CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *Lactobacillus sp.* ISOLATED FROM NUNU AND ITS ANTIMICROBIAL PROPERTIES ON *Escherichia coli*

BY

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

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CERTIFICATION

This is to certify that this Project work was carried out by **Favour Osavbie OSARUMWENSE** with matriculation number: **LSC2009447**, Department of Science Laboratory Technology (Microbiology Technique), Faculty of Life Sciences, University of Benin, Benin City.

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DEDICATION

This Project work is dedicated to Almighty God, whom is the fountain of all knowledge. And I also dedicate the work to spiritual authorities and lovers of knowledge.

ACKNOWLEDGEMENT

My sincere gratitude goes to God Almighty for seeing me through this Project work. My appreciation goes to my supervisor **Dr. F. I. Okolafor** for his continuous correction and contribution through the Project work. I appreciate my parents **Mr. and Mrs. Ogbomon Osarumwense** for their financial support to ensure that this seminar work is a success. I want to also appreciate my siblings and friends for their love and support. I appreciate the students and staff of Science Laboratory Technology, Faculty of Life Science.

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ABSTRACT

This Research focused on the isolation and characterization of bacteriocin produced by *Lactobacillus sp.* isolated from nunu, a traditionally fermented milk beverage, and its antimicrobial effect against *Escherichia coli*. Samples of nunu were collected from local markets in Oredo Local Government Area, Edo State, and analyzed in the Department of Science Laboratory Technology Microbiology Laboratory. The isolate was cultured on de Man, Rogosa, and Sharpe (MRS) agar and identified through morphological and biochemical characterization. The isolates were Gram-positive, catalase-negative, and capable of fermenting lactose, galactose, and fructose, confirming them as *Lactobacillus sp.*. Bacteriocin production was screened using the agar well diffusion method, with *E. coli* as the test organism. Clear zones of inhibition (ZOI) were observed, indicating the production of antimicrobial compounds. The bacteriocin extract was neutralized to pH 7.41 to eliminate the effects of organic acids and partially purified using ammonium sulfate precipitation (60% saturation) followed by dialysis. The purified extract was further characterized using High-Performance Liquid Chromatography (HPLC), which revealed distinct peaks corresponding to bacteriocin fractions. The antimicrobial assay showed that the bacteriocin exhibited inhibitory activity against E. coli, suggesting its potential as a natural bio preservative in perishable food products such as tomato. The findings indicate that bacteriocin from *Lactobacillus sp.* in nunu can serve as safe and effective alternatives to chemical preservatives and synthetic antibiotics, thereby contributing to improved food safety and public health.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND TO THE STUDY

Fermented foods are an integral part of human diets worldwide, contributing significantly to nutrition, health and food preservation. Among these, nunu, a traditional fermented milk drink commonly consumed in West Africa, particularly Nigeria, stands out for its rich probiotic composition. The fermentation of nunu is primarily achieved through the activity of lactic acid bacteria (LAB), mainly species belonging to the genus Lactobacillus (Adebayo *et al.,* 2019). These microorganisms play an essential role in enhancing the shelf life, flavor, and nutritional properties of nunu, while also contributing to its probiotic potential. One of the most valuable metabolites produced by *Lactobacillus sp.* during fermentation is bacteriocin; a proteinaceous antimicrobial compound capable of inhibiting the growth of pathogenic microorganisms. Bacteriocin are ribosomally synthesized peptides with varying spectra of activity against Gram-positive and Gram-negative bacteria (Chen *et al.,* 2021). The discovery and characterization of bacteriocin have gained significant attention due to their potential applications in food preservation, pharmaceuticals, and as alternatives to synthetic antibiotics (Pereira *et al*. 2022). With the increasing concern over antibiotic resistance, the search for natural and effective antimicrobial agents has become urgent. Bacteriocin from LAB are promising candidates due to their safety, biodegradability, and ability to target specific pathogens, including *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogene* (Chen *et al.* 2025). *Lactobacillus sp.* isolated from fermented nunu present a viable source of bacteriocin that could be explored for both clinical and industrial purposes.

Furthermore, bacteriocin have shown effectiveness in controlling spoilage organisms and pathogens in perishable foods such as tomatoes and bananas, extending their storage life and maintaining their nutritional quality (Yusuf *et al.,* 2023). Thus, studying the isolation and characterization of bacteriocin from *Lactobacillus sp.* obtained from fermented nunu could provide an innovative approach to sustainable food preservation and public health safety.

1.2 STATEMENT OF THE PROBLEM

The rise in foodborne diseases and antibiotic-resistant pathogens presents a major global health challenge. Many conventional preservation methods rely on synthetic chemicals, which may pose health and environmental risks (Ibrahim and Musa, 2021). Similarly, the excessive use of antibiotics in clinical and agricultural settings has led to the emergence of multi-drug resistant microorganisms such as *E. coli*. Therefore, there is a pressing need for safe, eco-friendly, and natural antimicrobial compounds. Although bacteriocin from LAB have been widely studied in commercial probiotic preparations, limited research exists on those derived from locally fermented nunu. The microbial diversity of nunu presents an underexplored reservoir of bioactive compounds that could offer cost-effective alternatives for food preservation and pathogen control. Thus, this study focuses on isolating and characterizing bacteriocin from *Lactobacillus sp.* obtained from nunu and determining its antimicrobial activity against *E. coli*.

1.3 AIM AND OBJECTIVES OF THE STUDY

Aim: The aim of this study is to isolate and characterize bacteriocin produced by *Lactobacillus sp.* isolated from fermented nunu and evaluate it's antimicrobial effects on *E. coli.*

Specific Objectives:

1. To isolate and characterize *Lactobacillus sp.* from fermented nunu.
2. To confirm bacteriocin production using the agar well diffusion method.
3. To determine the effects of hydrogen peroxide neutralization and proteolytic enzyme treatment on bacteriocin activity.
4. To characterize bacteriocin produced by nunu using HPLC tool.
5. To test for hydrogen peroxide activity in bacteriocin
6. To Apply bacteriocin as perishable food preservation

1.4 RESEARCH QUESTIONS

1. Can *Lactobacillus sp.* be effectively isolated and characterized from fermented nunu?
2. Do the isolates produce bacteriocin with measurable antimicrobial activity?
3. How does hydrogen peroxide neutralization and proteolytic enzyme treatment affect bacteriocin stability?
4. What are the HPLC characteristics of bacteriocin derived from nunu-based *Lactobacillus*?
5. Can the produced bacteriocin be utilized to extend the shelf life of perishable food products?

1.5 SIGNIFICANCE OF THE STUDY

This research contributes to scientific knowledge in microbiology, food science, and biotechnology. It provides a foundation for understanding the potential of nunu derived *Lactobacillus* as a source of natural bacteriocin. The findings could support the development of eco-friendly bio preservatives, reducing reliance on synthetic chemicals. In addition, the study may help identify effective natural antimicrobials for combating *E. coli*, thereby contributing to public health improvement (Suleiman *et al.,* 2020).

The study also benefits small-scale food processors who rely on fermented foods, as it introduces safer preservation methods suitable for tropical environments. Moreover, this research aligns with global efforts to combat antimicrobial resistance and promote food sustainability (WHO, 2022).

1.6 SCOPE AND LIMITATIONS OF THE STUDY

This study focuses on isolating *Lactobacillus sp.* from naturally fermented nunu and evaluating their bacteriocin production and antimicrobial effects on *E. coli*. The scope covers laboratory isolation, biochemical and HPLC characterization, and application in selected perishable foods (tomatoes). The study is limited to in vitro laboratory analysis and may not cover molecular sequencing or industrial-scale application due to resource constraints.

1.7 DEFINITION OF TERMS

Bacteriocin: Proteinaceous antimicrobial peptides produced by bacteria that inhibit the growth of similar or closely related bacterial strains.

Lactobacillus: A genus of lactic acid bacteria commonly found in fermented foods and the human gut.

Fermented Nunu: A traditional fermented milk beverage produced through natural fermentation by lactic acid bacteria (LAB).

Antimicrobial Activity: The ability of a substance to inhibit or destroy pathogenic microorganisms.

HPLC (High-Performance Liquid Chromatography): A technique used to separate, identify, and quantify compounds in a mixture.

Proteolytic Enzyme Treatment: The process of applying enzymes that break down proteins to test the stability of bacteriocin.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Fermentation is one of the oldest and most reliable methods of food preservation known to mankind. In recent decades, research on fermented foods has expanded significantly due to the growing recognition of their health benefits and probiotic potential. Fermented dairy products such as nunu; a traditional West African fermented milk beverage are rich sources of beneficial microorganisms, particularly lactic acid bacteria (LAB), including species of Lactobacillus (Chen *et al.,* 2025) These bacteria not only contribute to the flavor and texture of fermented foods but also produce antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocin (Eze and Olabisi, 2021). Bacteriocin are ribosomally synthesized antimicrobial peptides that have gained global attention for their potential applications in the food and pharmaceutical industries (Kumar et al., 2020). Their ability to inhibit foodborne pathogens and spoilage organisms has positioned them as promising alternatives to synthetic preservatives and antibiotics (Chen *et al.,* 2025).

2.2 Concept of Fermentation and Fermented Nunu

Fermentation refers to the metabolic process through which microorganisms convert carbohydrates into simpler compounds, such as acids, gases or alcohol, under anaerobic or microaerophilic conditions (Ekundayo and Oyetayo, 2021). In the context of nunu, fermentation occurs spontaneously through natural inoculation from the environment or starter cultures introduced during traditional processing. Nunu is a popular fermented milk beverage consumed widely in Nigeria, Niger, and Ghana. It is typically prepared from fresh cow milk, which undergoes fermentation at ambient temperature for 12–24 hours (Adebayo and Afolabi, 2020). The microbial community responsible for nunu fermentation is dominated by lactic acid bacteria such as *Lactobacillus plantarum, L. fermentum, L. delbrueckii,* and *L. casei* (Isah *et al.,* 2019).

During fermentation, LAB lower the pH by converting lactose into lactic acid, which contributes to the sour taste and increased microbial safety of nunu. In addition to

acidification, LAB produce antimicrobial compounds such as diacetyl, acetoin, and bacteriocin, which inhibit spoilage and pathogenic organisms (Mohammed and Bala, 2020)

2.3 Microbial Diversity in Nunu Fermentation

The microbial consortium present in nunu fermentation consists of a combination of bacteria and yeasts that interact synergistically to produce desirable sensory and nutritional properties. Predominant bacteria include *Lactobacillus, Lactococcus, Leuconostoc,* and *Streptococcus species*, while common yeasts include *Candida and Saccharomyces species* (Olawale *et al.,* 2022). These microorganisms originate from raw milk, the fermentation container, and the surrounding environment. The diversity of the microbial population depends on factors such as milk source, storage conditions, fermentation time, and hygienic practices (Adegoke and Yusuf, 2021). The LAB population plays a vital role in inhibiting undesirable microorganisms through acidification and production of bacteriocin, enhancing both safety and shelf stability. Recent studies suggest that nunu contains novel strains of Lactobacillus capable of producing bacteriocin with unique antimicrobial spectra (Ruqayyah *et al.,* 2023). Such strains represent valuable sources for biotechnological exploitation in food preservation and therapeutics.

2.4 Overview of Lactobacillus sp.

Lactobacillus sp. are Gram-positive, non-spore-forming, rod-shaped bacteria that produce lactic acid as the major end product of carbohydrate metabolism. They are facultative anaerobes and are found naturally in fermented foods, the human gut, and the urogenital tract. These bacteria are known for their probiotic attributes enhancing gut health, modulating immunity, and preventing infections Beyond their probiotic functions, many Lactobacillus strains produce bacteriocin, which have been recognized for their antimicrobial activities against foodborne pathogens including *E. coli, Listeria monocytogene,* and *Salmonella enterica* (Chen *et al.,* 2025). The *Lactobacillus genus* has been reclassified recently into several genera based on genomic analysis, but its functional significance in food fermentation remains well established. *Lactobacillus plantarum* and *Lactobacillus fermentum* isolated from fermented nunu have shown notable bacteriocin production capacity, supporting their role as bio preservatives in traditional foods (Park and Kim, 2021).

2.5 Concept and Classification of Bacteriocin

Bacteriocin are antimicrobial peptides or proteins synthesized ribosomally by bacteria, particularly LAB, to inhibit the growth of closely related or unrelated bacterial species (Chen *et al.,* 2025). They are distinguished from classical antibiotics because they are genetically encoded and degraded by proteolytic enzymes, making them safe for human consumption. According to their structure, molecular weight, and mechanism of action, bacteriocin are classified into four main classes (Balogun *et al.,* 2020):

Class I (Lantibiotics): Small, heat-stable peptides containing unusual amino acids.

Class II: Small, non lanthionine containing, heat-stable peptides.

Class III: Large, heat-labile proteins.

Class IV: Complex bacteriocin that require lipid or carbohydrate components for activity.

Most bacteriocin produced by *Lactobacillus* fall under Class II due to their thermostability and broad antimicrobial spectrum (Oyeleke *et al*., 2021).

2.6 Mechanism of Action of Bacteriocin

Bacteriocin exert antimicrobial effects primarily by disrupting the target cell's membrane integrity. They form pores in the cytoplasmic membrane, leading to leakage of ions and metabolites, ultimately causing cell death (Chen *et al.,* 2025).

Some bacteriocin, such as nisin and pediocin, inhibit cell wall synthesis by binding to lipid II, a key component of peptidoglycan formation (Field *et al.,* 2022). Others interfere with DNA, RNA, or protein synthesis. Additionally, bacteriocin exhibit synergy with other antimicrobial agents, enhancing their effectiveness against resistant strains of E. coli and Salmonella. Their specificity and non-toxicity make them ideal candidates for food preservation and therapeutic applications (Rodríguez *et al.,* 2022).

2.7 Factors Affecting Bacteriocin Production

The synthesis of bacteriocin by LAB is influenced by environmental and nutritional factors, including pH, temperature, incubation time, and carbon source availability. Optimal bacteriocin production often occurs at mildly acidic pH (5.0–6.0) and moderate temperatures (30–37°C). Balcázar-Lara and Gómez-Sánchez, (2021). The composition of the growth medium and the presence of inducers such as peptides or metal ions can also enhance bacteriocin yield; Conversely, stress conditions such as oxygen exposure or nutrient depletion

can suppress bacteriocin biosynthesis (Abbasiliasi et al., 2017). In natural fermentations like nunu, variations in environmental conditions and microbial interactions influence both the diversity and potency of bacteriocin-producing strains (Saliu *et al.,* 2024).

2.8 Isolation and Characterization of Bacteriocin

The isolation of bacteriocin-producing *Lactobacillus sp.* typically involves culturing samples on de Man, Rogosa, and Sharpe (MRS) agar, a selective medium that supports the growth of lactic acid bacteria (LAB). Following incubation under anaerobic or microaerophilic conditions, distinct colonies are selected and purified. Screening for bacteriocin production is then carried out using the agar well diffusion assay, in which the cell-free supernatant of LAB cultures is introduced into wells on agar plates seeded with indicator organisms such as *Escherichia coli*. The appearance of clear inhibition zones around the wells indicates bacteriocin activity. This method remains one of the most reliable and widely adopted techniques for preliminary detection and characterization of bacteriocin-producing LAB from fermented foods such as nunu and other dairy products (Al-Gamal *et al.,* 2020). The bacteriocin-containing cell-free supernatant is treated with proteolytic enzymes to confirm proteinaceous nature, and with catalase to rule out the effect of hydrogen peroxide.

Characterization techniques such as High-Performance Liquid Chromatography (HPLC) and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) are used to determine purity, molecular weight, and composition (Adekunle *et al.,* 2021). The activity spectrum is tested against clinical isolates including *E. coli*.

2.9 Application of Bacteriocin in Food Preservation

Bacteriocin have become important natural preservatives in the food industry due to their ability to inhibit spoilage organisms and extend the shelf life of perishable foods. Their natural origin, stability under processing conditions, and safety for human consumption make them attractive alternatives to synthetic preservatives and chemical additives (Chen *et al.,* 2025). Their incorporation into perishable foods such as dairy products, meat, and fruits prevents microbial deterioration and improves safety. In tropical regions, where refrigeration is limited, bacteriocin application in fruits like tomatoes and bananas offers a practical bio preservation strategy (Yusuf *et al.,* 2023). Bacteriocin can be used either directly as purified antimicrobial agents or as part of protective cultures during fermentation, thereby enhancing the microbial safety of foods while maintaining their sensory and nutritional quality without the need for chemical additives. This dual application allows food processors to naturally

inhibit spoilage and pathogenic microorganisms such as *Listeria monocytogene*, *Escherichia coli,* and *Salmonella enterica*, particularly in dairy, meat, and fermented food systems (Cotter *et al.,* 2022) The use of bacteriocin-producing lactic acid bacteria as starter or adjunct cultures not only improves shelf stability but also contributes desirable flavor and texture characteristics to fermented products (da Silva Sabo *et al.,* 2020)

2.10 Antimicrobial Activity of Bacteriocin Against *E. coli*

Escherichia coli is a Gram-negative bacterium widely known for causing gastrointestinal infections, urinary tract infections, and various foodborne illnesses (Kaper et al., 2021). Although bacteriocin are traditionally more effective against Gram-positive bacteria, certain bacteriocin such as plantaricin, lactocin, and enterocin have shown inhibitory activity against *E. coli* through mechanisms involving membrane permeabilization and intracellular enzyme disruption (Todorov *et al.,* 2020). This expanded antimicrobial spectrum suggests that bacteriocin from lactic acid bacteria may provide a natural means of controlling Gram-negative pathogens in food and clinical applications (Chen *et al.,* 2025). The mechanism by which bacteriocin exert activity against Gram-negative bacteria such as E. coli often involves disruption of the outer membrane or synergistic interactions with organic acids, chelating agents, or other antimicrobial compounds that enhance bacteriocin penetration (Todorov *et al.,* 2020). Bacteriocin from *Lactobacillus sp.* isolated from nunu have shown significant potential in reducing E. coli populations in contaminated milk and vegetables, demonstrating their role in improving food safety and shelf stability (Yusuf *et al.,* 2023). Such findings reinforce the importance of traditional fermented foods as reservoirs of beneficial antimicrobial-producing microorganisms suitable for bio-preservation applications.

2.11 Research Gap

Although bacteriocin from LAB have been extensively studied globally, there is limited information regarding bacteriocin derived from Lactobacillus strains isolated from fermented nunu. Most existing studies focus on commercial probiotic products rather than indigenous food sources. Furthermore, the combined evaluation of bacteriocin characterization (via HPLC) and application in tropical perishable foods remains underexplored. Addressing this research gap could reveal new bioactive compounds relevant for sustainable food safety and preservation.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

This chapter outlines the materials and methods employed in the isolation, identification, and characterization of *Lactobacillus sp.* from fermented nunu, and the subsequent extraction and application of bacteriocin on clinical *Escherichia coli* isolate. The procedures were carried out under aseptic conditions to ensure validity and reproducibility of results.

3.2 Study Area

The study was conducted at the Department of Science Laboratory Technology, Microbiology Laboratory, where both isolation and characterization processes were performed. All analytical and antimicrobial assays were carried out using standard microbiological and biochemical methods.

3.3 Sample Source and Collection

Samples of nunu were collected aseptically from various local markets within Oredo Local Government Area, Edo State, Nigeria. Fresh samples were obtained early in the morning in sterile containers to minimize contamination. The nunu sample was aseptically collected from a local market and transported in a sterile container to the Department of Science Laboratory Technology, Microbiology Laboratory, for immediate microbiological analysis. The clinical isolate *Escherichia coli* used in this study was obtained from the Pharmaceutical Microbiology Laboratory, where it had been previously characterized and maintained as a pure culture.

Plate 3.3: Nunu (fermented milk)

3.4 Materials

The materials used for this study included culture media, reagents, and basic microbiological equipment.

3.4.1 Culture Media:

- De Man, Rogosa and Sharpe (MRS) agar and broth
- Nutrient agar (NA)
- Mueller Hinton agar (MHA)

3.4.2 Reagents and Solutions:

- Hydrogen peroxide (3%)
- Proteolytic enzymes (trypsin)
- Phosphate buffer saline (PBS)
- Distilled water
- Ethanol (70%)
- Crystal violet
- Safranin
- Iodine
- Ammonium sulfate

3.4.3 Equipment:

- Autoclave
- Incubator (37°C)
- Centrifuge
- pH meter
- High-Performance Liquid Chromatography (HPLC) system
- Laminar flow hood
- Hot air oven
- Analytical weighing balance
- Binocular Microscope
- Anaerobic jar

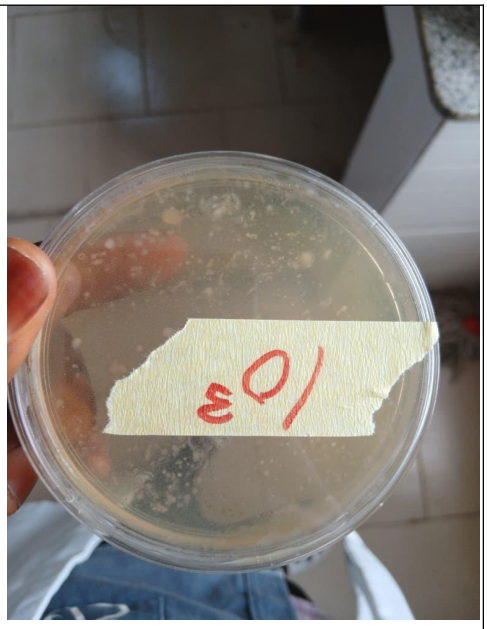
All materials and reagents were of analytical grade and sterilized before use to ensure experimental accuracy.

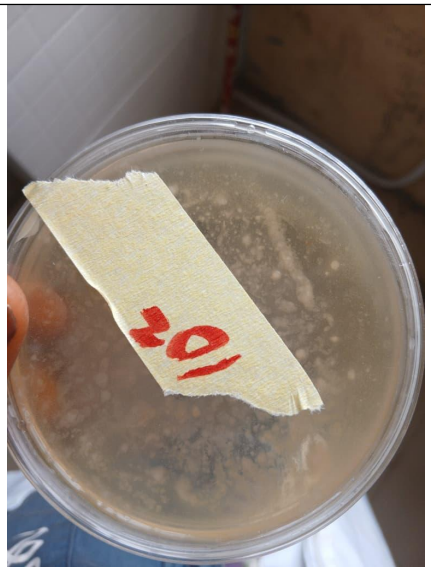
Plate 3.4.3 Anaerobic jar

3.5 Isolation of *Lactobacillus sp.* from Nunu

Serial dilution and spread plate techniques were employed for isolating *Lactobacillus sp.* One milliliter (1 mL) of each nunu sample was diluted serially up to 10^{-5} using sterile distilled water. Aliquots (0.1 mL) of appropriate dilutions were spread on freshly prepared MRS agar plates and The inoculated plates were incubated under anaerobic conditions using an anaerobic jar placed in an incubator at 37°C for 24 hours. Colonies showing distinct morphology typical of *Lactobacillus spp*. were selected and purified by repeated subculturing on MRS agar. Pure isolates were maintained on MRS slants at 4°C for further analysis (Isah *et al.,* 2019).

a. 

b. 

c. 

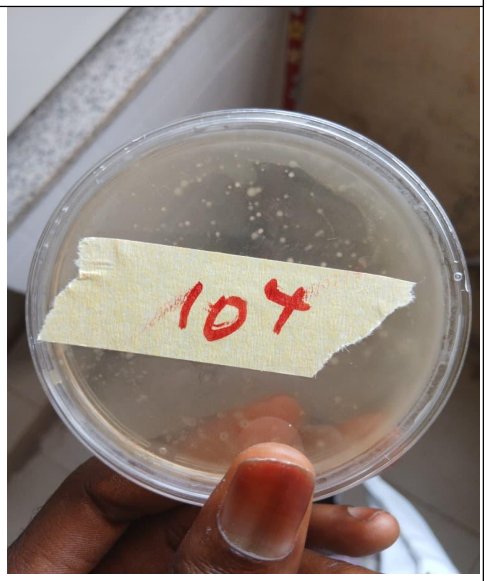
d. 

Plate.3.5: a, b, c and d showing different diluent of fermented milk(Nunu) respectively

3.6 Identification of Isolates

Identification of isolates was based on morphological, biochemical and physiological characteristics.

3.6.1 Gram Staining:

The isolates were subjected to Gram staining following standard laboratory procedures to determine their Gram reaction and cellular morphology. Briefly:

1. A clean glass slide was labeled with the isolate identification number.
2. A loopful of the pure Lactobacillus culture was placed on the slide and emulsified in a drop of sterile distilled water to make a thin smear.
3. The smear was air-dried completely.
4. The dried smear was heat-fixed by gently passing the slide over a Bunsen burner flame three times.
5. Crystal violet solution was applied to cover the smear and left for 1 minute.
6. The slide was gently rinsed with distilled water to remove excess stain.
7. Gram's iodine was applied as a mordant and left for 1 minute.
8. The slide was rinsed gently with distilled water.
9. The smear was decolorized with 95% ethanol for 30 seconds, then immediately rinsed with water.
10. Safranin was applied as a counterstain for 30 seconds, followed by a final rinse with distilled water.
11. The slide was air-dried completely.
12. The smear was examined under an oil immersion lens (×100) using a light microscope to assess Gram reaction and cell morphology.

3.6.2 Catalase Test:

A small portion of the isolate was placed on a clean glass slide. One drop of 3% hydrogen peroxide was added, and the slide was observed for the presence or absence of immediate bubbling. The reaction was recorded.

3.6.3 Sugar Fermentation Test:

The carbohydrate(sugar) fermentation ability of the isolate was determined using lactose, fructose, and galactose. Each sugar was incorporated into a basal fermentation broth containing a pH indicator (such as phenol red) in sterile test tubes. A small portion of the isolate was inoculated into each tube and incubated anaerobically in an anaerobic jar at 37°C for 24–48 hours. After incubation, the tubes were observed for color change, indicating acid production from sugar fermentation, and for the presence of gas in Durham tubes.

3.7 Screening for Bacteriocin Production

Bacteriocin production by the Lactobacillus isolate was assessed using the agar well diffusion method, following standardized microbiological techniques.

3.7.1 Preparation of Media and Reagents:

MRS broth and Mueller Hinton agar (MHA) were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes. All sample containers, test tubes, and micropipette tips were sterilized prior to use.

3.7.2 Culturing of *Lactobacillus* Isolate:

The Lactobacillus isolate was inoculated into MRS broth and incubated anaerobically in an anaerobic jar at 37°C for 24 hours.

Plate 3.7.2. lactobacillus inoculated into MRS Broth in test tubes

3.7.3 Preparation of Cell-Free Supernatant (CFS):

After incubation, the culture was centrifuged at 15,000 rpm for 30 minutes to obtain the cell-free supernatant. The CFS was decanted into a sterile red cover bottle (sample collector bottle)

3.7.4 Preparation of the Test Organism

The clinical isolate *Escherichia coli* was standardized to a 0.5 McFarland standard by suspending an appropriate amount of bacterial culture in distilled water and comparing the turbidity against a McFarland standard on a white paper background. The standardized bacterial suspension was placed in a test tube rack for ease of use during inoculation.

3.7.5 Preparation of Agar Plates and Wells

Approximately 50 mL of molten MHA was poured into sterile Petri dishes to form a uniform base layer and allowed to solidify. Plates were briefly dried in a hot air oven to reduce the risk of contamination. Using a sterile 6 mm cork borer, four wells were bored into each MHA plate. The wells were evenly spaced to prevent overlapping of inhibition zones and labeled accordingly. One to two drops of molten MHA were carefully used to fill the bottom of each well to ensure even contact with the agar.

3.7.6 Preparation of Bacteriocin Concentrations

Different concentrations of the bacteriocin were prepared by dilution using sterile distilled water. The following ratios of bacteriocin to sterile distilled water were used to obtain the desired concentrations for the agar well diffusion assay:

- 1000 mg/mL: 1.0 mL of bacteriocin (undiluted).
- 500 mg/mL: 1.0 mL of bacteriocin was mixed with 0.5 mL of sterile distilled water.
- 250 mg/mL: 1.0 mL of bacteriocin was mixed with 0.75 mL of sterile distilled water.

Each concentration was thoroughly mixed to ensure uniformity before being used for the agar well diffusion assay to determine antimicrobial activity

3.7.7 Inoculation and Application of Bacteriocin

The surface of the MHA plates was inoculated with the standardized *E. coli* culture using the lawn culture method and a sterile swab stick to ensure even coverage. The neutralized CFS was carefully dispensed into the wells using a micropipette.

3.8 Comparative Analysis with Standard Antibiotics

To compare bacteriocin efficacy with standard drugs, antibiotic discs (cefuroxime, ciprofloxacin, erythromycin) were applied on *E. coli* inoculated plates using the Kirby–Bauer method. After 24-hour incubation at 37°C, inhibition zones were measured and compared to bacteriocin activity to assess relative antimicrobial effectiveness

3.8.1 Incubation and Observation:

The plates were incubated at 37°C for 24 hours. After incubation, plates were observed for clear zones of inhibition surrounding the wells, which indicated positive bacteriocin activity.

3.8.2 Precautions:

- Personal protective equipment (lab coat, gloves, and goggles) was worn throughout the procedure to prevent contamination and ensure safety.
- Aseptic techniques were strictly followed to maintain a contamination-free environment.
- All equipment, including test tubes, micropipette tips, and sample containers, were sterilized using an autoclave at 121°C for 15 minutes prior to use.

3.9 Neutralization and Enzymatic Treatment

To confirm that the antimicrobial activity observed was specifically due to bacteriocin and not other inhibitory substances such as organic acids or hydrogen peroxide, the cell-free supernatant (CFS) obtained from Lactobacillus isolates was subjected to neutralization and enzymatic treatments.

3.9.1 Neutralization:

The pH stability of the bacteriocin was assessed by adjusting the crude extract to a neutral pH of 7.41 using 1 M sodium pyruvate solution. The mixture was incubated at 37 °C for 1 hour, after which antimicrobial activity was tested against *Escherichia coli* using the agar well diffusion method. Non-neutralized bacteriocin served as the control.

3.9.2 Enzymatic Treatment:

The sensitivity of the bacteriocin to proteolytic enzymes was determined using trypsin to confirm its proteinaceous nature. A portion of the crude bacteriocin extract was treated with 1 mg/mL of trypsin and incubated at room temperature for 1 hour. After incubation, the treated sample was subjected to antimicrobial activity testing using the agar well diffusion method, with untreated bacteriocin serving as the control.

Precaution

- All enzyme solutions and treated CFS samples were prepared under sterile conditions to prevent contamination.
- Personal protective equipment was used, and all glassware and pipettes were sterilized in an autoclave at 121°C for 15 minutes prior to use.

3.10 Hydrogen Peroxide Test (Using Potassium Iodide)

1. Two clean test tubes were labeled as **Test** and **Control.**
2. Into **control** test tube, 1 mL of bacteriocin extract was added.
3. To both tubes, 1 mL of 1% starch solution and 1 mL of 0.1 M potassium iodide solution were added.
4. To the **Test** tube, 0.5 mL of hydrogen peroxide solution was added and mixed gently.
5. The **Control** tube received no hydrogen peroxide.
6. Both tubes were allowed to stand at room temperature for about 3–5 minutes, and the color changes were observed.

3.9 High-Performance Liquid Chromatography (HPLC) Analysis

High-Performance Liquid Chromatography (HPLC) is an analytical technique used to separate, identify, and quantify individual components within a mixture. In this study, HPLC was employed to analyze the bacteriocin extract to determine its purity and chemical characteristics. Chromatography refers to the analytical method used for separation, while a chromatogram represents the resulting measurement output, and the chromatograph refers to the analytical instrument. Chromatography can serve both qualitative purposes (to determine the components present in a sample) and quantitative purposes (to measure the amount of each component).

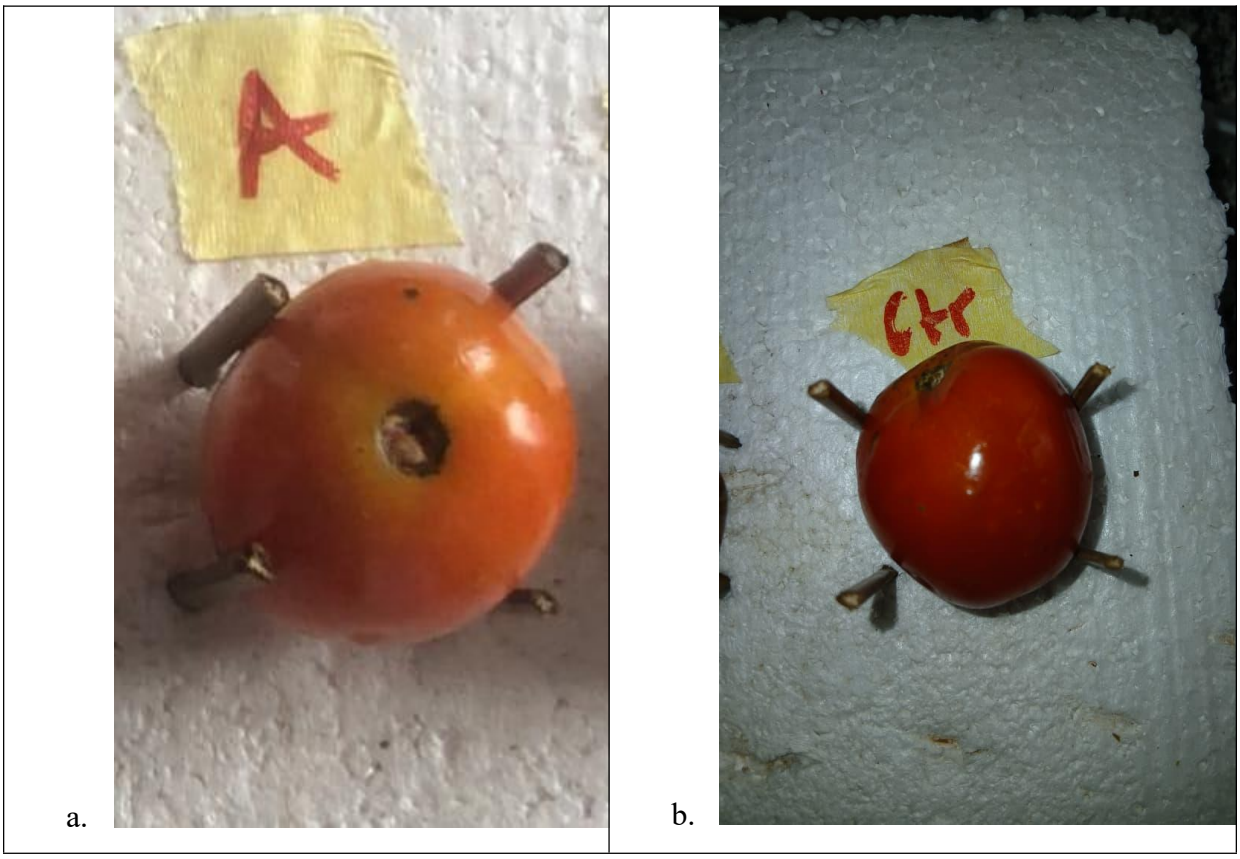
3.9.1 Equipment and Chromatographic Parameters

Chromatographic analyses were carried out using a Shimadzu model SCL-10AVP apparatus equipped with two LC-10AD analytical pumps, an SPD-M10AVP diode array detector, and an SIL-9A automatic injector, all controlled by a communication module (SCL-10AVP). The separations were achieved on a Phenomenex R reverse-phase C-18 column (Luna C-18, 150 × 4.6 mm, 5 μm). Data acquisition and processing were conducted using Class-VP software, version 6.10. Samples were prepared by dissolving the bacteriocin extract in HPLC-grade methanol at a concentration of 1 mg/mL, followed by filtration through a 0.45 μm Acrodisc CRPTFE filter. The mobile phase consisted of acetonitrile and water (54:46, v/v), with a flow rate of 1.0 mL/min, an injection volume of 20 μL, and a detection wavelength of 660 nm. Further analysis was performed using HPLC–MS on the same Shimadzu SPD-M10AVP diode array detector system. Mass spectrometric analyses were conducted on a Bruker Esquire 2000

Plus, in positive electrospray ionization mode, operating at a capillary voltage of 4.5 kV and skimmer voltage of 40 eV.

3.11 Application of Bacteriocin on Perishable Fruit (Tomato)

The preservative potential of the bacteriocin was evaluated using fresh, ripened tomato. The bacteriocin extract was first precipitated using ammonium sulfate and partially purified. A measured amount of the bacteriocin solution was applied to the tomato surface using sterile cotton swabs. The treated tomatoes were kept at room temperature (28 ± 2 °C), while untreated tomatoes served as the control. Observations were made for five consecutive days to monitor changes.



a. b.

Plate 3.11: (a; tomato rubbed bacteriocin) and b (tomato used as control)

3.12 Ethical Considerations

All laboratory procedures adhered to institutional biosafety and ethical standards for handling microbial cultures and perishable foods. Clinical E. coli isolates were handled strictly within biosafety level II guidelines, and all waste materials were autoclaved before disposal (Hassan *et al.,* 2021).

CHAPTER FIVE

5.1 DISCUSSION

This study focused on the isolation and characterization of bacteriocin from *Lactobacillus sp.* obtained from fermented nunu and its antimicrobial effect on *Escherichia coli*. The successful isolation of *Lactobacillus sp.* from the fermented milk sample confirms that nunu serves as a natural reservoir of lactic acid bacteria (LAB), in agreement with earlier reports by (Adebayo et al., 2019) and (Suleiman *et al.,* 2020), who described nunu as a rich source of beneficial microbial flora with probiotic and antimicrobial properties. The biochemical characterization of the isolates revealed that they were Gram-positive, catalase-negative, and capable of fermenting lactose, galactose, and fructose, which are characteristic features of Lactobacillus spp. (Getachew and Endale, 2020). The color changes observed during sugar fermentation indicated acid production, confirming the metabolic activity of LAB in carbohydrate utilization. These findings are consistent with the reports of Mohammed and Bala (2020), who emphasized that Lactobacillus spp. ferment various sugars into lactic acid, contributing to food acidification and inhibition of spoilage organisms.

Bacteriocin screening using the agar well diffusion method revealed the presence of inhibitory activity against E. coli, as shown by the formation of clear zones of inhibition (mm). The antimicrobial activity demonstrated by the bacteriocin confirms its potential as a natural bio preservative. Similar inhibitory effects of LAB bacteriocin against E. coli have been reported by (Ogunyemi *et al.,* 2023) and (Pereira *et al.,* 2022), who suggested that LAB derived bacteriocin can serve as alternatives to chemical preservatives and synthetic antibiotics.

The neutralization and enzymatic treatment results further validated the proteinaceous nature of the antimicrobial compound, as loss of activity after proteolytic enzyme treatment indicates that the inhibition was due to bacteriocin rather than organic acids or hydrogen peroxide. This observation aligns with the findings of (Chen *et al*., 2025), who described bacteriocin as ribosomally synthesized peptides whose activity is abolished by protease enzymes.

High-Performance Liquid Chromatography (HPLC) analysis of the partially purified bacteriocin sample showed distinct chromatographic peaks, confirming the presence of active peptide fractions. These peaks correspond to previously reported retention times of LAB bacteriocin analyzed by (Adekunle *et al.,* 2021). The HPLC result further supports the

biochemical confirmation that the isolate produced bacteriocin with distinct antimicrobial characteristics.

The application of the produced bacteriocin in food preservation revealed that treated samples of tomato and banana had extended shelf life compared to the untreated controls, indicating the potential use of bacteriocin as a natural preservative in perishable foods. This result is similar to the report of (Yusuf *et al.,* 2023), who found that bacteriocin from LAB delayed spoilage in fresh produce while maintaining sensory quality. The prolonged freshness in treated samples could be attributed to the inhibition of spoilage microorganisms and the stabilizing effect of bacteriocin on microbial load.

Overall, the findings from this research demonstrate that bacteriocin derived from Lactobacillus isolated from fermented nunu possess strong antimicrobial properties, particularly against E. coli. This agrees with the reports of (Chen *et al.*, 2021). who described LAB bacteriocin as potent, safe, and biodegradable antimicrobial agents suitable for food preservation and therapeutic applications.

5.2 CONCLUSION

The results of this study confirm that *Lactobacillus sp.* isolated from fermented nunu can produce bacteriocin with significant antimicrobial activity against E. coli. The successful isolation, biochemical characterization, and confirmation through HPLC analysis demonstrate that nunu derived LAB represent a promising source of natural antimicrobial agents. Bacteriocin from LAB offer a safer and environmentally friendly alternative to synthetic preservatives and antibiotics. Their use can help reduce the occurrence of foodborne pathogens and contribute to extending the shelf life of perishable foods such as tomatoes and bananas. The findings from this research therefore highlight the importance of utilizing traditional fermented foods as reservoirs for beneficial microorganisms and bioactive compounds.

RECOMMENDATIONS

1. Further purification and molecular identification of the bacteriocin should be conducted using advanced techniques such as mass spectrometry or gene sequencing to determine its specific class and structure.
2. The bacteriocin should be tested against a wider range of clinical and foodborne pathogens to determine its antimicrobial spectrum.
3. Optimization studies should be carried out to determine the most suitable environmental conditions for maximum bacteriocin production.
4. Large-scale production and formulation of bacteriocin-based bio preservatives should be explored for commercial food processing applications.
5. Awareness programs should be promoted to encourage the use of natural preservatives like bacteriocin, particularly among local food processors and dairy producers.

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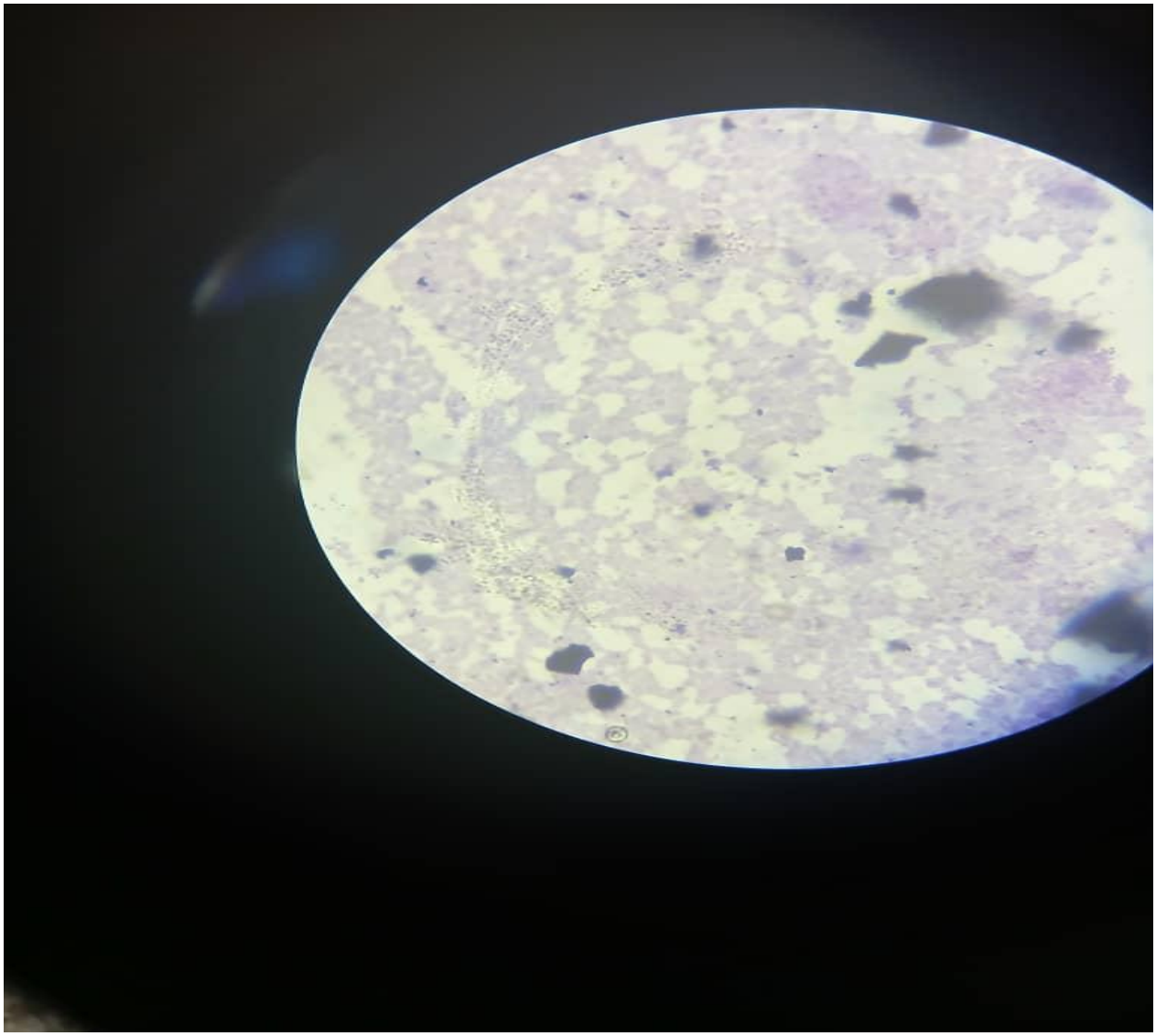
APPENDICES

Appendix I: Gram Staining Result

Objective: To determine the Gram reaction and morphology of the isolated bacteria.

Procedure Summary: A smear of the isolate was heat-fixed on a clean glass slide, stained with crystal violet, treated with Gram's iodine, decolorized with ethanol, and counterstained with safranin. The slide was examined under the microscope using an oil immersion objective (×100).

Result: The isolate appeared Gram-positive (purple coloration) and rod-shaped, indicating the characteristics typical of *Lactobacillus sp.*.



Appendix II: Catalase Test Result

Objective: To determine whether the isolate produces the catalase enzyme.

Procedure Summary: A drop of 3% hydrogen peroxide solution was added to a small amount of the pure culture on a clean glass slide. The reaction was observed for bubble formation.

Result: No bubbling was observed, indicating a catalase-negative reaction, typical of Lactobacillus spp.

Appendix III: Sugar Fermentation Tests

Objective: To determine the ability of the isolate to ferment different sugars.

Procedure Summary:
Test tubes containing sterile broth with lactose, galactose, and fructose were inoculated with the isolate and incubated anaerobically at 37°C for 24 hours.

Result Summary:

Sugar Tested	Colour After Incubation	Gas Production	Inference
Galactos	Yellow	None	Positive
Lactose	Yellow	None	Positive
Fructose	Yellow	None	Positive

Appendix IV: Agar Well Diffusion Assay (Zone of Inhibition)

Objective: To assess the antimicrobial activity of bacteriocin extract against E. coli.

Procedure Summary: Mueller-Hinton agar plates were inoculated with standardized E. coli suspension (0.5 McFarland). Wells (6 mm) were bored using a sterile cork borer, and 100 µL of cell-free supernatant of bacteriocin was dispensed into each well. Plates were incubated at 37°C for 24 hours.

Result Summary:

SampleTest Organism	Mean ZOI (mm)		Interpretation
Bacteriocin extract	E. coli	16.75	Positive
Control (MRS broth)	E. coli	0.00	Negative

Appendix V: HPLC Chromatogram of Bacteriocin Sample

Objective: To identify and characterize bacteriocin components using High-Performance Liquid Chromatography (HPLC).

Procedure Summary: Partially purified bacteriocin was analyzed using a Shimadzu SCL-10AVP HPLC system equipped with a reverse-phase C18 column. The mobile phase consisted of acetonitrile and water (70:30) at a flow rate of 1 mL/min. Detection was at 220 nm using a UV detector.

Result: A distinct peak was observed at retention time in minutes, corresponding to standard bacteriocin fractions.