABB16	γ-haemolytic	-	102.34±0.48 ^{bc}	106.19±0.26 ^{bc}	87.36±0.51 ^{bcd}
5)	JULABB30	γ-haemolytic	-	79.25±0.35 ^h	87.10±0.14 ^e	88.14±0.20 ^{bcd}
6)	JULABB35	γ-haemolytic	-	100.10±0.15 ^c	105.11±0.16 ^{bc}	87.63±3.63 ^c
7)	JULABB39	γ-haemolytic	-	70.46±0.65 ^{hij}	82.37±0.53 ^{fg}	77.39±0.55 ^{ef}
8)	JULABB45	γ-haemolytic	-	65.63±0.89 ^{ijk}	80.27±0.38 ^{fgh}	86.12±0.17 ^{bcd}
9)	JULABB56	γ-haemolytic	-	61.65±1.91 ^{jk}	71.38±0.54 ^h	88.33±0.46 ^{bcd}
10)	JULABBK02	γ-haemolytic	-	98.09±0.13 ^{cd}	101.37±0.53 ^{bcd}	80.27±0.38 ^e
11)	JULABBK06	γ-haemolytic	-	95.38±0.54 ^d	95.12±0.12 ^d	84.15±0.22 ^d
12)	JULABBK15	γ-haemolytic	-	82.27±0.38 ^{fgh}	90.45±0.63 ^{def}	84.15±0.22 ^d
13)	JULABBK21	γ-haemolytic	-	77.19±0.27 ^{hi}	86.20±0.28 ^{ef}	81.33±0.47 ^{df}
14)	JULABBK29	γ-haemolytic	-	70.62±0.31 ^{hij}	82.23±0.33 ^{fg}	63.26±0.36 ^g
15)	JULABBK32	γ-haemolytic	-	64.55±0.63 ^j	77.39±0.55 ^g	67.39±0.22 ^{fgh}
16)	JULABBK33	γ-haemolytic	-	61.92±0.51 ^{kl}	83.06±0.06 ^{fg}	88.43±0.05 ^{bcd}
17)	JULABBK39	γ-haemolytic	-	45.35±0.50 ^m	55.37±0.52 ^j	73.43±0.81 ^f
18)	JULABBK40	γ-haemolytic	-	73.16±0.22 ^{hij}	83.43±0.61 ^{fg}	68.37±0.28 ^{fgh}
19)	JULABBR03	γ-haemolytic	-	95.26±0.26 ^d	100.49±0.69 ^c	82.06±0.09 ^d
20)	JULABBR04	γ-haemolytic	-	95.08±0.11 ^d	99.49±0.70 ^c	80.48±0.69 ^e
21)	JULABBR05	γ-haemolytic	-	82.13±0.19 ^{fgh}	88.33±0.46 ^e	64.13±0.18 ^g
22)	JULABBR12	γ-haemolytic	-	75.10±0.14 ^{hi}	86.12±0.17 ^{ef}	86.20±0.28 ^{bcd}
23)	JULABBR17	γ-haemolytic	-	70.27±0.39 ^{hij}	82.06±0.09 ^{fgh}	83.43±0.61 ^d
24)	JULABBR21	γ-haemolytic	-	64.68±0.46 ^j	77.35±0.49 ^g	82.37±0.53 ^d
25)	JULABBR33	γ-haemolytic	-	98.10±0.14 ^c	103.27±0.38 ^{bcd}	85.19±0.28 ^{de}
26)	JULABBR39	γ-haemolytic	-	56.31±0.44 ^k	66.02±0.02 ⁱ	77.35±0.49 ^{ef}
27)	JULABBr40	γ-haemolytic	-	45.19±0.27 ^m	54.08±0.12 ^j	69.36±0.32 ^{fgh}
28)	JULABE01	γ-haemolytic	-	80.39±0.56 ^{fgh}	88.14±0.20 ^e	82.23±0.33 ^d
29)	JULABE05	γ-haemolytic	-	93.47±0.66 ^{de}	99.29±0.40 ^c	95.29±0.20 ^{bc}
30)	JULABE07	γ-haemolytic	-	93.43±0.61 ^{de}	78.10±0.10 ^g	65.26±0.37 ^g
31)	JULABE12	γ-haemolytic	-	74.41±0.58 ^{hi}	85.19±0.28 ^{efg}	76.43±0.61 ^{efg}
32)	JULABE23	γ-haemolytic	-	93.03±0.04 ^{de}	99.18±0.26 ^c	55.37±0.52 ^{hij}
33)	JULABE31	γ-haemolytic	-	69.29±1.00 ⁱ	81.33±0.47 ^{fgh}	90.31±0.04 ^{bc}
34)	JULABE33	γ-haemolytic	-	63.74±0.36 ^j	76.43±0.61 ^{gh}	67.43±0.12 ^{fgh}
35)	JULABE35	γ-haemolytic	-	55.25±0.36 ^k	65.26±0.37 ⁱ	65.26±0.71 ^g
36)	JULABE36	γ-haemolytic	-	67.92±0.12 ^{ij}	80.31±0.44 ^{fgh}	82.06±0.09 ^d

S/N	Isolates	Hemolytic activity	DNase Activity	Auto-aggregation	Co-aggregation	Hydrophobicity
37)	JULABK07	γ-haemolytic	-	80.08±0.11 ^{fg}	78.30±0.30 ^g	83.43±0.61 ^d
38)	JULABK29	γ-haemolytic	-	74.30±0.43 ^{hi}	84.15±0.22 ^f	77.39±0.55 ^{ef}
39)	JULABK30	γ-haemolytic	-	69.57±0.30 ⁱ	80.48±0.69 ^{fgh}	80.27±0.38 ^e
40)	JULABK37	γ-haemolytic	-	63.78±0.31 ^{jk}	75.23±0.33 ^{gh}	82.37±0.53 ^d
41)	JULABK39	γ-haemolytic	-	51.24±0.33 ^{kl}	64.13±0.18 ⁱ	87.36±0.21 ^{bcd}
42)	JULABK41	γ-haemolytic	-	79.38±0.54 ^h	87.36±0.52 ^e	75.23±0.33 ^{fg}
43)	JULABK46	γ-haemolytic	-	92.38±0.54 ^{de}	98.13±0.18 ^{cd}	80.31±0.44 ^e
44)	JULABK49	γ-haemolytic	-	92.30±0.43 ^{def}	97.45±0.64 ^{cde}	71.38±0.54 ^f
45)	JULABK50	γ-haemolytic	-	91.07±0.10 ^{def}	97.14±0.19 ^{cde}	89.14±0.12 ^{bc}
46)	JULABS01	γ-haemolytic	-	90.46±0.65 ^e	97.07±0.10 ^{cde}	74.46±0.65 ^f
47)	JULABS12	γ-haemolytic	-	63.85±0.21 ^{jk}	74.46±0.65 ^{gh}	87.10±0.14 ^{bcd}
48)	JULABS13	γ-haemolytic	-	89.12±0.17 ^{ef}	96.28±0.40 ^{cde}	54.08±0.12 ^{hij}
49)	JULABS30	γ-haemolytic	-	49.43±0.61 ^l	63.26±0.36 ⁱ	80.31±0.44 ^e
50)	JULABS31	γ-haemolytic	-	88.48±0.68 ^{ef}	95.09±0.12 ^d	66.02±0.02 ^{gh}
51)	JULABS36	γ-haemolytic	-	85.19±0.27 ^f	94.18±0.26 ^d	80.48±0.69 ^e
52)	JULABS38	γ-haemolytic	-	84.40±0.56 ^f	79.11±0.11 ^{fgh}	81.33±0.47 ^{df}
53)	JULABS41	γ-haemolytic	-	84.29±0.42 ^f	93.20±0.28 ^{de}	77.35±0.49 ^{ef}
54)	JULABS43	γ-haemolytic	-	84.26±0.36 ^{fg}	91.42±0.59 ^{de}	89.43±0.29 ^{bc}

‘-’ = no activity. Mean values denoted by the same letter within a column are not significantly different at $p < 0.05$.

4.4.DISCUSSION

A study on probiotics from Ethiopian fermented foods and beverages found that some bacteria exhibited strong probiotic and antimicrobial properties, suggesting potential use in promoting digestive health and preventing infections. The study identified 54 LAB isolates as promising candidates, providing a foundation for further research and development of functional food products to improve overall wellness in Ethiopia and beyond. Probiotic LAB, with their antimicrobial properties and nutritional benefits, are gaining interest in the food industry as natural preservatives. However, further research is needed to understand their mechanisms and optimize their use in various food systems. Despite these challenges, the potential benefits of probiotic LAB could lead to a more sustainable and healthier food industry.

All the 54 isolates tolerated 4% and 6.5% salt concentrations and varied temperatures (15°C and 37°C), with only a few thriving at 45°C. This aligns with earlier studies on LAB from Ethiopian fermented foods such as *Ergo*, *Shamita*, *Borde*, and fermented teff (Azadnia & Khan Nazer, 2009; Negasi et al., 2017). In addition, Mulaw et al. (2020), found that 34 probiotic LAB from kocho, *ergo*, and teff grew well at 4% and 6.5% salt concentrations and at 10°C and 15°C, closely resembling our findings.

The potential probiotic LAB isolates (n=54) exhibited the highest survival rates at pH 2, 2.5, and 3, although rates declined as pH dropped to 2. Survival rates ranged from 33.82% to 98.82%, indicating significant differences in their acid tolerance. Studies by Abushelaibi et al. (2017); Ribeiro et al. (2014) and Song et al (2021) noted *Pediococcus* species with a 70% survival rate at pH 2 for 3 h, while Akbar et al. (2019), reported that *L. lactis* with a 65% to 70% survival rate under similar conditions, but lower than our findings. Specific strains of bacteria and experimental conditions can affect survival rates, emphasizing the need for further research. These results underscore the importance of selecting probiotics based on strain-specific characteristics, especially for acid-sensitive applications. The LAB isolates showed reduced survival rates at low pH initially but increased over time. Isolates with full tolerance at pH 2.5 and 3.0 for 3 h and 6 h are a potential candidate to use as probiotics or food preservatives in the future (Argyri et al., 2013).

All the 54 LAB isolates in this study demonstrated high resistance to 0.3% w/v bile salt concentration, indicating their survivability in the small intestine for up to 4 h. Their survival rates ranged from 51.82% to 98.82%, which is consistent with the findings by Mulaw et al (2019), where 34 LAB isolates showed survival rates ranging from 82.58% to 99.44%. Similar results were observed by Dutra Rosolen et al (2021) and Harnentis et al (2020), who found that most *Pediococcus* and *Lactococcus* species exhibit bile tolerance, with survival rates ranging from 42.50% to 85.30%. The isolates' ability to resist 0.3% bile salt is attributed to their production of bile salt hydrolase, which de-conjugates bile acid, reducing its solubility and reabsorption efficiency. These findings underscore the potential of these LAB as probiotics, given their ability to withstand the digestive process and potentially benefit human health (de Melo Pereira et al., 2018).

Probiotic isolates vary in antagonistic activity, suggesting specificity against pathogens whose inhibition effect attributed to bioactive compounds like organic acids, hydrogen peroxide, and bacteriocins. These compounds aid probiotic LAB survival and resistance by inhibiting food-borne pathogens growth in order to ensure food safety. Probiotic LAB also offer health benefits such as reducing inflammation and improving mental well-being, highlighting their importance in overall health maintenance.

Out of 125 probiotic LAB isolates, only 39 showed evidence of bacteriocin production and antimicrobial activity. This aligns with the findings of Zhang et al. (2013), who found six out of 300 LAB isolates that produced bacteriocins with antagonistic activity against *Listeria monocytogenes*. Similarly, Mulaw et al. (2019) have reported that nine out of 34 potential probiotic LAB isolates produced bacteriocins and exhibited antagonistic activity against several pathogens. These results indicate that traditional Ethiopian fermented foods may harbor probiotics capable of producing bacteriocins. Further research is necessary to uncover the potential health benefits of these probiotic strains.

In this study, we assessed the survival and tolerance of probiotic LAB in the gastrointestinal tract and pancreatic juice simulation. All the 54 isolates displayed survival rates ranging from 30.09% to 90.24%. To be considered as a good probiotic candidate, LAB must withstand barriers like low pH, gastric juice, bile salts, and pancreatin (Pérez Montoro et al., 2018). *P. acidilactici* strains have shown a 68% survival rate in gastric juice at pH 3 as reported by Doğan & Ay (2021) and (Sarkar et al., 2020), indicating their

potential as probiotic LAB strains. Similarly, *Lactococcus lactis* from various sources exhibited potential survival rates in simulated gastric juice at pH 3 (Dobson et al., 2011; Naissinger da Silva et al., 2021). Studies have also highlighted the survivability of certain *Pediococcus* and *Lactococcus* strains in gastric juice at low pH levels, suggesting their probiotic potential for gut health (Gueimonde et al., 2006; S. Wang et al., 2024). However, survival in gastric juice alone does not ensure probiotic efficacy, and further research is necessary to assess the health benefits of these strains.

The gastric juice secretion with a pH of 2.0 is hostile to many pathogens, causing their demise upon entering the gastrointestinal tract (Vizoso Pinto et al., 2006). In our study, all 54 LAB isolates survived simulated gastric juice with rates ranging from 30.07% to 90.24%. This aligns with findings of Blajman et al. (2015) and García-Hernández et al. (2016) who reported the survival rates of 20% to 80%. It's crucial for LAB to withstand gastrointestinal conditions and reach the small intestine alive to provide health benefits, highlighting resistance to gastric and intestinal juice as essential for probiotic selection (Larsen et al., 2018). Our isolates demonstrated potential to survive and tolerate GI tract stimulation and intestinal juice, suggesting their suitability as starter cultures and natural food preservatives.

According to FAO guidelines, probiotic strains with hemolytic activity are discouraged due to safety issues. In this study, all the LAB isolates were tested for hemolytic activity on Columbia blood agar, with none exhibiting α - or β -hemolytic activity, aligning with prior findings (Oyewole et al., 2018; Pisano et al., 2014). This underscores that all of 54 LAB isolates are non-hemolytic, indicating their safety and potential as probiotic candidates. Auto and co-aggregation are key traits in identifying potential probiotic LAB, affecting adhesion to intestinal mucosa and biofilm formation (Krausova et al., 2019). LAB's auto-aggregation varied significantly ($P < 0.05$), from 45.19% to 120.39%. *P. pentosaceus* JULABB01 exhibited the highest auto-aggregation (120.39%), followed by *P. pentosaceus* JULABB16 (102.34%). *L. lactis* JULABB showed the least auto-aggregation (45.19%). Studies by (Mathara et al., 2008) and (Nallala & Jeevaratnam, 2015; Taheri et al., 2009) also found LAB with auto-aggregation above 40% as reported in this study. Auto-aggregation aids biofilm formation, enhancing probiotic adhesion and colonization in the host's intestinal cells.

Co-aggregation prevents enteric pathogen adhesion to intestinal cells. This binding is essential for pathogen omission and immunomodulation (Nallala & Jeevaratnam, 2015). This adhesion of probiotics to the intestinal cell is essential for pathogen omission and immunomodulation (Russell et al., 2011).

The cell surface hydrophobicity was ranged from 54.08 % to 116.20%, with significance variation at $p < 0.05$. Similar to this study Iñiguez-Palomares et al. (2008) reported that large numbers of probiotic LAB showed the cell surface hydrophobicity greater than 40% against xylene. The study on cell surface hydrophobicity is very important, because it helps in studying the colonization and adhesion of probiotic bacteria to epithelial cells in the gastrointestinal tract that leads to the prevention of colonization by pathogens through their interaction (Abushelaibi et al., 2017).

Antibiotic resistance is a significant concern in the food industry due to foodborne diseases and the spread of multidrug-resistant pathogens. While intrinsic resistance in bacteria poses minimal horizontal spread risk, acquired resistance is more problematic. The safety of probiotic LAB strains is often attributed to their possession of acquired resistance genes, which contribute to their Generally Recognized as Safe (GRAS) status. These acquired resistance genes provide probiotic LAB strains with the ability to survive and thrive in various environments, including the gastrointestinal tract, where they exert their beneficial effects. Additionally, these resistance genes help protect probiotic LAB strains from the effects of antibiotics and other antimicrobial agents, further enhancing their safety profile for human consumption. A study showed that many LAB isolates were resistant to kanamycin but sensitive to tetracycline and chloramphenicol, while being both resistant and susceptible to ampicillin and streptomycin. These patterns align with prior research in Ethiopia (Mulaw et al., 2019; Negasi et al., 2017; Tigu et al., 2021), where LAB from fermented foods were resistant to kanamycin yet susceptible to tetracycline, ampicillin, and erythromycin. Similarly, LAB from traditional fermented milk in Spain (Niazi Amraii et al., 2014; Rajoka et al., 2019), showed susceptibility to chloramphenicol, erythromycin, tetracycline, and ampicillin. These consistent antimicrobial profiles highlight LAB as potential assets in food industry and human health.

4.5.CONCLUSION

The lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages exhibit significant probiotic potential and safety characteristics. These LAB strains have demonstrated survival rates in simulated gastrointestinal conditions, indicating their resilience as potential probiotics. Importantly, they do not exhibit hemolytic activity, aligning with safety guidelines for probiotic strains.

Furthermore, these LAB strains show promising features such as auto-aggregation, co-aggregation with pathogens, antimicrobial properties, and strong adhesion to intestinal cells. These characteristics suggest their efficacy in preventing gastrointestinal infections, promoting gut health, and potentially improving food shelf life as natural preservatives and starter cultures.

However, further research is necessary to validate their probiotic effects in vivo, understand their mechanisms of action, assess their full spectrum of antimicrobial resistance genes, and evaluate their potential health impacts comprehensively. Overall, the LAB strains isolated from Ethiopian fermented foods and beverages represent valuable candidates for probiotic use pending further investigation and validation.

5.

ISOLATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCING LACTIC ACID BACTERIA ISOLATED FROM ETHIOPIAN TRADITIONAL FERMENTED FOODs AND BEVERAGES

The finding of this paper is submitted to **BMC Research Notes**, as:

Amenu, D., Bacha, K, (2024). Isolation and Characterization of Bacteriocin Producing *Enterococcus* species from Ethiopian Traditional Fermented Food and Beverages, BMC Research Note (Under Review)

ABSTRACT

Lactic acid bacteria (LAB) produce antimicrobial substances like organic acids, hydrogen peroxides, and bacteriocins that inhibit food-borne pathogens and spoilage microorganisms. Hence, this study aimed to isolate and characterize bacteriocin-producing lactic acid bacteria (LAB) from Ethiopian traditional fermented foods and beverages for their antimicrobial properties. Cell-free supernatants (CFS) were prepared from 18-24-hour LAB cultures grown in MRS broth, and the effect of organic acid and hydrogen peroxide was eliminated by adjusting the pH and adding catalase (5 mg/ml), respectively. The efficacy of bacteriocin was determined using a bacteriocin bioassay at various temperatures, pH, organic solvents, additives, and enzymes. A solvent extraction method was used to concentrate bacteriocin from LAB, and various extraction procedures were developed and tested for successful recovery. A total of 39 putative bacteriocin LAB were screened. Accordingly, all the potent LAB isolates showed strong inhibition against the selected human pathogens. The crude extracts of bacteriocin were deactivated by enzymes like proteinase K, pepsin, trypsin, and lysozyme, confirming their proteinaceous nature. The antimicrobial activity remained stable at 65°C for 30 minutes over a pH range of 2-8, and addition of chemicals did not affect the antibacterial activity of bacteriocin. Chloroform alone and in combination with isopropanol and ethyl acetate provided rapid and efficient bacteriocin recovery from CFS. Optimum bacteriocin production and strong inhibition activity were achieved at 37°C with a pH range of 5.5 to 6.5 and incubated for 16-18 h. Optimal bacteriocin production was examined by supplementing MRS broth with various growth media, with the Modified Brain Heart Infusion being the best media for optimal bacteriocin production. The addition of glycerol and vitamins had no effect on bacteriocin production, but carbon and nitrogen sources significantly impacted bacteriocin production. MRS broth supplemented with 5% lactose and 5-10% yeast extract showed the maximum bacteriocin inhibitory potency. Overall, these findings provide valuable insights into optimizing the production and recovery of bacteriocins from LAB cultures, laying the groundwork for their potential use as natural preservatives in the food industry. Further research and development efforts should focus on scaling up production processes and evaluating the efficacy and safety of bacteriocins in various food applications.

Key words: Antimicrobial activity, bacteriocin producing lactic acid bacteria, bacteriocin optimization, Ethiopian traditional fermented foods and beverage, solvent extraction

5.1. INTRODCUTION

Foodborne diseases (FBDs) are a global concern, necessitating diverse approaches like probiotics for proper intervention strategy (Sivapalasingam et al., 2004). Despite modern preservation methods, FBD rates rise, especially in areas lacking robust food safety systems. Over 75% of the world's population faces FBDs from contaminated foods, urging stricter regulations and consumer awareness (Akhtar et al., 2013). Food safety is crucial by combating pathogens and economic losses (Parada et al., 2007). Effective measures across the food supply chain are essential. Antimicrobial agents are vital, addressing concerns about additives. Natural products like bacteriocin from LAB meet demand for minimally processed foods without synthetic additives (Molognoni et al., 2019). GRAS bacteriocin-producing LAB are explored for bio-preservation in the food industry (O'Sullivan et al., 2002).

The main LAB in traditional fermented foods are *L. lactis*, *P. pentosaceus*, and *E. faecium*. These LAB show potential against foodborne pathogens such as *L. monocytogenes*, *Bacillus cereus*, and methicillin-resistant *S. aureus*. Their preservative effecciny is related to their ability to produce antimicrobial substances like organic acids, hydrogen peroxide, diacetyl, and bacteriocin by offering natural preservation effects (Fugaban et al., 2021). LAB bacteriocins effectively combat food spoilage and pathogens, offering safe preservation. They're especially valuable for their broad antimicrobial spectrum. Research targets robust LAB species like *Lactobacillus*, *Streptococcus*, and *Enterococcus*, each producing unique bacteriocins. Screening and stability assessments are crucial for using these LAB as bio-preservatives, meeting the need for natural safeguards in food production (Rashid et al., 2023).

Isolating, screening, and characterizing bacteriocin-producing LAB is crucial for identifying optimal stability conditions and potential bio-preservative use against foodborne pathogens. Scientists are developing new bio-preservatives using LAB as starter cultures to combat harmful microorganisms in order to meet the demand for natural preservatives. Bacteriocin-producing LAB isolated from fermented products also hold promise for treating drug-resistant infections and preserving food. Studies on lactic acid bacteria from Ethiopian fermented foods/beverages show strong antibacterial activity, suggesting their potential as natural preservatives(Amenu & Bacha, 2024).A recent study (Amenu and Bacha, 2024) evaluated LAB's bacteriocin-producing capacity from Ethiopian traditional fermented foods and beverages by assessing their effectiveness against foodborne pathogens like *S.*

aureus, *S. Typhimurium*, *E. coli*, *P. aeruginosa*, , and *C. albicans*. Thus, the current study was initiated was to characterize bacteriocin producing LAB from Ethiopian traditional fermented foods and beverages to assess their antimicrobial activities.

5.2. MATERIAL AND METHODS

5.2.1. Source and characterization of Bacteriocin-producing LAB

LAB were previously isolated and characterized from Ethiopian traditional fermented foods and beverages in section 3.2.7. The antagonistic activities of LAB isolates s were first investigated using the direct spot-on-lawn method, which was then validated using the agar well diffusion method(Amenu & Bacha, 2024)

5.2.2. Preliminary screening of bacteriocins producers

For bacteriocin detection, LAB culture supernatants were prepared by inoculating a 24-hour-old LAB culture into MRS broth. The cultures were incubated at 32°C for 18, 24, and 36 h without shaking. After centrifugation at 10,000 rpm for 20 minutes, the cell-free supernatants were filter-sterilized (0.22 µm) and stored at 4°C for antagonistic testing against standard strains for further characterization. The agar overlay method involved spotting 20 µl of 48-hour LAB cultures onto MRS agar and incubating them aerobically at 37°C for 24 h(Barboza et al., 2007).Soft agar containing the test bacterial suspension was then poured as an overlay onto the plates. Growth inhibition zones around pathogenic microorganisms with clear zones of inhibition reveals bacteriocin producers (Barboza et al., 2007). In the Agar Well Diffusion method, bacteriocin producers were cultured in MRS broth for 18 h at 37°C, then centrifuged to obtain cell-free supernatants. Adding 100 µl of this supernatant to wells on seeded plates with the selected foodborne pathogens incubated ---temperature for ----h to assess inhibition zones formation around the wells, indicating positive bacteriocin activity (Reinheimer et al., 1990).

5.2.3. Evaluating bacteriocins extraction methods

5.2.3.1.Convectional bacteriocins extraction

To extract bacteriocin, all potential probiotic isoaltes were and CFS was extracted and adjusted to pH 6.5 using a 1 M NaOH solution, and then the culture was stirred using magnetic stirrer at 200 rpm at room temperature for 30 minutes to increase bacteriocin absorption into the producer cells(Wray et al.,

2018). The culture was then heated for 30 minutes at 70°C in 250ml flask. LAB cells were extracted by centrifugation at 10,000 rpm for 20 minutes at 4°C, then washed twice with 5 mM sodium phosphate (pH 6.5) and centrifuged again. The LAB cells were then re-suspended in 50 mL of 100 mM NaCl at pH 2.0 (adjusted with 5% phosphoric acid) and stirred with a magnetic stirrer for 1 hour at 4°C. After centrifuging the cell suspension at 10,000 rpm for 20 minutes at 4°C, the cell-free supernatant was adjusted to pH 6.5 with a 1 M NaOH solution. The bacteriocin extract was filtered through a 0.22 µm pore-size cellulose acetate filter, and the bacteriocins' bioactivity against selected human pathogens was examined (Wray et al., 2018). Standard bacterial pathogens for this experiment were obtained from EPHI (Amenu & Bacha, 2024)

5.2.3.2. Ammonium sulphate precipitation

The cell-free supernatant was saturated with solid ammonium sulfate to 60-80% saturation and stirred (200 rpm) continuously overnight at 4°C. The precipitated protein was then collected in a cooling centrifuge by spinning at 10,000 rpm for 20 minutes at 4°C. Bacteriocins were precipitated by gently adding ammonium sulfate to the supernatant fluid until 40% saturation was attained and stirring (at 200 rpm) for 24 h at 4°C. The precipitate was collected by centrifugation at 4°C and 10,000 rpm for 20 minutes. The particle was resuspended in 10-20 mL of pH 7 Tris buffer (0.1 mol/L). Additional precipitation for 40-60% and 60-80% ammonium sulfate saturation was done using the same culture supernatant fluid. The 0-40% fraction pellet was dissolved in Tris buffer and dried. Bacteriocin activity was measured after re-suspending the dried material in 0.1 mol/L Tris buffer, pH 7.

5.2.4. Bacteriocin extraction using solvents

5.2.4.1. Solvent extraction

MRS broth with 1% LAB culture was incubated at 37°C for 16-18 h. The resulting culture was centrifuged at 10,000 rpm for 20 minutes at 4°C to obtain cell-free supernatant (CFS). This CFS (100 ml) was mixed with various organic solvents (1:1, v/v) such as methanol, petroleum ether, chloroform, isopropanol, chloroform-isopropanol (2:1, v/v), and chloroform-methanol (2:1, v/v) for 20 minutes. Acidified CFS (0.25% acetic acid) was also extracted with chloroform-methanol. Bacteriocin activity was primarily observed in chloroform-containing mixes, forming bactericidal precipitates at the solvent-aqueous interface.

For ethyl acetate extraction, culture broth and an equal volume of ethyl acetate were shaken vigorously in a separating funnel for 10 minutes, yielding an upper organic layer containing bacteriocins. The extract was evaporated, dissolved in methanol (adjusted to pH 6 with 1M NaOH), treated with catalase (5 mg/ml) and filtered through a 0.20 µm pore size membrane. Butanol extraction involved mixing n-butanol (65%) and NaCl (0.85%) with CFS, followed by centrifugation (at 4°C for 20 min) and evaporation of n-butanol. The residue was re-suspended in saline, and the resulting crude extract was tested for bacteriocin activity against specific pathogens (*S.aureus* and *E.coli*). Similarly, petroleum ether was used for extraction, and the final extract was treated and filtered. Chloroform extraction involved agitation, centrifugation, and multiple re-suspensions to obtain a bacteriocin assay against foodborne pathogens.

5.2.5. Physico-chemical characterization of bacteriocins

The extracted crude bacteriocins from potent LAB isolates were characterized for stability under different conditions.

Temperature Stability

The crude bacteriocins were exposed to various temperatures (40°C, 60°C, 80°C, and 100°C for different durations. Autoclaving was also done at 121°C for 15 minutes. Residual antagonistic activity against *Staphylococcus aureus* was assessed using the agar well-diffusion method (Ogunbanwo et al., 2003).

pH Stability

The pH of bacteriocin preparations was adjusted to pH 2.5, 6.5, 8.5, and 12, incubated for 4 h, and then tested against *Staphylococcus aureus* to determine residual activity (Kelleher et al., 2003).

Enzyme Sensitivity

Crude bacteriocin preparations were treated with amylase, lipase, and protease [1mg/ml] and then incubated to evaluate residual activity (Hassan et al., 2020).

Solvent Sensitivity

Bacteriocin sensitivity to different solvent concentrations (methanol, ethanol, acetone, and ethyl acetate) was investigated by incubating samples with and without solvents and assessing residual activity (Chopra et al., 2014).

Surfactant Sensitivity

The sensitivity of bacteriocins to surfactants was tested by incubating samples with and without surfactants and determined residual activity after 12 h. The crude bacteriocins extracted from all the selected LAB isolates with strong inhibition zone was characterized with respect to stability to temperature, pH, and enzymes and others parameters (Amenu & Bacha, 2023).

5.2.6. Optimization for enhanced bacteriocins production

5.2.6.1. Media selection for bacteriocins optimization

Commercially available media such as nutrient broth, tryptone soy broth, brine heart infusion broth, MRS broth, were prepared to optimize the carbon and nitrogen sources in MRS medium to find the potential medium for bacteriocin recovery and optimization. In a 250 ml conical flask, 100 ml of broth was made, 10% inoculum was introduced, and the flask was held at 35°C without agitation for 16 h. The fermented broth was centrifuged and the antibacterial potential of bacteriocins was determined.

5.2.6.2. Effect of carbon sources on bacteriocins production

To test the influence of carbon sources on the development and production of bacteriocin-producing lactic acid bacteria, 1% dextrose, glucose, maltose, fructose, sucrose, starch, and lactose were added to MRS broth and incubated at 37°C for 48 h. Bacteriocin activities and cell biomass were assessed (Abbasiliasi et al., 2017).

5.2.6.3. Effect of nitrogen sources on bacteriocins production

MRS broth was supplemented with 2% nitrogenous compounds (meat extract, yeast extract, tryptone, and peptone) and cultured at 37°C for 48 h to examine the influence of different nitrogen sources on the growth of bacteriocin-producing LAB and bacteriocins yield. Bacteriocin activities and cell biomass production were evaluated (Abbasiliasi et al., 2017).

5.2.6.4.Effect of Incubation Period on Bacteriocins Production

The influence of incubation time on bacteriocin synthesis was investigated. The media was incubated for various lengths of time that included 16 h, 18 h, 24 h, 32 h, and 37 h. Finally, the antibacterial activity of bacteriocins was tested to determine their efficiency.

5.2.7. Data analysis

All experiments were carried out in triplicate. The data is displayed as mean standard deviation. SPSS, soft ware, version 26 was used. A one-way ANOVA was employed to evaluate statistical differences, followed by Tukey's multiple comparison tests at $p < 0.05$ was used to determine whether a difference was significant.

5.3. RESULTS

5.3.1. Evaluating bacteriocins producing potential

This study identified **39** putative bacteriocin producers from the total isolates, with JULABB01 being the most potent (Table 5.1). The average inhibition zone of a crude extract containing bacteriocins inhibited foodborne pathogens with diameters that ranged from 13.00 mm to 35.94 mm. JULABBk02, JULABB35, and JULABB45 showed strong antagonistic effects against *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, and *Candida albicans*. The bacteriocins' proteinaceous nature was confirmed by testing their sensitivity to proteolytic enzymes, revealing their loss of antibacterial activity after proteinase K treatment (Table 5.1).

5.3.2. Antimicrobial activity of bacteriocins crude extract

5.3.2.1.Determination of antimicrobial substance

The study reveals that antimicrobial compounds produced by bacteriocin-producing LAB were inhibited by proteolytic enzymes like pepsin, trypsin, and proteinase K (Table 5.2). The inhibition zone values from suspected bacteriocin-producing LAB showed significant variation at $p < 0.05$, suggesting that the effectiveness of these compounds may depend on the specific type of proteolytic enzyme present. Further research is needed to understand the mechanisms behind this variability to optimize their use in various applications (Table 5.2).

Table 5. 1. Antimicrobial activity of putative bacteriocins producing LAB against food borne pathogens

S/N	LAB isolates	<i>E. coli</i> ATCC®25922	<i>S. aureus</i> ATCC®25923	<i>L.monocytogenes</i> ATCC®7644	<i>P. aeuroginosa</i> ATCC®27853	<i>S. Tphimurium</i> ATCC®13311	<i>C.albicans</i> ATCC®14053
1.	JULABB01	17.75±0.35 ^c	30.29±0.41 ^a	35.94±1.32 ^a	34.13±0.18 ^a	33.49±0.69 ^a	18.50±0.71 ^{de}
2.	JULABB10	19.50±0.71 ^{abc}	15.50±0.71 ^d	15.33±0.46 ^{de}	15.50±0.71 ^{ef}	24.88±0.18 ^b	18.13±0.18 ^{de}
3.	JULABB15	15.00±0.57 ^{cde}	12.00±0.00 ^{efg}	15.33±0.34 ^e	15.33±0.34 ^{ef}	23.34±0.34 ^b	21.34±0.34 ^c
4.	JULABB16	18.25±0.35 ^c	30.00±0.00 ^a	30.13±0.18 ^b	13.25±0.35 ^e	24.82±0.69 ^b	14.50±0.71 ^e
5.	JULABB30	23.75±0.35 ^b	17.50±0.71 ^{def}	20.07±0.10 ^{de}	17.13±0.18 ^d	18.82±0.69 ^{bcd}	15.38±0.53 ^{ef}
6.	JULABB35	23.88±0.18 ^b	18.40±0.57 ^{de}	17.95±0.07 ^d	18.50±0.71 ^d	19.88±0.18 ^{bc}	15.13±0.18 ^{ef}
7.	JULABB45	14.75±0.35 ^{cde}	18.00±0.00 ^{de}	23.75±0.35 ^{cd}	19.13±0.18 ^{de}	20.13±0.18 ^{bc}	30.26±0.37 ^a
8.	JULABB56	23.75±0.35 ^b	14.75±0.35 ^d	20.29±0.41 ^{de}	12.13±0.18 ^{fg}	16.16±0.69 ^c	15.38±0.53 ^{ef}
9.	JULABBK02	28.25±0.35 ^a	15.00±0.00 ^d	18.25±0.35 ^d	22.13±0.18 ^c	20.88±0.18 ^{bc}	18.26±0.37 ^{de}
10.	JULABBK21	19.75±0.35 ^{abc}	20.75±1.06 ^c	20.29±0.41 ^{de}	17.13±0.18 ^d	19.49±0.69 ^{bc}	25.38±0.53 ^{cd}
11.	JULABBK33	15.67±0.66 ^{cde}	13.00±0.00 ^e	14.67±0.34 ^e	19.33±0.34 ^{bd}	16.67±0.34 ^c	19.67±0.34 ^{de}
12.	JULABBK39	19.67±0.82 ^{abc}	17.00±0.00 ^{def}	25.33±0.33 ^c	11.33±0.33 ^{fg}	17.34±0.07 ^{bcd}	23.33±0.33 ^c
13.	JULABBK40	20.02±0.58 ^{ab}	18.50±0.58 ^{de}	23.050±0.87	15.26±0.89 ^{ef}	17.00±0.05	22.30±0.36
14.	JULABBR03	18.60±0.85 ^c	20.50±0.71 ^c	17.15±0.44 ^d	17.13±0.18 ^d	18.13±0.18 ^{bcd}	19.38±0.53 ^{de}
15.	JULABBR12	21.33±1.76 ^{ab}	15.00±0.00 ^d	16.33±0.66 ^{de}	16.33±0.33 ^{ef}	16.67±0.33 ^c	17.33±0.33 ^{de}
16.	JULABBR33	13.50±0.71 ^d	16.00±1.41 ^{def}	35.31±0.44 ^a	20.50±0.71 ^c	18.88±0.18 ^{bcd}	16.13±0.18 ^d
17.	JULABBr39	21.75±0.62 ^{abc}	17.25±0.25 ^{def}	20.0±0.28 ^{de}	14.25±0.25 ^e	16.25±0.47 ^c	13.75±1.43 ^e
18.	JULABBR40	17.75±0.35 ^c	17.50±0.71 ^{def}	28.32±0.45 ^{bc}	16.13±0.18 ^{ef}	15.13±0.18 ^c	24.38±0.53 ^{cd}
19.	JULABE01	17.75±0.35 ^c	20.50±0.71 ^c	23.25±0.35 ^{cd}	17.13±0.18 ^d	20.13±0.18 ^{bc}	14.26±0.37 ^e
20.	JULABE05	17.75±0.35 ^c	17.50±0.71 ^{def}	20.26±0.37 ^{de}	19.13±0.18 ^{de}	20.13±0.18 ^{bc}	20.38±0.53 ^c
21.	JULABE07	17.25±0.35 ^c	20.50±0.71 ^c	18.43±0.60 ^d	19.13±0.18 ^{de}	18.13±0.18 ^{bcd}	16.26±0.37 ^d
22.	JULABE12	19.93±0.11 ^{abc}	14.50±0.71 ^d	15.18±0.25 ^e	20.50±0.71 ^c	20.88±0.18 ^{bc}	18.13±0.18 ^{de}
23.	JULABE23	18.50±0.21 ^c	22.20±0.50 ^c	18.50±0.41 ^d	18.50±0.33 ^d	22.50±0.33 ^{bc}	18.36±0.36 ^{de}
24.	JULABE31	19.20±0.35	22.50±0.25 ^{abcd}	17.36±0.41 ^d	19.60±0.69 ^{de}	21.50±0.36 ^{bd}	23.00±0.00 ^c
25.	JULABE33	20.12±0.36 ^{ab}	19.25±0.58 ^{de}	18.63±0.50 ^d	20.36±0.34 ^{abc}	19.50±0.25 ^{bcd}	19.38±0.76 ^{de}
26.	JULABE35	20.00±0.00 ^{abc}	13.00±0.0 ^e	12.00±0.00 ^f	17.00±0.00 ^{cd}	13.00±0.00 ^d	23.00±0.00 ^c
27.	JULABE36	22.50±1.76 ^b	17.50±0.25 ^{def}	15.69±0.25 ^e	18.90±0.33 ^d	20.58±0.25 ^{bc}	18.26±0.37 ^{de}
28.	JULABK07	18.75±1.06 ^c	14.50±0.71 ^d	18.38±0.53 ^d	22.13±0.18 ^c	19.88±0.18 ^{bc}	20.26±0.37 ^c

S/N	LAB isolates	<i>E. coli</i> ATCC®25922	<i>S. aureus</i> ATCC®25923	<i>L.monocytogenes</i> ATCC®7644	<i>P. aeruginosa</i> ATCC®27853	<i>S. Typhimurium</i> ATCC®13311	<i>C.albicans</i> ATCC®14053
29.	JULABK30	24.50±0.71 ^b	20.50±0.71 ^c	25.00±0.00 ^c	30.25±0.35 ^a	25.83±0.71 ^b	19.50±0.71 ^{de}
30.	JULABK37	20.87±0.18 ^{abc}	14.75±0.35 ^d	14.13±0.18 ^e	18.50±0.71 ^d	19.88±0.18 ^{bc}	18.13±0.18 ^{de}
31.	JULABK46	18.50±0.71 ^c	18.00±0.00 ^{de}	28.25±0.35 ^{bc}	13.25±0.35 ^e	20.17±0.71 ^{bc}	21.50±0.71 ^c
32.	JULABK49	16.00±0.57 ^{cd}	15.00±0.00 ^d	17.33±0.33 ^d	17.33±0.33 ^d	16.67±0.34 ^c	15.33±0.34 ^{ef}
33.	JULABK50	20.87±0.18 ^{abc}	20.25±0.35 ^{abc}	20.20±0.35 ^{abc}	19.26±0.37 ^{bd}	20.25±0.35 ^{abc}	20.87±0.18 ^{abc}
34.	JULABS01	17.93±0.11 ^c	15.50±0.71 ^d	20.13±0.18 ^{de}	14.13±0.18 ^e	18.13±0.18 ^{bcd}	30.26±0.37 ^a
35.	JULABS12	18.25±0.35 ^c	19.75±0.35 ^c	14.75±0.35 ^e	18.50±0.71 ^d	19.88±0.18 ^{bc}	18.13±0.18 ^{de}
36.	JULABS13	18.93±1.31 ^{abc}	17.50±0.71 ^{def}	17.75±0.35 ^d	19.25±0.35 ^{bd}	18.83±0.71 ^{bcd}	16.50±0.71 ^d
37.	JULABS31	20.50±0.71 ^{abc}	17.50±0.71 ^{def}	20.26±0.37 ^{de}	21.25±0.35 ^c	20.17±0.71 ^{bc}	15.50±0.71 ^{ef}
38.	JULABS36	20.25±0.35 ^{abc}	17.50±0.71 ^{def}	25.25±0.35 ^c	16.13±0.18 ^{ef}	19.13±0.18 ^{bcd}	19.26±0.37 ^{de}
39.	JULABS43	20.25±0.35 ^{abc}	19.26±0.37 ^{de}	17.33±0.33 ^d	20.87±0.18 ^{abc}	19.26±0.37 ^{de}	20.25±0.35 ^{abc}
	Gentamycin(30 mg/ml)	18.75±1.06 ^c	17.50±0.71 ^{def}	18.38±0.53 ^d	14.13±0.18 ^e	20.13±0.18 ^{bc}	25.26±0.37 ^{cd}

Note: JU= Jimma University, B=*Bulla*, S=Cabbage-shamita, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*. Mean values denoted by the same letter within a column are not significantly different at p< 0.05.

Table 5. 2. Antimicrobial activity of crude bacteriocins produced by lactic acid bacteria

S/N	LAB isolates	Diameter of inhibition zone (mm)		
		<i>E. coli</i> ATCC® 25922	<i>S. aureus</i> ATCC® 25923	<i>L. monocytogenes</i> ATCC® 7644
1)	JULABB01	24.5±0.18 ^{ab}	28.2±0.69 ^b	33.52±0.71 ^b
2)	JULABB10	24.00±0.07 ^{ab}	17.25±0.18 ^e	18.00±0.18 ^{fg}
3)	JULABB15	24.00±0.13 ^{ab}	30.58±0.69 ^a	30.13±0.7 ^c
4)	JULABB16	24.00±0.33 ^{ab}	18.47±0.34 ^{de}	16.33±0.34 ^g
5)	JULABB30	20.52±0.01 ^{bc}	25.00±0.69 ^{cde}	21.00±0.53 ^{efg}
6)	JULABB35	20.5±0.18 ^{bc}	18.4±0.18 ^{de}	18.22±0.18 ^{fg}
7)	JULABB45	20.5±0.18 ^{bc}	16.00±0.18 ^e	24.5±0.37 ^{dfg}
8)	JULABB56	20.5±0.34 ^{bc}	18.78±0.69 ^{de}	25.00±0.53 ^{df}
9)	JULABBK02	20.25±0.33 ^{bc}	15.88±0.18 ^{ef}	19.22±0.37 ^f
10)	JULABBK21	20.14±0.1 ^{bc}	21.5±0.69 ^d	18.00±0.53 ^{fg}
11)	JULABBK33	20.14±0.34 ^{bc}	16.78±0.07 ^e	18.00±0.34 ^{fg}
12)	JULABBK39	20.14±0.33 ^{bc}	18.57±0.07 ^{de}	27.00±0.33 ^{df}
13)	JULABBK40	20.22±0.18 ^{bc}	24.00±0.71 ^{cde}	24.00±0.71 ^e
14)	JULABBR03	20.00±0.18 ^{bc}	22.5±0.18 ^d	15.00±0.53 ^{gh}
15)	JULABBR12	19.5±0.33 ^{bca}	19.7±0.33 ^{de}	18.00±0.33 ^{fg}
16)	JULABBR33	19.25±0.25 ^c	18.5±0.47 ^{de}	22.00±1.43 ^{ef}
17)	JULABBr39	19.00±0.71 ^c	20.22±0.18 ^d	36.00±0.18 ^a
18)	JULABBR40	18.93±0.18 ^{cd}	18±0.18 ^{de}	27.00±0.53 ^d
19)	JULABE01	18.75±0.18 ^{cd}	20.89±0.18 ^d	24.00±0.37 ^e
20)	JULABE05	18.75±0.18 ^{cd}	20.14±0.01 ^d	21.00±0.53 ^{efg}
21)	JULABE07	18.5±0.18 ^{cd}	22.55±0.18 ^d	18.00±0.37 ^{fg}
22)	JULABE12	24.00±0.71 ^{ab}	16.78±0.35 ^e	18.00±0.33 ^{fg}
23)	JULABE23	25.25±0.37 ^a	16.00±0.33 ^e	22.00±1.43 ^{ef}
24)	JULABE31	18.55±0.00 ^{cd}	20.14±0.34 ^d	24.11±0.37 ^e
25)	JULABE33	18.5±0.00 ^{cd}	18.55±0.00 ^{de}	17.00±0.00 ^{fgh}
26)	JULABE35	22.55±0.18 ^b	20.14±0.1 ^d	15.69±0.35 ^{gh}

27)	JULABE36	18.5±0.71 ^{cd}	16.77±0.18 ^e	16.36±0.18 ^g
28)	JULABK07	18.25±0.18 ^{cd}	14.5±0.18 ^f	20.12±0.37 ^f
29)	JULABK30	17.93±0.35 ^{cde}	20.55±0.71 ^d	25.00±0.71 ^{df}
30)	JULABK37	17.58±0.71 ^{cde}	14.00±0.18 ^f	18.00±0.18 ^{fg}
31)	JULABK46	16.78±0.35 ^{cde}	21.00±0.71 ^d	30.00±0.07 ^c
32)	JULABK49	16.00±0.33 ^{cde}	16.00±0.34 ^e	18.22±0.34 ^{fg}
33)	JULABK50	16.77±0.18 ^d	25.25±0.37 ^{cde}	16.00±0.18 ^g
34)	JULABS01	16.00±0.18 ^{cde}	16.00±0.18 ^e	24.11±0.37 ^e
35)	JULABS12	15.75±0.71 ^d	20.55±0.18 ^d	16.00±0.18 ^g
36)	JULABS13	15.69±0.35 ^{cdf}	17±0.07 ^e	16.00±0.71 ^g
37)	JULABS31	14.75±0.35 ^d	17.055±0.71 ^e	24.00±0.71 ^e
38)	JULABS36	14.5±0.18 ^d	17.5±0.18 ^e	25.25±0.37 ^{df}
39)	JULABS43	18±0.18 ^{cd}	25.25±0.37 ^{cde}	18±0.18 ^{fg}

Note: JU= Jimma University, B=*Bulla*, S=Cabbage *Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*. Mean values denoted by the same letter within a column are not significantly different at $p < 0.05$.

5.3.2.2.Preparation of bacteriocin crude extract

The crude bacteriocin extract, precipitated by ammonium sulfate, showed an antibacterial activity that increased with concentrations of ammonium sulfate. Further purification and characterization may be necessary to fully understand its properties and potential applications (Table 5.3).

5.3.2.3.Preparation of bacteriocin crude extract

The inhibitory zone of the ammonium sulfate precipitated crude bacteriocin extract had a diameter of less than 18 mm (Table 5.3). The diameter of the inhibitory zone increased when an 80% ammonium sulfate and pH 7-phosphate buffer solution was reconstituted. This suggests that the bacteriocin may be more stable at higher concentrations of ammonium sulfate. Further purification and characterization of the bacteriocin may be necessary to fully understand its properties and potential applications.

Table 5. 3. Antimicrobial activity of bacteriocin LAB (ammonium sulfate extraction)

S/N	LAB isolates	<i>E. coli</i>				<i>S. aureus</i>				<i>L. monocytogenes</i>			
		<i>ATCC® 25922</i>				<i>ATCC® 25923</i>				<i>ATCC® 7644</i>			
		40%	50%	60%	80%	40%	50%	60%	80%	40%	50%	60%	80%
1)	JULABB01	—	—	*	*****	—	—	*	*****	—	—	*	*****
2)	JULABB10	—	—	*	*****	—	—	*	*****	—	—	*	*****
3)	JULABB15	—	—	*	*****	—	—	*	*****	—	—	*	*****
4)	JULABB16	—	—	*	*****	—	—	*	*****	—	—	*	*****
5)	JULABB30	—	—	*	*****	—	—	*	*****	—	—	*	*****
6)	JULABB35	—	—	*	*****	—	—	*	*****	—	—	*	*****
7)	JULABB45	—	—	*	*****	—	—	*	*****	—	—	*	*****
8)	JULABB56	—	—	*	*****	—	—	*	*****	—	—	*	*****
9)	JULABBK02	—	—	*	*****	—	—	*	*****	—	—	*	*****
10)	JULABBK21	—	—	*	*****	—	—	*	*****	—	—	*	*****
11)	JULABBK33	—	—	*	*****	—	—	*	*****	—	—	*	*****
12)	JULABBK39	—	—	*	*****	—	—	*	*****	—	—	*	*****
13)	JULABBK40	—	—	*	*****	—	—	*	*****	—	—	*	*****
14)	JULABBR03	—	—	*	*****	—	—	*	*****	—	—	*	*****
15)	JULABBR12	—	—	*	*****	—	—	*	*****	—	—	*	*****
16)	JULABBR33	—	—	*	*****	—	—	*	*****	—	—	*	*****
17)	JULABBr39	—	—	*	*****	—	—	*	*****	—	—	*	*****
18)	JULABBR40	—	—	*	*****	—	—	*	*****	—	—	*	*****
19)	JULABE01	—	—	*	*****	—	—	*	*****	—	—	*	*****
20)	JULABE05	—	—	*	*****	—	—	*	*****	—	—	*	*****
21)	JULABE07	—	—	*	*****	—	—	*	*****	—	—	*	*****
22)	JULABE12	—	—	*	*****	—	—	*	*****	—	—	*	*****
23)	JULABE23	—	—	*	*****	—	—	*	*****	—	—	*	*****
24)	JULABE31	—	—	*	*****	—	—	*	*****	—	—	*	*****
25)	JULABE33	—	—	*	*****	—	—	*	*****	—	—	*	*****
26)	JULABE35	—	—	*	*****	—	—	*	*****	—	—	*	*****
27)	JULABE36	—	—	*	*****	—	—	*	*****	—	—	*	*****
28)	JULABK07	—	—	*	*****	—	—	*	*****	—	—	*	*****
29)	JULABK30	—	—	*	*****	—	—	*	*****	—	—	*	*****
30)	JULABK37	—	—	*	*****	—	—	*	*****	—	—	*	*****
31)	JULABK46	—	—	*	*****	—	—	*	*****	—	—	*	*****
32)	JULABK49	—	—	*	*****	—	—	*	*****	—	—	*	*****

S/N	LAB isolates	<i>E. coli</i>				<i>S. aureus</i>				<i>L. monocytogenes</i>			
		<i>ATCC® 25922</i>				<i>ATCC® 25923</i>				<i>ATCC® 7644</i>			
		40%	50%	60%	80%	40%	50%	60%	80%	40%	50%	60%	80%
33)	JULABK50	—	—	*	*****	—	—	*	*****	—	—	*	*****
34)	JULABS01	—	—	*	*****	—	—	*	*****	—	—	*	*****
35)	JULABS12	—	—	*	*****	—	—	*	*****	—	—	*	*****
36)	JULABS13	—	—	*	*****	—	—	*	*****	—	—	*	*****
37)	JULABS31	—	—	*	*****	—	—	*	*****	—	—	*	*****
38)	JULABS36	—	—	*	*****	—	—	*	*****	—	—	*	*****
39)	JULABS43	—	—	*	*****	—	—	*	*****	—	—	*	*****

Note: - no inhibition zone, *=<10 mm of diameter of inhibition zone, -**=10-20 diameter of inhibition zone, ***=20-30 diameter of inhibition zone, ***** =>30 mm. Note: JU= Jimma University, B=*Bulla*, S=Cabbage *Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*. Mean values denoted by the same letter within a column are not significantly different at p< 0.05.

The study analyzed bacteriocin extraction using various solvents, with zones of inhibition exceeding 18 mm (Table 5.4). Among the different solvents that utilized to concentrate and recover bacteriocin, three solvents, chloroform, Iso-propyl and Ethyl acetates showed the best potential to recovery bacteriocins like antimicrobial peptides (Table 5.4) The crude bacteriocin extract from rehydrating solvents in phosphate buffer solution showed the best antibacterial activity, with an inhibition zone diameter of over 28 mm. The extract from chloroform, isopropanol, and ethyl acetate extraction had higher antibacterial activity.

Table 5. 4. Antimicrobial activity of crude bacteriocin extracted with different organic solvents

S/N	LAB isolates	Chloroform	Iso-propaly	Ethyl Acetate	Petroleum ether	Methanol	Butanol	Ethanol
1.	JULABB01	****	***	***	**	**	*	*
2.	JULABB10	****	***	***	**	**	*	*
3.	JULABB15	****	***	***	**	**	*	*
4.	JULABB16	****	****	***	****	**	*	*
5.	JULABB30	****	****	***	**	*	*	*
6.	JULABB35	****	****	***	**	**	*	*
7.	JULABB45	****	***	**	**	*	*	—
8.	JULABB56	****	***	**	**	*	*	—
9.	JULABBK02	****	****	***	**	**	*	*
10.	JULABBK21	****	***	***	****	*	*	—
11.	JULABBK33	****	***	**	**	*	*	*
12.	JULABBK39	****	***	**	**	*	*	*
13.	JULABBK40	****	***	**	**	*	*	*
14.	JULABBR03	****	****	**	**	**	*	*
15.	JULABBR12	****	***	***	**	*	*	*
16.	JULABBR33	****	***	***	**	**	*	*
17.	JULABBr39	****	***	***	**	*	*	*
18.	JULABBR40	****	***	***	**	*	*	*
19.	JULABE01	****	****	***	**	**	*	*
20.	JULABE05	****	***	***	**	*	*	—
21.	JULABE07	****	***	***	**	*	*	—
22.	JULABE12	****	***	***	**	*	*	—
23.	JULABE23	****	***	***	**	*	*	—
24.	JULABE31	****	***	***	**	*	*	*
25.	JULABE33	****	***	***	**	**	*	*
26.	JULABE35	****	***	***	**	*	*	*
27.	JULABE36	****	***	**	**	*	*	*
28.	JULABK07	****	***	***	**	*	*	*
29.	JULABK30	****	***	**	**	*	*	*
30.	JULABK37	****	***	***	****	*	*	*
31.	JULABK46	****	****	**	****	**	*	*
32.	JULABK49	****	****	***	****	**	*	*
33.	JULABK50	****	***	***	**	**	*	*
34.	JULABS01	****	***	***	**	*	*	—
35.	JULABS12	****	***	**	**	*	*	—
36.	JULABS13	****	***	**	**	*	*	—
37.	JULABS31	****	***	**	**	*	*	—
38.	JULABS36	****	***	***	**	**	*	*
39.	JULABS43	****	***	***	**	**	*	*

Note: -= no inhibition zone, *=<10 mm diameter, **+10-18 mm diameter, ***19-28 mm diameter, ****=>28 mm diameter Note: JU= Jimma University, B=Bulla, S=Cabbage *Shamita*, Bk=Bukuri, Br= *Borde*, E=Ergo.

5.3.2.4.Characterization of the partially purified supernatants

5.3.2.4.1. Effects of Temperature

Bacteriocin activity was significantly influenced by temperature, with decreased activity at higher temperature values (Table 5.5). Bacteriocins from JULABB01, JULABB16, JULABB35, JULABB39, JULABB33, JULABBk39, and JULABS36 remained active over wide range of temperatures (45-100°C), indicating their heat-stable nature (Table 5.5). However, no action was observed in autoclaved supernatant after 10 minutes.

5.3.2.4.2. Effect of enzymes

Table 5.6 demonstrates the effects of introducing supernatants from each isolate to the test enzymes that included proteinase-k, pepsin, trypsin, and -amylase before using them to limit the growth of some selected pathogens (*E. coli*, *S. aureus*, and *L. monocytogenes*). It can be seen that no inhibition was produced by any of the cell-free supernatants in the presence of protein-digesting enzymes, indicating that the inhibitory substance was completely digested in the supernatants and confirming its proteinaceous nature. Nevertheless, no reduction in activity was observed in the presence of carbohydrate-degrading amylase.

5.3.2.4.3. Effect of pH on the inhibitory activity

The effects of varying the pH of the supernatants on their inhibitory effectiveness against the pathogenic bacterium are shown in Table 5.6. After treatment and pH adjustment, the supernatant obtained from bacteriocin-producing LAB had the highest inhibitory activity (inhibition zone diameter) at pH 5.0, but decreased slightly at pH 7.0 and pH 3.0. When pH further increased to 9.0, the activity dropped to 41% from pH 5.0.

Table 5. 5. Effects of enzymes on the potency of antimicrobial activity of bacteriocins produced by LAB isolates from Ethiopian traditional fermented foods and beverages

S/N	LAB isolates	Pepsin (5 mg/1ml)	Trypsin (5 mg/1ml)	Proteinase K (5 mg/1ml)	Amylase(x/1 ml)	Enzyme-free cell supernatants
1)	JULABB01	-	-	-	+	+
2)	JULABB10	-	-	-	+	+
3)	JULABB15	-	-	-	+	+
4)	JULABB16	-	-	-	+	+
5)	JULABB30	-	-	-	+	+
6)	JULABB35	-	-	-	+	+
7)	JULABB45	-	-	-	+	+
8)	JULABB56	-	-	-	+	+
9)	JULABBK02	-	-	-	+	+
10)	JULABBK21	-	-	-	+	+
11)	JULABBK33	-	-	-	+	+
12)	JULABBK39	-	-	-	+	+
13)	JULABBK40	-	-	-	+	+
14)	JULABBR03	-	-	-	+	+
15)	JULABBR12	-	-	-	+	+
16)	JULABBR33	-	-	-	+	+
17)	JULABBr39	-	-	-	+	+
18)	JULABBR40	-	-	-	+	+
19)	JULABE01	-	-	-	+	+
20)	JULABE05	-	-	-	+	+
21)	JULABE07	-	-	-	+	+
22)	JULABE12	-	-	-	+	+
23)	JULABE23	-	-	-	+	+
24)	JULABE31	-	-	-	+	+
25)	JULABE33	-	-	-	+	+
26)	JULABE35	-	-	-	+	+
27)	JULABE36	-	-	-	+	+
28)	JULABK07	-	-	-	+	+
29)	JULABK30	-	-	-	+	+
30)	JULABK37	-	-	-	+	+
31)	JULABK46	-	-	-	+	+
32)	JULABK49	-	-	-	+	+
33)	JULABK50	-	-	-	+	+
34)	JULABS01	-	-	-	+	+
35)	JULABS12	-	-	-	+	+
36)	JULABS13	-	-	-	+	+
37)	JULABS31	-	-	-	+	+
38)	JULABS36	-	-	-	+	+
39)	JULABS43	-	-	-	+	+

Note: -= no inhibition zone, +=<10 mm diameter, ++10-18 mm diameter, +++19-28 mm diameter, ++++=>28 mm diameter
Note: JU= Jimma University, B=*Bulla*, S=*Cabbage Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*.

Table 5. 6. Effects of temperatures, pH, solvents and additives of the antimicrobial activity of bacteriocins

S/N	LAB isolates	Temperature					pH				Solvents			Additives		surfactant
		40°C	60°C	80°C	100°C	120°C	2	5	7	10	Butanol (10%(v/v))	Ethanol (10%(v/v))	Methanol (10%(v/v))	Potassium chloride	Sodium citrate	
1)	JULABB01	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
2)	JULABB10	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
3)	JULABB15	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
4)	JULABB16	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
5)	JULABB30	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
6)	JULABB35	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
7)	JULABB45	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
8)	JULABB56	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
9)	JULABBK02	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
10)	JULABBK21	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
11)	JULABBK33	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
12)	JULABBK39	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
13)	JULABBK40	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
14)	JULABBR03	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
15)	JULABBR12	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
16)	JULABBR33	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
17)	JULABBr39	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
18)	JULABBR40	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
19)	JULABE01	+++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
20)	JULABE05	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
21)	JULABE07	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
22)	JULABE12	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
23)	JULABE23	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++

S/N	LAB isolates	Temperature					pH				Solvents			Additives		surfactant
		40°C	60°C	80°C	100°C	120°C	2	5	7	10	Butanol (10%(v/v))	Ethanol (10%(v/v))	Methanol (10%(v/v))	Potassium chloride	Sodium citrate	
24)	JULABE31	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
25)	JULABE33	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
26)	JULABE35	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
27)	JULABE36	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
28)	JULABK07	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
29)	JULABK30	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
30)	JULABK37	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
31)	JULABK46	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
32)	JULABK49	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
33)	JULABK50	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
34)	JULABS01	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
35)	JULABS12	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
36)	JULABS13	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
37)	JULABS31	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
38)	JULABS36	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++
39)	JULABS43	++	++	+	–	–	–	++	++	–	++	++	++	++	++	++

Note: -= no inhibition zone, +=<10 mm diameter, ++10-18 mm diameter, +++19-28 mm diameter, ++++=>28 mm diameter. JU= Jimma University, B=*Bulla*, S=Cabbage *Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*.

5.3.3. Bacteriocins production

Although the production of bacteriocin began in the late log phase and early stationary phase, the concentration of bacteriocin fell during the stationary phase. This could be the result of on-produced cells' incomplete adsorption or breakdown. In reference to the physico-chemical characteristics of bacteriocins, they exhibit a broad temperature and pH tolerance. However, bacteriocins became inactive at pH levels of 2 and 10. Most of the organic solvents and surfactants did not reduce the activity of bacteriocins.

5.3.3.1. Optimization of bacteriocin production produced by lactic acid bacteria

It was assessed how the parameters for increased bacteriocin production were optimized. After adjusting the pH from 5.5 to 6.00, the greatest bacteriocin synthesis with possible antibacterial action was noted at 32°C and an incubation period of 18–24 h (Table 5.7).

5.3.3.2. Effects of carbon and nitrogen sources on bacteriocin production

The study evaluated the effects of carbon and nitrogen sources on bacteriocin production using MRS broth supplemented with 1.0% of different carbon and nitrogen sources (Table 5.7). The results showed that glucose, sucrose, and lactose had the highest bacteriocin activity, with lactose producing more bacteriocins. In addition, the highest bacteriocin production was also observed in MRS broth supplemented with 1.00% yeast extract, suggesting yeast as a sufficient nitrogen source for optimal bacteriocin production (Table 5.7). These findings highlight the importance of selecting appropriate carbon and nitrogen sources for maximizing bacteriocins production in MRS broth. Further research could explore the potential synergistic effects of combining different carbon and nitrogen sources on bacteriocin activity.

Table 5. 7. Effects of different carbon and nitrogen sources for the enhanced bacteriocin production

S/N	LAB isolates	Bacteriocins activity								
		Carbon sources						Nitrogen sources		
		Glucose	Dextrose	Sucrose	Starch	Maltose	Lactose	Yeast Extract	Meat Extract	Peptone Tryptone
1)	JULABB01	+	+	+	+	+	++	++	+	+
2)	JULABB10	+	+	+	+	+	++	++	+	+
3)	JULABB15	+	+	+	+	+	++	++	+	+
4)	JULABB16	+	+	+	+	+	++	++	+	+
5)	JULABB30	+	+	+	+	+	++	++	+	+
6)	JULABB35	+	+	+	+	+	++	++	+	+
7)	JULABB45	+	+	+	+	+	++	++	+	+
8)	JULABB56	+	+	+	+	+	++	++	+	+
9)	JULABBK02	+	+	+	—	—	++	++	+	—
10)	JULABBK21	+	+	+	—	—	++	++	+	—
11)	JULABBK33	+	+	—	+	+	++	++	+	+
12)	JULABBK39	+	+	—	+	+	++	++	+	+
13)	JULABBK40	+	+	—	+	+	++	++	+	+
14)	JULABBR03	+	+	—	+	+	++	++	+	+
15)	JULABBR12	+	+	—	+	+	++	++	+	+
16)	JULABBR33	+	+	+	+	+	++	++	+	+
17)	JULABBr39	+	+	+	+	+	++	++	+	+
18)	JULABBR40	+	+	+	+	+	++	++	+	+
19)	JULABE01	+	+	+	+	+	++	++	+	+
20)	JULABE05	+	+	+	+	+	++	++	+	+
21)	JULABE07	+	+	+	+	+	++	++	+	+
22)	JULABE12	+	+	+	+	+	++	++	+	+
23)	JULABE23	+	+	—	+	+	++	++	+	+
24)	JULABE31	+	+	—	+	+	++	++	+	+
25)	JULABE33	+	+	—	+	+	++	++	+	+
26)	JULABE35	+	+	+	—	—	++	++	+	—
27)	JULABE36	+	+	+	—	—	++	++	+	—
28)	JULABK07	+	+	+	—	—	++	++	+	—

S/N	LAB isolates	Bacteriocins activity		Carbon sources					Nitrogen sources		
		Glucose	Dextrose	Sucrose	Starch	Maltose	Lactose	Yeast Extract	Meat Extract	Peptone	Tryptone
29)	JULABK30	+	+	+	+	+	++	++	+	+	+
30)	JULABK37	+	+	+	+	+	++	++	+	+	+
31)	JULABK46	+	+	+	+	+	++	++	+	+	+
32)	JULABK49	+	+	+	+	+	++	++	+	+	+
33)	JULABK50	+	+	+	+	+	++	++	+	+	+
34)	JULABS01	+	+	+	+	+	++	++	+	+	+
35)	JULABS12	+	+	+	+	+	++	++	+	+	+
36)	JULABS13	+	+	+	+	+	++	++	+	+	+
37)	JULABS31	+	+	+	+	+	++	++	+	+	+
38)	JULABS36	+	+	+	+	+	++	++	+	+	+
39)	JULABS43	+	+	+	+	+	++	++	+	+	+

Note. -=no bacteriocin activity, += low/moderate bacteriocin activity, ++= high bacteriocin activity. JU= Jimma University, B=*Bulla*, S=Cabbage *Shamita*, Bk=*Bukuri*, Br= *Borde*, E=*Ergo*.

In this study, clearly to see the effects of carbon sources on bacteriocin production, *E. faecium* strains were selected and the effect of the carbon source on the antimicrobial activity of bacteriocin producing *E. faecium* was determined using MRS medium supplemented with 2% of different carbon sources (glucose, lactose, fructose, Maltose, and sucrose). The strongest antimicrobial activity of bacteriocin producing *E. faecium* was observed in the MRS medium supplemented with glucose, sucrose and maltose after, their inhibitory activity tested against *L. monocytogens* (Table 5.8).

Table 5. 8. Antimicrobial activity of bacteriocin producing *Enterococcus faecium* after MRS broth supplemented with different carbon sources, against *Listeria monocytogens*

<i>Enterococcus faecium</i>	Glucose	Sucrose	Maltose	Fructose	Lactose	MRS broth
JULABBR39	31.33±0.41 ^a	29.67±1.70 ^a	25.00±0.82 ^{ab}	18.00±0.82 ^c	16.00±0.82 ^b	15.67±1.70 ^{abc}
JULABBR40	30.67±0.47 ^{ab}	28.00±0.82 ^a	27.33±0.47 ^a	28.00±0.00 ^a	17.33±0.94 ^a	17.00±0.82 ^a
JULABE23	29.00±0.81 ^{ab}	25.00±0.83 ^{ab}	25.67±0.47 ^{ab}	25.00±0.85 ^b	15.00±0.82 ^b	13.67±2.36 ^{bc}
JULABE36	25.00±0.82 ^b	22.00±0.85 ^{abc}	21.00±0.82 ^c	18.00±0.00 ^c	13.00±1.41 ^{bc}	10.67±0.94 ^d
JULABS36	21.00±0.82 ^{bc}	18.90±0.97 ^b	15.00±0.74 ^d	13.00±0.82 ^{de}	15.33±1.89 ^b	16.33±1.25 ^{ab}
JULABS43	17.50±0.40 ^c	18.33±0.48 ^b	20.33±0.52 ^c	14.83±0.24 ^d	12.33±0.47 ^{bd}	15.00±0.82 ^{abc}

Note: Mean values denoted by the same letter within a column are not significantly different at $p < 0.05$, JU= Jimma University, B=Bulla, S=Cabbage Shamita, Bk=Bukuri, Br= Borde, E=Ergo.

Similarly, the effects of nitrogen sources on the antimicrobial activity of *E. faecium* was investigated, hence the MRS broth supplemented with yeast and meat extract produced potential antagonistic effects against *L. monocytogenes* followed by tyryptone, while MRS broth supplemented with peptone produced the least inhibition results (Figure 5.1).

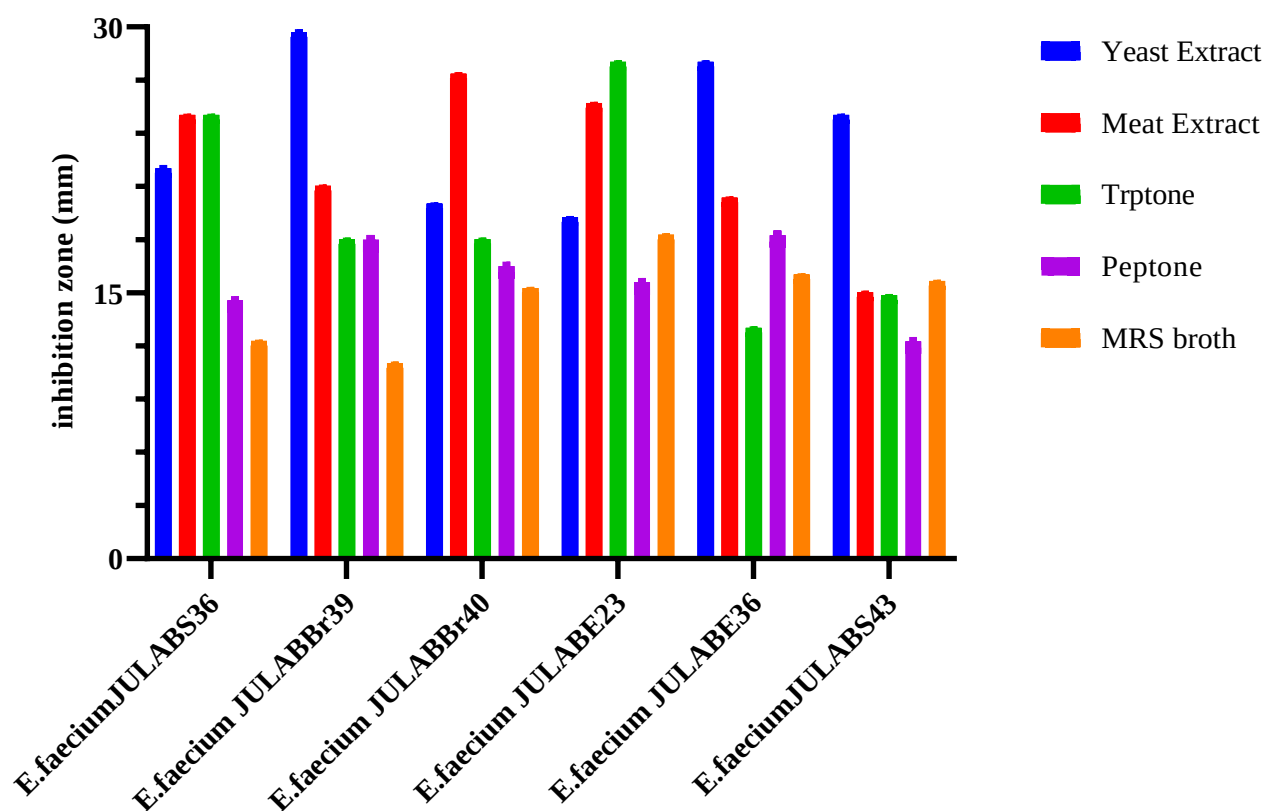


Figure 5. 1 Effects of Nitrogen sources on antimicrobial activity of bacteriocin producing *E. faecium* after MRS broth supplemented with different carbon sources, against *Listeria monocytogenes*

5.4. DISCUSSION

. The investigation identified 39 bacteriocin-producing LAB isolates from traditional Ethiopian foods, all highly effective against selected pathogens. Similarly, Bromberg et al. (2004) found that LAB from fermented meat produced effective bacteriocins against *S. aureus*. In this study, the common bacteriocin-producing LAB identified included *P. pentosaceus*, *P. acidilactici*, *L. lactis*, and *E. faecium*, all exhibiting strong inhibitory activity against pathogens. These strains showed potential natural antimicrobial activity against potential human pathogens.

Similar to this result, Balla et al. (2000) found that *E. faecium* produced bacteriocin that had broad antagonistic activity against *L. monocytogenes*. In addition, Gaaloul et al. (2015) have reported that bacteriocin-like substances produced by *E. faecium* GGN7 also showed potential antagonistic effects against *L. monocytogenes*. Furthermore, Cocolin et al. (2007), Ghairi et al. (2008), Ohmomo et al. (2000), and Rehaem et al. (2010) have shown that *E. faecium* isolated from various sources has potential antimicrobial effects against foodborne pathogens as a result of its capacity to produce bacteriocins. These findings confirm that *E. faecium* could be used as food preservative agent due to its ability to produce various antimicrobial peptides and bacteriocins.

Bacteriocin-producing *E. faecium* has shown broad antimicrobial activity against gram-positive and gram-negative pathogens, with inhibition zones ranging from 13.00 to 35.31 mm. Similarly, Abanoz & Kunduhoglu (2018) have found that *E. faecium* from fermented cheese showed potential antagonistic activity against *L. monocytogenes*, *S. aureus*, *S. Typhimurium*, and *E. coli* with inhibition zones ranging from 14 to 18 mm. This study suggests that *E. faecium* may have potential as a natural antimicrobial agent in food preservation and healthcare settings.

The investigation found that *P. pentosaceus* JULABB01 and JULABB16 have the most promising inhibitory effects against *E. coli* and *S. aureus*. Their ability to produce bacteriocin-like peptides against both gram-negative and gram-positive foodborne pathogens is defined by their antagonistic impact. This finding aligns with previous research by Millette et al. (2007) and Pinto et al. (2007) have indicated that bactericidal effects of LAB are primarily due to the synthesis of bacteriocin-like antimicrobial peptides. On other hand, according to Ghosh et al. (2019) and Simsek et al. (2006), *P. pentosaceus* E5 and GS4 were capable of exerting possible antagonistic effects against both gram-positive and gram-negative foodborne pathogens due to their potency to produce bacteriocin like proteins. Similarly, *P. acidilactici*, isolated from fermented meat (Mandal et al., 2011) and milk products (Abbasiliasi et

al., 2012; Franz et al., 2014), showed potential bacteriocin-producing capacity with effective antagonism against both gram positive and gram negative food borne pathogens. This finding suggests that *P. pentosaceus* and *P. acidilactici* isolates could be potential candidates for use as natural preservatives in the food industry.

This study identified *L. lactis* JULABBk39 and JULABE35 as the top bacteriocin producers among LAB isolates, highlighting *L. lactis* as a potential natural preservative in the food industry. The antimicrobial activity of *L. lactis* is mainly attributed to its bacteriocins. Similar studies by Enan et al. (2013) and Wray et al. (2018) also noted that *L. lactis*'s antagonistic effects against *L. monocytogenes* and *S. aureus*. Using these bacterial isolates could be a promising strategy for controlling food-borne pathogens during food processing and preservation.

The antimicrobial peptides from *E. faecium* were deactivated by pepsin, trypsin, and proteinase K treatments but remained active when exposed to amylase. This indicates their nature as bacteriocin-like antimicrobial peptides. Similar findings were reported for bacteriocin-producing *E. faecium* MMT21 by other researchers like Ghairi et al.(2008) and Zommiti et al.(2018). Enzyme treatments completely removed their antimicrobial activity, confirming them as bacteriocin-like substances. Similarly, the antimicrobial activity of *P. pentosaceus* JUBk33, JUB01, JUB16, JU31, JUE05, JUE33, and JUK37, as well as *P. acidilactici* JUBk40 and JUK50, is deactivated after enzyme treatments (pepsin, trypsin, and proteinase K), indicating that their inhibitory effects are primarily due to bacteriocin-like substances. A number of studies reported similar results, stating that bacteriocin produced by *P. pentosaceus* and *P. acidilactici* were particularly sensitive to proteolytic enzymes due to their proteinase nature (Altuntaş et al., 2014;Todorov et al., 2022; Yao., 2022). This suggests that the inhibitory action of these species is due to the production of bacteriocin-like substances.

The study identified *L. lactis* JUBk39 and JUE35 as producers of potent antimicrobial substances against various pathogens. However, their antimicrobial activity decreased after enzyme treatment, indicating that their effectiveness is primarily due to bacteriocin production rather than hydrogen peroxide or organic acid production. Similarly, other studies by Enan, & Negm,(2013), Suzuki & Suzuki, (2021), Yang et al. (2012) also showed that enzyme treatment led to a loss of antimicrobial activity in *L. lactis*, stressing the importance of bacteriocin production for their antimicrobial effects.

The study assessed bacteriocin-producing LAB's antibacterial activity against *S. aureus* ATCC@25923 across different temperatures (40-120°C) and pH levels (2-10). The results indicated stability up to

80°C but reduced effectiveness at 100°C. Bacteriocin activity remained unaffected by pH levels (2-10), suggesting applicability in acidic and alkaline food products. These findings highlight LAB isolates' promising antimicrobial properties and their potential as natural food preservatives. Additionally, a bacteriocin-like substance from *E. faecium* was found to be stable and effective in inhibiting foodborne pathogens even at high temperatures.

The bacteriocin produced by *E. faecium* M241 and GHB21 remained stable after a 30-minute heat treatment at 100°C, but activity was decreasing at higher temperatures as noted in previous studies by Abanoz & Kunduhoglu (2018) and Cocolin et al. (2007). Similar stability findings were observed for bacteriocin-producing *E. faecium* EF478 and *E. faecium* Q1 by Ben Braïek et al. (2017) and Phumisantiphong et al. (2017) at 60°C for specific durations. Due to their thermal stability and proteinaceous nature, these *E. faecium* isolates hold promise as effective food preservatives in industrial settings, especially given common heating steps in food processing (Abanoz & Kunduhoglu, 2018).

Similarly, the antimicrobial activity of bacteriocin producing *Pediococcus* isolates, was evaluated and all *P. pentosaceus* and *acidilactici* demonstrated stable antimicrobial activity at wide temperature ranges (45°C-100°C), though their antagonistic potential began to decrease after 100°C and at autoclave temperatures. In agreement with this findings, several authors reported that, *P. pentosaceus* (Kaur & Tiwari, 2016; Naresh et al., 2018) were capable to form potential antimicrobial activity and remain active over a wide temperature ranges. As a result of their small size and heat tolerance capacity, bacteriocins are good candidates for use as food preservatives. The antibacterial activity of bacteriocin-producing *L. lactis*, remains effective up to 80°C, but their potency lost after 30 minutes of treatment at 100 or 121°C. Likewise, Lei et al. (2022), Jebur & Auda (2020), Souza et al. (2017), Ribeiro et al. (2016) have reported that the antimicrobial activity of bacteriocin produced by *L. lactis* MMFII and *L. lactis* C15, *L. lactis* HJ202, *L. lactis* 481, and *L. lactis* CECT-4434 remained effective after heat treatment at 45, 60, and 80°C. Normally, temperatures for pasteurization during food manufacturing and processing are between 65 and 80°C for 15 minutes, and the antibacterial compounds produced by the LAB identified in this investigation were effective under these environments. As a result, these bacteriocin-producing LAB strains and their active metabolites have the potential to be used as food preservatives and food processing agents for fermented foods.

The study revealed that *E. faecium* exhibited maximum bacteriocin activity at pH 5 and 7. In line to this, Kumar et al. (2010) Sarkar et al., (2020), observed active antimicrobial activity in *E. faecium* LR/6 within pH ranges of 2 to 7. Likewise, (Kang & Lee, 2005) and (Moreira et al., 2020), found that bacteriocin-producing *E. faecium* strains maintained antagonistic activity across a wide pH range from 2.00 to 8.00. Consequently, the bacteriocin-producing *E. faecium* from Ethiopian traditional fermented foods and beverages showed active antimicrobial properties over a pH range of 2–8.50, suggesting their suitability as food preservatives in acidic and alkaline conditions (Isleroglu et al., 2012).

Additionally, antimicrobial peptides from *Pediococcus* (*P. pentosaceus* and *P. acidilactici*) exhibited strong antagonistic activity and stability at pH 2–12, with maximum activity observed at pH 5–6.5. Fugaban et al. (2021) and (Ladha & Jeevaratnam, 2020), noted potential bacteriocin production by *Pediococcus* strains like *P. pentosaceus* ST44AM, *P. pentosaceus* LJR1, and *P. pentosaceus* PB2, which remained active within pH ranges of 2–12. Furthermore, the study investigated the impact of various surfactants and food additives on bacteriocin effectiveness. Despite exposure to different additives and alcohols, bacteriocin-producing LAB strains maintained potency against *S. aureus*, reflecting similar resilience noted by Santos et al. (2018) against organic solvents and detergents. The resistance of bacteriocin-producing *E. faecium* to these substances allows for versatile antimicrobial use and maintains integrity during purification, enhancing their suitability for food processing and preservation (Kumar et al., 2016). Overall, LAB strains like *E. faecium*, *L. lactis*, *P. pentosaceus*, and *P. acidilactici* produce robust and stable bacteriocins effective against food-borne pathogens, making them valuable for use in fermented food production and preservation.

The study assessed different media for bacteriocin production and found that modified MRS broth and BHI broth were the most effective for LAB isolates with optimal antimicrobial activity. Modified MRS broth is rich in carbon and complex nitrogen sources, while BHI broth is a nutrient rich medium with excellent suitability to various microorganisms. Growth kinetics revealed that LAB thrived best in BHI broth with an initial pH of 7.4, leading to potential antimicrobial activity. Therefore, modified MRS broth and BHI broth, supplemented with diverse carbon and nitrogen sources are ideal for bacteriocin production.

This study developed techniques to efficiently recover bacteriocin production from LAB using ammonium sulfate precipitation and solvent extraction methods. Results indicated that bacteriocin

precipitated at 80% w/v ammonium sulfate exhibited the most effective antagonistic activities compared to lower concentrations. Studies by Barale et al. (2022) and Xu et al. (2009) have also supported this observation by noting higher antimicrobial activity with 80% ammonium sulfate precipitation. Solvent extraction was also explored with chloroform and its mixtures showing the highest recovery followed by isopropanol and ethyl acetate. These solvents are promising for extracting bacteriocin-like compounds with strong antibacterial activity from LAB in MRS broth. Combining these methods can efficiently purify and recover bacteriocin from LAB isolated from various traditional fermented foods and beverages, as suggested by previous research (Barale et al., 2022).

5.5. CONCLUSION

In conclusion, the isolation and characterization of bacteriocin-producing lactic acid bacteria (LAB) from Ethiopian traditional fermented foods and beverages have yielded promising results. The identified LAB strains such as *E. faecium*, *L. lactis*, *P. pentosaceus*, and *P. acidilactici*, have demonstrated potent antimicrobial activity against various food-borne pathogens. These LAB strains have shown stability and efficacy across a wide range of pH levels and temperatures, making them suitable candidates for natural food preservatives

Furthermore, the bacteriocins produced by these LAB strains have shown resilience against detergents, organic solvents, and other additives commonly used in food processing. This resilience ensures the integrity and effectiveness of bacteriocins even under challenging conditions, highlighting their potential for industrial applications in food processing industry.

Overall, the isolation and characterization of bacteriocin-producing LAB from Ethiopian traditional fermented foods and beverages offer promising avenues for enhancing food safety and shelf-life extension through natural antimicrobial agents. Further research and development in this area could lead to the widespread adoption of LAB-derived bacteriocins as natural and effective food preservatives in the food industry.

6.

STUDY ON SHELF-LIFE ENHANCEMENTS OF FRUITS AND VEGETABLES USING LACTIC ACID BACTERIA AND EVALUATING THEIR BIO PRESERVATIVE EFFICIENCY

Amenu, D., & Bacha, K. (2024). Shelf Life Enhancement of Fruits and Vegetables Using Lactic Acid Bacteria isolated Ethiopian traditional fermented foods and beverages. Probiotics and Antimicrobial Proteins (Under review)

ABSTRACT

*Lactic acid bacteria (LAB) are widely used to preserve foods and beverages to extend their shelf life added to quality improvement. These bacteria produce lactic acid through the fermentation of carbohydrates that lowers the pH of the food and inhibits the growth of harmful microorganisms. LAB are commonly found in fermented foods and beverages such as Kocho, Bulla, Ergo, Borde, Bukuri, and Cabbage Shamita. Bio-preservation of fruits and vegetables using LAB isolated from traditional Ethiopian fermented foods and beverages is an innovative approach. This method can be used as an alternative to chemical preservatives, which can have negative health effects. Additionally, this technique can help to promote the consumption of traditional foods and support local communities. Therefore, this study was conducted to investigate the application of LAB as a potential food preservative agent to enhance fruit and vegetable shelf life by inhibiting spoilage microorganisms. LAB were isolated from traditional fermented beverages and foods using MRS medium. The antagonistic activity, probiotic properties, and bacteriocin-producing ability of LAB were tested. The agar-well diffusion method was used to screen the potential LAB against *E. coli*, *S. aureus*, *S. Typhimurium*, *P. aeruginosa*, and *C. albicans*. The antagonistic activity of LAB was evaluated against fruit and vegetable pathogens. Of the total LAB isolates, three isolates, *Lactococcus lactis* (*L. lactis*), *Enterococcus faecium* (*E. faecium*), and *Pediococcus pentosaceus* (*P. pentosaceus*) showed a wide range of antibacterial activity against fruit and vegetable spoilage microorganisms. Hence, they were able to extend the fruits and vegetables' shelf life. *Lactococcus lactis* and its combination were identified as the most effective isolates for enhancing the shelf-life of all the fruits and vegetables within a minimum of three weeks. The potency of the LAB isolates to extend the fruits and vegetables showed a significant variation at $p < 0.05$. Therefore, all the three isolates had potential preservative effects for three to four weeks. Furthermore, the study also revealed that the combination of *L. lactis* with other LAB strains could further enhance the preservative effects on fruits and vegetables, indicating the possibility of developing a more effective preservation method for these perishable products. Probiotic and bacteriocin-producing *L. lactis*, *E. faecium*, and *P. pentosaceus* found in Ethiopian traditional fermented food and beverages may have potential applications to be used as bio-preservatives of fruits and vegetables to increase their shelf life by decreasing the percentage of spoilage. Researching probiotics and bacteriocin-producing LAB for food preservation could revolutionize the industry and public health. These approaches may yield safer preservation methods, cutting back on chemical additives, enhancing food quality, and supporting healthier choices. Furthermore, using probiotics could boost gut health, tapping into the trend for functional foods with added benefits.*

Key words: Bio preservative, Food preservation, Fruits and vegetables, LAB, Shelf life enhancements and Spoilage.

6.1. INTRODUCTION

Fresh fruits and vegetables are key components of the human diet, offering vital nutrients like carbohydrates, proteins, vitamins, minerals, and fiber. Regular consumption of these foods is globally recognized for their health benefits (Mohanty et al., 2014). However, their susceptibility to bacterial contamination poses significant challenges for the food industry, leading to financial losses and health risks due to pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, and associated toxins (Damania et al., 2016). Additionally, the physiological changes and microbial degradation during marketing result in a short shelf life and diminished quality, impacting color, aroma, taste, and promoting microbial growth. Proper handling and storage are crucial to minimize these risks and prolong shelf life, especially considering the increasing consumer demand for natural and chemical-free food preservation methods (De Corato, 2020; Salazar et al., 2017).

Since cutting procedures are essential for freshly cut fruits and vegetables and there is a chance of microbial development, it is important to prevent the contamination of food with food-borne pathogens without producing potentially harmful compounds. Consumers today desire healthier, more natural, safe, and non-chemically contaminated meals; hence, chemical compounds employed in fresh-cut fruit and vegetable preservation are not preferred while eating fresh-cut fruits and vegetables (Agriopoulou et al., 2020; Hasan et al., 2019). The preservation of fruits and vegetables faces challenges due to chemical additives and fumigation. Synthetic chemicals are commonly used to extend shelf life, but consumers prefer natural preservation methods. Biopreservation with LAB offers a promising solution for food safety and shelf life. LAB, known for producing natural antimicrobials like organic acids and bacteriocins, can enhance food quality without harmful additives. While LAB's role in food preservation is established, its specific use for extending the shelf life of fresh produce is a novel area, especially in regions like Ethiopia. This study focused on isolating LAB from traditional fermented foods and testing their antimicrobial effects against spoilage microorganisms in fruits and vegetables, aiming to enhance preservation methods naturally.

6.2. MATERIAL AND METHODS

6.2.1. Bacterial Strains and Growth Condition

The sources of LAB isolates used in this study are listed in Table 6.1. These strains were identified as *Lactococcus*, *Pediococcus*, and *Enterococcus* species based on their morphological, biochemical, and physiological characterization and confirmation with MALDI-TOF system (Amenu & Bacha, 2024). Selected food borne pathogens were obtained from the Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia (Table 6.1).

Table 6. 1. Sources of potential probiotic LAB used in this study

No.	Sources	Designation	Strains
1.	<i>Bulla</i>	JULABB01	<i>Pediococcus pentosaceus</i>
3.	<i>Bukuri</i>	JULABBk39	<i>Lactococcus lactis</i>
	<i>Borde</i>	JULABBr39	<i>Enterococcus faecium</i>
5.	EPHI	ATCC@25923	<i>Staphylococcus aureus</i>
6.	EPHI	ATCC@259222	<i>Escherichia coli</i> ,

6.2.2. Application of LAB as food preservative

6.2.2.1. Analysis microbial load and shelf life determination

LAB isolates were tested on grains for antibacterial effects, with regular pathogen level checks. After inoculation of fruits and vegetables samples underwent microbiological analysis. LAB isolates with extended food shelf life were re-inoculated, and the sensory qualities of these foods (fruits and vegetables) were evaluated. Bacterial and fungal colony counts were done after 24 h at 37°C on 0, 5, and 10 days for five days interval times

6.2.2.2. Percent spoilage

To evaluate the percent spoilage of vegetables and fruits per cent disease index (Rani et al., 2018) was calculated by applying the formula given below.

$$\text{Percent spoilage (\%)} = \frac{\text{Number of vegetables spoilt}}{\text{Total number of vegetables}} \times 100$$

6.2.2.3. Shelf life study of vegetables and fruits

Freshly harvested samples were brought to the lab within 24 h in sterile bags, washed gently with sterile water, and soaked in a 3% sodium hypochlorite solution for 10 minutes. Afterward, the items were aseptically dried under laminar hoods and treated with selected probiotic LAB isolates (Souza et al., 2017).

6.2.2.4. Efficacy of LAB isolates against fruits and vegetable spoilage

Vegetables like carrot, potato, and tomato and fruits like banana, orange, and avocado were brought to postgraduate and microbiology research laboratory. The samples were gently washed in sterile water and soaked in a sodium hypochlorite solution of 3% for 10 minutes (Saha et al., 2014). Later, a marked area (1 sq cm) was surface sterilized by 70% alcohol and inoculated by making nine wounds per vegetable, and single or mixed culture of LAB were separately or in combination applied to these wounds. Similarly, the LAB-isolated cell free supernatant was sprayed on each fruit and vegetable. Finally, the microbial loads were monitored at regular intervals before and after inoculation of the LAB isolates. Spoilage microorganisms were isolated and characterized from infected fruits and vegetables. These organisms were then used as test organisms to assess the ability of LAB isolates to extend the shelf life of fruits and vegetables. In this study fruits and vegetables samples were selected based on their susceptibility, availability and their wide usage among the communities. Finally, the fruits and vegetables that are expected to represent the commonly consumed and distributed in the market, ensuring that the research outcomes can be applied widely were selected.

Table 6. 2. In vitro experimental design to test the efficacy of LAB against pathogens

S/N	Parameters	Description
1)	Design	Complete random design
2)	Replication	Three
3)	Treatments	T1= <i>L.lactis</i>+ pathogenic+ fruits/vegetables T2=<i>P.pentosaceus</i>+ pathogenic+ fruits/vegetables T3=<i>E.faecium</i> + pathogenic+ fruits/vegetables T4= <i>L.lactis</i>+ <i>P.pentosaceus</i> +pathogenic+ fruits/vegetables T5= <i>L.lactis</i>+ <i>E.faecium</i> +pathogenic+ fruits/vegetables T6= <i>E.faecium</i>+ <i>P.pentosaceus</i> +pathogenic+ fruits/vegetables T7= <i>E.faecium</i>+ <i>L.lactis</i>+ <i>P.pentosaceus</i> +pathogenic+ fruits/vegetables T8= Control; fruits /vegetables without LAB strains
4)	Fruits	Avocado Orange Banana
5)	Vegetables	Tomato Potato Carrot

6.3. RESULTS

6.3.1. Bio-control of fruits spoilage microorganisms

The study evaluated the effects of fungal pathogens and probiotic LAB treatments on specific fruits and vegetables. Avocados treated with three LAB strains remained mold-free for 32 days, while oranges treated with *L. lactis* and *P. pentosaceus* showed no mold growth for 24 days. All the isolates, whether single or in mixed probiotic cultures, extended fruit shelf life to three weeks without fungal decay, with *P. pentosaceus* being the most effective. This suggests probiotic LAB treatments can significantly prolong fruit and vegetable shelf life by inhibiting mold growth. However, bananas treated with three combinations of bacterial isolates had shorter shelf lives compared to oranges and avocados. Single bacterial isolates preserved bananas for up to three weeks, with *L. lactis* and *P. pentosaceus* combination showing the best preservation for 24 days (Table 6. 3).

6.3.2. Bio-control of vegetables spoilage microorganisms

The study examined LAB strains for their ability to combat pathogens on tomato surfaces. While the control group decayed by day 10, tomatoes treated with individual strains stayed healthy for 32 days (Table 6.4). Combinations of *L. lactis* + *P. pentosaceus* + *E. faecium* showed no mold growth for 36 days, indicating potential for extending tomato shelf life. For potatoes, the combined strains were most effective, keeping them healthy for 36 days with minimal spoilage, whereas other treatments were less successful. Additionally, carrots treated with various LAB combinations remained healthy and attractive for up to five weeks (36 days) (Table 6.4).

Table 6. 3. Evaluating the efficiency of LAB from Ethiopian traditional fermented foods and beverages to prevent fungal spoilage

Samples	Treatments	Percent of fruits Spoilage (% of spoilage).								
		W1 4days	W2 8days	W3 12days	W4 16days	W5 20days	W6 24days	W7 28days	W8 32days	W9 36days
Avocado	T1	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00
	T2	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00	100.00
	T3	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00	100.00
	T4	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00
	T5	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00	100.00
	T6	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00
	T7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33
	T8	0.00	0.00	0.00	16.67	33.33	50.00	66.66	83.33	100.00
Orange	T1	0.00	0.00	0.00	0.00	0.00	00.00	00.00	16.66	33.33
	T2	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00
	T3	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00
	T4	0.00	0.00	0.00	0.00	0.00	00.00	00.00	16.66	33.33
	T5	0.00	0.00	0.00	0.00	0.00	00.00	8.33	16.66	33.33
	T6	0.00	0.00	0.00	0.00	0.00	0.00	8.33	16.66	33.33
	T7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T8	0.00	0.00	0.00	16.67	33.33	50.00	66.66	83.33	100.00
Banana	T1	0.00	0.00	0.00	0.00	0.00	8.33	16.66	33.33	50.00
	T2	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00	66.67
	T3	0.00	0.00	0.00	0.00	0.00	16.67	33.33	50.00	66.67
	T4	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00
	T5	0.00	0.00	0.00	0.00	0.00	8.33	16.66	33.33	50.00
	T6	0.00	0.00	0.00	0.00	0.00	8.33	16.66	33.33	50.00
	T7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33
	T8	0.00	0.00	0.00	16.67	33.33	50.00	66.66	83.33	100.00

Note: T1=*L.lactis*+Fruits+Pathogens (*A. flavus*), T2=*P.pentosaceus*+fruits+Pathogens (*A. flavus*), T3= *E. faecium*+Fruits+ Pathogens (*A. flavus*), T4= *L. lactis*+ *P. pentosaceus*+ Fruits + Pathogens (*A. flavus*), T5= *L. lactis*+*E.faecium* + Fruits + Pathogens (*A. flavus*), T6= *P. pentosaceus* ++*E.faecium* +fruits + Pathogens (*A. flavus*), T7=*L.lactis* + *P. pentosaceus*+*E.faecium* +fruits+ Pathogens (*A. flavus*) and T8= Control, fruits treated without any probiotic strains.

Table 6. 4. Evaluating the efficiency of LAB from Ethiopian traditional fermented foods and beverages to prevent fungal spoilage

Samples	Treatments	Percent of Vegetables Spoilage								
		W1 4days	W2 8days	W3 12days	W4 16days	W5 20days	W6 24days	W7 28days	W8 32days	W9 36days
Tomato	T1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.66
	T3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.66
	T4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T8	0.00	0.00	0.00	16.67	33.33	50.00	66.66	83.33	100.00
Potato	T1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33
	T3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33
	T4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T8	0.00	0.00	0.00	16.67	33.33	50.00	66.66	83.33	100.00
Carrot	T1	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	33.33
	T2	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00	50.00
	T3	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33	50.00
	T4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.66
	T5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33
	T6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.66	33.33
	T7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T8	0.00	0.00	16.66	33.33	50.00	66.66	83.33	100.00	100.00

Note: T1=*L.lactis*+vegetables+Pathogens (*A. flavus*), T2=*P.pentosaceus*+Vegetables+Pathogens (*A. flavus*), T3=*E.faecium*+vegetables + Pathogens (*A. flavus*), T4= *L. lactis*+ *P. pentosaceus*+vegetables + Pathogens (*A. flavus*), T5= *L. lactis*+*E.faecium* + vegetables + Pathogens (*A. flavus*), T6= *P. pentosaceus* ++*E.faecium* +vegetables + Pathogens (*A. flavus*), T7=*L.lactis* +*999 *P. pentosaceus*+*E.faecium* +vegetables + Pathogens (*A. flavus*) and T8= Control, fruits treated without any probiotic strains.

6.3.3. Extending and preserving fruits shelf life using Lactic Acid Bacteria

The study proved that combining *L. lactis* JULABBk39 and *P. pentosaceus* JULABB01 could prolong fruit shelf life by over a month (Figure 6.1). Avocados without probiotic LAB allowed mold growth on day 16, but the *L. lactis*, *P. pentosaceus*, and *E. faecium* combination preserved them for 36 days without spoilage. Oranges without LAB showed mold growth by day 13, while LAB-treated ones lasted healthy for 36 days, especially with *P. pentosaceus* and *E. faecium*. Similarly, untreated bananas spoiled by day 12, but the three-strain combination extended their shelf life by 24 days (Figure 6.2).

The study revealed that combining *L. lactis* JULABBk39 and *P. pentosaceus* JULABB01 extends fruit shelf life by more than a month (Figure 6.3). Avocados without probiotic LAB showed mold and spoilage by day 16, but a combination of three isolates (*L. lactis*, *P. pentosaceus*, and *E. faecium*) prevented fungal spoilage for 36 days, maintaining texture and appearance. Oranges without LAB had mold by day 13, while LAB-treated ones lasted 36 days, especially with *P. pentosaceus* and *E. faecium*. Similarly, untreated bananas spoiled by day 12, but the three-strain combination extended shelf life by 24 days (Figure 6.3).

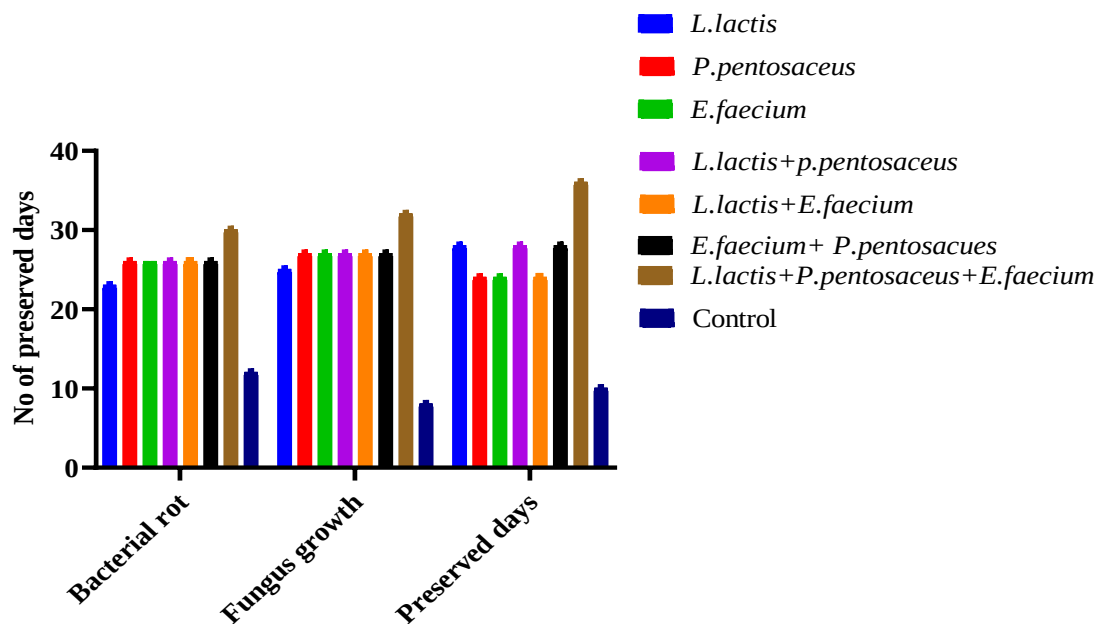


Figure 6. 1. Shelf life study of Avocado using probiotic LAB isolates isolated from Ethiopian traditional fermented food and beverage.

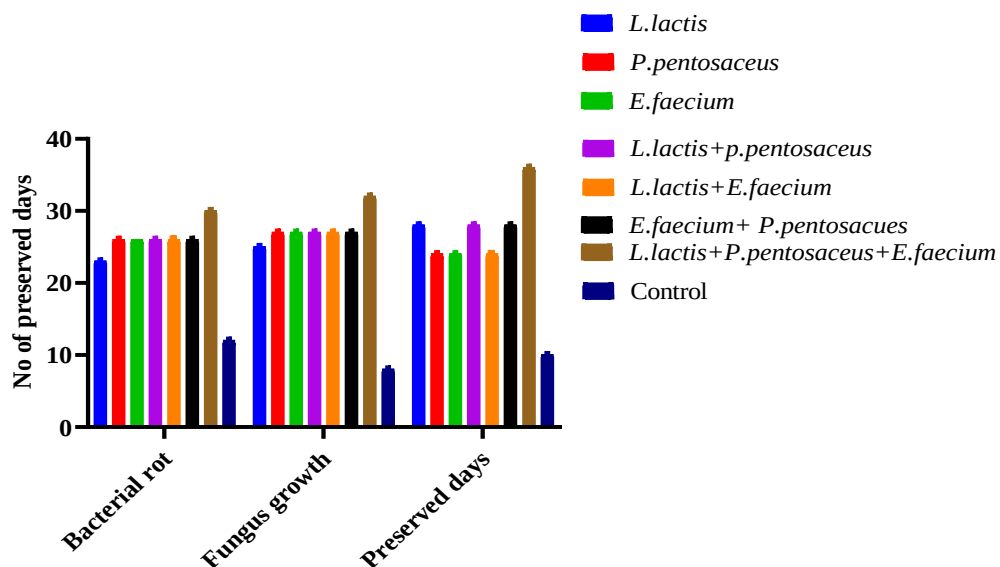


Figure 6. 2. Shelf life study of Orange using probiotic LAB isolates isolated from Ethiopian traditional fermented food and beverage.

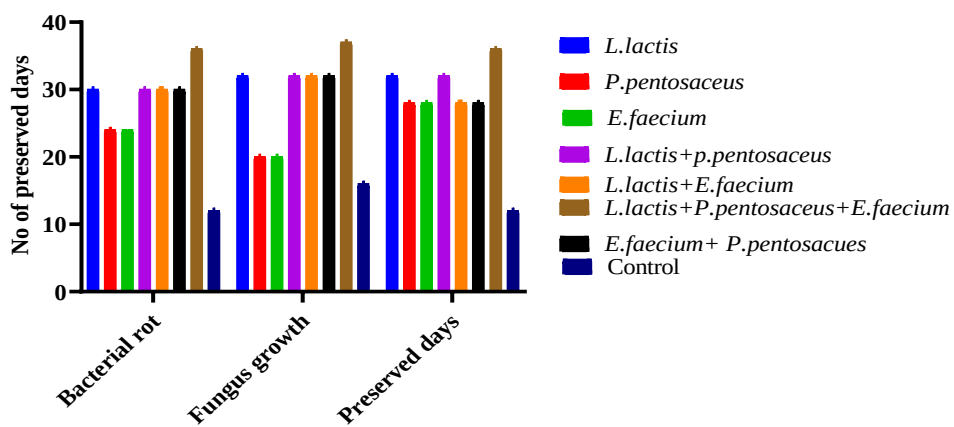


Figure 6. 3. Shelf life study of banana using probiotic LAB isolates isolated from Ethiopian traditional fermented food and beverage.

6.3.4. Extending and preserving vegetables shelf life using Lactic acid bacteria

The effectiveness of probiotic LAB isolates in extending vegetable shelf life was investigated and the results showed that these isolates demonstrated potential antifungal activity and controlled fungal spoilage, extending vegetable shelf life. Carrots treated with a combination of three strains (*L.lactis*, *P.pentosaceus*, and *E.faecium*), preserved for 36 consecutive days without visible mold growth, resulting in a significant increase in carrot shelf life by 16 days compared to the control (Figure 6.4). In addition, the tomatoes shelf life was studied and the control was spoilt on the 12th day, while treatments with *L.lactis*, *P.pentosaceus*, and *E.faecium* alone and their combination kept the tomatoes healthy up to the 28th and 36th days, increasing the shelf life by a minimum of 35 days (Figure 6.5). Potatoes were treated with various LAB isolates to prevent fungal spoilage and extend their shelf life. Therefore, with a 16.66% spoiling percentage, treatments 4, 5, and 6 were the most successful in keeping potatoes healthy for up to 36 days. The three synergistic effects produced the best and maximum preservation (Figure 6.6).

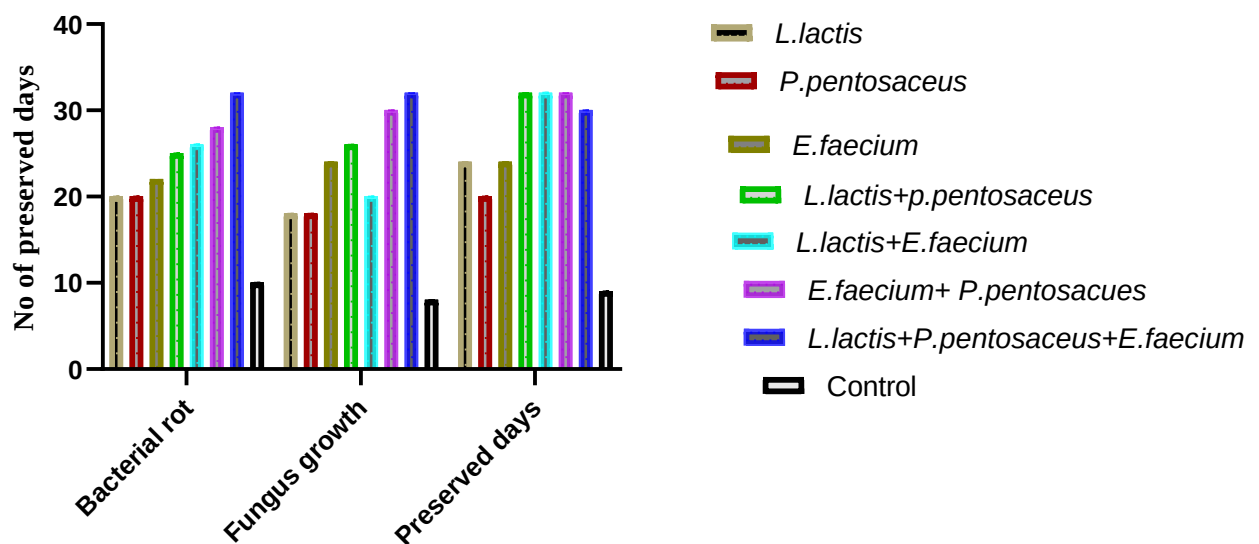


Figure 6. 4. Shelf life study of vegetables (carrot) using probiotic LAB isolates

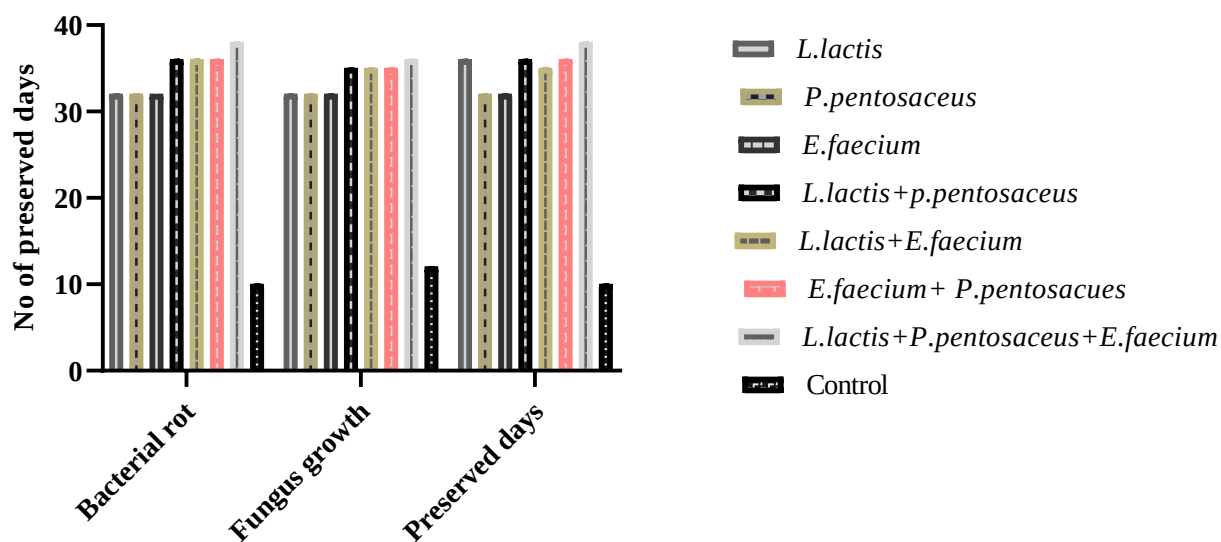


Figure 6. 5. Shelf life study of vegetables (tomato) using probiotic LAB isolates

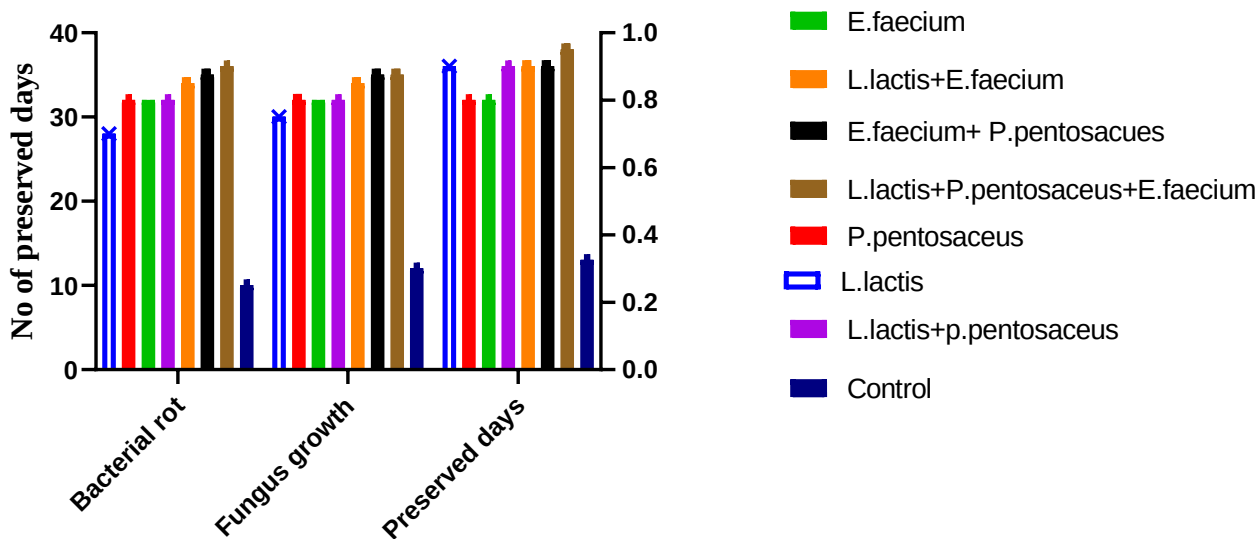


Figure 6. 1. Shelf life study of vegetables (tomato) using probiotic LAB isolates

6.3.5. Microbiological analysis of Fruits treated with LAB as bio-preservative

To investigate the microbiological analysis of fruits treated with these potential LAB isolates, the loads of indicators (AMB, *Staphylococci*, *E. coli* and Yeast and mold counts) were conducted on the first day, 7th, 15th, 21st, 28th, 35th day respectively (Table 6.5). Accordingly, in all treatments the LAB isolates were capable to preserve the fruits without any visible molds growth as well as no colonies of indicator organisms for two weeks. In case of avocado, up to five weeks $<4.06 \text{ Log}_{10}/1\text{cm}^2$ of AMB, *E. coli*, *Staphylococci* and yeast and molds counts were recorded. In addition, the microbiological analysis of orange showed that there were less than $4.06 \text{ Log}_{10}/1\text{cm}^2$ observed. On other hands, the microbiological analysis of banana revealed that, the LAB inoculum was capable to extend and preserve the fruits for consecutive two weeks by inhibiting the banana's spoilage (Table 6.5).

6.3.6. Microbiological analysis of Vegetables treated with LAB as bio-preservative

The microbiological analysis of vegetables treated with different probiotic LAB isolates used in this study revealed that almost all the isolates inoculated on each vegetable were capable of extending all the vegetables' shelf life by preventing fungal spoilage (Table 6.6). There were no AMBC, *E. coli*, staphylococci, yeast, or mold counts for two weeks in a row. As a result of these findings, all the LAB isolates, *L. lactis*, *P. pentosaceus*, and *E. faecium*, were effective at controlling and inhibiting fungal spoilage, allowing them to extend and maintain the freshness of vegetables for more than two weeks (Table 6.6).

Table 6. 5. Microbial analysis of fruits treated with LAB isolates to extend and enhance shelf life (avocado and orange)

Tt.	Week	Loads of indicator organisms per treated samples (log10-CFU/1cm ²)							
		AMBC	<i>E.coli</i>	<i>Staphyococci</i>	YMC	AMBC	<i>E.coli</i>	<i>Staphyococci</i>	YMC
		Avocado				Orange			
T1	W1	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W2	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W3	3.50±0.02 ^b	3.24±0.04 ^{bd}	3.27±0.03 ^{cd}	2.84±0.09 ^{cd}	3.46±0.02 ^{bc}	3.00±0.07 ^d	3.04±0.06 ^d	2.98±0.07 ^{cde}
	W4	3.66±0.01 ^b	3.44±0.02 ^{bc}	3.50±0.02 ^{ba}	3.25±0.03 ^{bc}	3.72±0.01 ^b	3.37±0.03 ^{ce}	3.40±0.03 ^{cd}	3.35±0.03 ^{bde}
	W5	3.75±0.01 ^{ac}	3.71±0.01 ^{ab}	3.53±0.02 ^{ba}	3.52±0.02 ^{abc}	4.14±0.60 ^a	4.03±0.66 ^a	4.01±0.61 ^{ab}	3.97±0.58 ^{ab}
T2	W1	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W2	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W3	2.73±0.11 ^{cd}	3.24±0.04 ^{bd}	2.84±0.09 ^{de}	3.00±0.06 ^c	3.55±0.02 ^{bc}	2.88±0.09 ^{de}	2.78±0.12 ^{de}	3.02±0.07 ^{cd}
	W4	3.20±0.04 ^c	3.54±0.02 ^b	3.27±0.03 ^{cd}	3.34±0.03 ^b	4.33±0.94 ^a	4.12±1.20 ^a	4.10±1.22 ^{ab}	4.16±1.12 ^a
	W5	3.50±0.02 ^b	3.72±0.01 ^{ab}	3.53±0.02 ^{ba}	3.54±0.02 ^{ab}	4.00±0.43 ^{ab}	3.86±0.45 ^{ab}	3.80±0.38 ^b	3.75±0.24 ^b
T3	W1	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W2	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W3	3.02±0.06 ^{cd}	3.48±0.02 ^b	3.32±0.03 ^c	3.02±0.06 ^c	3.42±0.01 ^{bc}	2.85±0.10 ^{de}	2.85±0.10 ^{de}	2.74±0.13 ^d
	W4	3.36±0.03 ^{bc}	3.72±0.01 ^{ab}	3.47±0.02 ^{ba}	3.34±0.03 ^b	4.28±0.94 ^a	4.00±1.20 ^a	4.09±1.16 ^{ab}	4.05±1.21 ^a
	W5	3.56±0.02 ^b	3.72±0.01 ^{ab}	3.68±0.01 ^b	3.53±0.02 ^{abc}	3.63±0.02 ^b	3.52±0.02 ^{cd}	3.56±0.02 ^c	3.52±0.02 ^{bcd}
T4	W1	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W2	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W3	2.53±0.18 ^d	3.06±0.05 ^c	2.90±0.08 ^d	2.73±0.11 ^{cde}	3.18±0.05 ^c	3.30±0.04 ^{ce}	3.08±0.06 ^d	3.21±0.04 ^c
	W4	3.18±0.04 ^c	3.47±0.02 ^b	3.31±0.03 ^c	3.22±0.04 ^{bc}	4.19±1.00 ^a	4.18±0.99 ^a	4.10±1.07 ^{ab}	4.16±0.96 ^a
	W5	3.53±0.02 ^b	3.66±0.01 ^{ab}	3.56±0.02 ^b	3.52±0.02 ^{abc}	3.60±0.02 ^{bc}	3.52±0.02 ^{cd}	3.52±0.02 ^c	3.51±0.02 ^{bcd}
T5	W1	<2 ^f	<2 ^e	<2 ^f	<2 ^e	<2 ^e	<2 ^f	<2 ^f	<2 ^e
	W2	3.20±0.04 ^c	3.23±0.04 ^{bd}	3.32±0.03 ^c	3.20±0.04 ^{bcd}	3.40±0.03 ^{bc}	3.36±0.03 ^{ce}	3.56±0.02 ^c	3.21±0.04 ^c
	W3	3.82±0.01 ^{ac}	4.06±0.01 ^a	3.81±0.01 ^{ab}	3.32±0.03 ^b	3.64±0.02 ^b	3.59±0.02 ^c	3.60±0.02 ^{cd}	3.33±0.03 ^c
	W4	3.98±0.01 ^{ab}	4.06±0.01 ^a	3.99±0.00 ^{ab}	3.66±0.01 ^{ab}	4.40±0.60 ^a	4.29±0.66 ^a	4.34±0.52 ^a	4.16±0.65 ^a
	W5	4.14±0.00 ^a	4.29±0.00 ^a	4.26±0.00 ^a	3.84±0.01 ^a	4.19±0.00 ^{ab}	4.15±0.01 ^a	4.16±0.00 ^{ab}	3.87±0.01 ^{abc}

Legends: Tt. =treatments, T1= *L. lactis*+fruits/vegetables, T2= *P. pentosacues*+fruits/vegetables, T3= *L. lactis* + *P. pentosaceus*+fruits/vegetables, T4== *L. lactis* + *P. pentosaceus*+ *E. faecium* + fruits/vegetables, T5= control. The mean values represented by the same letter are statically not shown significance variation at P<0.05 across the column. W=week.

Table 6. 6. Microbial analysis of fruits and vegetables treated with lab isolates to extend and enhance shelf life

Treatment	Week	Loads of indicator organisms per treated samples (log10-CFU/1cm2)							
		AMBC	<i>E.coli</i>	Staphyococci	YMC	AMBC	<i>E.coli</i>	Staphyococci	YMC
		Banana				Tomato			
T1	W1	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W2	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W3	3.28±0.04 ^{ce}	2.85±0.10 ^e	2.85±0.10 ^e	2.98±0.07 ^c	3.28±0.04 ^d	2.85±0.10 ^{ef}	2.85±0.10	2.98±0.07
	W4	3.58±0.02 ^{be}	3.26±0.04 ^{cd}	3.30±0.04 ^{de}	3.37±0.03 ^{bc}	3.58±0.02 ^{bc}	3.26±0.04 ^{ce}	3.30±0.04	3.37±0.03
	W5	3.66±0.02 ^{bd}	3.52±0.02 ^{bc}	3.57±0.02 ^{cde}	3.59±0.02 ^{abc}	3.66±0.02 ^{bc}	3.52±0.02 ^{be}	3.57±0.02	3.59±0.02
T2	W1	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W2	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W3	3.59±0.02 ^{bd}	2.85±0.10 ^e	2.82±0.11 ^{ef}	3.04±0.06 ^{bc}	3.59±0.02 ^{bc}	2.85±0.10 ^{ef}	2.82±0.11	3.04±0.06
	W4	3.77±0.01 ^{bc}	3.30±0.04 ^{cd}	3.27±0.03 ^{df}	3.41±0.04 ^b	3.77±0.01 ^{abc}	3.30±0.04 ^{cd}	3.27±0.04	3.41±0.03
	W5	3.81±0.01 ^b	3.57±0.02 ^{bc}	3.55±0.02 ^{cde}	3.60±0.02 ^{abc}	3.81±0.01 ^{abc}	3.57±0.02 ^{be}	3.55±0.02	3.60±0.02
T3	W1	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W2	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W3	3.21±0.04 ^d	2.88±0.09 ^e	2.88±0.09 ^e	3.04±0.06 ^{bc}	3.21±0.04 ^{de}	2.88±0.09 ^e	2.88±0.09	3.04±0.06
	W4	3.45±0.03 ^c	3.34±0.03 ^{cd}	3.32±0.03 ^{de}	3.43±0.03 ^b	3.45±0.03 ^c	3.34±0.03 ^{cd}	3.32±0.03	3.43±0.03
	W5	3.55±0.02 ^c	3.60±0.02 ^b	3.58±0.02 ^{cde}	3.63±0.02 ^{ab}	3.55±0.02 ^{bcd}	3.60±0.02 ^{bd}	3.58±0.02	3.63±0.02
T4	W1	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W2	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W3	3.37±0.03 ^{cd}	3.12±0.05 ^d	3.43±0.03 ^d	3.43±0.03 ^b	3.37±0.03 ^{cd}	3.12±0.05 ^d	3.43±0.03	3.43±0.03
	W4	3.66±0.02 ^{bd}	3.46±0.02 ^c	3.62±0.02 ^{cd}	3.67±0.01 ^{ab}	3.66±0.02 ^{bc}	3.46±0.02 ^c	3.62±0.02	3.67±0.01
	W5	3.75±0.01 ^{bc}	3.63±0.02 ^b	3.67±0.01 ^{cd}	3.70±0.01 ^{ab}	3.75±0.01 ^b	3.63±0.02 ^{bc}	3.67±0.01	3.70±0.01
T5	W1	<2 ^f	<2 ^g	<2 ^g	<2 ^e	<2 ^g	<2 ^g	<2	<2
	W2	3.36±0.03 ^{cd}	3.42±0.03 ^c	3.43±0.03 ^d	3.18±0.05 ^{bc}	3.36±0.03 ^{cd}	3.42±0.03 ^{ce}	3.43±0.03	3.18±0.05
	W3	3.74±0.01 ^{bc}	3.67±0.01 ^b	3.71±0.01 ^c	3.65±0.02 ^{ab}	3.74±0.01 ^b	3.67±0.01 ^b	3.71±0.01	3.65±0.02
	W4	3.99±0.01 ^{ab}	3.99±0.01 ^a	3.96±0.01 ^b	3.74±0.01 ^a	3.99±0.01 ^{ab}	3.99±0.01 ^{ab}	3.96±0.01	3.74±0.01
	W5	4.18±0.00 ^a	4.17±0.00 ^a	4.18±0.00 ^a	3.88±0.01 ^a	4.18±0.00 ^a	4.17±0.00 ^a	4.18±0.00	3.88±0.01

Legends: Tt. =treatments, T1= *L. lactis*+fruits/vegetables, T2= *P. pentosacues*+fruits/vegetables, T3= *L. lactis* + *P. pentosaceus*+fruits/vegetables, T4== *L. lactis* + *P. pentosaceus*+ *E. facecium* + fruits/vegetables, T5= control. The mean values represented by the same letter are statically not shown significance variation at P<0.05 across the column.W=week

Table 6. 7. Microbial analysis of vegetables treated with LAB isolates to extend and enhance shelf life (potato and carrot).

Loads of indicator organisms per treated samples (log10-CFU/1cm ²)									
Treatment	Week	AMBC Potato	<i>E.coli</i>	<i>Staphyococci</i>	YMC	AMBC Carrot	<i>E.coli</i>	<i>Staphyococci</i>	YMC
T1	W1	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W2	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W3	3.41±0.01 ^{bd}	2.81±0.05 ^{cd}	3.02±0.03 ^e	3.02±0.03 ^{df}	3.74±0.01 ^{cd}	3.64±0.01 ^c	3.49±0.01 ^c	3.42±0.01 ^{cd}
	W4	3.68±0.01 ^{ad}	3.29±0.02 ^{bd}	3.36±0.01 ^{cd}	3.37±0.01 ^{cd}	3.93±0.00 ^{bc}	3.84±0.00 ^{bd}	3.72±0.01 ^b	3.67±0.01 ^{bc}
	W5	3.70±0.01 ^{ac}	3.56±0.01 ^{ac}	3.55±0.01 ^{bc}	3.56±0.01 ^{bd}	3.89±0.00 ^{bc}	3.85±0.00 ^{bd}	3.72±0.01 ^b	3.71±0.01 ^b
T2	W1	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W2	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W3	3.15±0.02 ^{cd}	3.02±0.03 ^c	3.04±0.03 ^{df}	3.20±0.02 ^d	3.75±0.01 ^{cd}	3.76±0.01 ^{be}	3.10±0.02 ^d	3.59±0.01 ^{bd}
	W4	3.48±0.01 ^{bc}	3.41±0.01 ^{bc}	3.42±0.01 ^c	3.51±0.01 ^{be}	3.79±0.00 ^c	3.89±0.00 ^{bd}	3.48±0.01 ^c	3.81±0.00 ^{abc}
	W5	3.60±0.01 ^b	3.61±0.01 ^{ac}	3.62±0.01 ^b	3.63±0.01 ^{bc}	3.96±0.00 ^b	3.94±0.00 ^{bc}	3.66±0.01 ^{bc}	3.84±0.00 ^{abc}
T3	W1	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W2	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W3	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W4	3.27±0.02 ^c	3.06±0.03 ^c	3.23±0.02 ^d	3.10±0.02 ^{de}	3.61±0.01 ^d	3.02±0.03 ^{df}	3.10±0.02 ^d	3.04±0.03 ^{de}
	W5	3.56±0.01 ^{bc}	3.44±0.01 ^{bc}	3.51±0.01 ^{bc}	3.48±0.01 ^{cd}	3.81±0.00 ^c	3.41±0.01 ^{ce}	3.43±0.01 ^{ce}	3.41±0.01 ^{cd}
T4	W5	3.74±0.01 ^{ac}	3.63±0.01 ^{ab}	3.62±0.01 ^b	3.67±0.01 ^b	3.80±0.00 ^c	3.55±0.01 ^{cd}	3.54±0.01 ^{be}	3.57±0.01 ^{bd}
	W1	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W3	3.24±0.02 ^c	3.19±0.02 ^{bd}	3.16±0.02 ^{de}	3.19±0.02 ^d	3.11±0.02 ^e	3.16±0.02 ^{de}	2.93±0.04 ^{de}	2.47±0.10 ^{df}
	W4	3.57±0.01 ^b	3.51±0.01 ^b	3.50±0.01 ^{bc}	3.50±0.01 ^c	3.50±0.01 ^{de}	3.41±0.01 ^{ce}	3.27±0.02 ^{cf}	3.11±0.02 ^d
	W5	3.69±0.01 ^{ad}	3.65±0.01 ^{ab}	3.65±0.01 ^{ac}	3.63±0.01 ^{bc}	3.63±0.01 ^d	3.51±0.01 ^{cd}	3.52±0.01 ^{be}	3.52±0.01 ^c
T5	W1	<2 ^e	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f	<2 ^g	<2 ^f
	W2	3.27±0.02 ^{bd}	3.35±0.01 ^{bd}	3.29±0.02 ^{ce}	3.06±0.03 ^{df}	3.48±0.01 ^{df}	3.39±0.01 ^d	3.35±0.01 ^{cd}	3.19±0.02 ^{ce}
	W3	3.77±0.01 ^{ab}	3.70±0.01 ^{ab}	3.75±0.01 ^{ab}	3.52±0.01 ^{be}	3.69±0.01 ^{cf}	3.76±0.01 ^{bf}	3.60±0.01 ^{bd}	3.58±0.01 ^{bd}
	W4	3.80±0.00 ^{ab}	3.76±0.01 ^a	3.91±0.00 ^{ab}	3.83±0.00 ^{ab}	3.98±0.00 ^b	3.98±0.00 ^b	4.01±0.00 ^{ab}	3.87±0.00 ^{ab}
	W5	3.96±0.00 ^a	3.86±0.00 ^a	3.93±0.00 ^a	3.98±0.00 ^a	4.23±0.00 ^a	4.26±0.00 ^a	4.25±0.00 ^a	3.92±0.00 ^a

Legends: Tt. =treatments, T1= *L. lactis*+fruits/vegetables, T2= *P. pentosacues*+fruits/vegetables, T3= *L. lactis* + *P. pentosaceus*+fruits/vegetables, T4=*L.lactis* + *P. pentosaceus*+ *E. facecium* + fruits/vegetables, T5= control. The mean values represented by the same letter are statically not shown significance variation at P<0.05 across the column.

6.4. DISCUSSION

The study showed that combining *L. lactis* with *P. pentosaceus* or *E. faecium* effectively preserved fruits and vegetables, suggesting their potential as natural preservatives. However, more research is needed to confirm their safety and effectiveness across different food types. LAB strains, especially *Lactococcus* species like *L. lactis* with bacteriocin production, have proven antifungal properties, making them attractive for biopreservation. Further investigation is required to understand their mechanisms and optimize their application in diverse foods. *L. lactis* stands out among *Lactococcus* species for its antifungal activity and ability to inhibit various fungi on fruits and vegetables (Siroli et al., 2015). Other LAB species that have been found to produce bacteriocins with antifungal activity include *E. faecium*, and *P. acidilactici*. These bacteriocins have potential applications in food preservation and crop protection.

The study showed that probiotic LAB treatments can significantly extend the shelf life of fruits and vegetables by preventing mold growth. Avocados and oranges treated with specific LAB strains remained mold-free for 32 and 24 days, respectively. Regardless of whether the LAB strains were used alone or in combination, they consistently extended shelf life to three weeks without fungal decay, with *P. pentosaceus* being the most effective. Several authors have reported similar findings, highlighting the efficacy of LAB and metabiotics in food preservation (Agriopoulou et al. 2020; Badea, Diguță, et al., 2022; Dhundale et al. 2018). Hence, these LAB strains are classified as food-grade and are employed as preservatives to inhibit pathogen growth. They also serve as potential alternatives to antibiotics in preventing or treating various food-related issues (Terpou et al., 2019).

The findings of this study align with previous research demonstrating the effectiveness of LAB strains in extending the shelf life of fruits and vegetables. For instance (Ebah et al., 2019) showed that individual LAB strains significantly prolonged the shelf life of tomatoes, similar to the results found in this study. Additionally, the synergistic effect observed when combining multiple LAB strains for food preservation, as highlighted in this study, has been reported by other researchers as well (İrkin et al., 2015). Regarding potatoes, the success of combined LAB strains in maintaining freshness for an extended period also corresponds with previous findings that emphasized the importance of strain selection and combination for specific produce types (Badea et al., 2022; Linares et al. 2018). Moreover, the remarkable preservation of carrots with various LAB combinations aligns with previous

studies showcasing the versatility of LAB treatments in extending the shelf life of different vegetables (Barbosa et al., 2017a). Overall, these comparative discussions highlight the consistency and reliability of LAB-based treatments in preserving the freshness and quality of various fruits and vegetables, as evidenced by multiple studies in the field.

The study demonstrates the substantial potential of LAB strains in extending the shelf life of fruits, showcasing the effectiveness of combining *L. lactis* JULABBk39 and *P. pentosaceus* JULABB01. Notably, avocados without probiotic LAB developed mold by day 16, whereas the combination of *L. lactis*, *P. pentosaceus*, and *E. faecium* preserved them for 36 days without spoilage, maintaining both texture and appearance (Figure 6.1). Similarly, untreated oranges showed mold growth by day 13, but LAB-treated ones, particularly those treated with *P. pentosaceus* and *E. faecium*, remained healthy for 36 days. For bananas, which spoiled by day 12 without treatment, the three-strain LAB combination extended their shelf life by 24 days (Figure 6.2).

These findings are consistent with previous research indicating that LAB strains can significantly prolong the freshness of various fruits and vegetables (Cirat et al., 2024; Tenea et al., 2024). The observed effectiveness of combining multiple LAB strains, particularly in the case of avocados and oranges, underscores the synergistic effects of these bacteria in inhibiting mold growth and spoilage (De Simone et al., 2021). Moreover, the versatility of LAB treatments in preserving different types of fruits, as shown in this study, aligns with earlier studies that have highlighted the broad applicability of LAB in food preservation (Aguirre et al., 2024).

The study highlights the effectiveness of LAB strains in extending the shelf life of fruits, specifically avocados, oranges, and bananas. A combination of *L. lactis* JULABBk39 and *P. pentosaceus* JULABB01 significantly prolonged the shelf life of these fruits by inhibiting mold growth. For instance, avocados without LAB showed mold by day 16, whereas the combination of *L. lactis*, *P. pentosaceus*, and *E. faecium* prevented spoilage for 36 days. Similarly, untreated oranges developed mold by day 13, while LAB-treated oranges lasted 36 days, particularly with *P. pentosaceus* and *E. faecium*. Untreated bananas spoiled by day 12, but the three-strain combination extended their shelf life by 24 days.

These findings align with previous research demonstrating the antimicrobial properties of LAB strains and their ability to extend the shelf life of various produce (Agriopoulou et al., 2023; Linares-Morales et al. 2018). The synergistic effect observed with combined LAB strains in this study supports earlier reports that multiple LAB strains can be more effective than single strains in preserving food (Barbosa et al. 2017). Additionally, the study's results confirm the versatility of LAB treatments, which have been shown to preserve a wide range of fruits and vegetables in previous studies.

The microbiological analysis of fruits and vegetables treated with LAB isolates highlights their significant role in preserving produce, aligning with previous studies on the antimicrobial properties of LAB. In this study, avocados, oranges, and bananas treated with LAB remained mold-free and maintained low microbial counts for up to five weeks, particularly avocados, which stayed well-preserved the longest. This result is comparable to earlier findings where LAB strains effectively extended the shelf life of various fruits by inhibiting spoilage organisms (Ebah, Wusuum, 2019). For vegetables, the LAB isolates (*L. lactis*, *P. pentosaceus*, and *E. faecium*) demonstrated a similar efficacy. They prevented fungal spoilage and maintained microbiological safety for over two weeks, with no detectable levels of AMBC, *E. coli*, Staphylococci, yeast, or molds. This effectiveness is consistent with prior research showing LAB's capacity to enhance the shelf life and quality of vegetables through their antimicrobial activity (P. Ibrahim et al., 2021).

Comparatively, this study reinforces the findings of previous research while extending the understanding of LAB's effectiveness across different types of produce. Both fruits and vegetables benefited significantly from LAB treatments, highlighting the versatility and broad applicability of these probiotics in food preservation. These results support the potential of LAB as natural bio-preservatives, offering a sustainable alternative to chemical preservatives and contributing to improved food safety and reduced waste.

The study demonstrated that combining *Lactococcus lactis* with *Pediococcus pentosaceus* or *Enterococcus faecium* effectively preserved fruits and vegetables, significantly extending their shelf life by inhibiting mold growth. Avocados and oranges treated with specific LAB strains remained mold-free for 32 and 24 days, respectively. All LAB isolates, whether used singly or in combination, consistently extended the shelf life of produce to three weeks without fungal decay, with *P. pentosaceus* being particularly effective. These findings align with previous studies that have shown the

antimicrobial properties of LAB and their potential as natural preservatives in food preservation (Agriopoulou et al., 2020; Badea et al., 2022; Dhundale et al., 2018).

Moreover, the study confirmed that LAB strains are food-grade and can be used as preservatives to inhibit pathogen growth, serving as potential alternatives to antibiotics (Terpou et al., 2019). This study's results also support the findings of Ebah et al. (2019), who reported that individual LAB strains significantly prolonged the shelf life of tomatoes. Additionally, the synergistic effects observed when combining multiple LAB strains for food preservation were consistent with earlier reports by Irkin et al. (2015).

7.

BIO-PRESERVATION OF MILK USING BACTERIOCIN PRODUCING LACTIC ACID BACTERIA ISOLATED FROM ETHIOPIAN TRADITIONAL FERMENTED DAIRY PRODUCTS (*ERGO*).

The findings of this paper is published under probiotics and antimicrobial proteins as

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ABSTRACT

Bacteriocin, a key antimicrobial peptide produced by lactic acid bacteria, inhibits food-borne pathogens and maintains a healthy microbial balance. It targets closely related strains, protecting food from pathogens and enhancing shelf life. This natural preservation mechanism aligns with safe label products, sustainable practices, and reduces reliance on synthetic preservatives. Therefore, the main objective of this study is to assess the antibacterial activity and bio-preservation potential of bacteriocin-producing LAB isolated from Ethiopian traditional fermented dairy products in raw milk from Jimma town. Bacteriocin-producing LAB were tested for antimicrobial activity against food-borne pathogens like *S. aureus*, *E. coli*, *L. monocytogenes*, and *S. Typhimurium*. Milk samples were inoculated with potent LAB isolates to improve shelf life, and food quality parameters like physiochemical, microbiological, and proximate analysis that were examined before and after the sample inoculation. The study examined food quality parameters after inoculation with bacteriocin-producing LAB. Results showed that probiotic LAB isolates effectively inhibited foodborne pathogens like *E. coli*, *S. aureus*, and *L. monocytogenes*, with inhibition zones ranging from 22.00 ± 0.57 to 34.13 ± 0.57 . *E. faecium* and *L. lactis* demonstrated strong antagonistic effects against *E. coli*, with inhibition zones of 33.00 ± 0.58 and 30.33 ± 0.34 , respectively. The isolates demonstrated co-aggregation potential with pathogens, with *L. lactis* isolates and their combinations with other probiotic LAB demonstrated potential aggregation. Additionally, after 60 minutes of treatment, all the probiotic isolates were able to demonstrate the potential decrease and destruction of extracellular materials (NA) from pathogens as seen by the effects of CFS on the release of extracellular material from pathogens at 260 nm. The putative probiotic isolates (*Lactis* JULABE35, *E. faecium* JULABE23, and *P. pentosaceus* JULABE05) were effective in reducing *L. monocytogenes* in raw milk, as demonstrated by the results of the bio-preservative assay. After 7-8 days, milk samples diagnosed with *L. lactis* JULABE35, *E. faecium* JULABE23, and *P. pentosaceus* JULABE05 isolates showed a complete reduction of *L. monocytogenes*. The bio-preservation capability of bacteriocin-producing LAB on raw milk, revealed that inoculation of *L. lactis* JULABE35, *E. faecium* JULABE 23, and *P. pentosaceus* JULABE05 isolates prolonged the shelf life of milk at 4°C storage for ten days. As a result, milk samples inoculated with these three probiotic isolates were kept for a maximum of ten days, while milk samples without probiotic LAB were kept for six days. The pH of milk samples inoculated with probiotic isolates ranged from 6.40 to 3.99, whereas milk titrate acidity ranged from 0.0227 ± 0.0015 to -0.215 ± 0.013 . Apart from these, milk samples preserved with probiotic and bacteriocin-producing isolates demonstrated the most acceptable and promising sensory evaluation, with an overall good look and acceptance. Furthermore, milk samples inoculated and preserved with these potential bacteriocin-producing LAB isolates showed good proximate analysis: moisture content (59.67 ± 0.58 to 77.67 ± 0.58), ash content (4.23 ± 0.01 to 4.99 ± 0.01), and protein content (15.94 ± 0.01 to 25.00 ± 0.26), all of which showed significant variation with milk kept without bacteriocin-producing isolates at $P < 0.05$. As a result, the isolated chemicals employed in this study can be used as food additives or as food preservatives. As a consequence, this investigation found potential bacteriocin-producing LAB isolates in Ethiopian traditional fermented dairy products (Ergo). As a result, bacteriocin-producing LAB has been found to be effective against food-borne infections and may be employed as a promising food preservative, often used in response to customer requests for natural products.

Key words: Bacteriocin, food borne pathogens, Food preservative, LAB, Natural products

7.1. INTRODUCTION

Customers are becoming more concerned about the potential side effects and health impacts of traditional food preservatives, which is why they prefer fresh, naturally preserved foods with few synthetic ingredients (Balciunas et al., 2013). Microorganisms can withstand different temperatures, raising concerns about food safety and contamination. This drives the preference for fresh, naturally preserved products to avoid food-borne diseases (Casaburi et al., 2016; Ricci et al., 2019). Food fermentation, which involves the use of microbes, is a traditional technique of creating and preserving food, with many people relying on fermented foods as staples (Sharma et al., 2020). Bio preservation uses natural microflora and antimicrobial compounds like bacteriocins to extend food shelf life and improve food safety (Castellano et al., 2017). Lactic acid bacteria (LAB) regulate the microflora of many foods during storage because they are non-toxic and antibacterial.

LAB, recognized as safe, are utilized in food fermentation and preservation, offering various benefits like proteolytic activity, fermentation abilities, and antimicrobial production. These qualities make them suitable for human health and ideal for food preservation (Cholakov et al., 2017; Gıda et al., 2016). They also produce organic acids, ethanol, aromatic compounds, and bacteriocins, improving food quality, maturation, and bio-preservation (De Martinis et al., 2002). Studies highlight LAB's potential in extending shelf life and promoting consumer health benefits, making them attractive for industrial use in functional foods and beverages (Cholakov et al., 2017; Kagkli et al., 2016; Tinello et al., 2017). Bacteriocins produced by LAB exhibit broad-spectrum antimicrobial activity against various pathogens, offering a natural alternative to antibiotics and synthetic preservatives (Cholakov et al., 2017; Gıda et al., 2016; Kagkli et al., 2016).

The growing demand for natural preservatives has led to the exploration of LAB-derived bacteriocins, which prevent pathogenic and spoilage bacteria, ensuring healthier food products with increased shelf life and reduced health risks (Daba & Elkhateeb, 2020; Hassan et al., 2020; Soltani et al., 2021). This research opportunity could provide insights into bacteriocins' usefulness in food preservation, leading to the development of novel natural preservatives and contributing to food safety and quality standards.. Identifying specific LAB isolates producing bacteriocins in Ethiopian fermented products can provide natural alternatives and improve food safety standards. This study evaluated bacteriocin-producing LAB from Ethiopian traditional fermented dairy products for antimicrobial activity and bio-preservative potential on raw milk samples.

7.2. MATERIALS AND METHODS

7.2.1. Bacterial isolates and growth conditions

The LAB isolates (*L. lactis* E05, *E. faecium* E23, and *P. pentosaceus* E35) were obtained from Jimma University culture collection centre (Amenu and Bacha-----). From where? Pathogenic bacteria (*S. aureus*, *E. coli*, and *L. monocytogenes*) were sourced from the Ethiopian Health and Nutrition Research Institute (EHNRI). These pathogens were identified and subcultured in their respective selective culture media. Cell-free supernatant from the samples was placed in wells and allowed to diffuse for 1 hour. Plates were then incubated at 37°C for 48 h, and clear zones of inhibition were observed (Babatunde et al., 2014).

7.2.2. Antibacterial activity of live culture

Bacteriocin-producing LAB were tested for antibacterial activity using the agar spot method on MRS agar plates layered with pre-inoculated BHI soft agar containing pathogens. Clear zones around LAB colonies indicated antibacterial action (Hwanhlem et al., 2011). For co-culture ability assessment, live probiotic cells (500 µl) were mixed with an equal volume of pathogenic cells (1:1) and incubated at 37°C for 24 h. Viable cell counts were determined using selective media: MRS for LAB and MacConkey for *E. coli*, Mannitol salt for other pathogens (Afolayan & Ayeni, 2017).

7.2.3. Co-aggregation potential of LAB isolates

To accomplish this, 500 µl of probiotic LAB isolates and bacterial pathogens were combined and incubated at 37°C for 24 h. The absorbance at 600 nm of probiotic monocultures and bacterial pathogen co-cultures was then measured using a UV spectrophotometer. The co-aggregation percentage was

calculated as follows (Bao et al., 2012).
$$\frac{[\frac{A_{\text{pathogens}} + A_{\text{probiotic}}}{2} - (A_{\text{mix}})]}{(A_{\text{pathogens}} + A_{\text{probiotic}})/2} * 100$$

Where A= absorbance

7.2.4. Effects of LAB isolates on bacterial (pathogens) growth

To assess LAB isolates' effectiveness against food-borne pathogens, their cell-free supernatants (CFS- 10 ml, in 90 ml of pathogenic bacteria were mixed with pathogenic strains and incubated. The LAB cells were then centrifuged, filter sterilized, and mixed (MRS broth) with pathogenic cells for incubation. The inhibitory effect of LAB CFS on bacterial growth was monitored by counting viable

cells of each pathogen at 2-hour intervals on selective media, using pathogen cell cultures without CFS as controls (Kalhor et al., 2023).

7.2.5. Bio preservation potential of LAB isolates

The bio-preservative potential of LAB isolates was tested on raw milk contaminated with *L. monocytogenes*. Each 100 ml milk sample received 2 ml of probiotic LAB and *L. monocytogenes*. The samples were stored at 37°C for 7 days, and daily milk samples were taken for total bacterial count assessments. Counts were conducted on Listeria selective agar and MRS agar, with milk samples containing only *L. monocytogenes* used as controls (Fernandes et al., 2017).

7.2.6. Bio preservation Study of milk bacteriocin producing LAB isolates

7.2.6.1 pH Determination

A conical flask containing 10 ml of the milk samples was filled with the samples, and the pH meter was used to measure the pH over the course of seven days (Didari et al., 2014).

7.2.6.2.Determination of Total Titratable Acidity (TTA)

Milk's total titratable acidity was measured by adding 1ml of phenolphthalein indicator to 200ml of boiled distilled water. The solution was neutralized with 0.1M Sodium hydroxide and transferred to separate sterile volumetric pipette. Total acidity was calculated using the formula (Yang et al., 2016).

$$Lactic\ acid\ (\%) = \frac{Titre\ value * normality\ of\ alkali * 9}{volume\ of\ samples}$$

7.2.6.3.Proximate analysis

7.2.6.3.1.Determination of Moisture Content

Metalic dishes were dried at 80°C for 20 minutes before being weighed in a desiccator. Five milliliters of milk were weighed in the dishes. After 24 h of drying, the dishes were transferred to a desiccator to cool. The moisture content, or weight loss of milk during drying, was calculated using the moisture percentage method (Yang et al., 2016). $\frac{W_2 - W_3}{W_2 - W_1} * 100$

7.2.6.3.2.Determination of Ash Content

Crucibles were cleansed and dried in the oven, after drying they were cooled in the desiccator and weighed (W1). Two millileter of the milk sample was placed in the crucibles and weighed (W2). They were transferred into the muffle furnace at 550° C for 2 h then removed and cooled in the desiccator and weighed (W3)(Perring & Tschopp, 2019). The % ash was calculated as: $\frac{W3 - W1 \times 100}{W2 - W1}$.

7.2.6.3.3.Protein determination

The Kjeldahl method is a procedure commonly used for determining the protein content in samples. Here are the steps involved: Samples containing protein was accurately weighed and placed into a Kjeldahl digestion flask. Concentrated sulfuric acid and a catalyst, often copper sulfate, are added to the flask with the sample. The mixture is then heated until it becomes clear, indicating complete digestion of the sample. After digestion, the sample is allowed to cool down. It is then diluted with distilled water to reduce the acidity. The diluted sample is transferred to a distillation apparatus. Sodium hydroxide (NaOH) solution is added to neutralize the excess sulfuric acid, resulting in the release of ammonia. The released ammonia is distilled and collected in a boric acid solution containing an indicator. This causes the solution to change color, indicating the presence of ammonia. The boric acid solution containing absorbed ammonia is titrated with a standard acid solution, typically hydrochloric acid (HCl), to determine the volume of acid required to neutralize the ammonia. Using the volume of acid used, the normality of the acid, and the molecular weight of nitrogen, the nitrogen content of the sample is calculated as a percentage. This nitrogen content is then multiplied by a specific factor, often 6.25 for most proteins, to determine the protein content as a percentage.

7.2.7.Potential of bacteriocin producing LAB to extend the shelf life of milk sample

The study examined the shelf life of milk treated with bacteriocin-producing Lactic acid bacteria. Milk samples were combined with 5 ml of bacteriocin-producing CFS and refrigerated at various temperatures (4 and 25 °C) for 13 days. Every 24 h, samples were assessed for viable count, color, and aroma changes. Shelf life extension was gauged by comparing total viable counts and subjective appearance over each 24-hour interval of inoculation, using a control milk sample for comparison (Ali et al., 2018).

7.2.8.Sensory Property of Milk

Sensory quality attributes such as colour, aroma, texture, taste and general acceptability of the bacteriocin incorporated milk was evaluated using a five-point Hedonic scale. The milk sample were served to ten panelists for rating on five-point scale ranging from score1 (dislike much), 2 (dislike), 3 (like), 4(like much), 5(like very much) Compared with the control without bacteriocin. Sensory scores generated were statistically analyzed using ANOVA (Zhi et al., 2016).

7.3. RESULTS

Ethiopian dairy product probiotics were tested for antimicrobial activity and bio-preservation potential. Live cells and their CFS showed antimicrobial effects against food-borne pathogens and extended raw milk shelf life. Characterization and purification of antibacterial compounds from LAB isolates revealed strong activity across a broad pH (2-10) and temperature range (40-80°C). Selected isolates, *L. lactis*, *E. faecium*, and *P. pentosacues*, were potent against pathogens like *L. monocytogenes* and survived simulated gut conditions. All isolates displayed antimicrobial potential against *E. coli*, *S. aureus*, and *L. monocytogenes*, with *P. pentosacues* JULAB E05 showing the highest inhibition zone against *S. Typhimurium* (33.33 mm) (Table 7.1.).

Table 7. 1. Antimicrobial activity of probiotic **LAB** isolates from Ethiopian traditional fermented diary product (*Ergo*) against food borne pathogens as indicated by zones of inhibition (mm)

No.	Pathogens	LAB isolates (probiotics)		
		<i>P. pentosaceus</i> JULAB E35	<i>E. faecium</i> JULABE23	<i>L. lactis</i> JULABE05
1.	<i>Escherichia coli</i>	29.00±0.57 ^{bc}	33.00±0.58 ^a	30.33±0.34 ^a
2.	<i>Staphylococcus aureus</i>	27.00±0.58 ^{cd}	31.67±0.88 ^a	24.33±0.82 ^{bc}
3.	<i>Salmonella Typhimurium</i>	33.00±1.12 ^a	22.00±0.57 ^c	24.00±0.56 ^{bc}
4.	<i>Listeria monocytogenes</i>	28.33±0.82 ^{bcd}	20.67±0.67 ^c	25.00±1.00 ^{bc}
5.	<i>Bacillus cereus</i>	26.06±0.34 ^d	26.00±0.57 ^b	22.00±1.16 ^c
6.	<i>Pseudomonas aeruginosa</i>	34.33±0.57 ^a	31.67±1.120 ^a	24.67±0.34 ^{bc}
7.	<i>Klebsella pneumonia</i>	30.00±0.57 ^a	22.33±1.86 ^c	24.33±0.95 ^{bc}
	P value	0.0130	0.000	0.001

Note: the data for this study was analysis using One Way Anova variance using post hoc multiple comparison (Duncan), the mean represented by different letter are significantly different at p<0.05.

7.3.1. Co-aggregation potential of probiotic isolates

Probiotic LAB isolates were assessed for their co-aggregation ability with *E. coli*, *S. aureus*, and *L. monocytogenes*. They all showed promising co-aggregation rates ranging from 55.53% to 101.00%. Notably, *L. lactis* exhibited high co-aggregation rates with all thee pathogens, with rates of 101.00%, 80.33%, and 74.00%, respectively. This indicates the potential for these isolates to colonize the gut effectively (Figure 7.1.).

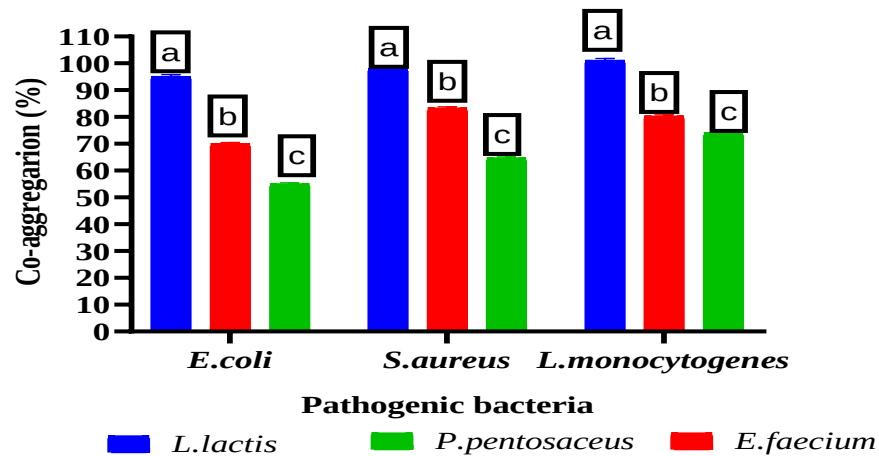


Figure 7. 1. The co-aggregation potential of probiotic LAB isolates from Ethiopian traditional fermented dairy product (*Ergo*) with selected food borne pathogens (*Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*).

Note: the data for this study was analysis using One Way Anova variance using post hoc multiple comparison (Duncan), the mean represented by different letter are significantly different at $p < 0.05$.

7.3.2. Co-culture potential of probiotic with pathogens.

It was observed that the survival levels of *E. coli*, *S. aureus*, and *L. monocytogenes* decreased significantly, indicating the potential of probiotics to inhibit these pathogens. Among the probiotic isolates, *P. pentosaceus* exhibited the most substantial reduction in pathogen survival, suggesting a notable effect on preventing food-borne infections (Figure 7.2).

7.3.1. Effects of CFS on 260nm releasing material (NA) from pathogens

The study evaluated LAB isolates' impact on the release of extracellular material from *E. coli*, *S. aureus*, and *L. monocytogenes* at 260 nm absorbance, as shown in Figure 7.3. Compared to the control, all LAB isolates increased the release of extracellular components from these pathogens. This suggests that the isolates' cell-free supernatant (CFS) can kill pathogens, release extracellular components, and accelerate cell death, as indicated by the 260 nm optical density. This indicates that these probiotic isolates have the potential to inhibit pathogenic bacterial growth by releasing extracellular material (Figure 7.3.).

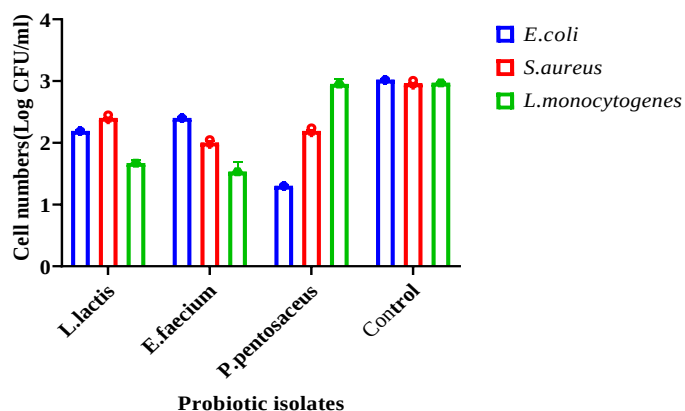


Figure 7. 2. Evaluating the co-culture potential and the interaction of probiotic LAB isolated from Ethiopian traditional dairy products with some selected food borne pathogens.

Note: The data for this study was analysis using One Way Anova variance using post hoc multiple comparison (Duncan), the mean represented by different letter are significantly different at $p < 0.05$.

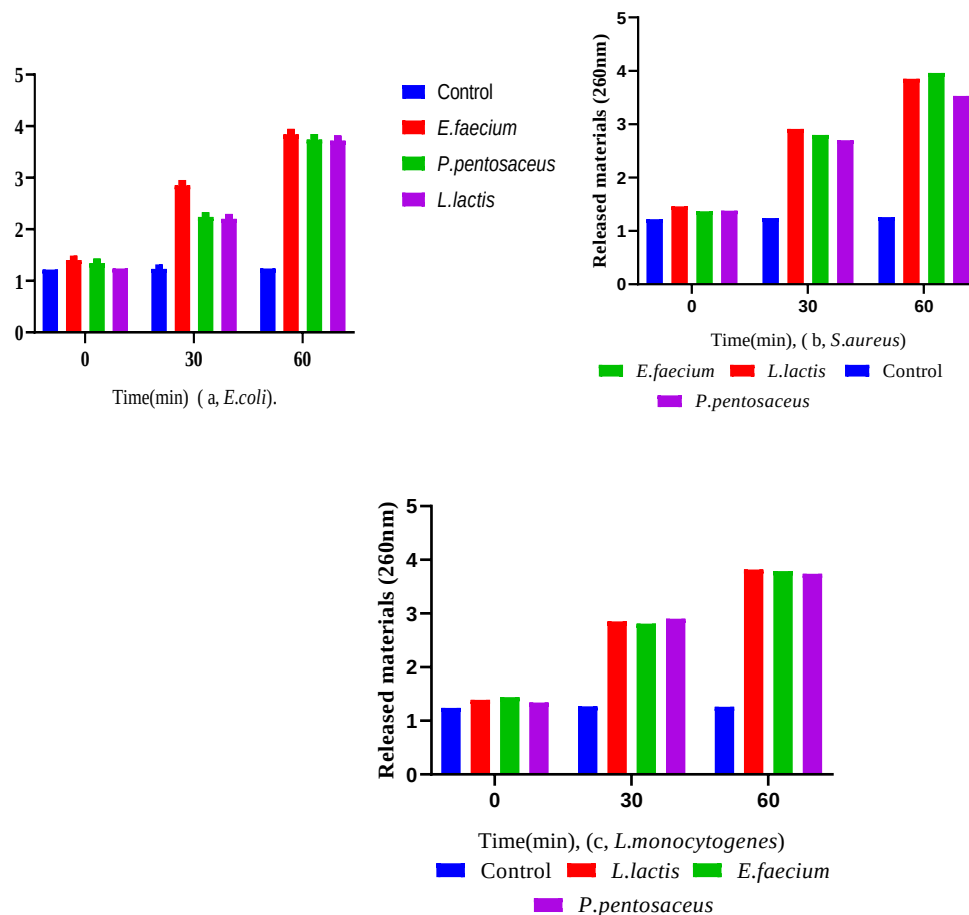


Figure 7. 3. Evaluating the effects of CFS on releasing materials (nucleic acid and other materials) (260nm) some selected food borne pathogens.

7.3.2. Effects of CFS on growth of food borne pathogens

All three LAB isolates tested positive for antagonistic activities against food-borne pathogens in this investigation (Figure 7.4). As a result of the CFS inoculation, there was a reduction in the proliferation of food-borne infections. All of the probiotic isolates demonstrated a broad antibacterial range against gram positive and gram negative foodborne pathogens. *L.lactis* CFS demonstrated promising antibacterial activity against all three foodborne pathogens. The formation of antimicrobial compounds such as organic acids, hydrogen peroxide, carbon iodides, bacteriocins, and bacteriocin-like molecules may be responsible for these antimicrobial actions (Figure 7.4).

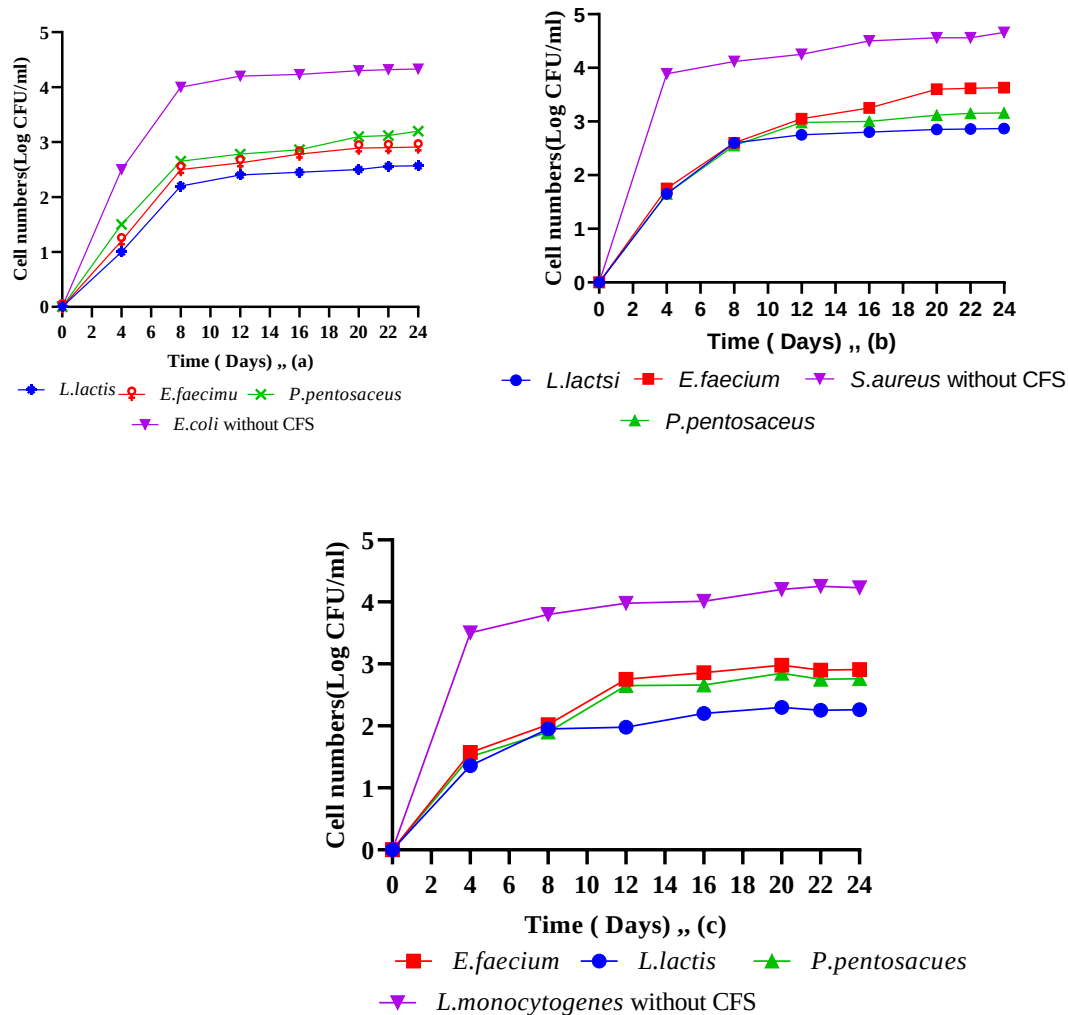


Figure 7. 4. Effects of cfs produced by probiotic isolates on the growth of food borne pathogens (*E. coli*, *S. aureus*, and *L.monocytogenes*).

7.3.3. Bio-preservation of Milk

Potential probiotic LAB isolated from *Ergo*, Ethiopian traditional fermented dairy product showed potential preservative effects on the raw milk against *L. monocytogenes*. The growth of *L. monocytogenes* was gradually reduced with time increases. There was no growth observed after 8 days on raw milk inoculated with three probiotic LAB isolates (*L. lactis* JULABE35, *E. faecium* JULABE 23 and *P. pentosaceus* JULABE05) (Figure 7.5).

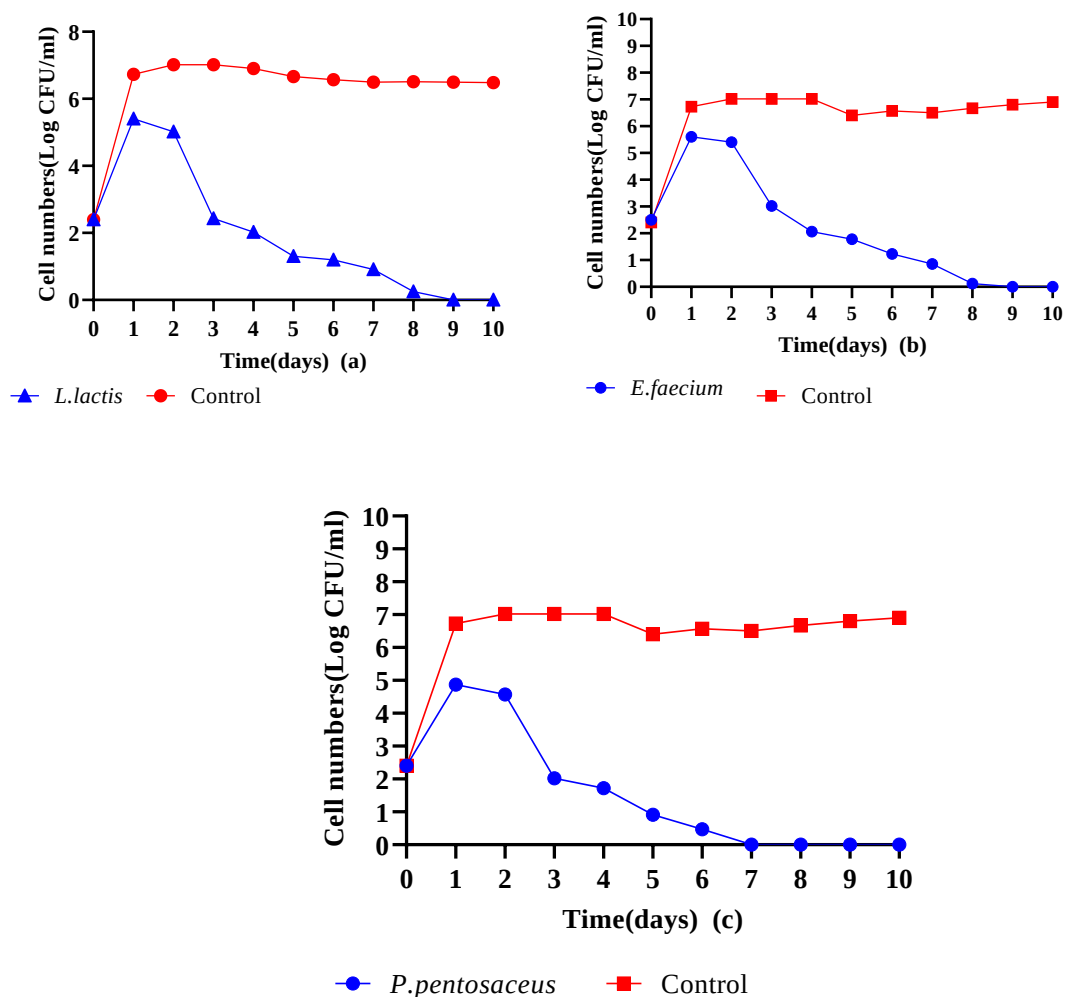


Figure 7. 5 .Bio-preservation effects of probiotic LAB isolates, (a), *L. lactis*, (b), *E. faecium* and, (c), *P.pentosaceus* during the ten days' storage of raw milk.

7.3.4. Effects of probiotic isolates on pH and TTA of raw milk

As shown figure below (Figure 7.6 (a, b)), demonstrated that the effects of the probiotic LAB isolates on milk pH, and the pH. Figure 6, showed the pH of bacteriocin inoculated milk sample. A gradual decrease in pH was observed throughout the period of storage. Hence, pH decreases from initial 5.4 ± 0.1 to 3.9 ± 0.1 while that of the control (milk without bacteriocin) decreases from 5.93 ± 0.11 to 5.0 ± 0.1 . On the other hand, the treatable acidity of milk inoculated with probiotic LAB isolates increased throughout the study of the storage time. The total treatable acidity of the milk samples increased from 0.02 to 0.214 percent of lactic acid (Figure 7.6).

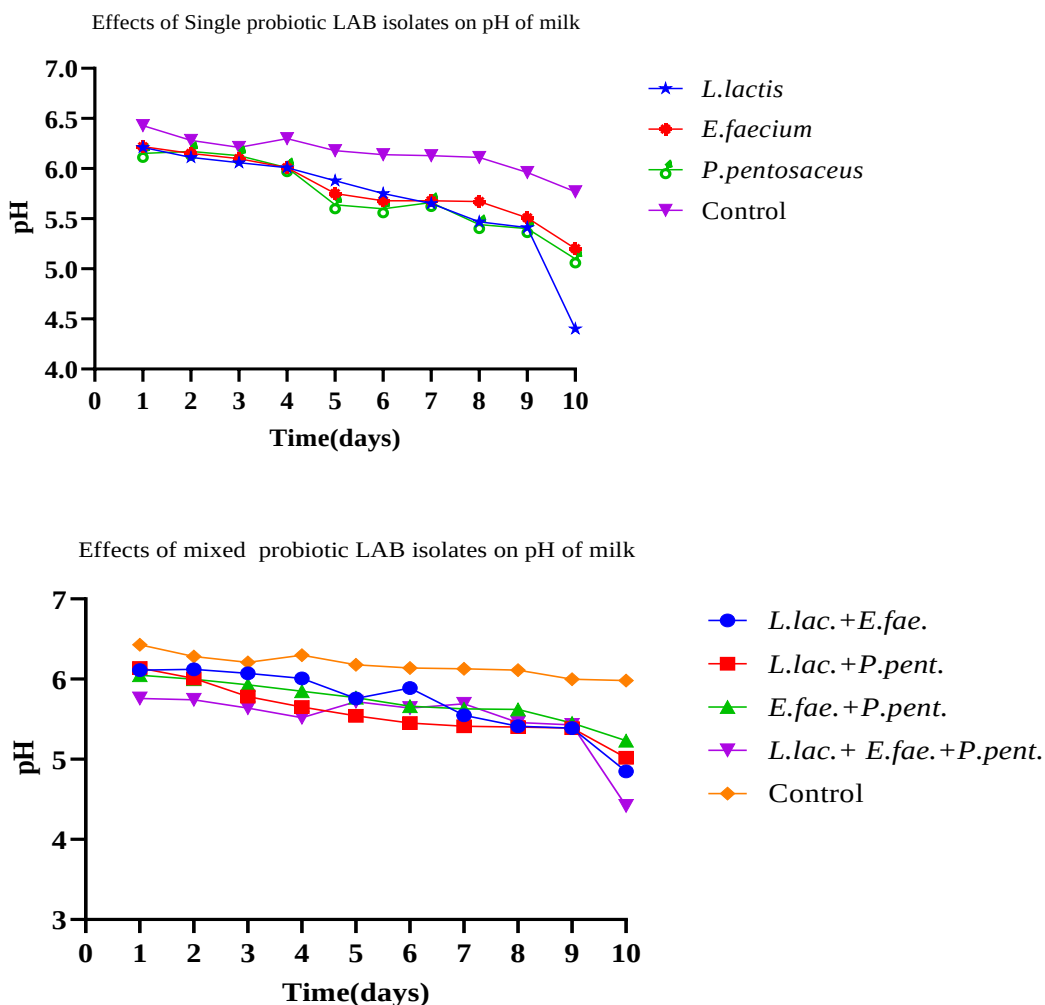


Figure 7. 6. pH of the milk samples inoculated with single and mixed cultures of probiotic LAB isolated from Ethiopian traditional fermented foods (*Ergo*).

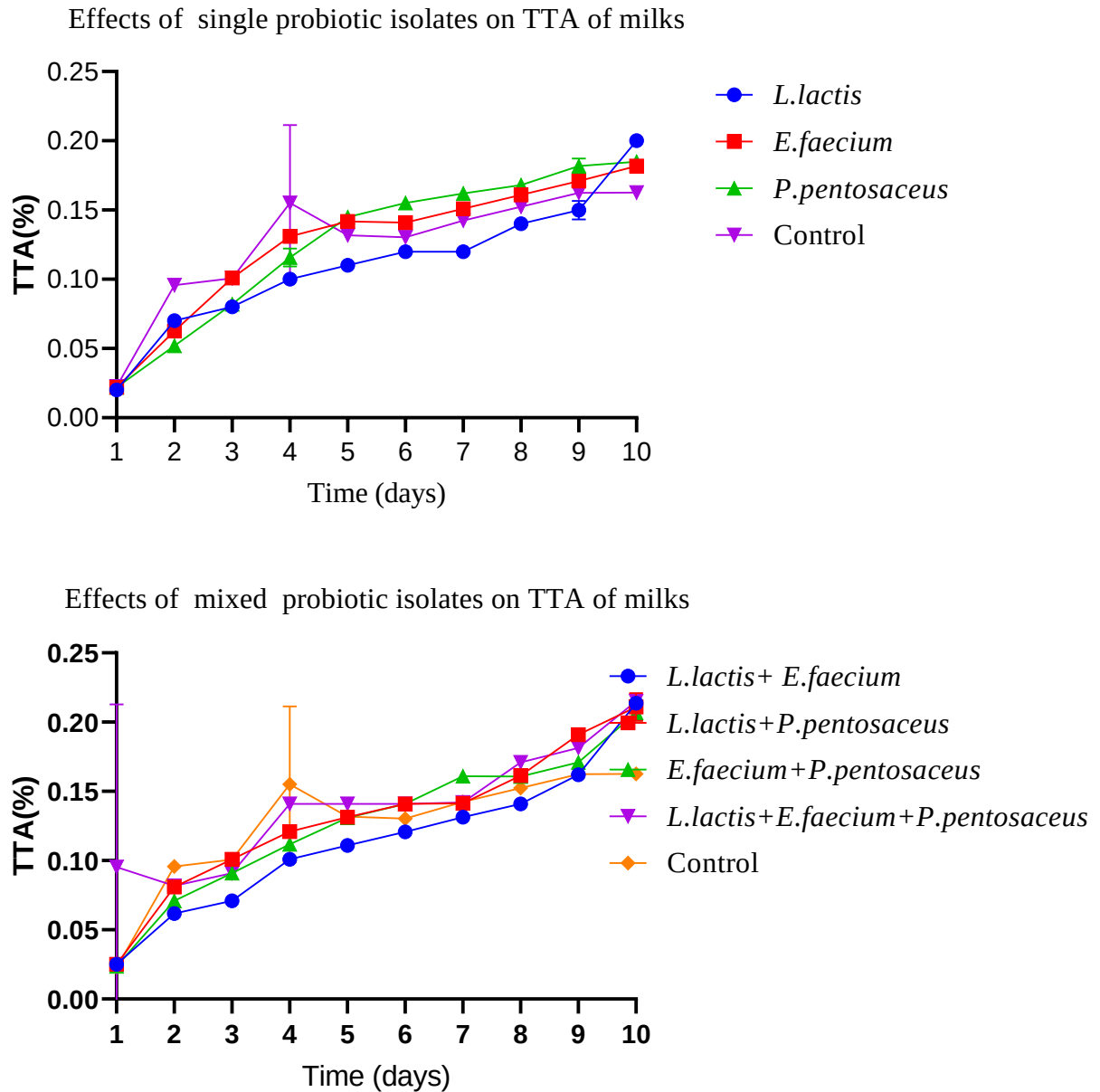


Figure 7. 7. pH of the milk samples inoculated with single and mixed cultures of probiotic LAB isolated from Ethiopian traditional fermented food (*Ergo*)

7.3.5. Proximate analysis of milk samples

In this section, the proximate analysis of milk samples treated with probiotic and bacteriocin producing LAB isolates were evaluated and the milk samples inoculated with bacteriocin producing LAB isolates demonstrated good proximate composition. The proximate composition of milk samples treated with bacteriocin producing LAB showed that there was a statistical difference between the moisture contents of milk samples inoculated with bacteriocin producing probiotic LAB isolates. Accordingly, the milk samples preserved with the three combinations (*L. lactis*, *E. faecium* and *P. pentosacues*) showed the most influential moisture composition followed by the two combinations (*L. lactis* + *E. faecium*).

On other hand, the ash composition of milk samples treated with probiotic LAB isolates ranged 4.23 ± 0.01 to 4.99 ± 0.01 , with the milk inoculated with the milk inoculated with *L. lactis*, *E. faecium* and *P. pentosacues* showed the highest ash composition, which accounted 4.99 ± 0.01 and the milk sample without probiotic showed the least ash composition. Furthermore, the milk samples inoculated with *Lactococcus lactis* and its composition showed the maximum protein composition in compared to the milk samples inoculated without probiotic LAB isolates (Figure 7.2).

Table 7. 2. Proximate analysis (moisture, ash content and protein composition) of milk samples inoculated by probiotic LAB isolates as food preservatives.

S/N	Treatments	Moisture	Ash contents	Proteins
1)	T1= <i>Lactococcus lactis</i>	64.33 ± 0.58^b	4.88 ± 0.03^c	19.82 ± 0.14^d
2)	T2= <i>Enterococcus faecium</i>	64.67 ± 0.58^b	4.79 ± 0.01^d	17.10 ± 0.66^e
3)	T3= <i>P. pentosacues</i>	64.33 ± 0.58^b	4.88 ± 0.01^c	22.54 ± 0.16^c
4)	T4= <i>L. lactis</i> + <i>E. faecium</i>	61.67 ± 0.58^c	4.96 ± 0.05^{ab}	23.78 ± 0.43^b
5)	T5= <i>L. lactis</i> + <i>P. pentosaceus</i>	62.67 ± 0.58^c	4.90 ± 0.08^{bc}	23.50 ± 0.00^b
6)	T6= <i>E. faecium</i> + <i>P. pentosa</i>	64.67 ± 0.58^b	4.68 ± 0.01^e	16.33 ± 0.26^f
7)	T7= <i>L. lac</i> + <i>E. fae</i> + <i>P. pent.</i>	59.67 ± 0.58^d	4.99 ± 0.01^a	25.00 ± 0.26^a
8)	T8= Control	77.67 ± 0.58^a	4.23 ± 0.01^f	15.94 ± 0.11^f

7.3.6. Sensory property

Similarly, Table 7.3, showed the sensory property of milk samples inoculated with probiotic and bacteriocin producing LAB isolates and the milk samples inoculated with probiotic LAB isolates had shown the most acceptable sensory quality incopmared to the milk preserved without probiotic isolates. The milk inoculated with thee combinations (*L. lactis* +*E. faecium* +*P. pentosaceus*) had shown good aroma, texture, taste and overall acceptability and appearance, the results showed a statistical significance difference (P-value < 0.05) between the milk samples treated with different probiotic isolates and milk samples stored without LAB isolates. Hence, the milk inoculated by probiotic and bacteriocin producing isolates had better acceptable, in terms of aroma, texture, appearance, and taste.

Table 7. 3. Sensory property (aroma, taste, texture, acceptability and appearance) of milk samples inoculated by probiotic LAB isolates as food preservatives.

S/N	Treatments	Aroma	Taste	Texture	Aptability	Appearance
1	<i>Lactococcus lactis</i>	4.33±0.58	4.00±0.00 ^{bc}	4.22±0.01 ^c	4.67±0.58 ^c	4.67±0.58 ^c
2	<i>Enterococcus faecium</i>	4.00±0.00 ^{bc}	5.00±0.00 ^a	4.87±0.01 ^a	4.33±0.58 ^d	4.67±0.58 ^c
3	<i>P. pentosacues</i>	4.67±0.58 ^{ab}	4.00±0.00 ^{bc}	4.66±0.56 ^{bc}	4.67±0.58 ^c	4.67±0.58 ^c
4	<i>L.lactis</i> + <i>E.faecium</i>	4.33±0.58 ^{ab}	4.67±0.58 ^{ab}	4.83±0.29 ^a	4.33±0.58 ^d	4.33±0.58 ^d
5	<i>L.lactis</i> + <i>P.pentosaceus</i>	5.00±0.00 ^a	4.67±0.58 ^{ab}	4.63±0.56 ^b	4.03±0.02 ^{de}	4.67±0.58 ^c
6	<i>E.faecium</i> + <i>P.pentosa</i>	4.67±0.58 ^{ab}	4.67±0.58 ^{ab}	4.65±0.58 ^b	4.88±0.01 ^{ab}	5.00±0.00 ^a
7	<i>L.lac</i> + <i>E.fae</i> + <i>P.pent.</i>	4.67±0.58 ^{ab}	5.00±0.00 ^a	4.95±0.05 ^a	4.99±0.01 ^a	5.00±0.00 ^a
8	Control	3.33±0.58 ^c	3.33±0.58 ^c	3.68±0.51 ^d	3.90±0.00 ^{de}	3.33±0.58 ^f

7.4. DISCUSSION

Lactic acid bacteria from Ethiopian fermented foods have potential antibacterial activity and bio-preservative efficiency. Live probiotic cultures and their CFS were investigated for their effects on food-borne pathogen growth. Raw milk shelf life was extended using probiotic and bacteriocin-generating LAB, while co-culture of bacteriocin-producing LAB isolates was used to investigate their impact on food-borne pathogen proliferation.

This finding indicated that, bacteriocin producing LAB can inhibit the growth of food-borne pathogens. As a consequence, all isolates were able to lower the log of CFU/ml for *E. coli*, *S. aureus*, and *L. monocytogenes* from 3.02 to 1.33, 2.97 to 2.00, and 2.97 to 1.53 Log CFU/ml. In line to this study, Afolayan & Ayeni (2017); Hae et al. (1997) and Monteagudo-Mera et al. (2012) reported that, bacteriocin producing LAB isolated from a variety of traditional fermented foods had demonstrated potential co-cultures with food borne pathogens by demonstrating significant reduction of food borne pathogens starting from 6 log of CFU/ml to less than 3 Log CFU/ml.

In addition, all bacteriocin-producing LAB isolates also showed significant co-aggregation potential with *E. coli*, *S. aureus*, and *L. monocytogenes*, with *L. lactis* (JULAB-E35) having the highest levels of co-aggregation (87.30, 93.45, and 101.00%, respectively). In agreement with these findings, *L. lactis* subsp. *lactis* DF04Mi isolated from goat milk shown co-aggregation capacity with *L. monocytogenes* (Furtado et al., 014). Additionally, *E. faecium* showed possible co-aggregation ability against *E. coli* (Lodemann et al., 2015). As a result, the co-aggregation potential of LAB isolates that produce bacteriocin is a good indication of their ability to colonize the gut and prevent the invasion of pathogens (Kalhor et al., 2023). The cellular aggregation that promotes gut colonization with probiotic bacteria will often be promoted and enhanced by co-aggregation of the LAB isolates with pathogens.

Furthermore, CFS produced by *L. lactis* and its combination with *E. faecium* and *P. pentosacues* demonstrated potential pathogenic reduction. Because, these CFS may possess potential antagonistic effects due to their capacity to produce various antimicrobial metabolites like hydrogen peroxides, organic acid, carbon dioxides, and bacteriocins (Abushelaibi et al., 2017; Drider et al., 2010; Kalhor et al., 2023; Sahoo et al., 2010; Todorov & Dicks, 2006; Trivedi et al., 2013). This results indicated that, bacteriocin producing LAB isolates has the most preservative efficiency to be used as the promising preservative potential in food industry.

Bacteriocin producing LAB isolates showed potential antagonistic effects against *L. monocytogenes*. This indicated that, these LAB isolates have shown potential preservative effects on raw milk sample against *L. monocytogenes*. The growth of *L. monocytogenes* was gradually decreased as time increased as compared to control. No growth of *L. monocytogenes* was observed after 8 days in raw milk samples. In this study, the effects of bacteriocin producing LAB isolates were used to compare the effects of their preservative effects on raw milk inoculated with *L. monocytogenes*. Bacteriocin producing LAB and their antimicrobial peptides are used and applied as bio-preservative in raw milk. Similar to this study, Cintas et al. (1995); Fernandes et al. (2017) and Kalhor et al. (2023) reported that probiotic LAB isolated from traditional fermented dairy products had shown potential preservative effects on raw milk by reducing the numbers of *Listeria monocytogenes*.

Due to their adoption in some conditions and affinity for conventionally fermented dairy products, these bacteriocin-producing LAB isolates have been utilized as the most effective food preservative (Mezaini et al., 2009; Verma et al., 2022). Moreover, due to their ability to produce a number of antimicrobial compounds, LAB isolated from traditional fermented products have reportedly demonstrated potential antagonistic effects against foodborne pathogens, including *L. monocytogenes* (Ammor et al., 2005; Arici et al., 2004; Isleroglu et al., 2012). These findings highlight the potential of bacteriocin-producing LAB isolates as natural alternatives to chemical preservatives in the food industry. Additionally, their ability to inhibit the growth of foodborne pathogens makes them a promising tool for improving food safety and reducing the risk of foodborne illnesses.

According to several research, Ammor et al. (2005); Arici et al. (2004) and Isleroglu et al. (2012), LAB isolated from traditionally fermented items may have antagonistic effects against foodborne pathogens, including *L. monocytogenes*. As a result, LAB isolates that have the capacity to act as probiotics and produce bacteriocins are able to inhibit the growth of *L. monocytogenes* in raw milk samples. This suggests that LAB could have potential to be used as natural preservatives in the food industry to prevent the growth of harmful bacteria.

Additionally, the total bacterial counts at 4° C for ten days were reduced by the inoculation of bacteriocin-producing *L. lactis*, *E. faecium*, and *P. pentosaceus*. This shown that bacteriocin-producing LAB isolates can increase the shelf life of milk by reducing the risks of food borne pathogens. On the same manner, inoculation with bacteriocin-producing LAB isolates, certain bacteriocin-producing

LAB isolates are reported to inhibit a number of foodborne pathogens, resulting in a decrease in the overall bacterial counts of raw milk (Mezaini et al., 2009; Silva et al., 2018). In this investigation, bacteriocin-producing LAB isolates were capable of preventing the growth of foodborne pathogens and extending the milk shelf life for ten days because they produce various antimicrobial peptides that prevent the growth of food borne pathogens.

L. lactis, a bacteriocin-producing LAB isolate, has been found to prolong milk shelf life by inhibiting harmful bacteria like *Escherichia coli* and *S. aureus*. This natural antimicrobial compound offers a safer alternative to chemical preservatives, extending milk's shelf life (Goyal et al., 2018). In addition, the inoculation of *P. pentosaceus* into raw milk has been shown to reduce or inhibit the growth of *L. monocytogenes* in dairy products. This is due to the production of bacteriocins by *P. pentosaceus*, which are antimicrobial compounds that can effectively control the growth of *L. monocytogenes*. Furthermore, the inoculation of *P. pentosaceus* has been found to improve the overall quality and shelf life of dairy products by preventing spoilage caused by other harmful bacteria (Mezaini et al., 2009; Silva et al., 2018).

The moisture contents of milk samples inoculated with bacteriocin-producing LAB ranged from 59.67 to 64.67. Low moisture concentrations of raw milk, on the other hand, did not support the growth of food borne pathogens and increased the milk shelf life due to low water activity and superior food quality. High moisture contents supported the growth of food borne pathogens, which decreased the milk shelf life.

Therefore, inoculating milk with *L. lactis* and its combination of two and three isolates, which generate bacteriocin, produced the lowest content and led to a decrease in moisture composition as well as a reduction in the microbial load, both of which further enhanced the food's quality. In accordance to this study, Goyal et al. (2018) found that the *L. lactis* subsp. *lactis* strain isolated from Indian dairy product produced a bacteriocin with broad spectrum inhibition against a number of food pathogens, such as *L. monocytogenes*. These findings demonstrate that the bacteriocin-producing LAB isolates identified in this study as the most effective probiotic isolates also showed potential antimicrobial activity and preservative potential. In addition, the study also highlighted the potential application of this strain in the development of novel antimicrobial agents for food safety purposes.

In milk samples treated with bacteriocin-producing LAB isolates (*L. lactis*, *E. faecium*, and *P. pentosaceus*), a decrease in pH with a rise in TTA was observed. Due to the milk's ability to act as a

buffer, these characteristics increase the acidity of fermenting milks. Since lactic acid fermentation lowers pH, pH also has a significant impact on how long milk samples can remain on the shelf during fermentation.

The environment is adverse for the majority of pathogenic bacteria because pathogenic bacteria cannot grow, endure, or survive in this acidic environment at low pH levels. As a result, milk samples injected with *L. lactis* and its combination with *E. faecium* and *P. pentosaceus* produced low pH and high TTA, thus extending the shelf life of milk.

Similar to this finding, Uaboi et al. (2010) reported that the milk samples inoculated with bacteriocin-producing LAB isolates demonstrated low pH (5.83–3.20) and high TTA (0.049–0.137), which is more similar to the current findings. These results suggest that bacteriocin-producing LAB isolates have a consistent effect on the pH and TTA of milk samples. This further supports the potential use of these isolates in preserving and extending the shelf life of dairy products (D’Incecco et al., 2021; Ibarra-Sánchez et al., 2020).

Sensory characteristics such as the milk's color, acceptability, smell, texture, and flavor provided information about its quality and safety. According to this principle, LAB isolates that produce bacteriocin are the most likely to exhibit positive sensory properties. In this investigation, milk samples that had been inoculated with *L. lactis* and its offspring *E. faecium* and *P. pentosaceus* displayed favorable sensory characteristics. This significant difference between milk samples that included bacteriocin-producing isolates and those that did not may be fermented using bacteriocin-producing LAB isolates that inhibit a variety of food-borne pathogens. Therefore, these bacteria, bacteriocin-producing LAB isolates, can provide positive sensory qualities to the milk sample, such as a good scent, satisfying textures, and tasty flavor.

7.5. Conclusion and recommendation

This study examined the bio-preservation potential of bacteriocin-producing lactic acid bacteria (LAB) from Ethiopian traditional fermented dairy products (*ergo*) for milk preservation. Key findings include LAB isolates, especially *L. lactis*, *E. faecium*, and *P. pentosaceus*, showed strong antimicrobial activity against pathogens like *E. coli*, *S. aureus*, and *L. monocytogenes* through methods like agar spot assays and co-culture experiments. These LAB isolates significantly extended milk's shelf life, maintaining quality and safety for over three weeks under refrigeration. The bio-preservative effect was confirmed by reduced microbial counts in treated milk samples due to bacteriocins and other antimicrobial compounds (organic acids, hydrogen peroxide) that disrupted pathogen cell membranes. LAB isolates demonstrated good probiotic attributes, such as resistance to harsh conditions and co-aggregation with pathogens, indicating potential gut health benefits.

Future research should focus on scaling up LAB production, testing industrial-scale efficacy, conducting safety assessments, and obtaining regulatory approvals. Practical formulations like freeze-dried cultures or probiotic blends should be developed, and consumer studies on sensory acceptance should be conducted. Additionally, exploring LAB's potential in preserving other perishable foods is recommended.

7.6. Strength and limitation of the study

This study identified the most potent and promising probiotic and bacteriocin-producing LAB, which may be able to prolong the shelf life of milk. However, this study failed to investigate the detailed molecular, whole genome sequencing of LAB, which aids in identifying the gene responsible for bacteriocin production and antibiotic resistance gene determination. By conducting molecular and genomic sequencing, researchers can gain a deeper understanding of the mechanisms behind bacteriocin production and antibiotic resistance in LAB. This information could further enhance the development of effective strategies to improve milk shelf life and ensure food safety.

8. GENERAL DISCUSSION

Globally, gastrointestinal pathogen-related food-borne infections are becoming a serious public health concern due to the high mortality rate from severe diarrheal illnesses (Ayana et al., 2015; Girma & Aemiro, 2021). To combat these challenges, it's crucial to identify and disclose antimicrobial metabolites produced and used in traditional fermented foods and beverages. LAB, found in Ethiopian traditional fermented foods and beverages, produce antimicrobial compounds that can function antagonistically against various food-borne diseases and spoilage bacteria. These compounds have shown potent inhibition activity against various foodborne pathogens (Girma & Aemiro, 2021; Goa et al., 2022; Mulaw et al., 2020). This study found that six Ethiopian traditional fermented foods and beverages were dominated by *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and *Streptococcus*, with *Lactobacillus* being the most prevalent LAB isolate (Dejene et al., 2021; Girma & Aemiro, 2021; Mulaw et al., 2020). This finding aligns with previous research, which found that 95% of LAB isolates in these foods and beverages were homofermentative, with only a few being heterofermentative. The fermentation profile of these LAB isolates is consistent with previous studies.

Furthermore, the study identified 340 LAB isolates capable of producing strong inhibition zones against food-borne pathogens, with 125 identified as potential probiotic candidates. In vitro testing of 54 LAB isolates against six food-borne pathogens showed *Pediococcus pentosaceus* JULABB16 and *Enterococcus faecium* JULABBr39 had the highest inhibition zones. Inline to this study, Tadesse et al. (2015), reported that all LAB isolates originated from *Shamita* and *Borde* inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*, also Negasi et al. (2017) reported that nearby 54 isolates had strong inhibition activity against *Salmonella Typhimurium*. Moreover, this study found that *Lactococcus lactis* and *Pediococcus pentosaceus* isolated from Ethiopian fermented foods and beverages can produce potential antagonistic effects against food spoilage and foodborne pathogens. Correspondingly, Assefa et al. (2008) reported that *Pediococcus acidilactici* isolated from Ethiopian dairy products had antibacterial activity against *Staphylococcus aureus* ATCC@25923, and all *Lactococcus lactis* isolates had possible inhibitory effects on *Escherichia coli* ATCC 25922.

The antimicrobial substances produced by LAB isolated from Ethiopian traditional fermented foods and beverages demonstrated potential antimicrobial activity after heat treatment at 45-100° C, but lost their inhibition activity above 100° C. This study is similar to that of Dejene et al. (2021); Girma & Aemiro (2021) and Tilahun et al. (2018), who found that heating CFS at different temperature ranges (40-100°C) showed potential antimicrobial effectiveness. In addition, the antibacterial activity of 54 LAB strains was more effective in acidic environments, while *Pediococcus pentosaceus* JULABB01, JULABB16, *Enterococcus faecium* JULABBr39, and JULABBr40 showed promising antagonistic activity at pH 8. In agreement with this study, Girma & Aemiro (2021) and Ren et al. (2018) found that LAB strains' antibacterial activity is more effective at pH 6.5-7.5, hence it was found that the antimicrobial activity of LAB isolates was mostly due to the production of organic acids and partially as a result of bacteriocin like substances production.

The study examined the effects of food additives and enzymes on the antibacterial activity of LAB strains, and it discovered that some strains became partially inactivated when exposed to enzymes such as proteinase K, trypsin, and pepsin. The study also looked at the effects of food additives such as potassium, tween, and sodium citrate. Upon treatment with both, all 54 LAB isolates exhibited consistent antimicrobial activity; however, following Tween-80 treatment, several isolates showed decreased activity. In a similar vein, Heredia-Castro et al. (2015) and Ren et al. (2018) reported that following CFS treatment with sodium citrate and potassium sorbate, the majority of LAB isolates exhibited effective antagonistic activity.

The study evaluated the probiotic LAB survival rates at low pH 2, 2.5, and 3 for 3 and 6 h. All 54 LAB isolates showed potential survival rates ranging from **33.52% to 85.39%**. In this study, the isolates demonstrated a potential for longer-term survival at these various pH ranges. These findings are much more similar to those reported by (Negasi et al., 2017; Song et al., 2021), who reported that LAB isolated from various sources has demonstrated potential survival rate at low pH after being exposed to pH 2 for 3 h. This suggests that the probiotic LAB isolates may have robust survival mechanisms in acidic environments, which could be beneficial for their functionality in the gastrointestinal tract. Further research is needed to understand the specific mechanisms behind this enhanced survival capability. All 54 LAB isolates were bile salt tolerant, with a tolerance of 0.3% bile salt (81.33-99.2%). This is in line with a study by Mulaw et al. (2020) in which 34 BLA isolates had survival rates of

82.58-99.44 %, respectively. LAB's ability to resist and be tolerant to 0.3% bile salt is due to their ability to produce bile salt hydrolase, which reduces serum cholesterol(de Melo Pereira et al., 2018).

This study examined the survival rates of isolated LAB isolates simulated gastric and pancreatic digestion, with all isolates being viable, in which the survival rates ranging from 30.07% to 79.04%. Probiotics must be resistant to harsh gastrointestinal conditions for human health benefits. The LAB isolates used in this investigation were promising starter cultures, with survival rates ranging from 32% to 85%. Over 50% of the LAB isolates could tolerate the stomach environment and function efficiently as probiotics. Typically, the specific concentration of human bile in the duodenum, which is around 0.3%, is used to analyze and choose probiotic strains(Shehata et al., 2016).

A study found that all 54 probiotic lactic acid bacteria (LAB) were more resistant to kanamycin than tetracycline and chloramphenicol, while more sensitive to streptomycin. This finding aligns with previous studies showing LAB from Ethiopian traditional fermented foods and beverages to be more resistant to kanamycin and susceptible to certain antibiotic drugs (Mulaw et al., 2020; Negasi et al., 2017; Tesfaye et al., 2011). These results suggest a potential pattern in antibiotic resistance among LAB strains from different sources. Further research is needed to understand the mechanisms behind these differences and their implications for probiotic use in food and medicine.

The FAO guidelines recommend that probiotic microbial strains should be safe for the host, with no α - or β -haemolytic activity. All LAB isolates selected for evaluation were safe for use as probiotics, as none exhibited α - or β -haemolytic activity. This is consistent with previous studies, Pavli et al.(2016) and Oyewole et al. (2018), which have shown that most LAB strains are non-haemolytic and safe for use as probiotics. Adherence to the gut is crucial for probiotic selection, and all selected LAB isolates showed potential for cell surface hydrophobicity, which is a crucial criterion for probiotic selection. The study also found that the adhesion of probiotics to epithelial cells is influenced by factors such as pH, temperature, and the presence of other microorganisms in the gut, which can improve the efficacy of probiotic supplementation in promoting gut health, which is more similar with the previous studies (Valledor et al., 2022; H. Xu et al., 2009; Yadata, 2014).

The study found that probiotic LAB showed potential auto-aggregation and co-aggregation, which is essential for their survival and persistence in the gastrointestinal system. The auto-aggregation potential of LAB ranged from **45.19% to 120.39%**, with *Pediococcus pentosaceus* JULABB01 having the

highest auto-aggregation rate. All isolates showed both auto- and co-aggregation potential, suggesting they may be beneficial for human intestinal health. A good probiotic should be able to both auto-aggregate and co-aggregate with pathogens, with a co-aggregation capacity greater than 50% and exceptional hydrophobic characteristics (Fugaban et al., 2021; M. Li et al., 2020).

In this study, *Pediococcus pentosaceus* JUB 01 and JUB 16 demonstrated significant inhibitory effects against *E. coli* and *S. aureus*, indicating their ability to produce bacteriocin-like peptides against both gram-positive and gram-negative foodborne pathogens. This aligns with previous studies suggesting that bacteriocin-like protein production is primarily responsible for LAB's bactericidal actions (Millette et al., 2007; Vizoso Pinto et al., 2006). In addition, bacteriocin-like substances produced by *L. lactis* have a proteinase nature and remain active after treatment with enzymes. Likewise, Cocolin et al. (2007); Ghairi et al. (2008); Zommiti et al. (2018), reported that, bacteriocin like antimicrobial substances produced by *Enterococcus faecium* MMT22, *Enterococcus faecium* M241 and M249, *Enterococcus faecium* GHB21 was totally removed after treatment, and making them considered bacteriocin like antimicrobial substances.

In this study, various media were tested for their potential to produce bacteriocin, and the best antibacterial activity was obtained when *Enterococcus faecium* was grown in Modified MRS broth first and then BHI broth. According to growth kinetics findings, BHI broth with an initial pH of 7.4 was the best growth environment for LAB and could produce potential antibacterial activity (Gutiérrez-cortés et al., 2018; Moore et al., 2018; Weine & Kim, 2019). Chloroform and its combination produced the best recovery, which was then followed by isopropanol and ethyl acetate.

The fruit-processing industry is crucial globally, but microbe adherence and persistence on surfaces can lead to the spread of pathogens and spoilage microorganisms, affecting shelf life and safety. Alternatives to synthetic fungicides are being researched to increase fruit and vegetable shelf life. LAB have shown potential as a preservative, coating fruit surfaces to inhibit rotting bacteria and extend shelf life. Fresh fruits and vegetables are eaten raw without further processing, and microbiological safety is crucial to ensure quality standards. Application of LAB has shown potential as a source of fruit and vegetables preservative, forming a coating on the surface of fruit, inhibiting fruit and vegetables rotting bacteria, and extending fruit shelf life (Dhundale, Hemke, Desai, et al., 2018; Ongeng et al., 2006).

This study revealed that an antimicrobial compound produced by *Lactococcus lactis*, *Enterococcus faecium* and *Pediococcus pentosaceus* was able to prevent fungal growth on selected fruits and vegetables and maintain the health of the fruits and vegetables under storage. This is due to the ability of lactic acid bacteria to produce varieties of metabolites, which are widely used to control the attack of fungi on grains (Pawlowska et al., 2012, Tiwari et al., 2014 and Wray et al., 2018).

Lactic acid bacteria (LAB) from Ethiopian fermented foods have potential antibacterial activity and bio-preservative efficiency. Raw milk shelf life was extended using probiotic and bacteriocin-generating LAB, while co-culture of bacteriocin-producing LAB isolates was used to investigate their impact on food-borne pathogen proliferation. The findings indicated that bacteriocin-producing LAB can inhibit the growth of food-borne pathogens, lowering the log of CFU/ml for *E. coli*, *S. aureus*, and *L. monocytogenes*.

Bacteriocin-producing LAB isolates have shown potential antagonistic effects against *L. monocytogenes*, suggesting that LAB could have potential to be used as natural alternatives to chemical preservatives in the food industry. Their ability to inhibit the growth of foodborne pathogens makes them a promising tool for improving food safety and reducing the risk of foodborne illnesses. Inoculation of bacteriocin-producing *L.lactis*, *E.faecium*, and *P.pentosaceus* into raw milk has been shown to reduce total bacterial counts at 4°C for ten days. Inoculation with bacteriocin-producing LAB isolates can increase the shelf life of milk by reducing the risks of food-borne pathogens. In this investigation, bacteriocin-producing LAB isolates were capable of preventing the growth of foodborne pathogens and extending the milk shelf life for ten days because they produce various antimicrobial peptides that prevent the growth of food-borne pathogens.

Furthermore, *L. lactis* has been found to prolong milk shelf life by inhibiting harmful bacteria like *Escherichia coli* and *S. aureus*, offering a safer alternative to chemical preservatives. Inoculation of *P. pentosaceus* into raw milk has been shown to reduce or inhibit the growth of *L. monocytogenes* in dairy products, improving overall quality and shelf life by preventing spoilage caused by other harmful bacteria.

9. GENERAL CONCLUSION

This study identified 583 LAB isolates with strong antimicrobial activity against food-borne pathogens and spoilage microbes. Key points include:

- ✓ Predominant genera: *Lactobacillus*, followed by *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*.
- ✓ Species identification via MALDI TOF revealed *P. pentosaceus* as the most common, followed by *E. faecium*, *L. lactis*, and *P. acidilactici*.
- ✓ LAB strains *L. lactis*, *P. pentosaceus*, *P. acidilactici*, and *E. faecium* from Ethiopian fermented foods exhibited strong antagonistic effects against *S. aureus* and *E. coli*.
- ✓ LAB-driven fermentation in Ethiopian foods enhances flavor and nutrition through lactic acid production, with homofermentative LAB contributing significantly.
- ✓ LAB strains showed antimicrobial efficacy at various temperatures (45-80°C), pH levels (2.5-6.5), and in the presence of preservatives, alcohols, and surfactants, primarily due to organic acids and bacteriocin-like substances.
- ✓ LAB's environmental tolerance and adaptability make them promising for food industry applications, but further research is needed to optimize their use.
- ✓ LAB can also preserve fruits and vegetables by forming a protective coating, inhibiting spoilage bacteria, and extending shelf life.

10. GENERAL RECOMMENDATION

To discover effective antimicrobial-producing lactic acid bacteria (LAB) for commercial applications, molecular characterization and whole genome sequence analysis are essential. This helps identify resistance genes and bioactive peptide clusters in LAB strains, aiding in the development of reliable probiotics and starter cultures.

Understanding LAB's genetic basis for antimicrobial and probiotic properties can lead to targeted, efficient products for food preservation and human health. This research also provides insights into LAB genetic diversity and evolution, contributing to new probiotic products with enhanced health benefits and identifying potential safety concerns.

Using LAB probiotics can reduce the risk of multidrug resistance and other health issues related to imbalanced gut microbiota. Fermented foods and beverages, rich in probiotics, improve digestion and boost the immune system. Consuming traditional fermented products supports gut microbiota and prevents the growth of harmful microbes, countering the rise of multidrug-resistant pathogens.

11. STRENGTH AND LIMITATION

The study's strength is its identification and pursuit of the most promising natural food preservative, as well as its introduction of techniques for extension of the shelf life of fruits and vegetables using bacteriocin-producing lactic acid bacteria, a novel strategy, particularly in Ethiopia. Therefore, the shelf life of fruits and vegetables can be extended by more than three weeks. The characterization of probiotic candidates made in this study relied on more specific metrics that serve as the fundamental baseline for subsequent research. Additionally, crude bacteriocins generated by lactic acid bacteria were extracted and characterized using solvent extraction procedures.

Because MRS agar has been found to allow for the growth of various microorganisms such as yeast, molds, *Bacillus*, *Staphylococcus*, and *Streptococcus*, researchers have concluded that it is not a more sensitive selective medium. As a result, we changed the MRS media to be only selective for LAB by adding CaCO₃, Sodium Azide, Ascorbic Acid, and Bromocresol Green.

Due to a lack of well-organized and sophisticated material, such as high capacity centrifuge with more than 100,000rpm, and the capacity to hold a 250-500ml centrifuge tube or falcon tube, we were unable to identify the types of bacteriocins produced by lactic acid bacteria. We intended to use Whole genome sequencing to explore the gene responsible for bacteriocin production or bacteriocin clustering, as well as to determine antibiotic resistance genes; however, we were unable to proceed further due to a lack of access to the right facilities at institution level.

12. FUTURE PROSPECTIVES

The recent study made an essential contribution to the scientific community by prolonging the shelf life of fruits and vegetables and providing insight into the application of lactic acid bacteria as a prospective food preservative in a number of industries. The antibiotic resistance profile of these potential probiotic candidates must be researched further in order to evaluate the specific safety concern of the probiotics for optimum starter culture development. Furthermore, whole-genome sequencing, specifically amino acid sequencing, is required to discover the position, types, and properties of the gene responsible for bacteriocin production among the candidate isolates.

Furthermore, the microbiological, physiochemical, proximate analysis, and fermentation period dynamics of numerous traditional fermentation beverages, such as *Bukuri* and ***Cabbage-Shamita***, are still not effectively addressed, necessitating additional research.

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14. APPENDIXS

Appendix 1. Modified MRS agar developed at jimma university for isolation and purification of lactic acid bacteria, isolated from ethiopian traditional fermented foods and beverages

Ingredients	g/L
Proteose peptone	10.00
HM peptone B	10.00
Yeast extract	5.00
Glucose	20.00
Polysorbate 80(tween 80)	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulfate	0.10
Manganese sulfate	0.05
Dipotassium hydrogen phosphate	2.00
Final pH(6.50±0.2, at 25°C	
Calcium carbonate	3%
Sorbic acid/cylohexamide	0.2%
Sodium azide	0.01
Bromocresol green solution	0.05
Distilled water	1000ml

Appendix 2. Curriculum vitae (CV).

1. Personal information

Name: Desalegn Amenu Delesa

Sex: Male

Date of birth: November 1989 GC

Place of Birth: East Wollega Zone, Limu District

Nationality: Ethiopian

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2. Educational background

- | | |
|------------|---|
| Since 2017 | Ph.D candidate at at Biology Department, Applied Microbiology (Food Microbiology), College of Natural sciences, Jimma University, Ethiopia |
| 2011/12 | MSC in Microbiology from Biology Department, College of Natural and Computatioanl Sciences, Haramaya Univrersity, Harar, Ethiopia |
| 2007/2010 | B.Sc in Biology from Biology Department, College of Natural and Computatioanl Sciences, Mada Walabu University, Bale, Ethiopia |

3. Work Experince

- | | |
|--------------|--|
| 2013-to-date | Lecture at Wollega University, Nekemte, Ethiopia |
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Appendix 3. Publications

1. **Amenu, D and Bacha, K.** Probiotic potential and safety analysis of lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages. *Ann Microbiol* **73**, 37 (2023). <https://doi.org/10.1186/s13213-023-01740-9>.

Amenu and Bacha *Annals of Microbiology* (2023) 73:37
<https://doi.org/10.1186/s13213-023-01740-9>



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Probiotic potential and safety analysis of lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages

Desalegn Amenu^{1,2} and Ketema Bacha^{1*}

Abstract

Background Probiotics are live microorganisms that effectively combat foodborne pathogens, promoting intestinal health when consumed in sufficient amounts. This study evaluated the probiotic potential and safety of lactic acid bacteria isolated from selected Ethiopian traditional fermented foods and beverages (Kotcho, Bulla, Ergo, Cabbage-Shamita, Borde, and Bukuri). To assess the isolates' probiotic activity, tolerance, and survival rate under various stressful conditions, including low pH, intestinal inhibitory substances, salt concentration, bile salt, and simulated gastric/intestinal juice. The isolates were also tested for antagonistic activities against common bacterial and fungal pathogens (*Staphylococcus aureus*, *Salmonella Typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans*) and safety (auto-aggregation, co-aggregation, cell surface hydrophobicity, hemolytic activity, DNase, and antibiotic susceptibility). The best probiotic lactic acid bacteria (LAB) were characterized to species level following standard MALDI TOF/mass spectrometry analysis.

Results A total of 125 potentially probiotic LAB were isolated of which 17 (13.60%) isolates survived low pH (2, 2.5, and 3), bile salt (0.3%), intestinal inhibitory chemicals (phenol, bile, low acidity, pepsin, and pancreas), and simulated gastro-intestinal settings with near 60–94% survival rate. In addition, 11 best LAB isolates were further screened based on additional screening including their antimicrobial efficacy, preservative efficiency, bacteriocin production besides resistance to low acid and bile salts, and survival potential under simulated gastrointestinal conditions. All 11 LAB isolates were resistant to ampicillin, vancomycin, gentamicin, kanamycin, clindamycin, and chloramphenicol, while they were susceptible to streptomycin and tetracycline. The MALDI TOF mass spectrometry analysis result of efficient probiotic LAB grouped them under the genus *Pediococcus*, *Enterococcus*, and *Lactococcus* including *Pediococcus pentosaceus*, *Enterococcus faecium*, *Lactococcus lactis*, and *Pediococcus acidilactici*.

Conclusion Ethiopian traditional fermented foods and beverages are good sources of promising probiotic lactic acid bacteria. These isolates could serve as potential starter cultures and bio-preservative for the enhancement of the shelf life of foods. This study established the groundwork for the selection of excellent probiotics for the development and application of LAB for antibacterial action, starter culture production, and preservation activities.

Keywords Antimicrobial activity, Ethiopian, Lactic acid bacteria, Probiotic potential, Traditional fermented products

2. Amenu, D., & Bacha, K. (2024). Antagonistic Effects of Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Foods and Beverages Against Foodborne Pathogens. Probiotics and Antimicrobial Proteins, 0123456789. <https://doi.org/10.1007/s12602-024-10231-5>

Probiotics and Antimicrobial Proteins
<https://doi.org/10.1007/s12602-024-10231-5>

RESEARCH



Antagonistic Effects of Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Foods and Beverages Against Foodborne Pathogens

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Abstract

Lactic acid bacteria (LAB) found in Ethiopian traditional fermented foods and beverages have potential antagonistic effects against foodborne pathogens due to their capacity to produce various antimicrobial metabolites. This study evaluated the antagonistic activity of LAB isolated from Ethiopian traditional fermented foods and beverages against foodborne pathogens and characterized their antimicrobial substances. A total of 180 traditional fermented foods and beverages were collected, and the antagonistic activities of LAB were evaluated against selected foodborne pathogens. The effects of pH, temperature, enzymes, and food additives on the antagonistic effects of cell-free supernatant produced by LAB were investigated. LAB identification and characterization were conducted using an integrated phenotypic approach and MALDI TOF MS spectrum analysis, and data were analyzed using one-way ANOVA and Tukey post hoc analysis. A total of 956 LAB were isolated, of which seventeen (17 LAB) isolates of *Pediococcus pentosaceus* (*Pc. pentosaceus* ($n=7$)), *Pediococcus acidilactici* (*Pc. acidilactici* ($n=2$)), *Enterococcus faecium* (*Ec. faecium* ($n=6$)), and *Lactococcus lactis* (*Lc. lactis* ($n=2$))) were screened for antagonistic activity based on their ability to produce bacteriocins, probiotic activity, and preservative potential. *Pc. pentosaceus* JULABB16, *Pc. pentosaceus* JULABB01, and *Ec. faecium* JULABB39 showed strong antagonistic activity against all pathogens, with mean inhibition zone diameters ranging from 23.50 to 35.50 mm. *Lc. lactis*, *Pc. pentosaceus*, *Pc. acidilactici*, and *Ec. faecium* produced bioactive metabolites that were sensitive to proteolytic enzymes and capable of withstanding high temperatures (80–100 °C) and acid concentrations (pH 2–10). The CFS produced by *Lc. lactis*, *Pc. pentosaceus*, *Pc. acidilactici*, and *Ec. faecium* showed the most impending antagonistic activity against all pathogens. The bioactive substances produced by LAB isolates had promising effects against food spoilage and pathogenic bacteria, making them potential natural food preservatives.

Keywords Antagonistic activity · Ethiopian · Foodborne pathogens · Traditional fermented product · Lactic acid bacteria

3. Amenu, D., Bacha, K. (2024). Bio-Preservation Potential and Antimicrobial Activity of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Dairy Products. *Probiotics & Antimicro. Prot.* (2024). <https://doi.org/10.1007/s12602-024-10272-w>

Probiotics and Antimicrobial Proteins
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RESEARCH



Bio-Preservation Potential and Antimicrobial Activity of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Dairy Products

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Abstract

This study aimed to assess the antibacterial activity and bio-preservation capability of bacteriocin-producing LAB isolated from Ethiopian traditional fermented dairy products in raw milk from Jimma town. Bacteriocin-producing LAB were tested for their antimicrobial activity against various foodborne pathogens, including *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella typhimurium*. The results showed that probiotic LAB isolates inhibited foodborne pathogens (*E. coli*, *S. aureus*, and *L. monocytogenes*), with inhibition zones ranging from 22.00 ± 0.57 to 34.13 ± 0.57 . *Enterococcus faecium* and *Lactococcus lactis* demonstrated possible antagonistic effects against *E. coli*, while *Pediococcus pentosaceus* had a 34.13 ± 0.57 mm inhibitory zone against *Pseudomonas aeruginosa*. The isolates also showed co-aggregation potential with the pathogens, with *Lactococcus lactis* isolates and their combinations demonstrating the best co-aggregation capabilities against the investigated pathogens. The bio-preservative assay showed that putative probiotic isolates (*L. lactis* JULABE35, *E. faecium* JULABE 23, and *P. pentosaceus* JULABE05) were efficient in decreasing *Listeria monocytogenes* in raw milk. After 7–8 days, milk samples diagnosed with these isolates showed complete reduction of *Listeria monocytogenes*. The bio-preservation capability of bacteriocin-producing LAB on raw milk extended the shelf life of milk at 4 °C storage for ten days, compared to six days for milk samples without probiotic LAB. The milk samples preserved with probiotic and bacteriocin-producing isolates showed good proximate analysis, showing significant variation with milk kept without bacteriocin-producing isolates. The isolated chemicals employed in this study can be used as food additives or food preservatives, indicating potential applications in Ethiopian traditional fermented dairy products.

Keywords Bacteriocin · Foodborne pathogens · Food preservative · LAB · Natural products

Declaration

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