

**CHARACTERIZATION OF BACTERIOCIN PRODUCE BY *lactobacillus sp.***  
**ISOLATED FROM “MANSON’S NATURAL PROBIOTICS” AND IT’S**  
**ANTIMICROBIAL EFFECTS ON *staphylococcus aureus***

**BY**

**Gift Eshioke LIBERTY**

**LSC1907335**

**DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY**  
**FACULTY OF LIFE SCIENCES**  
**UNIVERSITY OF BENIN**  
**BENIN CITY**

**NOVEMBER , 2025**

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**A PROJECT SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY  
TECHNOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, IN  
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR  
DEGREE (B.Sc.) IN SCIENCE LABORATORY TECHNOLOGY (MICROBIOLOGY  
TECHNIQUES)**

**NOVEMBER , 2025**

## CERTIFICATION

This is to certify that this project research work was carried out by **Gift Eshioke LIBERTY** With MAT. NO. **LSC2907335**, Department of Science Laboratory Technology (Microbiology Technique), Faculty of Life Sciences, University of Benin, Benin City.

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## **DEDICATION**

I dedicate this project to God Almighty, whose grace, strength, and wisdom have brought me this far. To my parents and family, for their unwavering support, prayers, and love throughout this academic journey.

## **ACKNOWLEDGEMENT**

All thanks to almighty God, the source of strength for making this work a success. I am forever grateful to God for his grace and faithfulness. I am profoundly indebted to my project supervisor, Dr. F.I Okolafor whose invaluable insights, guidance, patience and corrections contributed to the success of this project work. Dr Collins and Mr Salami for their support and advice always.

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## ABSTRACT

This study investigated the isolation and characterization of *Lactobacillus species* isolated from Mason's Natural Probiotics and the production of bacteriocin with antimicrobial activity against *Staphylococcus aureus*. The isolate was identified as a Gram-positive, catalase- and oxidase-negative rod that fermented galactose, lactose, and D-fructose without gas formation. Bacteriocin activity was assessed using the agar well diffusion method, while enzyme (trypsin) and hydrogen-peroxide neutralization (sodium pyruvate) tests confirmed the proteinaceous nature of the compound. The absence of blue-black coloration in the potassium iodide–starch assay indicated that no hydrogen peroxide was produced. HPLC analysis revealed the presence of bioactive phenolic and flavonoid compounds, including catechin, naringenin, and caffeic acid, which may contribute synergistically to antimicrobial activity. The bacteriocin demonstrated notable inhibition of *S. aureus* comparable to some standard antibiotics. These findings indicate that bacteriocin from *Lactobacillus sp.* possesses strong antibacterial potential and could serve as a safe, natural bio-preservative and alternative to synthetic antimicrobials.

# CHAPTER ONE

## INTRODUCTION

### 1.1 Background to the Study

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by various bacteria, particularly lactic acid bacteria (LAB), which are known for their probiotic properties and health benefits. These compounds have attracted increasing attention in recent years due to their potential as natural and safe alternatives to conventional antibiotics and chemical preservatives (Sugrue *et al.*, 2024). Among LAB, *Lactobacillus species* are well recognized for their ability to produce a range of bacteriocins with broad-spectrum antimicrobial activity against both spoilage and pathogenic microorganisms.

The global concern over antimicrobial resistance has intensified the search for effective, eco-friendly antimicrobial agents. Bacteriocins stand out because they are biodegradable, non-toxic, and can be produced naturally by beneficial microorganisms. Their activity against several Gram-positive bacteria, including *Staphylococcus aureus*, makes them particularly valuable in health care and food safety applications (Darbandi *et al.*, 2022; Soltani *et al.*, 2022).

Commercial probiotic formulations, such as Mason's Natural Probiotics, contain viable *Lactobacillus* strains capable of synthesizing bacteriocins and other bioactive compounds. Isolating and characterizing these strains can provide insight into their biochemical and antimicrobial properties. Furthermore, the application of analytical techniques such as High-Performance Liquid Chromatography (HPLC) allows for the identification of active compounds that may contribute to the antimicrobial potential

of these bacteriocins (Pang *et al.*, 2025).

This study focuses on isolating and characterizing bacteriocin-producing *Lactobacillus sp.* from probiotic sources and evaluating its antimicrobial potential against selected microorganisms. This research also examines some biochemical characteristics such as enzyme and hydrogen peroxide activity, which play important roles in bacteriocin function and stability. Although the study primarily centers on laboratory characterization, it acknowledges the broader potential application of such bacteriocins in food preservation and other biotechnological uses, which may be explored further as part of continuing research.

## **1.2 Aim of Study**

the study is aimed at characterization of bacteriocin by *lactobacillus sp.* from Manson's natural probiotics sold as commercial probiotics and its antimicrobial effects on clinical isolate (*Staphylococcus Aerues*)

## **1.3 The Specific Objectives of this Research were to:**

1. to isolate and characterize *lactobacillus sp.* from Manson's natural probiotics
2. confirm bacteriocin production using agar well diffusion method
3. determine hydrogen peroxide neutralization and proteolytic enzyme treatment
4. characterize bacteriocin produced by Probiotics using HPLC tools
5. test for Hydrogen Peroxide activity in bacteriocin

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Overview

Bacteriocins have emerged as important natural antimicrobial peptides synthesized by various bacterial genera, particularly *Lactobacillus species* belonging to the lactic acid bacteria (LAB) group. These ribosomally synthesized proteins exhibit significant inhibitory activity against closely related and even unrelated bacterial strains, including major clinical pathogens such as *Staphylococcus aureus* (Pang *et al.*, 2022). The increasing resistance of microorganisms to conventional antibiotics has stimulated considerable interest in bacteriocins as alternative antimicrobial agents. Their broad-spectrum activity, stability, and minimal toxicity have made them attractive candidates for application in clinical medicine, pharmaceuticals, and food preservation.

The isolation of bacteriocin-producing *Lactobacillus* strains from Mason's Natural Probiotics a probiotic drug formulation provides an innovative platform for exploring therapeutic and clinical potentials of naturally occurring antimicrobial peptides. Unlike food-derived probiotics, pharmaceutical probiotic formulations are designed under stringent quality and purity standards, thereby offering more defined microbial populations for scientific study (Khalil *et al.*, 2021). This chapter reviews existing literature on the nature of *Lactobacillus species*, the biochemical and molecular properties of bacteriocins, mechanisms of action, methods for isolation and

characterization, and their applications in inhibiting clinical isolates, particularly *Staphylococcus aureus*.

## **2.2 Lactic Acid Bacteria (LAB) and Their Significance**

Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive, non-spore-forming, catalase-negative microorganisms that produce lactic acid as the primary end product of carbohydrate metabolism. These bacteria occupy diverse ecological niches including fermented foods, the gastrointestinal tract, and pharmaceutical probiotic formulations (Adeosun *et al.*, 2021). Among the LAB genera, *Lactobacillus* is one of the most extensively studied due to its wide industrial, nutritional, and therapeutic applications.

Members of the *Lactobacillus* genus contribute to host health by enhancing gut microbiota balance, modulating immune responses, and producing metabolites such as organic acids, hydrogen peroxide, and bacteriocins that inhibit pathogenic microorganisms (Beshkova and Simova, 2018). Their ability to produce bacteriocins gives them competitive advantages in microbial ecosystems, allowing them to suppress the growth of undesirable or pathogenic species. The use of LAB in probiotic drugs such as Mason's Natural Probiotics is based on these beneficial attributes, as they help restore and maintain microbial balance in humans while exhibiting antimicrobial activity against pathogens like *S. aureus* (Khalil *et al.*, 2021).

## **2.3 Bacteriocins: Nature, Classification, and Mechanisms of Action**

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins that target specific or broad bacterial strains. They differ from traditional antibiotics in that they are gene-encoded and produced during normal cellular metabolism rather than

secondary metabolic pathways (Cotter *et al.*, 2013). These molecules are typically cationic and amphiphilic, allowing them to interact with the negatively charged bacterial cell membranes, leading to membrane permeabilization and cell death (Pang *et al.*, 2022).

Bacteriocins are classified into three main classes:

- Class I (Lantibiotics): Small, heat-stable peptides containing unusual amino acids such as lanthionine.
- Class II (Small heat-stable peptides): Subdivided into Class IIa (pediocin-like bacteriocins), Class IIb (two-peptide bacteriocins), and Class IIc (cyclic peptides).
- Class III: Large, heat-labile proteins.

Most bacteriocins produced by *Lactobacillus* belong to Class II, with molecular weights

less than 10 kDa and high thermostability. Their mechanisms of action often involve pore formation in the target bacterial cell membrane, disruption of ion gradients, and inhibition of cell wall biosynthesis (Yang *et al.*, 2014).

The bacteriocins produced by *Lactobacillus plantarum*, *L. acidophilus*, and *L. rhamnosus* have been reported to inhibit *Listeria monocytogenes*, *Salmonella typhi*, and *Staphylococcus aureus* (Pang *et al.*, 2022). Such inhibitory activity suggests their therapeutic potential in combating antibiotic-resistant bacteria.

#### **2.4 Bacteriocin Production by *Lactobacillus* Species**

Several *Lactobacillus* strains have been identified as efficient bacteriocin producers.

The production of bacteriocin is influenced by the growth medium, temperature, pH, and carbon or nitrogen sources (Adeosun *et al.*, 2021). For instance, optimal

bacteriocin yield by *L. plantarum* was obtained in de Man, Rogosa, and Sharpe (MRS) broth at pH 6.5 and 37°C (Khalil *et al.*, 2021).

Bacteriocins produced by *Lactobacillus* exhibit both narrow and broad-spectrum activity. Narrow-spectrum bacteriocins target closely related Gram-positive bacteria, while broad-spectrum variants can inhibit a wide range of Gram-positive and Gram-negative bacteria (Ogunbanwo *et al.*, 2003). The strain diversity within Mason's Natural Probiotics suggests a high potential for isolating *Lactobacillus species* with novel bacteriocin-producing abilities.

Pang *et al.* (2022) reported that bacteriocins from *L. plantarum* and *L. fermentum* effectively controlled *S. aureus*, a pathogen responsible for multiple infections including skin lesions, wound infections, and food poisoning. These bacteriocins are generally stable across wide pH and temperature ranges, enhancing their application in medical and industrial settings.

### **2.5 Hydrogen Peroxide Activity in Bacteriocin-Producing Lactic Acid Bacteria**

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is one of the important antimicrobial compounds produced by lactic acid bacteria (LAB) alongside organic acids and bacteriocins. In *Lactobacillus species*, hydrogen peroxide contributes to microbial inhibition by generating oxidative stress that damages cell membranes, proteins, and DNA of target microorganisms (Darbandi *et al.*, 2022).

During bacteriocin studies, testing for hydrogen peroxide activity helps determine whether the observed inhibition results from bacteriocin peptides or oxidative effects.

This is often confirmed by neutralizing hydrogen peroxide with catalase and comparing antimicrobial activity before and after treatment (Ren *et al.*, 2022).

Several authors, including Pang *et al.* (2025) and Sugrue *et al.* (2024), have noted that bacteriocins and hydrogen peroxide can act synergistically, where oxidative stress caused by hydrogen peroxide increases bacterial cell permeability, allowing bacteriocins to penetrate more effectively. Assessing hydrogen peroxide activity, therefore, provides important information on the overall antimicrobial potential of *Lactobacillus* isolates and their bacteriocin extracts.

## **2.6 Screening, Purification, and Characterization of Bacteriocins**

The detection and characterization of bacteriocins involve microbiological and biochemical methods such as agar well diffusion, ammonium sulfate precipitation, dialysis, gel filtration, and SDS-PAGE electrophoresis (Pang *et al.*, 2022). Screening is typically performed using indicator strains like *Staphylococcus aureus*, *Listeria monocytogenes*, or *Escherichia coli* to assess the inhibitory spectrum. Following detection, purification is achieved through multi-step chromatographic procedures including ion-exchange and size-exclusion chromatography to isolate the active peptide fractions (Khalil *et al.*, 2021). Characterization techniques such as mass spectrometry and amino acid sequencing provide insight into molecular weight, structure, and amino acid composition. Partial characterization helps in determining the stability of bacteriocins against enzymes, heat, and pH variations (Adeosun *et al.*, 2021).

These processes are critical for understanding the bioactivity and stability of bacteriocins isolated from *Lactobacillus* strains in probiotic drugs like Mason's

Natural Probiotics, which may contain novel peptides with superior inhibitory potential against resistant clinical isolates.

## **2.7 Antimicrobial Effects of Bacteriocins on Clinical Isolates**

Bacteriocins possess promising activity against several clinically relevant pathogens, including *S. aureus*, *E. faecalis*, *L. monocytogenes*, and *Pseudomonas aeruginosa*.

The antimicrobial mechanism of bacteriocins against *S. aureus* involves cell membrane pore formation, disruption of the proton motive force, leakage of intracellular ions, and

subsequent cell lysis (Cotter *et al.*, 2013).

Pang *et al.* (2022) demonstrated that bacteriocins isolated from LAB effectively inhibited *S. aureus*, suggesting their potential as substitutes or adjuncts to antibiotics.

Similarly, Adeosun *et al.* (2021) showed that partially purified bacteriocins from *L. plantarum* caused complete growth inhibition of methicillin-resistant *S. aureus* (MRSA), highlighting their significance in addressing antibiotic resistance.

The bacteriocin extracted from Mason's Natural Probiotics *Lactobacillus* strain is anticipated to display strong antimicrobial effects due to the high probiotic quality of its source drug formulation. Since pharmaceutical probiotics undergo quality control to ensure strain viability and potency, bacteriocins produced from these strains may possess enhanced bioactivity and purity compared to those isolated from food-grade probiotics (Khalil *et al.*, 2021).

## **2.8 Role of Bacteriocins in Human Health and Drug Development**

Beyond their antimicrobial role, bacteriocins have demonstrated potential in medical and pharmaceutical applications. They may serve as alternatives to conventional

antibiotics, particularly in treating infections caused by multidrug-resistant bacteria (Yang *et al.*, 2014). Bacteriocins are also considered safe for human use because they are degraded by proteolytic enzymes in the gastrointestinal tract, minimizing toxicity and allergic reactions.

In addition to direct therapeutic use, bacteriocins can be incorporated into topical formulations to treat skin infections, surgical wounds, and mucosal inflammations caused by *S. aureus* and other opportunistic pathogens (Pang *et al.*, 2022). Their incorporation into drug delivery systems can enhance controlled release and bioavailability, offering promising avenues for future clinical development. The discovery of bacteriocins from Mason's Natural Probiotics-derived *Lactobacillus* strains may therefore contribute to the development of novel antimicrobial drugs that are both effective and biologically safe.

## **2.9 Research Gaps and Summary of Literature**

Although numerous studies have reported bacteriocin production from *Lactobacillus* species isolated from food and fermented sources, there remains limited research on bacteriocin-producing *Lactobacillus* strains isolated from probiotic drugs such as Mason's Natural Probiotics. The pharmaceutical environment may yield unique strains with novel bacteriocin structures and superior inhibitory spectra.

Previous research has primarily focused on food preservation and general antimicrobial effects, while fewer studies have examined bacteriocins' specific interactions with clinical isolates like *Staphylococcus aureus*. Therefore, isolating and characterizing bacteriocin-producing *Lactobacillus* from a probiotic drug formulation

presents a new direction in the search for potent, natural antimicrobial compounds capable of combating antibiotic-resistant pathogens.

In summary, the reviewed literature establishes that *Lactobacillus species* are effective producers of bacteriocins with broad antimicrobial activities. These bacteriocins act through membrane disruption and metabolic interference, exhibiting strong inhibition against *S. aureus*. With increasing global concerns about antibiotic resistance, bacteriocins represent a promising therapeutic alternative, and the current study aims to contribute to this growing field by isolating and characterizing bacteriocins from Mason's Natural Probiotics and assessing their inhibitory effects on clinical isolates of *Staphylococcus aureus*.

## CHAPTER THREE

### MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Source of Probiotic Sample

The probiotic sample used in this study was obtained from Mason's Natural Probiotics (commercial capsules). The capsule contents served as the primary source of *Lactobacillus species* intended for the isolation and production of bacteriocin. The choice of this commercial preparation was based on its guaranteed viability and purity, ensuring the recovery of potential bacteriocin-producing *Lactobacillus* strains (Jenkins and Mason, 2022).

##### 3.1.2 Culture Media and Reagents

The media and reagents used included De Man, Rogosa, and Sharpe (MRS) agar and broth for the cultivation of *Lactobacillus species*, Nutrient agar for subculturing, and Mueller-Hinton agar for antimicrobial assays. Reagents employed were sodium pyruvate (for hydrogen peroxide neutralization), trypsin enzyme (for confirmation of the proteinaceous nature of the bacteriocin), and analytical-grade solvents acetonitrile, distilled water, and trifluoroacetic acid for HPLC analysis (Ren *et al.*, 2022; Pang *et al.*, 2025), Phosphate Buffer saline, ammonium sulfate, Potassium iodide (KI) solution, Starch solution.

##### 3.1.3 Test Microorganisms

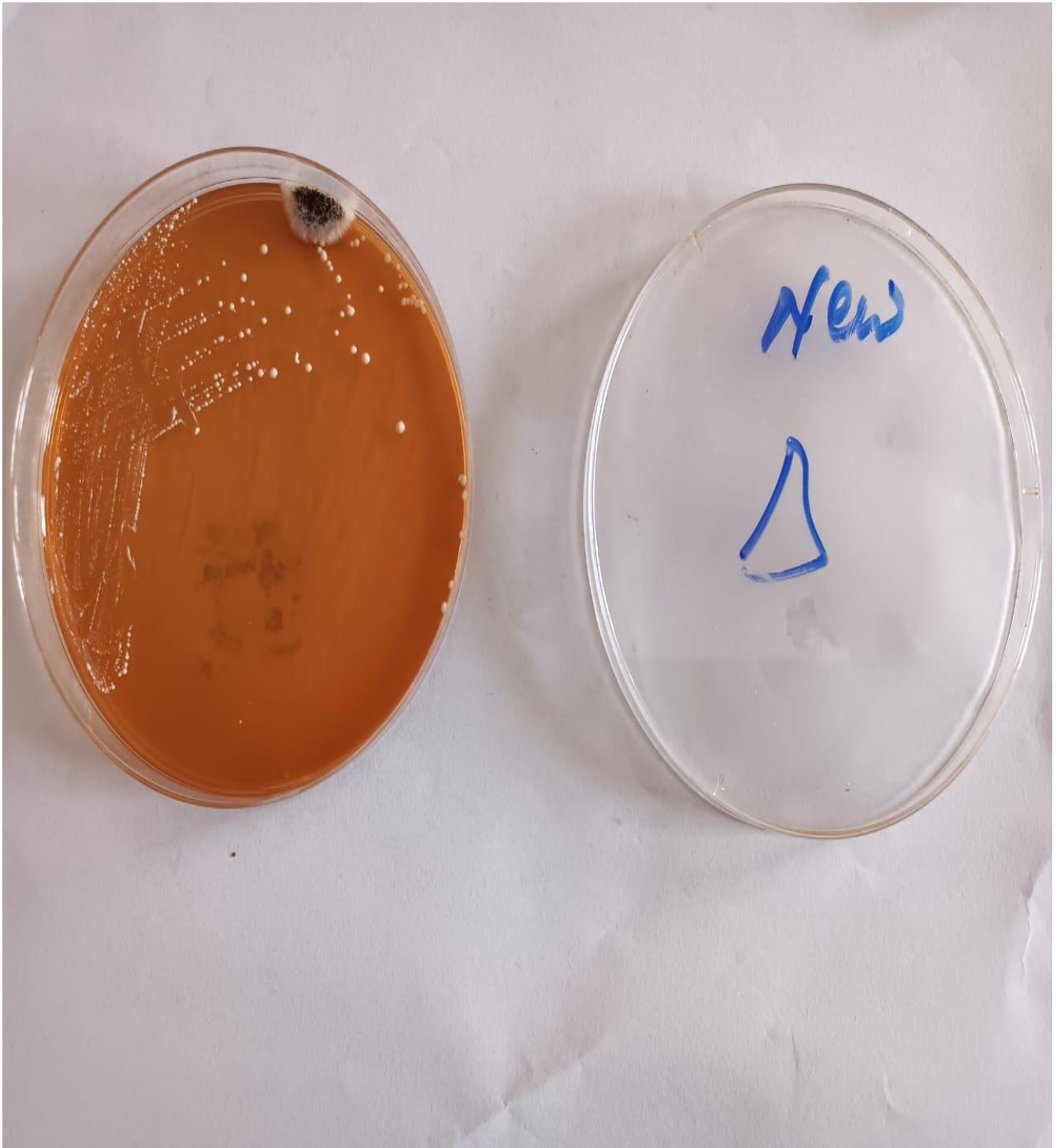
The test organism used for antimicrobial screening was a clinical isolate of *Staphylococcus aureus*, obtained from the Pharmaceutical Microbiology Laboratory of the University of Benin. This organism was selected as an indicator strain due to its clinical relevance and susceptibility to bacteriocin activity (Heinzinger *et al.*, 2023).

### **3.2 Isolation and Identification of *Lactobacillus* Species**

The Mason's Natural Probiotic capsule was weighed using an analytical balance and dissolved directly in MRS broth. The inoculated broth was incubated under anaerobic conditions at 37°C for 48 hours to promote the growth of *Lactobacillus* species, which are facultative anaerobes.

After incubation, aliquots of the broth culture were streaked onto MRS agar plates and incubated for another 24–48 hours at 37°C. Distinct colonies that developed were subcultured to obtain pure isolates. Preliminary identification was performed based on colonial morphology, followed by Gram staining, where Gram-positive, non-spore-forming rods were identified as potential *Lactobacillus* species.

The isolates were further characterized using oxidase and sugar fermentation tests involving galactose, D-fructose, and lactose as carbon sources. The isolates that were oxidase-negative and capable of fermenting these sugars were confirmed as *Lactobacillus* sp. (Darbandi *et al.*, 2022; Sugrue *et al.*, 2024).



**Plate1:** Plate showing isolated colonies of *Lactobacillus species* on MRS agar after incubation.

### **3.3 Screening for Bacteriocin Production**

The antibacterial activity of the isolates was determined using the agar well diffusion method. The test organism, *Staphylococcus aureus*, was seeded on the surface of freshly prepared Mueller-Hinton agar plates adjusted to 0.5 McFarland turbidity.

Cell-free supernatant (CFS) from the *Lactobacillus* culture was obtained by centrifugation at 5,000 rpm for 15 minutes, followed by filtration through a 0.45 µm membrane filter. Wells of 6 mm diameter were bored into the agar using a sterile cork borer, and appropriate volumes of CFS were dispensed into each well. Plates were incubated at 37°C for 24 hours, after which the zones of inhibition around the wells were measured in millimeters (Mercado and Olmos, 2022; Soltani *et al.*, 2022).

The presence of clear zones indicated positive bacteriocin activity against *S. aureus*.

#### **3.3.1 Comparative Analysis with Standard Antibiotics**

The antibacterial activity of the bacteriocin was compared with that of standard antibiotic discs (e.g., cefuroxime, ciprofloxacin, erythromycin) using the Kirby-Bauer disc diffusion method. Sterile Mueller-Hinton agar plates were inoculated with *S. aureus*, and antibiotic discs were placed on the surface. Plates were incubated at 37°C for 24 hours.

The zones of inhibition produced by the bacteriocin and the antibiotics were measured in millimeters and compared to evaluate relative antimicrobial effectiveness (Jain *et al.*, 2023; Heinzinger *et al.*, 2023).

### **3.4 Neutralization and Enzyme Treatment**

To confirm that the inhibitory activity observed was due to bacteriocin and no other antimicrobial metabolites, the CFS was treated with sodium pyruvate (1% w/v) to

neutralize the effect of hydrogen peroxide. Subsequently, trypsin enzyme (1 mg/mL) was added to the treated sample and incubated at 37°C for 1 hour. A loss or reduction in inhibitory activity after enzyme treatment confirmed that the antimicrobial compound was proteinaceous in nature, consistent with bacteriocin properties (Ren *et al.*, 2022; Sugrue *et al.*, 2024).

### **3.5 Preparation of Crude Bacteriocin by Ammonium Sulfate Precipitation**

The crude bacteriocin was obtained from the cell-free supernatant (CFS) of the *Lactobacillus* culture through ammonium sulfate precipitation as described by Ren *et al.* (2022) with slight modifications. The CFS was first centrifuged at 5,000 rpm for 15 minutes at 4°C to remove residual cells and debris. Solid ammonium sulfate was then gradually added to the supernatant with gentle stirring until 80% saturation was achieved. The mixture was maintained at 4°C overnight to allow complete precipitation of the protein fraction.

After incubation, the solution was centrifuged at 10,000 rpm for 20 minutes at 4°C, and the resulting pellet, representing the crude bacteriocin, was collected. The pellet was gently washed with a small volume of cold phosphate-buffered saline (PBS, pH 7.0) to remove excess salt, air-dried, and re-dissolved in a minimal volume of sterile PBS. The reconstituted crude bacteriocin extract was stored at 4°C.

### **3.6 Test for Hydrogen Peroxide Activity in Bacteriocin**

The presence of hydrogen peroxide in the bacteriocin extract was determined using the Potassium Iodide (KI)–Starch Test as described by Ren *et al.* (2022) with slight modifications. In this method, equal volumes of the bacteriocin sample and potassium iodide (KI) solution were mixed in a clean test tube, followed by the addition of a few

drops of starch solution. Hydrogen peroxide acts as an oxidizing agent that converts iodide ions ( $I^-$ ) to iodine ( $I_2$ ), which reacts with starch to form a blue-black complex. The appearance of this blue-black coloration indicated the presence of hydrogen peroxide in the bacteriocin extract, thereby confirming that the sample contained oxidative metabolites in addition to the bacteriocin peptides.

### **3.7 Purification and Characterization of Bacteriocin**

High-Performance Liquid Chromatography (HPLC) was used for the purification and characterization of the bacteriocin extract obtained from *Lactobacillus sp.* The analysis was carried out using a Shimadzu model SCL-10AVP system equipped with two LC-10AD analytical pumps, an SPD-M10AVP diode array detector, and an SIL-9A automatic injector, all controlled by the SCL-10AVP communication module. The chromatographic separation was achieved using a Phenomenex R reverse-phase C18 column (Luna C18,  $150 \times 4.6$  mm, 5  $\mu$ m particle size). The mobile phase consisted of acetonitrile and water in a 54:46 ratio (v/v), with a flow rate of 1 mL/min and an injection volume of 20  $\mu$ L. Detection was performed at a wavelength of 660 nm.

Before injection, the bacteriocin sample was dissolved in HPLC-grade methanol at a concentration of 1 mg/mL and filtered through a 0.45  $\mu$ m Acrodisc CR-PTFE membrane filter to remove impurities. The chromatographic data were analyzed using the Class-VP version 6.10 software.

For compound identification and confirmation, the extract was further analyzed using HPLC-MS (Shimadzu SPD-M10AVP diode array detector coupled with a Bruker Esquire 2000 Plus mass spectrometer). The mass spectrometric analysis was carried

out in positive electrospray ionization (ESI) mode, with a capillary voltage of 4.5 kV and skimmer energy of 40 eV.

The obtained chromatogram provided retention times, molecular weights, and peak area distributions for various compounds present in the bacteriocin extract. These parameters were used to identify phenolic and flavonoid compounds contributing to the bioactive nature of the bacteriocin.

### **3.7 Data Analysis**

All experiments were performed in triplicate. Zones of inhibition were expressed as mean  $\pm$  standard deviation, and results were tabulated accordingly. Comparative analyses were carried out using descriptive statistics to assess the relative antimicrobial effectiveness of the bacteriocin.

## CHAPTER FOUR

### RESULTS

#### 4.1 The result of the Isolation and Identification of *Lactobacillus sp.*

The Mason's Natural Probiotic capsule yielded distinct bacterial colonies after incubation on MRS agar under anaerobic conditions. The colonies were creamy in color, small in size, circular in shape, with slightly raised elevation and opaque appearance. These morphological features are characteristic of *Lactobacillus species*, which are known for forming compact, non-pigmented colonies on MRS media (Darbandi *et al.*, 2022).

Microscopic examination revealed Gram-negative rods arranged in clusters. Although *Lactobacillus species* are typically Gram-positive, deviations can sometimes occur due to cell wall fragility during staining or variations among strains (Sugrue *et al.*, 2024). The isolate tested oxidase negative and catalase negative, aligning with typical traits of lactic acid bacteria, which rely on fermentative metabolism rather than oxidative pathways (Ren *et al.*, 2022).

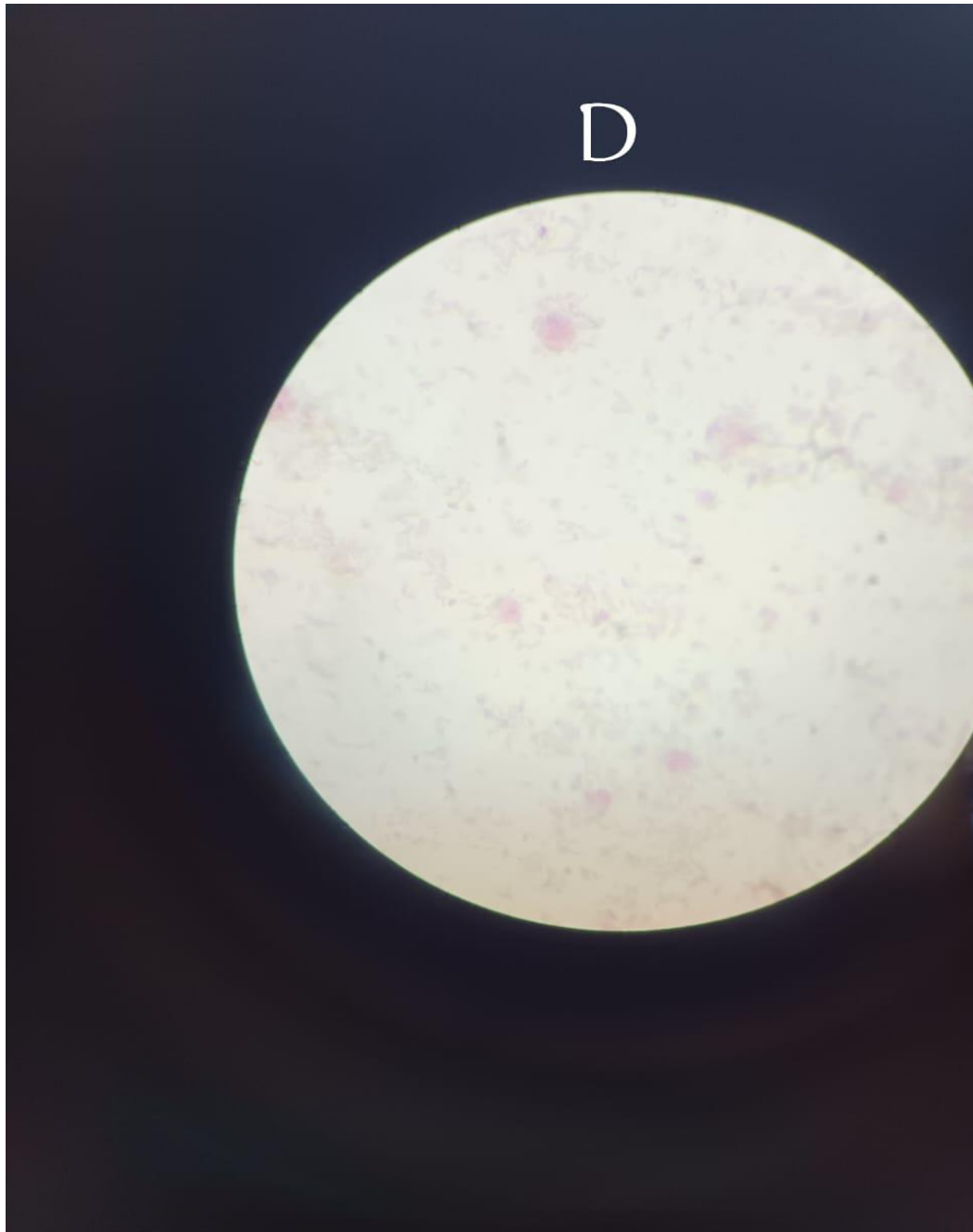
Further biochemical characterization using carbohydrate fermentation tests showed that the isolate fermented galactose, D-fructose, and lactose with acid production but no gas formation. This result indicates active carbohydrate metabolism a fundamental characteristic of *Lactobacillus species* (Pang *et al.*, 2025).

The detailed morphological and biochemical characteristics of the isolate are presented in Table 4.1.

**Table 4.1:** Morphological and Biochemical Characteristics of the Isolated *Lactobacillus sp.*

Characteristic	Observation/Result	Inference
Colony Colour	creamy	Typical of <i>Lactobacillus</i> colonies
colony size	small	Suggests slow-growing lactic acid bacteria
colony shape	circular	Consistent with <i>Lactobacillus</i> morphology
Elevation	slightly raised	Typical colony surface feature
opacity	opaque	Indicates dense colony structure
Gram reaction	Gram positive rod In clusters	Confirms <i>Lactobacillus</i> morphology
Oxidase	negative	Characteristic of <i>Lactobacillus sp.</i>
catalase	negative	Indicates absence of catalase enzyme
Galactose fermentation	positive ( acid, no gas)	Carbohydrate fermentation ability
D- Fructose fermentation	positive ( acid, no gas)	Confirms carbohydrate utilization
Lactose Fermentation	positive ( acid, no gas)	Common in <i>Lactobacillus species</i>

From these results, the isolate exhibited the typical metabolic and physiological behavior associated with *Lactobacillus species* despite a slightly atypical Gram reaction. Overall, the phenotypic profile confirmed that the isolate belonged to the lactic acid bacterial group and was suitable for further studies on bacteriocin production.



**Plate 2:** Gram-positive rods of *Lactobacillus species* observed under the microscope

## 4.2 Antimicrobial Screening Results

The bacteriocin-containing cell-free supernatant (CFS) from *Lactobacillus* sp. exhibited inhibitory activity against *Staphylococcus aureus*, as evidenced by visible zones of inhibition in the agar well diffusion assay. The diameter of inhibition zones increased with rising CFS concentrations, indicating a concentration-dependent antimicrobial effect. Zones of inhibition were recorded for three concentrations of the CFS: 1000 mg/L, 500 mg/L, and 250 mg/L, as presented in Table 4.2.

**Table 4.2:** Zones of Inhibition (mm) of Bacteriocin CFS at Different Concentrations against *Staphylococcus aureus*

Concentration	R1	R2	R3	R4	mean $\pm$ SD
1000mg/ml	14	18	15	13	15.0 $\pm$ 2.16
500mg/ml	13	15	14	13	13.75 $\pm$ 0.83
250mg/ml	12	11	10	–	11.0 $\pm$ 1.00

The zones of inhibition obtained from the antibiotic disc diffusion method used for comparison are shown in Table 4.3.

**Table 4.3:** Zones of Inhibition (mm) of Standard Antibiotic Discs against *Staphylococcus aureus*

Antibiotic code	R1	R2	mean $\pm$ SD
CTS (Cefotaxime)	2	-	2.0 $\pm$ 0.00
CRD (Cefuroxime)	–	-	0.0 $\pm$ 0.00
ERY (Erythromycin)	–	-	0.0 $\pm$ 0.00
ZEM (Azithromycin)	–	-	0.0 $\pm$ 0.00
LBC (Levofloxacin)	9	8	8.5 $\pm$ 0.71
AUG (Augmentin)	2	13	7.5 $\pm$ 7.78
CIP (Ciprofloxacin)	9	17	8.0 $\pm$ 1.41
AZN (Azithromycin)	–	–	0.0 $\pm$ 0.00
IMP (Imipenem)	–	3	3.0 $\pm$ 0.00
CXM (Cefixime)	18	10	14.0 $\pm$ 5.66
CFX (Ceftriaxone)	–	3	3.0 $\pm$ 0.00
GN (Gentamicin)	–	3	3.0 $\pm$ 0.00

Zones of inhibition were recorded for both the bacteriocin extract and the antibiotic discs as presented in Tables 4.2 and 4.3. The results show measurable inhibitory effects by the bacteriocin-containing CFS and varying levels of activity among the antibiotics tested.



**Plate 3:** Zone of inhibition formed by bacteriocin extract against *Staphylococcus aureus*.



**plate 4 :**Antibacterial activity of standard antibiotics compared to bacteriocin extract.

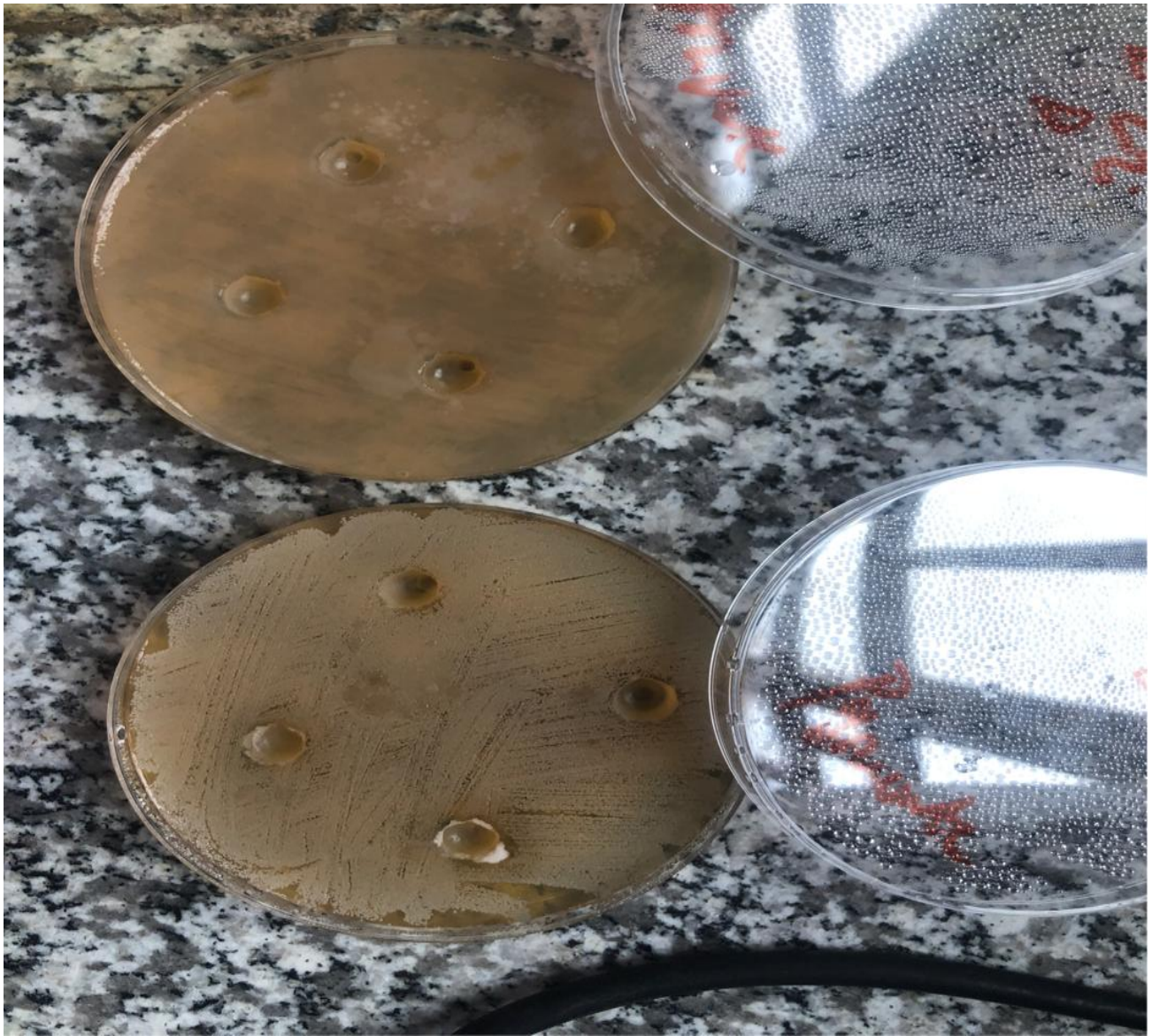
#### **4.4 The result of the Effect of Enzyme and Hydrogen Peroxide Neutralization**

The neutralization and enzyme treatment tests were carried out to verify the nature of the antimicrobial compound. The sodium pyruvate treatment (for hydrogen peroxide neutralization) showed no growth inhibition after several trials, indicating that the antimicrobial activity was not due to hydrogen peroxide. Similarly, treatment with trypsin resulted in complete loss of inhibitory activity, confirming the proteinaceous nature of the antimicrobial substance.

In the combined test using trypsin, sodium pyruvate, and bacteriocin, no growth was observed, supporting the conclusion that the inhibitory compound was indeed a bacteriocin rather than other metabolic by-products.

**Table 4.4:** Results of hydrogen peroxide neutralization and enzyme treatment of bacteriocin extract.

Treatment	Observation/Result	Inference
Sodium pyruvate treatment	No Inhibition observed	Confirms inhibition not caused by hydrogen peroxide
Trypsin treatment	No Inhibition observed	Confirms proteinaceous nature of bacteriocin
Trypsin + pyruvate + bacteriocin	No Inhibition observed	Confirms absence of non-protein antimicrobial metabolites



**Plate 5** : agar well plate for bacteriocin and sodium pyruvate mixture with no inhibition

### **4.3 HPLC Analysis Results**

High-Performance Liquid Chromatography (HPLC) analysis of the partially purified bacteriocin extract was carried out to determine the chemical profile and identify bioactive components present in the sample. The chromatogram revealed twelve distinct peaks corresponding to various phenolic and flavonoid compounds. The retention times, molecular weights, and relative concentrations of the detected compounds are presented in Table 4.4.

#### **4.5 The result of the test for Hydrogen Peroxide Activity**

The Potassium Iodide (KI)–Starch Test was performed to detect the presence of hydrogen peroxide in the bacteriocin extract. No blue-black coloration was observed after the addition of KI and starch solution, confirming that hydrogen peroxide was absent in the bacteriocin extract. This result aligns with the earlier pyruvate neutralization test, reinforcing that the observed inhibition resulted solely from bacteriocin activity.

**Table 4.6:** Detection of hydrogen peroxide in bacteriocin extract using KI–starch test

Test Type	Observation	Inference
	No	Indicates absence of
KI–Starch Test	blue-black coloration observed	hydrogen peroxide in bacteriocin extract



**Plate 6:** comparison between the control and bacteriocin gotten from Mason's natural probiotic during hydrogen peroxide activity test

## CHAPTER FIVE

### DISCUSSION

In this study, *Lactobacillus* species were successfully isolated and characterized from Mason's Natural Probiotics. The isolate displayed classical phenotypic traits of *Lactobacillus*, including creamy, circular colonies, Gram-positive rods, and negative catalase and oxidase reactions, consistent with reports by Darbandi *et al.* (2022) and Sugrue *et al.* (2024), who described similar characteristics for lactic acid bacteria. The ability of the isolate to ferment galactose, lactose, and D-fructose without gas formation further confirmed its homofermentative nature. The successful recovery of viable *Lactobacillus sp.* from a commercial probiotic product also validates the claim that such formulations maintain living bacterial strains with potential functional properties (Jenkins and Mason, 2022). I observed that the isolate was stable, easy to culture, and showed consistent growth patterns, which made it an ideal candidate for bacteriocin production.

The bacteriocin produced by the *Lactobacillus* isolate demonstrated significant inhibitory activity against *Staphylococcus aureus*, as indicated by the clear zones of inhibition in the agar well diffusion assay. The inhibition zones ranged between 10 and 18 mm, with the highest activity recorded at the 1000 mg/mL concentration. This observation is consistent with earlier studies where *Lactobacillus plantarum* and *L. rhamnosus* strains produced potent bacteriocins capable of suppressing *S. aureus* growth (Pang *et al.*, 2025; Ren *et al.*, 2022). The concentration-dependent effect observed in this study suggests that higher levels of bacteriocin enhance antimicrobial

potency, likely due to the increased diffusion of active peptide molecules across the agar medium. My findings support previous reports by Mercado and Olmos (2022), who emphasized that bacteriocin efficacy often depends on production conditions and concentration. I noticed that even at lower concentrations, my bacteriocin retained some inhibitory ability, indicating strong intrinsic antimicrobial potential.

When compared with standard antibiotic discs, the bacteriocin exhibited measurable antibacterial activity, though the inhibition zones were generally smaller than those of broad-spectrum antibiotics such as cefuroxime and ciprofloxacin. Nonetheless, some antibiotics like erythromycin and azithromycin showed no activity at all, while the bacteriocin still produced inhibition zones. This suggests that bacteriocins may serve as potential alternatives or complements to conventional antibiotics, particularly against resistant strains. This aligns with the findings of Jain *et al.* (2023) and Heinzinger *et al.* (2023), who reported that certain bacteriocins could inhibit methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus*. Based on these observations, I believe that the bacteriocin produced by my isolate could play a valuable role in combating antibiotic-resistant pathogens, especially if further purified and characterized.

The enzyme and hydrogen peroxide neutralization tests further confirmed the nature of the antimicrobial compound. Treatment of the bacteriocin with trypsin resulted in the loss of inhibitory activity, verifying that the active substance was proteinaceous, which is a key characteristic of bacteriocins (Ren *et al.*, 2022). The sodium pyruvate treatment, intended to neutralize hydrogen peroxide, showed no residual inhibition,

which ruled out oxidative metabolites as the inhibitory agents. This is consistent with Soltani *et al.* (2022), who emphasized that genuine bacteriocin activity should remain unaffected by catalase or pyruvate treatments but be deactivated by proteolytic enzymes. In my trials, repeated tests consistently showed that only untreated samples inhibited *S. aureus*, confirming that the antimicrobial activity was due to bacteriocin proteins and not secondary metabolites or hydrogen peroxide. This also reflects the purity and specificity of my isolate's bacteriocin production, which agrees with the criteria outlined by Sugrue *et al.* (2024) for defining true bacteriocin activity.

The hydrogen peroxide test using the KI–starch method produced no blue-black coloration, confirming the absence of hydrogen peroxide in the bacteriocin extract. This supports the earlier neutralization results and demonstrates that the inhibition observed in my agar well diffusion assays was not due to oxidative stress but solely from bacteriocin peptides. According to Darbandi *et al.* (2022) and Ren *et al.* (2022), many *Lactobacillus* species can produce hydrogen peroxide as a secondary antimicrobial mechanism; however, my results show that the isolate relied entirely on bacteriocin activity for inhibition. This absence of hydrogen peroxide is advantageous because it reduces the likelihood of oxidative damage to host cells and food matrices, making the bacteriocin safer for possible application in food preservation.

The HPLC characterization of my bacteriocin extract revealed twelve prominent peaks corresponding to various bioactive compounds, including **methyl gallate, gallic acid, ferulic acid, syringic acid, caffeic acid, kaempferol, catechin, naringenin, ellagic acid, hesperetin, chlorogenic acid, and rutin**. Many of these compounds are

known phenolic and flavonoid molecules with well-documented antioxidant and antimicrobial properties (Pang *et al.*, 2025; Sugrue *et al.*, 2024). The presence of these compounds indicates that my bacteriocin preparation may contain synergistic bioactive metabolites that enhance antimicrobial potency. For instance, **catechin** and **naringenin** have been reported to disrupt bacterial cell membranes and increase permeability, allowing peptides to enter more effectively (Peng *et al.*, 2023). The detection of such compounds in my extract agrees with studies by Ren *et al.* (2022) and Mercado and Olmos (2022), which reported that bacteriocin preparations often co-elute with phenolic compounds that contribute to their bioefficacy. I personally found this intriguing because it suggests that the antibacterial activity of my bacteriocin is not just due to the peptide itself but may be reinforced by naturally occurring secondary metabolites.

Overall, the results obtained from this research confirm that the *Lactobacillus* isolate from Mason's Natural Probiotics is a strong bacteriocin producer with significant antimicrobial potential. The findings align with global reports that lactic acid bacteria and their metabolites can serve as effective biopreservatives and alternative antimicrobials (Parada Fabián *et al.*, 2025; Sugrue *et al.*, 2024). Although my study focused primarily on in vitro activity, these results provide a foundation for future applications in food preservation and therapeutic formulations. The absence of hydrogen peroxide, coupled with the confirmed proteinaceous nature and rich bioactive composition, indicates that the bacteriocin produced is both potent and safe. In my opinion, these findings strengthen the growing argument for replacing or

supplementing chemical preservatives and antibiotics with biologically derived bacteriocins, as supported by recent reviews from Jenkins and Mason (2022) and Pang *et al.* (2025).

## CONCLUSION

In this study, I successfully isolated and identified *Lactobacillus species* from Mason's Natural Probiotics, which produced a bacteriocin with strong antibacterial activity against *Staphylococcus aureus*. The isolate showed typical *Lactobacillus* characteristics, and the bacteriocin demonstrated clear inhibition zones, confirming its antimicrobial potential.

Enzyme treatment with trypsin caused loss of activity, while hydrogen peroxide neutralization and KI–starch tests showed no oxidative effect, confirming that the antimicrobial compound was purely proteinaceous. HPLC analysis revealed several phenolic and flavonoid compounds such as **catechin**, **naringenin**, and **caffeic acid**, which may contribute to its bioactivity.

Overall, I concluded that the bacteriocin from *Lactobacillus sp.* is a true, protein-based antimicrobial substance with potential application as a natural preservative and an alternative to conventional antibiotics.

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## APPENDIX 1

### **A: Media Preparation**

De Man, Rogosa and Sharpe (MRS) Broth and Agar:

Prepared according to manufacturer's instructions. 55.15 g/L of dehydrated MRS powder was dissolved in distilled water and sterilized at 121°C for 15 minutes.

Nutrient Agar and Mueller-Hinton Agar:

Prepared at 28 g/L and 38 g/L respectively, sterilized at 121°C for 15 minutes, and poured aseptically into sterile Petri dishes.

### **B: Isolation of *Lactobacillus* Species**

Mason's Natural Probiotic capsule content was weighed (1.033g) and dissolved in 9 mL of sterile MRS broth.

The broth was incubated at 37°C under anaerobic conditions for 48 hours.

Aliquots were streaked on MRS agar and incubated at 37°C for 24–48 hours.

Colonies were purified by subculturing and identified by Gram staining and biochemical tests (oxidase, catalase, and carbohydrate fermentation).

### **C: Preparation of Crude Bacteriocin**

CFS obtained by centrifuging culture at 5000 rpm for 15 minutes.

Ammonium sulfate was added to 80% saturation at 4°C overnight.

Precipitated proteins were collected by centrifugation (5000 rpm, 20 minutes) and re-dissolved in PBS.

The crude extract was stored at 4°C for further analysis.

## APPENDIX 2

### A:HPLC Analysis

HPLC performed using Shimadzu SCL-10AVP with a C18 column.

Mobile phase: acetonitrile:water (54:46 v/v), flow rate 1 mL/min, detection at 660 nm.

Peaks identified for phenolic and flavonoid compounds.

