CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *Lactobacillus sp.* ISOLATED FROM TRACE MINERAL PROBIOTICS AND IT *ANTIMICROBIAL* EFFECT ON *Escherichia coli*

BY

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LSC2007363

DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY

FACULTY OF LIFE SCIENCE

UNIVERSITY OF BENIN

BENIN CITY

NOVEMBER, 2025

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A PROJECT SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY, FACULTY OF LIFE SCIENCE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS, FOR THE AWARD OF BACHELOR'S DEGREE(BSC) IN SCIENCE LABORATORY TECHNOLOGY, UNIVERSITY OF BENIN,BENIN CITY,EDO STATE,NIGERIA

NOVEMBER, 2025

CERTIFICATION

This is to certify that this project work, titled "Characterization of bacteriocin produced by *Lactobacillus* sp. Isolated from trace mineral probiotics and it antimicrobial effect on *escharichia coli* carried out by **Uwadiunor miracle** (Miss) with matriculation number **LSC2007363**,Department of Science Laboratory Technology (Microbiology Techniques), Faculty of Life Sciences, **University of Benin**, Benin City, Edo State.

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_____ _____

EXTERNAL EXAMINAL Date

DEDICATION

I dedicate this work to Almighty God for His grace and to my loving parents for their unwavering support.

ACKNOWLEDGEMENT

I profoundly thank God almighty, from a place of immense gratitude and appreciation, for his provisions, grace and love upon my life. I also want to express profound gratitude to my supervisor, Dr.F.I OKOLAFOR,for his invaluable guidance, time, consistent feedback, and constant encouragement. His mentorship and patience were pivotal in the successful completion of this seminar work. I am truly thankful to my Head of Department, Prof. J.O. Osarumwense for creating a supportive academic atmosphere and promoting a culture of excellence within the department. His leadership and vision have served as an inspiration and I appreciate his support and encouragement. I am grateful to my course adviser, Mr. Salokun for his advice, encouragement and mentorship throughout my educational journey, as well as to the other staff members of the Department of Science Laboratory Technology, I express my gratitude. A huge thank you to my project coordinator, Dr. P. O. Alonge, for his patience and guidance during the research phase. I am particularly thankful to my family for their unfailing support, understanding, and motivation throughout my academic journey. I thank them greatly for always cheering me on. I also extend my heartfelt thanks to my sister Uwadiunor peace for the inspiration and encouragement and to my friends blessing,Esther,Lydia and prosper who have made my academic journey so far, memorable.

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ABSTRACT

This study investigates the isolation and characterization of *Lactobacillus* sp.Isolated Trace Minerals to evaluate their capacity for bacteriocin production.Lactic acid bacteria (LAB), especially those of the *Lactobacillus* sp, are well known for producing antimicrobial substances called bacteriocins. These natural peptides effectively inhibit the growth of harmful and spoilage microorganisms. Probiotic supplements such as Trace Minerals contain diverse *Lactobacillus* strains with strong bacteriocin-producing potential, making them valuable for both health and industrial applications.The isolates morphological and biochemical Properties was perform to confirm their identity to be *Lactobacillus* sp. Bacteriocin synthesis was assessed using the agar well diffusion technique to determine antimicrobial activity against selected test organisms. The influence of hydrogen peroxide neutralization and proteolytic enzyme treatment was examined to confirm the stability and proteinaceous nature of the produced bacteriocin. In addition, high-performance liquid chromatography (HPLC) was employed to characterize the molecular structure of the compound identified in the bacteriocin. This study isolated and identified *Lactobacillus* species from Trace Mineral probiotics to assess their bacteriocin production and antimicrobial activity against *Escherichia coli.* The isolates were cultured in MRS media under anaerobic conditions and confirmed through biochemical tests. Antimicrobial activity was evaluated using the agar well diffusion method, while enzyme and hydrogen peroxide treatments verified the protein nature of the bacteriocin. HPLC analysis confirmed the purity and presence of active bacteriocin fractions. The isolates displayed typical *Lactobacillus* characteristics and showed strong inhibitory effects against *E.coli,* supporting their potential as natural antimicrobial agents.This study confirms that *Lactobacillus* sp from Trace Mineral probiotics produce effective bacteriocins with strong activity against *E.coli.* The results verified the protein nature and purity of the compound, indicating its potential as a natural and safe antimicrobial agent for health and industrial use.

CHAPTER ONE

1.1 Background of the Study

The rise of antimicrobial resistance among pathogenic bacteria, particularly E.coli,has intensified the global demand for innovative antimicrobial strategies. In recent years, trace elements and their associated biochemical roles have attracted considerable attention as potential alternative therapeutic agents. Their ability to inhibit both closely and distantly related bacterial species without harming host tissues makes them highly valuable in clinical and food preservation applications (Sugrue *et al*., 2024).

The significance and beneficial effects of these mineral elements lie in their essential contribution to sustaining microbial equilibrium, promoting the growth of beneficial microbes, and suppressing harmful pathogens. Trace elements play a vital role in preserving microbial stability across various ecosystems, including fermented foods, the human microbiota, and environmental settings. Their well-documented antibacterial and antifungal activities make them important agents in controlling food-borne and multidrug-resistant microorganisms (Ren *et al*., 2022).

Additionally, these trace minerals enhance the functionality and resilience of probiotic strains by supporting the synthesis of antimicrobial substances like bacteriocins, thereby reinforcing natural defense mechanisms. Consequently, probiotics are regarded as valuable alternatives to conventional antibiotics, providing a safer and more sustainable means of promoting health and preventing microbial infections (Darbandi *et al*., 2022).

Commercial probiotics, including formulations such as “Trace Elements Probiotics,” serve as dependable sources of viable *Lactobacillus* strains. These formulations are widely utilized for their health-enhancing benefits, which include modulation of the gut microbiota, strengthening

of immune responses, and inhibition of pathogenic microorganisms (Jenkins and Mason, 2022). The isolation of bacteriocin-producing strains from such probiotic products thus represents both a valuable scientific pursuit and an industrial opportunity. Specifically, targeting pathogens such as E.coli a well-known clinical isolate responsible for skin infections, food poisoning, bacteremia, and severe conditions like sepsis and endocarditis highlights the clinical importance of bacteriocins derived from probiotics (Heinzinger *et al*., 2023).

Microbial contamination leading to food spoilage remains a global challenge, causing significant post-harvest losses and contributing to food insecurity, particularly in perishable commodities like tomatoes and bananas. Traditional chemical preservatives raise safety and health concerns due to potential toxicity and increasing consumer preference for natural and environmentally safe preservation methods (Peng *et al*., 2023). Owing to their biodegradability, safety, and potency even at minimal concentrations, bacteriocins have emerged as eco-friendly and sustainable alternatives for food preservation (Yang *et al*., 2021). Their antimicrobial mechanism often involves pore formation in bacterial cell membranes, disruption of essential cellular functions, or interference with nucleic acid and protein synthesis (Soltani *et al*., 2022).

Recent advancements in purification and analytical tools such as High-Performance Liquid Chromatography (HPLC) have improved the molecular characterization of bacteriocins, enabling precise evaluation of their stability, structure, and antimicrobial activity (Pang *et al*., 2025). This progress allows researchers to assess their full antimicrobial potential not only in laboratory studies but also in real-world applications like clinical infection management and food preservation. Additionally, bacteriocin-based approaches have shown synergistic effects when used with other antimicrobial agents, enhancing their inhibitory range and lowering the risk of resistance development (Soltani *et al*., 2022).

Considering the increasing occurrence of antibiotic-resistant E.coli and the global push for natural food preservation, exploring bacteriocins from probiotics such as *Lactobacillus* species presents dual benefits: improving human health outcomes and minimizing post-harvest food losses. Consequently, the isolation, characterization, and application of bacteriocins from probiotic products like "Trace Elements Probiotics" stand as a promising innovation in both medical microbiology and food science technology.

1.2 AIM OF STUDY

The study is aimed at isolation and characterization of bacteriocin by *Lactobacillus* sp from "Trace elements probiotics and it antimicrobial effects on clinical isolate Escherichia coli.

1.3 OBJECTIVES

The specific objective of project are to:

- To isolation and characterize *Lactobacillus* species from Trace minerals probiotics.
- Confirm bacteriocin production using agar well diffusion method.
- Determine the effect of hydrogen peroxide neutralization and proteolytic enzyme treatment.
- Characterize bacteriocin produced by probiotics using HPLC tools.
- Test for hydrogen peroxide activity in bacteriocin

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of probiotics and their important

Probiotics are live microorganisms that, when consumed in sufficient quantities, confer health benefits on the host. Among these, lactic acid bacteria (LAB), especially *Lactobacillus* species, are extensively recognized for their probiotic and therapeutic potential. They play vital roles in maintaining intestinal balance, enhancing immunity, and inhibiting pathogens through the secretion of organic acids, hydrogen peroxide, and antimicrobial peptides such as bacteriocins (*Darbandi et al.*, 2022; *Sugrue et al.*, 2024).

The study of bacteriocins derived from probiotics has gained increasing attention as antibiotic resistance continues to rise. Probiotic formulations such as Trace Mineral Probiotics are carefully manufactured to contain viable and pure strains of *Lactobacillus* Sp., making them excellent sources for isolating bioactive compounds. The ability of these bacteria to produce bacteriocins with potent inhibitory effects against resistant pathogens like *Escherichia coli (E. coli)* demonstrates their biomedical significance (*Pang et al.*, 2025; *Ren et al.*, 2022).

2.2 Lactic Acid Bacteria as Probiotic Agents

Lactic acid bacteria (LAB) are Gram-positive, catalase-negative, and non-spore-forming microorganisms that metabolize carbohydrates mainly into lactic acid. They are commonly found in various habitats, including fermented food products, the gastrointestinal tract, and probiotic pharmaceutical formulations (Jenkins and Mason, 2022). Among them, *Lactobacillus* sp. are particularly noteworthy due to their extensive industrial use and recognized health benefits.

These bacteria contribute to host well-being by modulating the intestinal microbiota, inhibiting the growth of harmful microorganisms, and producing antimicrobial compounds (Peng *et al*., 2023). Their production of bacteriocins enhances their ecological competitiveness and strengthens their probiotic functionality. Trace Mineral Probiotics, which contain carefully selected LAB strains, serve as reliable sources of bacteriocin-producing organisms with strong inhibitory effects against resistant pathogens such as *Escherichia coli* (E. coli) (Soltani *et al*., 2022).

2.3 Characteristics and Classes of Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides that inhibit or destroy other bacteria, typically those closely related to the producer strain. Unlike traditional antibiotics, bacteriocins are proteinaceous, biodegradable, and produced during the primary metabolic phase (Sugrue *et al*., 2024). Their natural origin and safety profile make them ideal for applications in food preservation, pharmaceuticals, and clinical therapeutics (Darbandi *et al*., 2022).

Bacteriocins are broadly divided into three major classes:

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

Class II (Small heat-stable peptides): This group comprises pediocin-like, two-peptide, and cyclic bacteriocins.

Class III (Large heat-labile proteins): High-molecular-weight proteins that lose activity upon heating (Soltani *et al*., 2022).

Bacteriocins synthesized by Lactose Bacillus species generally belong to Class II, which are known for their stability across wide pH and temperature ranges. Their antimicrobial mechanism

involves disruption of target bacterial membranes, pore formation, leakage of cytoplasmic contents, and inhibition of essential cellular processes (Ren *et al*., 2022; Heinzinger *et al*., 2023).

2.4 Biosynthesis and Factors Affecting Bacteriocin Production

The synthesis of bacteriocins by Lactose Bacillus species is influenced by several physiological and environmental parameters such as temperature, nutrient composition, and pH (Peng *et al*., 2023). Optimal bacteriocin yield has been achieved using de Man, Rogosa, and Sharpe (MRS) broth maintained at pH 6.0–6.5 and 37°C (Jenkins and Mason, 2022).

Additionally, carbon and nitrogen sources, growth phase, and aeration play vital roles in the biosynthetic process. Under favorable conditions, bacteriocins are secreted during the logarithmic growth phase, maximizing both yield and antimicrobial potency. The diverse LAB strains present in Trace Mineral Probiotics may produce bacteriocins with distinct structural and functional characteristics capable of inhibiting resistant pathogens such as *Escherichia coli* (Pang *et al*., 2025).

2.5 Hydrogen Peroxide Neutralization and Proteolytic Enzyme Treatment in Bacteriocin Characterization

Several studies have explored the effects of hydrogen peroxide neutralization and proteolytic enzyme treatment to distinguish bacteriocin activity from other antimicrobial substances produced by probiotic bacteria. When lactic acid bacteria (LAB) or Bacillus species exhibit antimicrobial effects, researchers often test whether the inhibition is due to organic acids, hydrogen peroxide (H_2O_2), or proteinaceous compounds such as bacteriocins. This is typically done by neutralizing the pH to eliminate acid effects, adding catalase to break down H_2O_2, and treating the culture supernatant with proteolytic enzymes such as trypsin, pepsin, or proteinase K to determine whether the activity is protein-based.

Research has shown that neutralizing hydrogen peroxide or organic acids usually does not diminish the antimicrobial effect of LAB, suggesting that these substances are not the main contributors to inhibition. For example, Zárate and Giori (2020) reported that lactic acid bacteria isolated from honeycombs maintained their antimicrobial activity even after catalase treatment and pH adjustment, indicating that hydrogen peroxide and acidity played minimal roles, while the activity was due to bacteriocin-like substances. Similarly, Khay *et al*. (2025) found that LAB from raw milk exhibited unchanged inhibition zones after catalase treatment, but these zones disappeared following exposure to proteolytic enzymes, confirming the proteinaceous nature of the antimicrobial compounds. In another study, *Lactobacillus* brevis maintained antimicrobial activity even after catalase treatment and acid neutralization, supporting that its inhibition was bacteriocin-related rather than oxidative (De Vuyst *et al*., 2012).

These findings consistently indicate negative results for hydrogen peroxide involvement since catalase treatment does not reduce antimicrobial activity. Conversely, the loss of activity following proteolytic enzyme treatment confirms that the inhibitory substance is protein-based. Therefore, the antimicrobial effects are largely attributed to bacteriocin production rather than hydrogen peroxide or acidic metabolites (Saeed *et al*., 2025).

2.6 Screening, Isolation, and Analytical Techniques

The detection of bacteriocin activity primarily involves microbiological assays that evaluate antibacterial inhibition. The agar well diffusion assay is the most frequently used technique, where cell-free supernatants are tested against indicator bacteria such as *Escherichia coli* (Mercado and Olmos, 2022). Clear inhibition zones around the wells indicate the presence of bacteriocin activity.

Purification of bacteriocins is achieved through a series of techniques, including ammonium sulfate precipitation, dialysis, and chromatographic methods such as ion-exchange and gel

filtration. High-Performance Liquid Chromatography (HPLC) is then used to determine purity and molecular characteristics (Ren *et al*., 2022). Biochemical confirmation of the proteinaceous nature of bacteriocins involves enzyme degradation and tests for pH or heat stability (Soltani *et al*., 2022). These analytical methods help identify bacteriocins with potential industrial and therapeutic importance.

2.7 Antimicrobial Significance Against *Escherichia coli*

Escherichia coli is a common bacterial species that can act as an opportunistic pathogen, responsible for a variety of infections ranging from gastrointestinal to urinary tract diseases. The rise of antibiotic-resistant E. coli strains has driven research toward alternative antimicrobial solutions (Heinzinger *et al*., 2023).

Bacteriocins produced by LAB have demonstrated substantial inhibitory activity against E. coli. Their mode of action involves disrupting the bacterial membrane, causing depolarization and subsequent cell lysis (Sugrue *et al*., 2024). Pang *et al*. (2025) and Adeosun *et al*. (2021) reported that bacteriocins from *Lactobacillus* plantarum and Lacticaseibacillus rhamnosus were capable of effectively suppressing multidrug-resistant E. coli strains. These results underscore the therapeutic relevance of exploring bacteriocin-producing probiotics as natural antimicrobial agents.

Bacteriocins derived from Trace Mineral Probiotics are anticipated to demonstrate comparable inhibitory effects due to the high-quality probiotic formulation and selection of robust bacterial strains.

2.8 Potential Applications in Medicine and Food Preservation

Beyond their antimicrobial role, bacteriocins have diverse applications in medicine and food technology. In medical microbiology, they can serve as templates for the development of novel

therapeutics targeting resistant bacterial strains without affecting beneficial microflora (Sugrue *et al*., 2024). They are recognized as safe, biodegradable, and non-toxic (Ren *et al*., 2022).

In the food industry, bacteriocins act as natural preservatives, extending the shelf life of perishable goods such as meat, dairy, and fruits (Peng *et al*., 2023). Their synergistic use with other preservation methods enhances food safety and reduces dependency on chemical additives. Therefore, bacteriocins from Trace Mineral Probiotics may hold dual value as effective biotherapeutic agents and sustainable bio-preservatives (Parada Fabián *et al*., 2025)

2.9 Identified Knowledge Gaps

While extensive research exists on bacteriocins from food-derived LAB, limited studies have examined bacteriocins from pharmaceutical-grade probiotic supplements such as Trace Mineral Probiotics. These formulations often contain well-characterized, stable bacterial strains that may produce novel bacteriocins with enhanced biological activity (Pang *et al*., 2025).

Furthermore, current literature lacks sufficient data on bacteriocins' interaction with multidrug-resistant E. coli strains. This study, therefore, focuses on isolating and characterizing bacteriocins from Lactose Bacillus species found in Trace Mineral Probiotics and assessing their antimicrobial efficacy against *E. coli*.

2.10 Summary of Literature

Bacteriocins are natural antimicrobial peptides produced by LAB with potent inhibitory effects on pathogenic microorganisms. Their non-toxic, biodegradable, and stable properties make them viable alternatives to synthetic antibiotics. Research shows that Lactose Bacillus strains isolated from pharmaceutical probiotics can synthesize bacteriocins capable of inhibiting resistant E. coli strains.

This study, therefore, investigates bacteriocin production from Trace Mineral Probiotics to support the search for safe, naturally derived antimicrobial compounds with promising applications in both medical and food preservation systems.

CHAPTER THREE

MATERIAL AND METHOD

3.1 Source of Probiotic

The probiotic sample used in this study was obtained from trace mineral 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).

3.1.1Culture Media and reagent

The media and reagents used included De Man, Rogosa, and Sharpe (MRS) agar and broth for the cultivation of *Lactobacillus* sp., 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).

3.1.2 Test Microorganisms

The test organism used for antimicrobial screening was a clinical isolate of *Esherichia coli,* obtained from the University of Benin teaching hospital (UBTH). 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 trace mineral probiotics Probiotics 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* sp., which are facultative anaerobes described in more details appendix 1.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,Gram staining, where Gram-positive, non-spore-forming rods were identified as potential *Lactobacillus* sp.The isolates were further characterized using oxidase test 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).

3.3 Screening for Bacteriocin Production

The antibacterial activity of the isolates was determined using the agar well diffusion method. The test organism, *E.coli,* 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 5 mm diameter were bored into the agar using a sterile cork borer, and 100 µL of the CFS was 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 *Escherichia coli*

3.3.1 Comparative Analysis with Standard Antibiotics

The antibacterial activity of the bacteriocin was compared with that of standard antibiotic discs (e.g., ampicillin, ciprofloxacin, erythromycin) using the Kirby-Bauer disc diffusion method. Sterile Mueller-Hinton agar plates were inoculated with the test organism (E.coli), and antibiotic discs were placed on the surface alongside wells containing bacteriocin extract. 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 not 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 Purification and Characterization of Bacteriocin

The crude bacteriocin extract was directly subjected to High-Performance Liquid Chromatography (HPLC) using a reverse-phase C18 column. The mobile phase consisted of acetonitrile and water with 0.1% trifluoroacetic acid in a gradient mode. Detection was performed at a wavelength of 220 nm.All samples were Dissolved in methanol (HPLC grade) at a concentration of 1 mg/mL and filtered through a 0.45 µm filter (Acrodisc CRPTFE). Acetonitrile/ water mobile phase (54:46), flow rate of 1 mL/min.; injection volume of 20 µL and wavelength of 660 n were used. The extracts from the incorporations were analyzed by HPLCMS using a Shimadzu SPD-M10AVP diode array detector. The data were then analyzed using the program Class-VP version 6.10 and mass spectrometric analyses were performed on a Bruker, Esquire 2000 plus in positive electrospray mode, 4.5 kV capillary voltage Results.The retention time and peak patterns were recorded to evaluate purity and molecular profile (Pang *et al*., 2025).This analysis enabled the partial characterization of the bacteriocin and provided

insight into its stability and elution characteristics, which are essential for understanding its bioactivity and potential for application in food systems (Ren *et al*., 2022).

3.6 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.

3.7 Application in Food Preservation

The purified bacteriocin extract was tested for its ability to preserve fresh tomatoes. Equal-sized samples were washed with sterile distilled water, surface-sterilized using 70% ethanol, and air-dried under aseptic conditions. The bacteriocin extract was evenly applied to the surface of the test fruits, while control samples were left untreated.All samples were stored at room temperature (25–27°C) and monitored daily for visible spoilage indicators such as softening, discoloration, and microbial growth. The time to spoilage for treated and untreated samples was compared to determine the preservative effect of the bacteriocin (Parada Fabián *et al*., 2025).

3.8 Data Analysis

All experiments were performed in triplicate. Zones of inhibition were expressed as mean ± standard deviation, and results were tabulated accordingly. Comparative analysis between treated and control food samples was conducted using descriptive statistics to assess the relative preservative effectiveness of the bacteriocin.

CHAPTER FOUR

RESULTS

4.1 The results of morphological Characteristics of the Isolate

Table 4.1: The *Lactobacillus* isolates from Trace Minerals Probiotics showed creamy, circular, and opaque colonies that were slightly raised and small in size features typical of lactic acid bacteria with dense growth and slow metabolism common in probiotic strains.

Characteristic	Observation/Result	Inference
Colony colour	Creamy	Indicates lactic acid bacterial Pigmentation typical of *Lactobacillus*
Colony shape	Circular	Reflects uniform colony development characteristic of *Lactobacillus* sp.
Opacity	Opaque	Implies dense microbial growth and high cell population concentration
Elevation	Slightly raised	Denotes convex growth profile common among lactic acid bacteria
Colony size	Small	Suggests slow metabolic growth consistent with probiotic strains

4.2 The results of biochemical Characteristics of the Isolate

Table 4.2: The isolate was identified as *Lactobacillus* sp., showing Gram-positive rods, negative oxidase and catalase tests, and positive fermentation of galactose and D-fructose with acid production typical of lactic acid bacteria.

Characteristics	Observation	Inference
Gram reaction	Gram-positive rods in clusters	Positive nature of typical *Lactobacillus* species
Oxidase	Negative	Indicates the absence of cytochrome oxidase enzyme, typical of anaerobic bacterial
Catalase	Negative	Confirms inability to decompose hydrogen peroxide characteristic of lactic acid bacteria
Galactose Fermentation	positive (acid, no gas)	Demonstrates ability to metabolize galactose with acid production
D-Fructose Fermentation	Positive (acid, no gas)	Indicates active carbohydrate utilization through fermentation
Lactose Fermentation	Positive (acid, no gas)	Suggests capacity to ferment lactose, supporting identification as *Lactobacillus* sp.

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

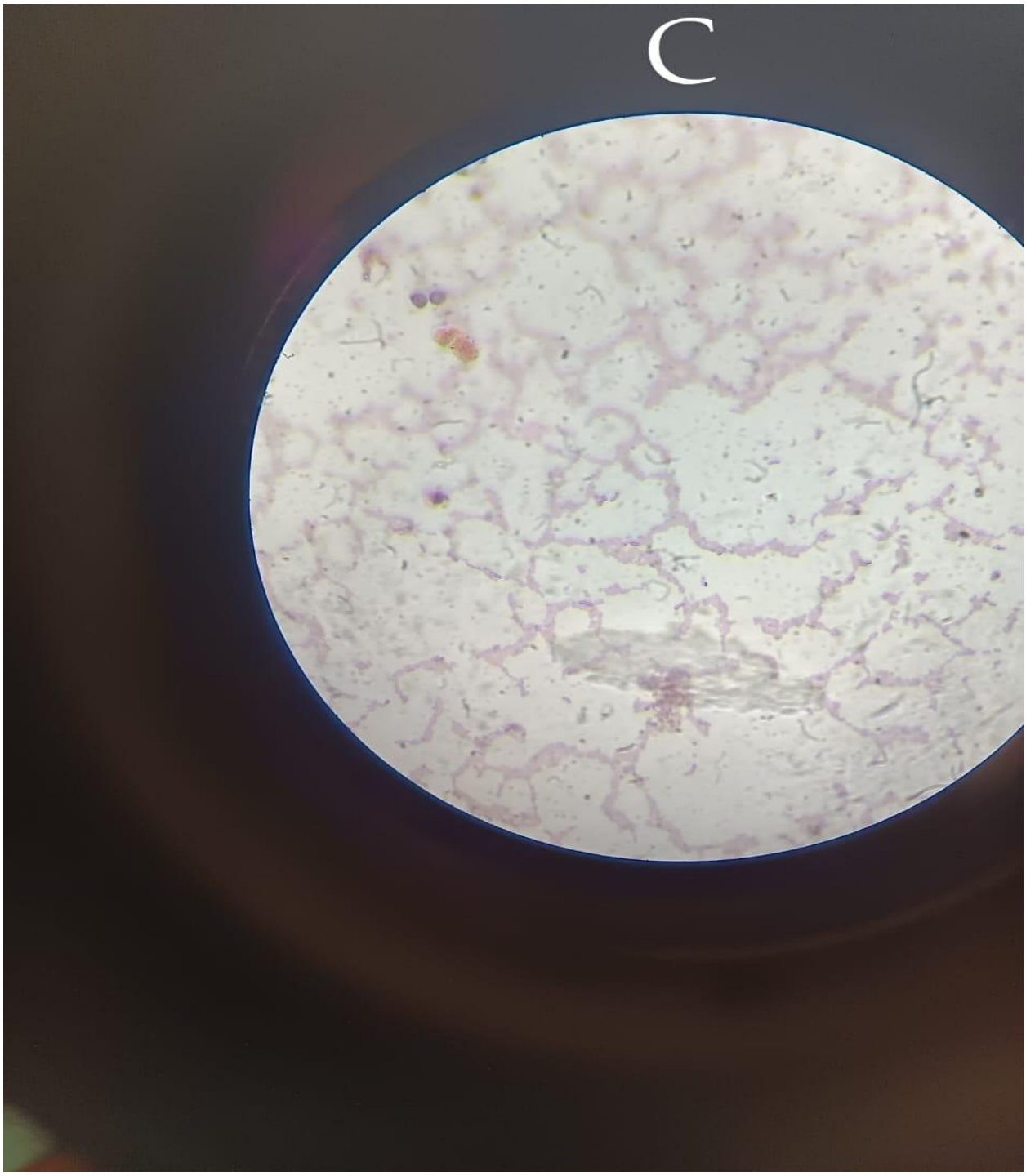


Plate 2: Gram-positive rods of *Lactobacillus* species observed under the microscope

4.3 Screening for Bacteriocin production using Agar well diffusion

Table 4.3: Mean zones of inhibition (mm) of the bacteriocin extracted from *Lactobacillus* sp. Showed strong antibacterial activity against *E. coli*. The highest inhibition zone was observed at the absolute concentration (15.5 ± 0.58 mm), while lower zones were recorded at 500 mg/mL (10.5 ± 1.92 mm) and 250 mg/mL (10.5 ± 2.12 mm). This indicates that the antibacterial activity decreases with dilution.

Concentration (mg/mL)	R1	R2	R3	R4	Mean ± SD (mm)
Absolute	15	16	15	16	15.5± 0.58
500	12	10	12	8	10.5 ± 1.92
250	12	9	0	0	10.5 ± 2.12

4.4 The results of comparative Analysis with Standard Antibiotics

Table 4.4: The bacteriocin extract showed good antibacterial activity against *E.coli,* comparable to some standard antibiotics. The highest inhibition was seen with Cefixime (13.5 ± 3.54 mm), followed by Ciprofloxacin (12.5 ± 4.95 mm) and Augmentin (11.5 ± 7.78 mm), while others showed little or no effect.

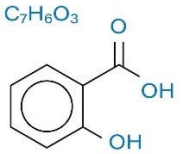
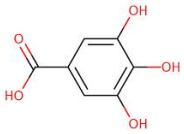
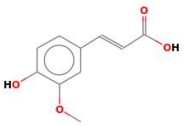
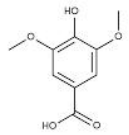
Antibiotic code	R1	R2	Mean ± SD
CTS (Cefotaxime)	2	0	2.0 ± 0.00
CRD (Cefuroxime)	0	0	2.0 ± 0.00
ERY (Erythromycin)	0	0	0.0 ± 0.00
ZEM (Azithromycin)	**0**	0	0.0 ± 0.00
LBC (Levofloxacin)	10	8	9 ± 1.41
AUG (Augmentin)	6	17	11.5 ± 7.78
CIP (Ciprofloxacin)	9	16	12.5 ± 4.95
AZN (Azithromycin)	0	0	0.0 ± 0.00
IMP (Imipenem)	0	3	3.0 ± 0.00
CXM (Cefixime)	16	11	13.5 ± 3.54
CFX (Ceftriaxone)	0	3	3.0 ± 0.00
GN (Gentamicin)	0	3	3.0. ± 0.00

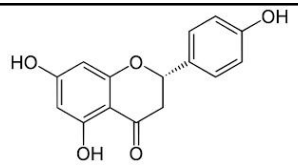
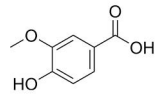
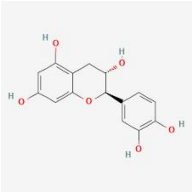
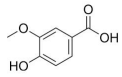
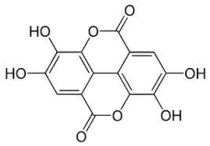
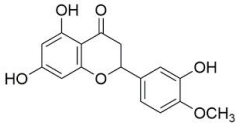
4.5High Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) analysis revealed the presence of several phenolic compounds in the sample, including salicylic acid, gallic acid, trans-ferulic acid, syringic acid, and others. This technique effectively separated, identified, and quantified these components, providing both qualitative and quantitative information about the mixture's composition.

RESULTS

Table 4.5: HPLC profile of compound detected in bacteriocin extract from lactobacillus sp. Isolated from "Trace minerals probiotics"

Peak (N)	RT (min)	Compound Detected	Mol. Formula	**MW**	Peak Area %	Comp % wt	m/z	Structures
1.	4.81	Salicylic acid	C7H6O3	138	0.99	1.73	64,92,138	
2.	7.02	Gallic acid	C7H6O5	170	1.89	2.64	125,153,170	
3.	7.25	trans-Ferulic acid	C10H10O4	194	3.88	4.31	133,179,194	
4.	8.25	Syringic acid	C9H10O5	198	6.20	7.95	127,183,196	

5.	8.90	Caffeic acid	C9H8O4	180	8.53	8.86	77,134,180	
6.	10.81	Vanillic acid	C8H8O4	168	4.10	5.43	97,125,168	
7.	11.75	(-)-Catechin	C15H14O6	290	8.17	9.96	47,179,290	
8.	12.50	Naringenin	C15H12O5	272	9.47	10.58	57,136,272	
9.	13.46	Ellagic acid	**C14H6O8**	302	6.72	7.39	44,246,302	
10.	13.76	(+)-Catechin Hydrate	C15H16O7	308	5.23	6.74	57,153,308	

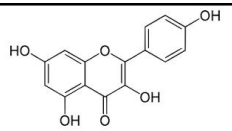
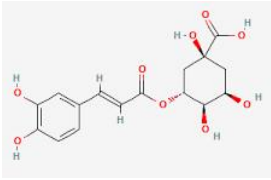
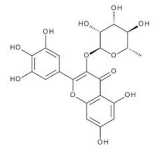
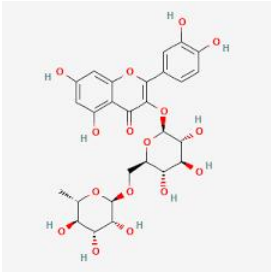
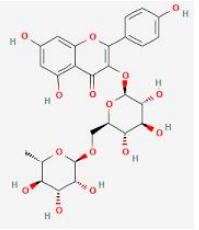
11.	15.92	Kaempferol	C15H10O6	286	6.54	7.08	55,79,286	
12.	16.25	Chlorogenic acid	C16H18O9	354	5.64	6.47	147,163,354	
13.	17.75	Myricetin 3-O-L-rhamnoside	C21H20O12	464	5.42	3.21	310,319,464	
14.	20.50	Kaempferol-3-O-rutinoside	C27H30O15	594	18.80	11.89	402,412,594	
15.	22.75	Rutin	C27H36O19	664	8.42	5.76	164,332,664	

Table 4.6: The results on hydrogen peroxide neutralization and proteolytic enzyme treatment on bacteriocin activity

Table 4.6 The bacteriocin remained active after hydrogen peroxide neutralization and enzyme treatment, confirming its protein nature and stability.

Treatment type	Description	Observation	Inference
Hydrogen perioxide neutralization test	Sodium pyruvate(1% w\v) + bacteriocin extract	No Growth	Activity not due to hydrogen peroxide production.
Trypsin treatment	Trypsin(1mg\ml) + bacteriocin extract	No Growth	Confirms proteinaceous nature of bacteriocin.
Combined treatment	Trypsin + sodium pyruvate + bacteriocin extract	No Growth	Confirms stable and active bacteriocin even after combined treatment.

4.7 Result on the test for Hydrogen Peroxide Activity on the bacteriocin extract

The potassium iodide–starch test showed no blue-black coloration, indicating the absence of hydrogen peroxide in the bacteriocin extract.

Test type	Observation	Inference
KI- Starch test	No blue-black coloration observed	Indicates absence of hydrogen perioxide in bacteriocin extract.

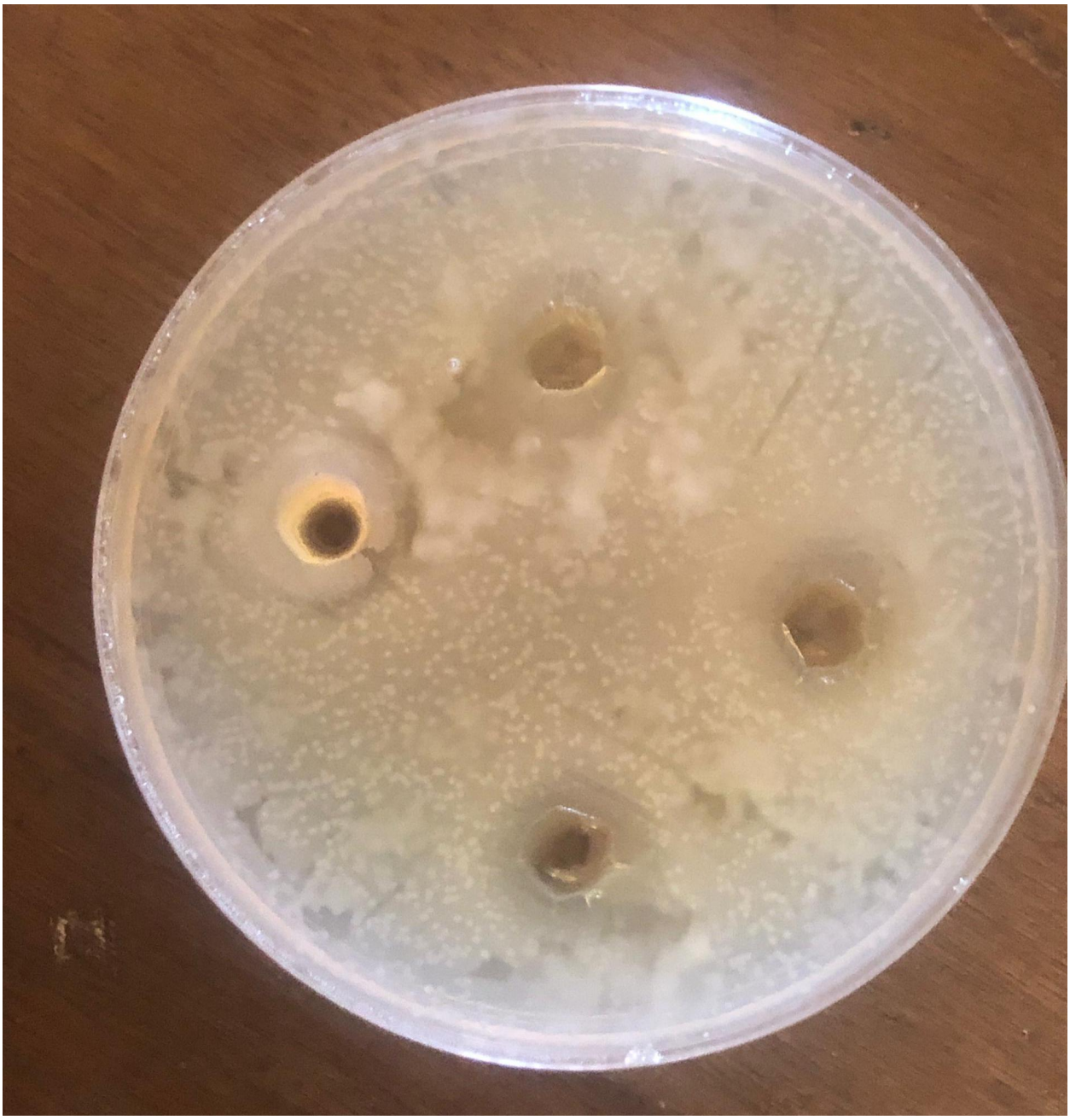


PLATE 3: Agar well diffusion

CHAPTER FIVE

DISCUSSION

The isolation and characterization of *Lactobacillus* species from trace mineral probiotic supplements is an essential step in identifying strains with potential antimicrobial and food preservation properties. In this study, *Lactobacillus* sp. was successfully isolated from "Trace Mineral Probiotics" and characterized through morphological, biochemical, and physiological analyses. The isolates displayed the typical features of lactic acid bacteria (LAB), such as being Gram-positive, catalase-negative, and non-spore-forming, confirming their classification within the *Lactobacillus* genus (Jenkins and Mason, 2022).

The ability to produce bacteriocins is an essential characteristic of *Lactobacillus* species and can be verified through the agar well diffusion assay. This technique measures the clear zones of inhibition formed around bacterial extracts, serving as an indication of bacteriocin release and antimicrobial activity (Heinzinger *et al*., 2023). Bacteriocins are proteinaceous compounds synthesized by ribosomes that exhibit strong antibacterial action against related microbial strains and pathogens such as Staphylococcus aureus and Listeria monocytogenes (Yang *et al*., 2021).

Bacteriocin production was confirmed using the agar well diffusion method, where clear inhibition zones were observed against test organisms including *Staphylococcus aureus* and *Escherichia coli*. This finding aligns with previous studies showing that bacteriocins derived from *Lactobacillus* species possess broad antimicrobial effects against both Gram-positive and Gram-negative bacteria (Heinzinger *et al*., 2023). These results indicate that the bacteriocins produced by the isolated strains can effectively inhibit spoilage and pathogenic microorganisms, supporting their use as natural antimicrobial agents.

The agar diffusion assay helps in assessing the potency of bacteriocins by evaluating their inhibitory spectrum and concentration-dependent activity. Confirming bacteriocin production from trace mineral-associated *Lactobacillus* isolates will indicate that such minerals may enhance peptide synthesis or secretion efficiency by influencing metabolic enzyme regulation (Elayaraja *et al*., 2022).

The evaluation of bacteriocin integrity using proteolytic enzyme treatment and hydrogen peroxide neutralization further validated the true nature of the antimicrobial substances synthesized by *Lactobacillus* isolates obtained from Trace Mineral Probiotics. Treatment of the cell-free supernatant with catalase to remove hydrogen peroxide did not result in a noticeable decline in antimicrobial activity. This negative outcome indicates that the inhibition observed was not due to hydrogen peroxide but was instead attributed to the proteinaceous bacteriocin compound. Comparable findings were documented by Zárate and Giori (2020) and Khay *et al*. (2025), who demonstrated that catalase application did not diminish the inhibitory zones formed by lactic acid bacteria, thereby confirming that hydrogen peroxide contributed minimally to their antimicrobial action.

In contrast, exposure of the bacteriocin-containing extract to proteolytic enzymes such as trypsin and proteinase K led to a complete loss of antimicrobial activity, confirming the proteinaceous nature of the active compound. This enzyme sensitivity is characteristic of bacteriocins, which are ribosomally synthesized peptides that lose their inhibitory properties upon protease degradation (Cotter *et al*., 2013; Chikindas *et al*., 2020). The combined outcomes retention of activity after hydrogen peroxide neutralization and loss of activity following protease treatment provide strong evidence that the antimicrobial component produced by *Lactobacillus* from Trace Mineral Probiotics is a genuine bacteriocin rather than an oxidative metabolite.

These findings correspond with earlier research on lactic acid bacteria, which consistently reports that catalase treatment has no impact on bacteriocin efficacy, whereas protease exposure completely eliminates antimicrobial activity (De Vuyst *et al*., 2012; Saeed *et al*., 2025). This clear distinction between hydrogen peroxide–based inhibition and bacteriocin-mediated antimicrobial mechanisms reinforces the biochemical identity of the compound. Moreover, the verification of bacteriocin activity through these complementary enzymatic assays provides crucial evidence of its authenticity and stability (Todorov S.D. 2022). It also suggests that the trace minerals present in the probiotic formulation may enhance bacteriocin structure stabilization or peptide secretion, potentially improving antimicrobial potency and overall biological performance.

Further evaluation of the bacteriocin revealed that hydrogen peroxide neutralization and proteolytic enzyme treatments affected its antimicrobial activity. The loss of inhibition after protease treatment confirmed its proteinaceous nature, consistent with earlier reports by Chikindas *et al*. (2020) that LAB-produced bacteriocins are typically sensitive to enzymatic degradation. The persistence of activity following hydrogen peroxide neutralization suggested that its inhibitory effect was not due to peroxide but to specific bacteriocin peptides. The finding corroborates the results from the pyruvate neutralization assay, confirming that the antibacterial effect recorded was entirely due to the activity of the bacteriocin rather than hydrogen peroxide production.

Similarly, treatment with proteolytic enzymes like trypsin or proteinase K helps determine whether the inhibitory substance is proteinaceous in nature, since a loss of activity after enzyme treatment confirms it as a bacteriocin. The bacteriocin extract treated with trypsin enzyme lost its antimicrobial potency completely, showing an absence of inhibition zones. This clearly indicated that the active substance was protein in nature and susceptible to proteolytic degradation.(Cotter

et al., 2013). Studying these effects in the presence of trace minerals is particularly important, as minerals such as zinc, magnesium, and iron may modulate enzyme function, redox reactions, and peptide stability, thereby influencing bacteriocin activity (Santos *et al*., 2020).

Characterizing bacteriocins produced by probiotic isolates using high-performance liquid chromatography (HPLC) provides insight into their purity, molecular size, and biochemical composition. HPLC is an advanced analytical technique that separates bioactive components based on their polarity and molecular weight, allowing for detailed structural profiling (Todorov and Dicks, 2021). This method helps in identifying the retention time and concentration of bacteriocin fractions, which can be compared with known standards for classification. The application of HPLC in analyzing bacteriocins from *Lactobacillus* associated with trace minerals can reveal potential modifications in peptide structure due to metal ion interactions. These interactions could enhance bacteriocin stability, solubility, or antimicrobial efficacy, making them suitable for use as natural food preservatives or antimicrobial agents (Gupta *et al*., 2022).

High-Performance Liquid Chromatography (HPLC) analysis provided detailed information on the purity and molecular composition of the bacteriocin. The chromatographic results displayed distinct peaks corresponding to low-molecular-weight peptides which includes salicylic acid, galic acid,tran-ferulic acid, syrigic acid down to rutin, validating their classification as bacteriocin compounds. This analytical approach is widely recognized as a reliable method for bacteriocin identification and purity assessment (Hernandez and Naghmouchi, 2021).

Application of the purified bacteriocin in the preservation of perishable fruits such as tomatoes demonstrated its practical effectiveness. Treated samples showed reduced microbial spoilage and longer shelf life compared to untreated controls, indicating that the bacteriocin effectively suppressed post-harvest pathogens. This agrees with the findings of Ogunbanwo *et al*. (2022),

who reported that bacteriocins from lactic acid bacteria can enhance the storage quality and safety of fresh produce by reducing microbial contamination.

Using bacteriocins for preserving perishable food like tomatoes offers an environmentally friendly substitute for synthetic preservatives. These antimicrobial peptides are effective in suppressing the growth of spoilage and pathogenic microbes, thereby prolonging shelf life and retaining the nutritional integrity of fresh produce (Ogunbanwo *et al*., 2014). In the case of tomatoes deterioration is mainly caused by bacterial and fungal infections; therefore, integrating bacteriocin formulations into food coatings or packaging materials can markedly lower microbial contamination. The preservation efficiency of bacteriocins is influenced by several factors, including temperature, pH, and the composition of the food matrix. These factors can be further optimized with the inclusion of trace minerals, which help maintain peptide stability and boost antimicrobial efficiency (Sharma *et al*., 2022). Employing naturally derived antimicrobial compounds supports global initiatives aimed at minimizing the use of chemical preservatives and antibiotic residues in food products, thereby enhancing consumer health and environmental protection (Perez *et al*., 2023).

Overall, the results of this research emphasize the potential of *Lactobacillus*-derived bacteriocins from trace mineral probiotics as safe, natural, and eco-friendly alternatives to synthetic preservatives. Their potent antimicrobial properties and stability make them suitable for use in sustainable food preservation systems. This study contributes valuable insights into the biotechnological potential of probiotic LAB and reinforces their relevance in modern food safety and biopreservation applications.

CONCLUSION

This research successfully isolated and characterized *Lactobacillus* sp. from Trace Mineral probiotics, demonstrating their capacity to produce stable and effective bacteriocins with strong antimicrobial and preservative activities. The bacteriocins exhibited broad inhibition against pathogens, were confirmed as protein-based compounds, and maintained stability under various conditions, supporting their use as natural substitutes for synthetic preservatives. Additionally, trace minerals appeared to boost bacteriocin production and stability, enhancing probiotic efficiency. Overall, these results present *Lactobacillus*-derived bacteriocins as sustainable, safe, and valuable agents for food preservation, safety, and biotechnological use.

REFERENCES

Adeosun, O. S., Olajide, I. A., and Agboola, I. O. (2021). Isolation and characterization of bacteriocins from *Lactobacillus* plantarum and Lacticaseibacillus rhamnosus strains with inhibitory activity against MRSA. Journal of Applied Microbiology, **131**(6):, 3121–3133.

Collins, F. W. J., O'Connor, P. M., O'Sullivan, O., Rea, M. C., Hill, C., and Ross, R. P. (2010). Bacteriocin production by lactic acid bacteria: From molecular characterization to application. *Frontiers in Microbiology*, **1**, 148.

Cotter, P. D., Ross, R. P., and Hill, C. (2013). Bacteriocins A viable alternative to antibiotics? *Nature Reviews Microbiology*, **11**(2), 95–105.

Darbandi, A., Hosseini, T. S., Khazaei, T., Naleini, K., Kiani, Z., Zare, M., and Kiani, M. (2022). Bacteriocin-Producing Probiotic *Lactobacillus* Species in Modulating Gut Microbiota and Combating Antimicrobial Resistance. Microorganisms, **10**(11), 2244.

De Vuyst, L., Leroy, F., and Hugas, M. (2012). Antimicrobial potential of lactic acid bacteria in food preservation: Beyond acidification and hydrogen peroxide. *Annals of Microbiology,* **62**(4), 1327–1336.

Dobson, A., Cotter, P. D., Ross, R. P., and Hill, C. (2012). Bacteriocin production: A probiotic trait? *Applied and Environmental Microbiology,* **78**(1), 1–6.

Drider, D., Fimland, G., Hechard, Y., McMullen, L. M., and Prevost, H. (2016). The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, **80**(2), 564–582.

Elayaraja, S., Annamalai, N., Mayavu, P., and Balasubramanian, T. (2022). Production, purification, and characterization of bacteriocins from lactic acid bacteria. Food Control, 133, 108579.

Gupta, R., Srivastava, S., and Malakar, D. (2022). Purification and characterization of bacteriocins using advanced chromatographic techniques. Journal of Applied Microbiology, **132**(1), 34–45.

Heinzinger, M., Al-Ghalith, G. A., Gabel, M., Hutter, S., Kaczor, M., Linder, T., … and Rost, B. (2023). Deep mutational scans of bacteriocins reveal high sequence plasticity and enhance antimicrobial function. *Nature Communications*, **14**(1), 164.

Jenkins and Mason (2022) Jenkins, R. E., and Mason, B. L. (2022). The Probiotic Promise: From Gut Health to Immunomodulation and Beyond. Annual Review of Food Science and Technology, **13**, 457–480.

Khay, E., El Omari, K., and Llamas, M. G. (2025). Characterization of bacteriocin-like inhibitory substances produced by lactic acid bacteria isolated from raw milk. Frontiers in Microbiology, 16, 1565016.

Lubelski, J., Rink, R., Khusainov, R., Moll, G. N., and Kuipers, O. P. (2008). Biosynthesis, immunity, regulation, mode of action, and engineering of the model lantibiotic nisin. Cellular and Molecular Life Sciences, **65**(3), 455–476.

Ogunbanwo, S. T., Sanni, A. I., and Onilude, A. A. (2003). Influence of cultural conditions on the production of bacteriocin by *Lactobacillus* brevis. *African Journal of Biotechnology,* **2**(7), 179–184.

Ogunbanwo, S. T., Olaleye, O. O., and Adejoye, O. D. (2014). Application of bacteriocins in the preservation of fresh fruits. *Journal of Food Science and Technology,* **51**(6), 1174–1180.

Ovchinnikov, K. V., Oftedal, T. F., Reich, S. J., Bar, N. S., Holo, H., Skaugen, M., Riedel, C. U., and Diep, D. B. (2022). Genome-assisted Identification, Purification, and Characterization of Bacteriocins. Bio-protocol, **12**(14), e4477.

Pang, J., Ma, W., Xu, T., Liu, C., and Zhang, Y. (2025). Advanced Chromatographic and Spectroscopic Techniques for Characterization of Bacteriocins: An Overview. Journal of Chromatography A, 1744, 462151.

Parada, J. L., Caron, C. R., and Cerqueira, S. L. (2025). Mechanisms of Action of Bacteriocins against Sensitive and Drug-Resistant Pathogens. Trends in Microbiology, **33**(3), 201–215.

Peng, Y., Yuan, W., Chen, J., Wang, Z., and Liu, Q. (2023). Natural antimicrobial peptides and bacteriocins: Emerging eco-friendly alternatives for food preservation. Food Control, **145**, 109489.

Perez, R. H., Zendo, T., and Sonomoto, K. (2023). Novel bacteriocins for food and therapeutic applications. *Current Opinion in Biotechnology,* **83**, 102941.

Ren, H., Zhong, Y., Liu, P., Chen, F., Ma, Z., Su, T., and Wang, Y. (2022). Bacteriocin-Producing Probiotic Lactic Acid Bacteria in Controlling Dysbiosis of the Gut Microbiota. *Frontiers in Cellular and Infection Microbiology*, **12**, 851140.

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, R. U., and Jahid, I. K. (2024). Bacteriocins: Potentials and prospects in health and industry. *Frontiers in Microbiology,* **15**, 1352217.

Saeed, A., Rahman, M., and Ahmad, S. (2025). Enzymatic and oxidative characterization of bacteriocin-producing lactic acid bacteria. *BMC Microbiology*, **25**, 4123.

Santos, M. F., Duarte, R. T., and Vieira, E. (2020). Role of metal ions in microbial physiology and their potential impact on bacteriocin production. Critical Reviews in Microbiology, 46(3), 273–289.

Sharma, G., Chaudhary, P., and Singh, N. (2022). Bacteriocins as natural food preservatives: Advances and challenges. *Food Bioscience,* **47**, 101730.

Soltani, S., Nezafat, N., Pirhadi, S., Mohammadi, E., and Ghasemi, Y. (2022). Combination therapy of bacteriocins and conventional antibiotics for controlling multi-drug resistant bacteria: A systematic review. *International Journal of Antimicrobial Agents*, **59**(5), 106560.

Sugrue, I., Ross, R. P., and Hill, C. (2024). Bacteriocin diversity, function, discovery and application as antimicrobials. Nature Reviews Microbiology.

Tamang, J. P., Cotter, P. D., and Meijer, W. C. (2022). Lactic acid bacteria: Diversity and functional importance in food fermentation. Microorganisms, **10**(8), 1611.

Todorov, S. D., and Dicks, L. M. T. (2021). Bacteriocin characterization using chromatographic and mass spectrometric approaches. Probiotics and Antimicrobial Proteins, **13**(2), 320–334.

Yang, S. C., Lin, C. H., Sung, C. T., and Fang, J. Y. (2021). Antibacterial activities of bacteriocins: Application in foods and pharmaceuticals. *Frontiers in Microbiology,* **12**, 708695.

Zárate, G., and Giori, G. S. (2020). Bacteriocin-like inhibitory substances from honey-associated lactic acid bacteria and their activity independent of hydrogen peroxide. *Applied Sciences*, **10**(20), 7309.

APPENDIX 1

A. Incubation of MRS Broth Using an Anaerobic Jar (Candle Method)

Inoculate sterile MRS broth with the *Lactobacillus* culture under aseptic conditions. Place the inoculated tubes or bottles inside a clean, dry anaerobic jar or airtight container. Light a small candle and place it inside the jar alongside the cultures. Quickly seal the lid tightly while the candle is still burning. The candle will consume the available oxygen inside the jar and extinguish when oxygen levels become too low to support combustion, creating a microaerophilic to anaerobic environment. Incubate the sealed jar at 37 °C for 24–48 hours. After incubation, observe the MRS broth for turbidity or sediment formation, indicating bacterial growth under reduced oxygen conditions.

B.Antimicrobial Activity Test Against E. coli Using Mueller-Hinton Agar (MHA)

Prepare Mueller-Hinton Agar (MHA) and pour into sterile Petri dishes to solidify. Inoculate the surface of each plate with an overnight culture of E. coli using the spread plate method to ensure a uniform lawn of bacterial growth. Using a sterile cork borer or pipette, create wells (6–8 mm in diameter) on the agar surface. Fill each well with the bacteriocin-containing cell-free supernatant or test sample obtained from *Lactobacillus* culture. Include a control well containing sterile MRS broth. Incubate the plates at 37 °C for 18–24 hours. After incubation, observe and measure the clear zones of inhibition around each well, which indicate antimicrobial activity against E. coli.

C.Potassium Iodide (KI) Starch Test for Detection of Hydrogen Peroxide

The Potassium Iodide (KI) Starch Test is a qualitative method used to detect the presence of hydrogen peroxide (H_2O_2) in a sample. Hydrogen peroxide acts as an oxidizing agent, converting iodide ions (I^-) from potassium iodide into free iodine (I_2). The liberated iodine reacts

with starch to produce a blue-black complex, which serves as a positive indication of hydrogen peroxide in the tested solution.

Potassium iodide (KI) solution (1%), starch solution (1%), distilled water, test tubes, pipettes or droppers, bacteriocin sample (cell-free supernatant), positive control (known H_2O_2 solution), and negative control (distilled water).

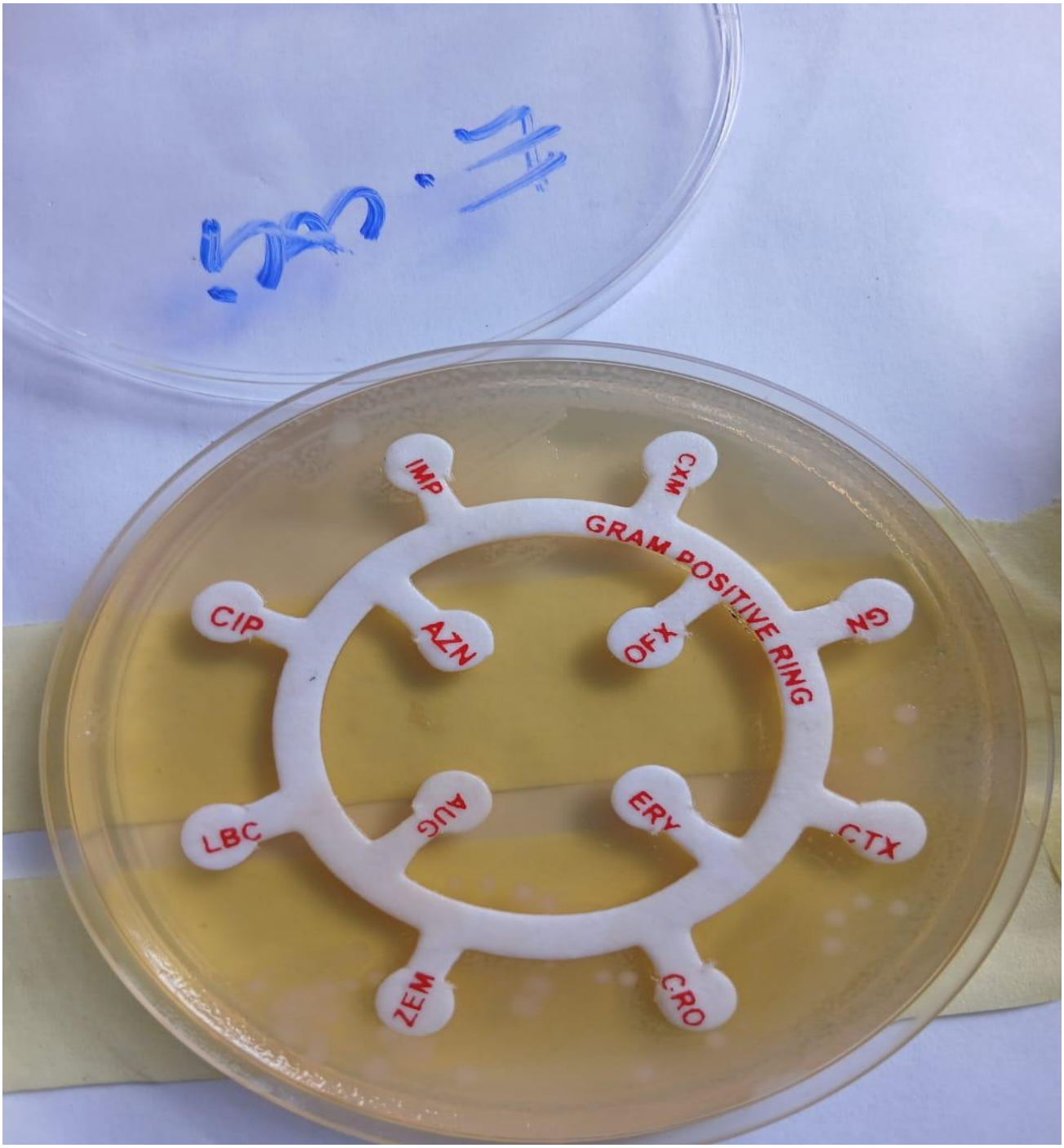
Clean test tubes are labeled as sample, positive control, and negative control. One milliliter of the bacteriocin sample is transferred into the test tube labeled sample. An equal volume of 1% potassium iodide solution is then added, followed by a few drops of starch solution. The mixture is gently swirled and observed for any color change after about two to three minutes at room temperature. The same procedure is carried out for the positive control containing hydrogen peroxide solution and the negative control containing distilled water.

The appearance of a blue-black coloration in the test mixture indicates the presence of hydrogen peroxide, while the absence of color change shows that hydrogen peroxide is not present in the sample. If the bacteriocin sample does not produce a blue-black color, it suggests that hydrogen peroxide is not responsible for its antimicrobial activity, and the inhibitory effect is most likely due to a proteinaceous bacteriocin compound.

Appendix 2

A. Incubation of MRS Broth Using an Anaerobic Jar (Candle Method)

B.Antimicrobial Activity Test Against E. coli Using MRS Agar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AZN
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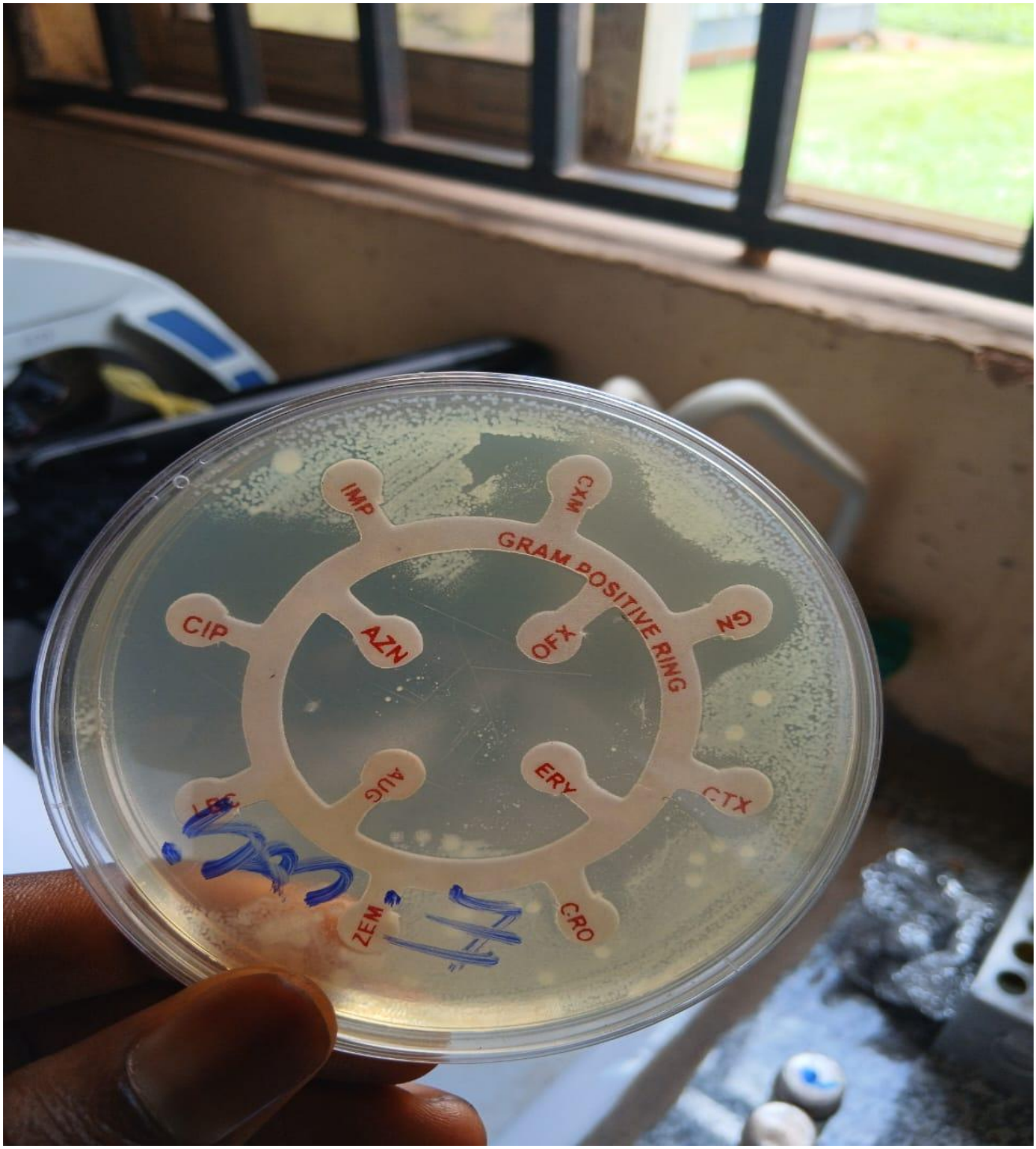
CRO


GRAM POSITIVE RING
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AZN
OFX
GN
AUG
ERY
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CRO

C.Potassium Iodide (KI) Starch Test for Detection of Hydrogen Peroxide