PRODUCTION AND CHARACTERIZATION OF PLANTARICIN Y11 FROM *Lactiplantibacillus plantarum* STRAIN Y11 (MW642245.1) ISOLATED FROM NIGERIAN FOOD SAMPLES AND THEIR APPLICATIONS IN PRESERVING MILK

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ABSTRACT

Bacteriocin from lactic acid bacterium (LAB) was characterized and its biopreservation assay carried out using conventional methods and integrative approaches. About 66.7% (n=180) of the bacterial strains isolated from twelve different fermented/non-fermented food products were potential LAB strains by biochemical characterization. The highest bacteriocin producing strain was selected and confirmed with 16S rRNA sequencing and submitted to GenBank of National Center for Biotechnology Information as *Lactiplantibacillus plantarum strain Y11* (Accession number: MW642245.1). Polymerase Chain Reaction (PCR) revealed the presence of virulence genes (cytotoxin K and haemolysin C) in *L. plantarum* Y11. The bacteriocin produced was labelled as plantaricin Y11 with molecular weight of 1540 Da, amino acid sequence was NSHGTADYCVMWLIXK, and possessed the merits of wide pH stability (2–10), high thermal stability and sensitivity to protease. The bacteriocin showed wide spectrum of activity against bacterial reference strains with bacteriocin activity ranging from 3200Au/mL to 6400Au/mL. Preservative potentials of the bacteriocin compared favourably with sodium benzoate in milk, inhibiting bacterial and fungal load compared to the control. There was log reduction of aerobic mesophilic bacterial count of 8.02±0.15 and 8.43±0.21 Log 10 CFU/mL in milk treated with plantaricin Y11 and sodium benzoate, respectively. The log reductions of fungal count in plantaricin Y11 and sodium benzoate treated milk ≥4.41±0.11 and ≥3.1±0.2 Log 10 CFU/mL respectively. The plantaricin Y11 was then classified as class IIa in bacteriocin classification schemes and it sustained some organoleptic conditions of milk making it a promising candidate for safe bio-preservatives.

Keywords: Bacteriocin, *Lactobacillus plantarum*, virulence genes, biopreservation,

INTRODUCTION

The preservation of perishable food products, such as milk, remains a critical concern to ensure their safety, quality, and prolonged shelf life (Amit *et al*., 2017; Teshome *et al*., 2022). The dairy industry faces significant challenges due to microbial contamination, which can lead to spoilage and potential health risks. In response to these challenges, bacteriocins, antimicrobial peptides produced by various bacterial species, have emerged as promising natural preservatives (Habanabakize *et al.,* 2022; Igoche *et al*., 2022; Teshome *et al*., 2022). Bacteriocins, which are antimicrobial peptides produced by various bacteria, have emerged as potential natural preservatives due to their ability to inhibit the growth of pathogenic and spoilage microorganisms (Cleveland *et al*., 2001). Bacteriocins offer a promising alternative to synthetic chemical preservatives, addressing the increasing consumer demand for safer and more natural food preservation methods (Galvez *et al*., 2007; Singh, 2018).

Lactobacillus plantarum, a lactic acid bacterium commonly found in various food matrices, has gained attention for its production of potent bacteriocins (Parada *et al*., 2007). These bacteriocins exhibit a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria, making them potential candidates for food preservation (Yang *et al*., 2014; Singh, 2018). Previous studies have highlighted the antimicrobial properties of bacteriocins from L. plantarum strains, demonstrating their effectiveness in inhibiting the growth of common foodborne pathogens such as Listeria monocytogenes and Staphylococcus aureus (Drider *et al*., 2006; Singh, 2018).

Nigeria, a country rich in culinary diversity and traditional fermented foods, offers a unique environment for the isolation and characterization of bacteriocin-producing strains. Nigerian food samples have been shown to harbor a diverse range of microorganisms, including lactic acid bacteria with potential probiotic and antimicrobial properties (Ogunbanwo and Sanni, 2003). Exploring the biodiversity of *L. plantarum* strains in Nigerian food samples and harnessing their bacteriocin-producing capabilities could contribute to the development of locally relevant and sustainable food preservation strategies.

Investigating the capacity of bacteriocins as biopreservatives holds significant importance within the field, given the rising occurrence of human infections associated with the consumption of dairy products over the last decade, as indicated by Kim *et al*. (2008). While the potential of bacteriocins from *L. plantarum* as natural preservatives is promising, further research is needed to fully understand their production, characterization, and application in preserving milk. This study aims to isolate *L. plantarum* strain Y11 from Nigerian food samples, characterize its bacteriocin, and assess its efficacy in extending the shelf life of milk. By elucidating the antimicrobial properties and practical applications of bacteriocins from *L. plantarum* strain Y11, this research contributes to the growing body of knowledge on natural food preservation methods.

MATERIALS AND METHODS

This study employs an experimental research design, grounded in the principles of natural science. The chosen experimental framework incorporates both 'control' and 'experimental' groups to facilitate rigorous investigation. Empirical data generated through this approach are subjected to statistical analysis, presented as mean ± standard deviation (SD), and visualized through graphs using the SPSS statistical software

Sample Collection

Study Samples: Multiple food samples including African locust beans (Iru), yogurt, goat milk, fish, ogi from yellow corn, millet, guinea corn and white corn, ogiri, honey, cheese, and fufu were procured from diverse vendors in Uyo, Akwa Ibom State. These samples were collected using aseptic techniques and placed within sterilized containers. Subsequently, the aseptically contained samples were transported under refrigerated conditions to the postgraduate laboratory within the Microbiology Department of the University of Uyo. The primary objective of this collection and transportation process was to facilitate the isolation of bacteriocin-producing lactic acid bacteria (LAB) and to enable subsequent comprehensive analyses.

Test Culture Collection and Maintenance of Test (Pathogenic) Cultures

For the investigation, the indicator microorganisms were carefully chosen from Gram-positive and Gram-negative foodborne pathogens. Their antagonistic potentials against the isolated strains was evaluated. These microorganisms were sourced from the culture collection housed within the microbiology laboratory division of the University of Uyo Teaching Hospital. To ensure viability, these organisms were preserved on MRS Agar medium at a temperature of 4°C and were subjected to subculturing every three weeks.

The specific test isolates encompassed *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, and *Enterococcus cloacae*. Upon acquiring these selected pathogenic organisms in freeze-dried ampoules, their cultures were meticulously cultivated on nutrient agar at a controlled temperature of 37°C for a duration of 24 hours. Subsequent to this incubation period, the cultures were consistently maintained at 4°C, with regular subculturing performed using the same nutrient agar medium at intervals of three weeks.

Microbiological Analysis of the Sample

The various samples including African locus beans, yogurt, goat milk, fish, ogi, honey, and cheese were meticulously weighed and homogenized, aiming to execute a 1:10 dilution procedure. Specifically, African locust beans (iru), cheese, ogi, and fish samples underwent a mashing process, where one gram (1 g) of each sample was dissolved in 9 ml of sterile distilled water. Utilizing this solution, a series of ten-fold serial dilutions were meticulously performed. A 1 ml aliquot of the dilutions (10^{-3}, 10^{-4}, and 10^{-5}) was subsequently introduced onto sterile petri dishes in duplicates through the pour plate technique. For this purpose, de Man Rogosa and Sharpe (MRS) agar medium, optimized for the growth and isolation of lactic acid bacteria, was employed. The incubation of the MRS agar plates occurred at 37°C under anaerobic conditions for a duration of 48 hours (Smith *et al*., 2022).

Likewise, serial dilutions were extended to the goat milk, honey, and yogurt samples. In this context, one milliliter (1 ml) of each sample was dissolved within 9 ml of sterile distilled water, followed by subsequent ten-fold serial dilutions. These dilutions were subjected to analogous analysis as described earlier. Upon completion of the incubation period, the enumeration of the total microbial load was performed. The colonies that emerged on the agar plates were meticulously counted, and the resultant data was documented as colony forming units per milliliter (CFU/mL) (Johnson *et al*., 2023).

Purification and Maintenance of Microbial Isolates

Following the incubation period, viable cells were meticulously isolated from each MRS agar plate. The colonies were selectively chosen and subjected to sub-culturing onto fresh MRS agar plates using the streak plating technique. This process aimed to attain pure cultures of the isolates. Subsequently, the obtained pure cultures were cultivated on MRS agar slants in duplicate, and incubated at 37°C for 48 hours. These slants were then stored at a temperature of 4°C to facilitate subsequent analyses (Smith *et al*., 2023).

Screening of Isolates Based on Morphological, Physiological, and Biochemical Characteristics

The identification of pure isolates was conducted in accordance with established protocols for cultural, morphological, and biochemical traits (Oyeleke and Manga, 2008). Essential attributes such as color, form, margin, elevation, and texture of each isolate were diligently observed. Furthermore, Gram staining and Catalase tests were meticulously performed on all isolates before proceeding to assess their antagonistic behavior.

Screening of Isolates Based on Antagonistic Pattern

Isolates displaying a Gram-positive characteristic and a negative catalase test were subjected to an antimicrobial activity assessment against various target organisms, including *E. coli*, *Salmonella typhii*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa*. The modified well diffusion assay method described by Ogunbanwo *et al*. (2003) and Reddy *et al*. (2006) was employed. Isolates exhibiting substantial and robust inhibition against the maximum number of respective test organisms were selected for further investigations.

Confirmation of Bacteriocinogenic Nature

Given the capability of Lactic Acid Bacteria (LAB) to generate diverse antimicrobial compounds, including organic acids and hydrogen peroxide (H_2O_2), measures were taken to eliminate any interference from these compounds,

ensuring that the observed inhibition was solely attributed to bacteriocins. The elimination of organic acids and hydrogen peroxide was achieved using the method outlined by Adesina *et al*. (2016). The antimicrobial activity, represented by zones of inhibition, was quantified in arbitrary units (AU/mL). The unit of antimicrobial activity was defined as the inverse of the highest dilution leading to a clear growth inhibition zone against the test strains, calculated per milliliter of bacteriocin. The antimicrobial activity was then expressed as a percentage, denoted as ab x 100, where "a" signifies the dilution factor and "b" represents the highest dilution resulting in a ≥2mm inhibition zone diameter. This methodology is in accordance with Todorov and Dicks (2007).

Biochemical Identification of Bacteriocin Producing Isolates

Characterization of the isolated bacteria encompassed a battery of tests, including motility, oxidase, indole production, methyl red, Voges Proskaur (VP) reaction, citrate utilization, and sugar fermentation assessments.

Molecular Identification of Selected Bacteriocin Producing LAB Isolates

DNA Extraction, Visualization, and Quantification

For DNA extraction and purification, the ZR Fungal/Bacterial DNA Miniprep kit (Zymo Research, cat number: D6005) was employed, following the manufacturer's guidelines. Agarose gel was supplemented with loading buffer (Tris/Borate/EDTA), stained with ethidium bromide, and solidified. The solidified gel was placed in a gel tank containing buffer solution, with stained samples loaded into wells created in the agar under buffer. Electrophoresis was carried out at 100 V for approximately 1 hour to ascertain the presence of extracted DNA. DNA fragments were visualized using a UV transilluminator (BIORAD, South Africa). Additionally, the quantification of extracted DNA was conducted using a Nano-Drop spectrophotometer 2000 (microvolume sample retention system) (Thermo Scientific, United Kingdom) (Ojo-Okunola *et al*., 2020).

Amplification and Sequencing of Genetic Materials

The polymerase chain reaction (PCR) mixture consisted of 12.5 µL of Taq 2X Master Mix (New England Biolabs, M0270), 1 µL of each 10µM forward and reverse primers, 2 µL of the DNA template, and was adjusted with 8.5 µL of high-quality molecular grade water. The primers employed for the amplification of the 16S rRNA gene to facilitate bacterial identification were as follows: Forward Primer 27F: AGAGTTTGATCMTGGCTCAG and Reverse Primer 1525R: AAGGAGGTGWTCCARCCGCA. The amplification protocol encompassed an initial denaturation phase at 94˚C for 5 minutes, followed by 36 cycles involving denaturation at 94˚C for 30 seconds, annealing at 55˚C for 30 seconds, and extension at 72˚C for 45 seconds. This was subsequently succeeded by a final extension stage at 72˚C for 7 minutes, with the temperature held at 10˚C indefinitely.

Visualization of Amplified Fragments

The gel electrophoresis of the amplified fragments was conducted following the previously outlined procedure, including the introduction of a molecular weight ladder into the first lane of the gel.

Gene Sequencing and Data Analysis

For gene sequencing, a cocktail mix was prepared, combining 9 µL of Hi di Formamide with 1 µL of the purified PCR product, resulting in a total volume of 10 µL. The prepared samples were loaded onto the 3130xl genetic analyzer (Applied Biosystems, United States of America), which generated data corresponding to the bases A, C, T, and G.

The sequences generated

Sequence Alignments and Phylogenetic Investigation

Sequence alignments and phylogenetic analysis were performed based on the 16S rRNA sequences that were generated. The sequences were subjected to a BLAST search in the public Genbank National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The outcomes of this search were utilized to identify the specific organisms under investigation. The alignment procedure was carried out using Bioedit (version 7.0.5.3), while the phylogenetic analysis was executed using MEGA 7 software (version 7.0.63) (Claridge, 2004).

Detection of Virulence Genes

The presence of specific virulence genes, namely Hemolysin C gene (*HblC*) and cytotoxin K gene (*CytK*), was determined using the PCR technique. The PCR mixture was composed of 12.5 µL of Taq 2X Master Mix (New England Biolabs, M0270), 1 µL each of 10µM forward and reverse primers (as detailed in Table 1), 2 µL of the DNA template, and adjusted with 8.5µL of high-quality molecular grade water. The PCR process commenced with an initial denaturation at 94˚C for 5 minutes, followed by 36 cycles of denaturation at 94˚C for 30 seconds, annealing at 56˚C for 20 seconds (*HblC*) (Rowan *et al*., 2003) or 30 seconds (*CytK*) (Ehling-Schulz *et al*., 2006), and extension at 72˚C for 45 seconds. A final extension step was executed at 72˚C for 7 minutes, after which the temperature was maintained at 10˚C indefinitely.

Table 1 The primers used for Genotypic characterization of Virulence Determinants

Virulence genes	Primers Sequence	Amplicon size (bp)	References
HblC-N	AAT AGG TAC AGA TGG AAC AGG	399	Rowan *et al*., 2003
HblC-C	GGC TTT CAT CAG GTC ATA CTC		
CytKF-2	ACA GAT ATC GGT CAA AAT GC	421	Ehling-Schulz *et al*., 2006
CytKR-5	CAA GTT ACT TGA CCT GTT GC		

Sequence Preparation and Deposition in GenBank

Sequence Acquisition and Preparation

Upon successful completion of the sequencing process and meticulous sequence quality assessment, the obtained

genetic sequence was subjected to a rigorous cleanup procedure. This preparation phase ensured the sequence's integrity, readability, and compliance with GenBank's submission guidelines.

Deposition at GenBank

The prepared sequence, identified as *L. plantarum* strain Y11, was meticulously documented in the accepted FASTA file format. Subsequently, the sequence was officially deposited within the GenBank database, operated by the NCBI. This deposition is essential for enabling wider access to the genetic information, fostering collaboration among researchers, and facilitating comparative studies.

Accession Number Assignment:

The culmination of the deposition process resulted in the assignment of a unique accession number to the *L. plantarum* strain Y11 sequence. The accession number, MW642245.1, serves as an identifier that distinguishes this particular sequence from others within the GenBank repository.

Bacteriocin Production and Purification

Production of Crude Bacteriocin

To initiate bacteriocin production, the selected isolates were cultivated in 100 ml of MRS broth under conditions that favored optimal bacteriocin synthesis. Subsequent to incubation, the cellular biomass was separated from the growth medium through centrifugation at 10,000 rpm for 15 minutes (Ogunbanwo *et al*., 2003). The resulting cell-free supernatant was then adjusted to a pH of 6.5 using 1 mol/L NaOH to neutralize acidic effects. Additionally, neutralization of hydrogen peroxide in the supernatant was achieved by introducing 10 mg of catalase enzyme. This treated supernatant was deemed as crude bacteriocin and was employed for evaluating antagonistic activity through the agar well diffusion assay as outlined earlier.

Bacteriocin Purification

The crude bacteriocin extracted from the culture supernatant was subjected to purification through the ammonium sulfate precipitation method, as detailed by Sapatnekar *et al*. (2010). This time-tested method is a classical approach for isolating and concentrating proteinaceous substances from natural sources. In summary, the crude bacteriocin (cell-free supernatant) was prepared as described previously. The supernatant was then subjected to heating at 90°C for 10 minutes on a controlled surface heater. The ensuing precipitation of proteins occurred through gradual addition of ammonium sulfate to the supernatant, achieving up to 40% saturation (123 g/L), and the mixture was held overnight at 4°C with continuous stirring. Subsequent centrifugation at 12,000 rpm for 30 minutes at 4°C resulted in a surface pellicle containing the bacteriocin. This pellicle, rich in bacteriocin content, was re-suspended in a 50 mM sodium phosphate buffer (pH 6.5). The bacteriocin fraction thus obtained was then subjected to treatment with a chloroform-methanol mixture (2:1, v/v) for 1 hour at 4°C. The resulting fine white precipitate was collected through centrifugation at 12,000 rpm for 30 minutes at 4°C. The resulting pellet was re-suspended in 1 ml of ultrapure (double-distilled) water. This partially purified bacteriocin was subsequently stored in a sterile vial at 4°C. The antagonistic activity of the purified bacteriocin was assessed against various indicator strains through the agar well diffusion assay, consistent with the earlier description.

Bacteriocin Characterization

The bacteriocin in its purified form underwent comprehensive characterization to assess its stability under diverse conditions, including variations in pH, heat exposure, proteolytic enzyme activity, and shelf life.

Heat Resistance Assessment

The crude bacteriocins were subjected to varying temperatures (40°C, 60°C, 80°C, 100°C, and 121°C) for 15 minutes to gauge their thermal sensitivity. Following each heat treatment, the samples were tested for bacteriocin activity against specific foodborne pathogens, utilizing the agar well diffusion assay as previously outlined. As a control, the crude bacteriocin was maintained at 37°C, and measurements of growth inhibition zones were recorded post-overnight incubation.

pH Sensitivity Evaluation

The pH sensitivity of the crude bacteriocin was determined by adjusting its pH to different levels (2, 4, 6, 8, 10, and 12) using either 1N hydrochloric acid (HCl) or 1N sodium hydroxide (NaOH), following the approach modified from Karaoglu *et al*. (2004). Subsequently, the bacteriocin samples were incubated for 4 hours at room temperature. After the incubation period, each sample was subjected to the well diffusion method to assess bacteriocin activity against specific foodborne pathogens. The diameters of growth inhibition zones were measured after overnight incubation. Treatment, organism, and media controls were also included in the experiment.

Enzymatic Activity Evaluation

To ascertain the impact of enzymes on purified bacteriocin activity, enzyme solutions were filter-sterilized and mixed with purified bacteriocin aliquots, followed by incubation at 37°C for 2 hours. A control sample without enzymes was also incubated under similar conditions. After incubation, the samples were subjected to boiling water for 5 minutes to deactivate the enzymes. Subsequently, antimicrobial activity was determined using the agar well diffusion method, as described earlier.

Molecular Mass and Amino Acid Sequence Determination

The amino acid composition and sequence of the partially purified bacteriocin were obtained through a method detailed elsewhere. Briefly, bacteriocin-producing lactic acid bacteria were cultured in 1L of MRS broth and incubated at 37°C for 16 hours. Following centrifugation at 8,000 rpm for 3 minutes, bacteriocin in the cell-free supernatant was precipitated using ammonium sulfate. The precipitates were collected, dissolved in double-distilled water, filtered, and subjected to molecular mass determination through high-performance liquid chromatography.

Application of Partially Purified Bacteriocin in Milk

To assess the effect of bacteriocin on milk shelf life, a bacteriocin-mediated bio-preservation strategy was employed. Fresh cow milk was procured and divided into three conical flasks, each containing 50 ml of milk. Pasteurization was carried out at 71.7°C for 15 seconds, and subsequent cooling ensured microbial sterility. The three conical flasks were inoculated with semi-purified bacteriocin, a chemical preservative (sodium benzoate) for comparison, and a control with no additives. The preservatives were introduced within permissible limits, following guidelines from Ogunbanwo and Okanlawon (2006). The samples were stored at room temperature for 10 days, with microbial assessments and sensory evaluations performed during this storage period.

Amino Acid Composition and Molecular Weight

The amino acid analysis performed by high-performance liquid chromatography (HPLC). This was done following the method of Tumbarski *et al*. (2018).

Statistical Analysis

Data pertaining to growth inhibition zones against various bacterial pathogens were analyzed and presented as mean ± standard deviation (SD). Graphical representation of the data was generated using SPSS statistical software.

RESULTS

Antagonistic Activity of Isolated Lactic Acid Bacteria

Fig. 1 showcases the remarkable antagonistic activity of the isolated Lactic Acid Bacteria on selected food pathogens, with the noteworthy formation of Y11 by *L. plantarum* Y11. This visually demonstrates the potent inhibitory effects of the LAB strains on the growth of various pathogenic microorganisms. *L. plantarum* Y11 showed better inhibitory/ antagonistic activity than all the others against *Listeria monocytogenes*, *Escherichia coli*, *Enterococcus cloacae*, *Salmonella typhi* and *Staphylococcus aureus*. Furthermore, Fig. 2 illustrates the broad antagonistic spectrum of the confirmed bacteriocin produced by the isolated Lactic Acid Bacteria. This spectrum underlines the potential of these LAB strains to exert inhibitory effects against a diverse range of foodborne pathogens, highlighting their possible application in food preservation and safety.

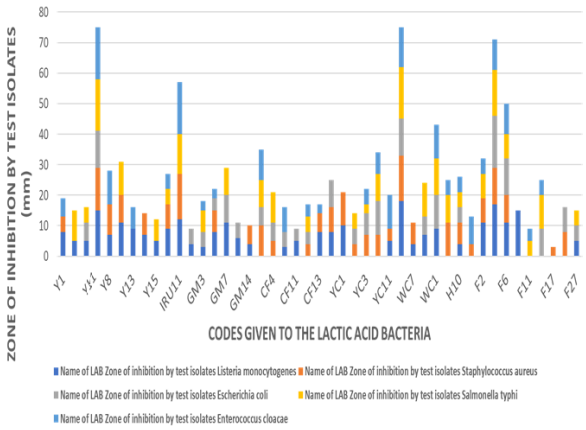

Fig. 1: Antagonistic Activity of Isolated Lactic Acid Bacteria on Selected Food Pathogens (Note: *L. plantarium* Strain Y11)

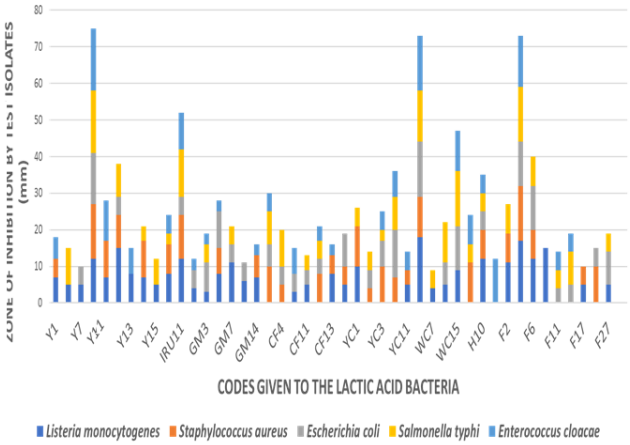

Fig. 2: Antagonistic Spectrum of Confirmed Bacteriocin of Isolated Lactic Acid Bacteria

Effects of Incubation Time and Environmental Factors on Bacteriocin Production

The investigation into the effects of incubation time on bacteriocin production by Isolate Y11 is depicted in Fig. 3. This informative graph depicts the relationship between incubation time and bacteriocin production, shedding light on the optimal conditions for enhanced bacteriocin synthesis. The peak bacteriocin activity was achieved above 10 h against all the test bacterial isolates (*Listeria monocytogenes*, *Escherichia coli*, *Enterococcus cloacae*, *Salmonella typhi* and *Staphylococcus aureus*).

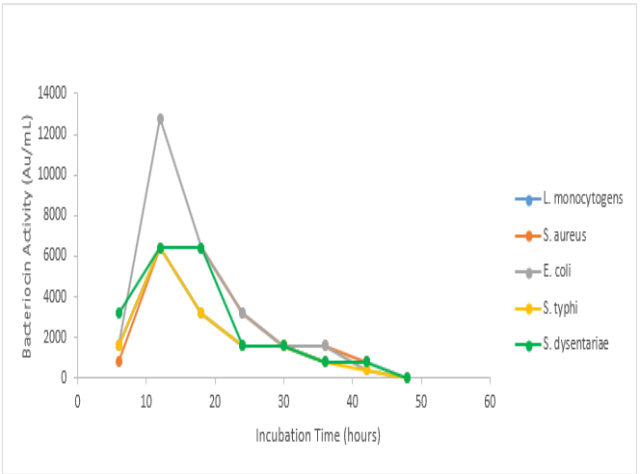

Fig. 3: Effects of Incubation Time on Bacteriocin Production by Isolate Y11

Temperature's role in bacteriocin production is exemplified in Table 2, which reveals the zone sizes and activity levels against different foodborne pathogens at varying temperatures. Optimum temperature that showed the best bacteriocin activity was 37ºC in which mean zone of 16 mm (6400 Au/ml) was observed for *L. monocytogenes*; 18 mm

(3200 Au/ml) for *S. aureus*; 15.5 mm (6400 Au/ml) for *E. coli*; 17 mm (6400 Au/ml) for *S. typhi* and 11 mm (1600 Au/ml) for S. *dysentariae.* The influence of pH on bacteriocin production is presented in Table 3, with zone sizes and activity against different pathogens shown for different pH levels. For the two Gram-positive test isolates (*Listeria monocytogenes* and *Staphylococcus aureus*) These tables provide valuable insights into the effects of temperature and pH on bacteriocin production, aiding in the determination of optimal conditions for enhanced antimicrobial activity.

Table 2: Effect of Temperature on Bacteriocin Production by Isolate Y11

Temp. (°C)	*L. monocytogenes*		*S. aureus*		*E. coli*		*S. typhi*		S. *dysentariae*	
	Zone (mm)	Au/ml	Zone (mm)	Au/ml	Zone (mm)	Au/ml	Zone (mm)	Au/ml	Zone (mm)	Au/ml
25	9	1600	10	800	11	800	12	1600	9	200
30	14	1600	15	3200	15	6400	13	6400	14.8	800
37	16	6400	18	3200	15.5	6400	17	6400	11	1600
40	14	3200	11	800	15	800	15	3200	10	800
45	10	800	10	400	13	800	13	800	8	400

Table 3: Effects of pH on Bacteriocin Production by Isolate Y11

pH	***L. monocytogenes***		***S. aureus***		***E. coli***		***S. typhi***		***S. dysentariae***	
	Zone size (mm)	**Au/ml**	**Zone size (mm)**	**Au/ml**	**Zone size (mm)**	**Au/ml**	**Zone size (mm)**	**Au/ml**	**Zone size (mm)**	**Au/ml**
3.0	7	200	-	-	9.2	200	-	-	-	-
3.5	-	-	-	-	10	200	-	-	-	-
4.0	-	-	8	400	10	200	10	400	-	-
4.5	10	200	9.8	400	8	200	10	400	-	-
5.0	10	200	11	200	12	1600	10	400	12	800
5.5	12	200	15	1600	12	400	8	1600	10	800
6.0	16	3200	16	6400	18	12800	15	3200	15	3200
6.5	18	1600	17.8	1600	15	3200	17	1600	13	3200
7.0	10	1600	17	1600	15.5	3200	10	1600	15	1600
7.5	8.5	4000	-	-	8.8	400	10	800	-	-
8.0	-	-	-	-	-	-	-	-	-	-

Characterization of Purified Bacteriocin

Table 4 presents the antagonistic activity of the partially purified Plantaricin Y11 against selected food pathogens, further emphasizing its inhibitory potential. Higher activity of Plantaricin Y11 was observed across all the test isolates than when it was in crude form. The mean zone of inhibition that ranged from 15 mm to 19 mm showed that the purer the bacteriocin Plantaricin Y11, the better the activity. Additionally, Tables 5 and 6 delve into the effect of temperature and pH, respectively, on the antagonistic activity of purified Plantaricin Y11 against different test pathogens. These tables offer a comprehensive view of how temperature and pH variations impact the antimicrobial effectiveness of the purified bacteriocin. Higher temperature than the previously observed optimum temperature of 37ºC by the crude bacteriocin from the producers affected the partially purified cell free bacteriocin, reducing their activity. The activity reduced with higher than 37ºC temperature Table 5. Optimum pH of 6.5 was also reported for bacteriocin activity. *L. monocytogenes*, *S. aureus*, *E. coli*, *S. typhi* and *Shigella* species showed peak activity with mean zone of inhibition that ranged from 15 mm to 19 mm at pH of 6.5 (Table 6).

Table 4: Antagonistic Activity of the Partially Purified Plantaricin Y11 against the Selected Food Pathogens

Parameters	***L. monocytogenes***		***S. aureus***		***E. coli***		***S. typhi***		***S. dysenteriae***	
	zone size (mm)	**Au/ml**	**zone size (mm)**	**Au/ml**	**zone size (mm)**	**Au/ml**	**zone size (mm)**	**Au/ml**	**zone size (mm)**	**Au/ml**
Time (h) 12 pH 6.0 Temp. 37°C	18	6400	15	6400	15	6400	19	6400	19	3200

Table 5: Effect of temperature on antagonistic activity of partially purified Plantaricin Y11 against different test pathogens

pH Range	Purified Plantaricin Y11				
	Zones of inhibition against different pathogens (mm)				
	L. monocytogenes	***S. aureus***	***E. coli***	***S. typhi***	***Shigella***
Control (37°C)	18	15	14	20	18
40	17	15	10.8	18.5	17.5
60	16.5	13.5	10	15	16.8
80	11	11.5	10	12	14
100	11.5	05	06	11.5	09
121	09	05	-	08	05

Table 6: Effect of pH of purified Plantaricin Y11 against different test pathogens

pH	Purified bacteriocin				
	Zones of inhibition against different pathogens (mm)				
	L. monocytogenes	***S. aureus***	***E. coli***	***S. typhi***	***Shigella***
2.0	10	09	09	10.5	10
4.0	16	11.5	10.8	15.8	14
6.0	17.8	15.5	13	17	16.8
6.5	18	16	15	19	17
8.0	11.5	14	11	11.5	09
10.0	09	05	06	08	05
12.0	-	-	-	-	-

Phylogenetic Tree and Genetic Analysis

Fig. 4 portrays the phylogenetic tree of *L. plantarum* strain Y11 constructed by Maximum Likelihood analysis on Mega 7. This tree offers insights into the genetic relationships and evolutionary connections of *L. plantarum* Y11 with other related species, contributing to our understanding of its genetic lineage. *L. plantarum* strain Y11 (MW642245.1) appears to be closely related to the

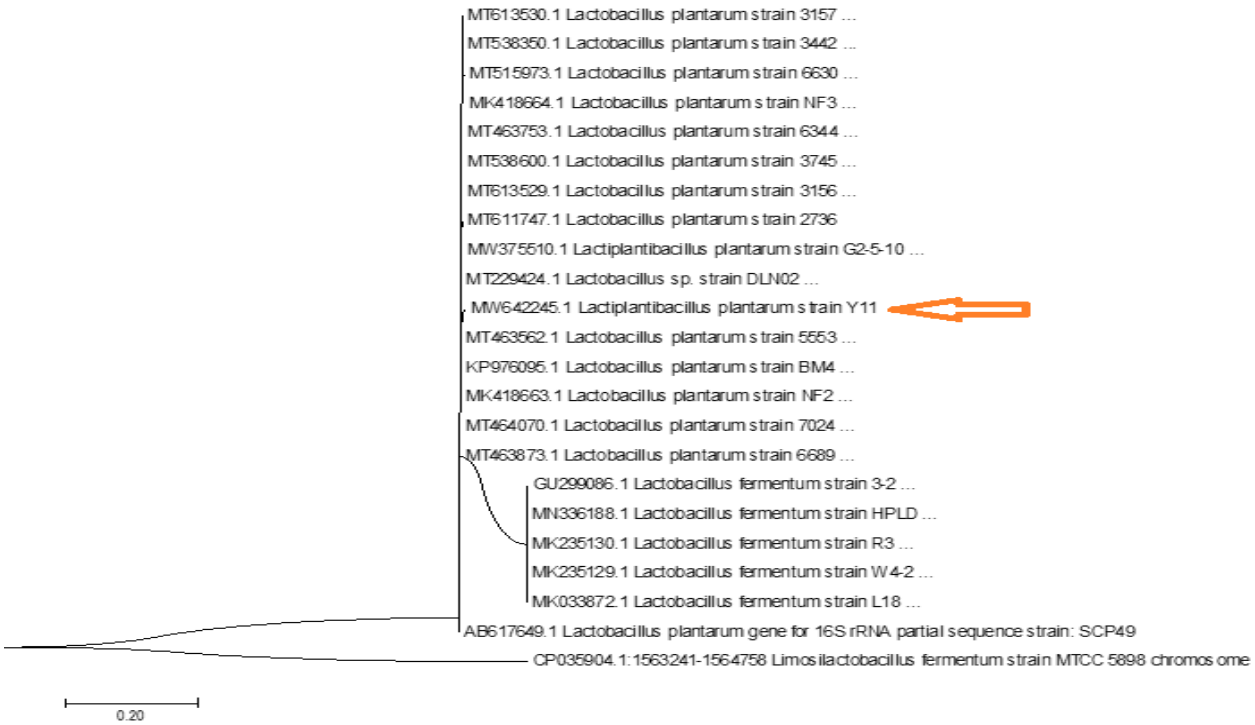

Fig. 4: Phylogenetic tree of *Lactiplantibacillus plantarum strain Y11*, MW642245.1 (←) constructed by Maximum Likelihood on Mega 7 (Version 7.0.26; 1993-2022)

Molecular Detection of Virulence Determinants

The successful amplification of Hemolysin C gene (*HblC*) and cytotoxin K gene (*CytK*) for *L. plantarum* Y11 and other Lactobacilli isolates at expected sizes of 399 bp and 421 bp respectively show the need for further studies on the safety

of the bacteriocin produced by *L. plantarum* strain Y11 (MW642245.1).

Preservative Assay Results

Plantaricin Y11 checked the growth of mesophilic bacteria in the milk preserved with it. Unlike the control which was too numerous to count from the day 2, the microbial population of potential spoilage bacteria in milk samples preserved with Plantaricin Y11 displayed a paltry initial log CFU/ml of 3.11 on Day 2. Table 7 showed how Plantaricin Y11 compared with sodium benzoate used as commercial chemical preservative and its promising results for the first 5 days. No fouling odour was perceived in the milk preserved with both Plantaricin Y11 and sodium benzoate for the first 5 days.

Amino Acid Composition and Molecular Mass of Plantaricin Y11 Revealed by HPLC Analysis

High-performance liquid chromatography (HPLC) analysis unveiled the amino acid composition of the bacteriocin generated by the Y11 strain of *L. plantarum*. This bacteriocin was found to encompass a total of 16 distinct amino acid types, with its amino acid makeup represented as NSHGTADYCVMWLIXK and molecular weight of 1540 Da. Figure 5 shows the HPLC chromatogram of Plantaricin Y11 showing the 16 peaks.

Table 7: Total Aerobic Mesophilic Bacterial Count (Log CFU/ml) of Milk Sample during Storage

Treatment	Storage days (log CFU/ml)									
	1	2	3	4	5	6	7	8	9	10
Plantaricin Y11	2.86	3.11	5.20	7.71	9.83	TNTC	TNTC	TNTC	TNTC	TNTC
Chemical preservative	2.45	3.11	3.56	3.92	5.34	6.71	7.06	7.85	10.88	12.09
Control	10.88	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

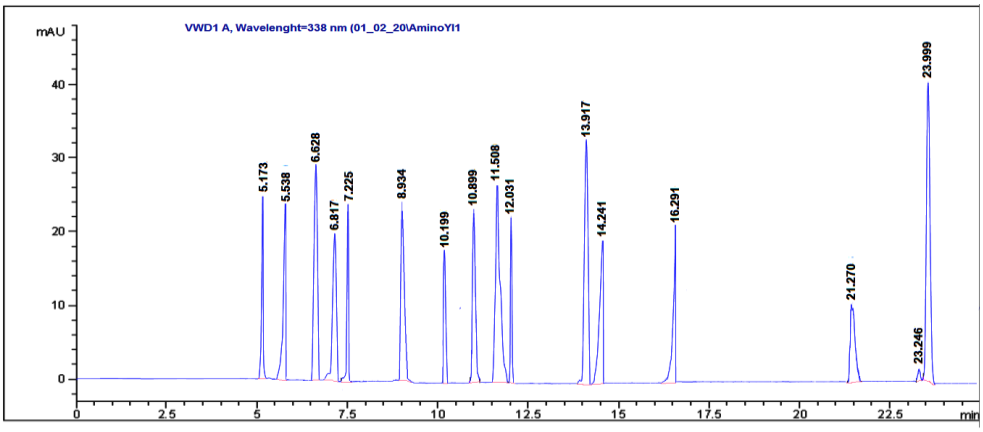

Fig. 5: High performance liquid chromatography (HPLC) chromatogram of Plantaricin Y11

DISCUSSION

The present study sheds light on the inhibitory potential of the isolated Lactic Acid Bacteria (LAB) against specific foodborne pathogens, accentuating their capacity to bolster food preservation and safety (Mokoena *et al*., 2021; Sharma *et al*., 2022; Zapaśnik *et al*., 2022). Figure 1 shows that the antagonistic effects of LAB strains are evident, with *L. plantarum* strain Y11. This particular strain demonstrates exceptional antagonistic activity against a gamut of pathogens, including *Listeria monocytogenes*, *Escherichia coli*, *Enterococcus cloacae*, *Salmonella typhi*, and *Staphylococcus aureus*, outperforming its LAB counterparts. This correlation is congruent with the earlier findings of Aljasir *et al*. (2019), which highlighted the inhibitory potency of *L. plantarum* strains against analogous pathogens.

Furthermore, Figure 2 accentuates the wide-ranging antagonistic spectrum of the verified bacteriocin produced by the isolated LAB strains. This phenomenon implies their potential applicability in counteracting diverse foodborne pathogens, thereby reaffirming their role in enhancing food safety measures. This observation aligns with the conclusions drawn by Fossi *et al*. (2016), who extensively explored the potential of LAB bacteriocins in the context of food preservation.

The influence of incubation time on bacteriocin production by isolate Y11 is elucidated in Figure 3. Optimal bacteriocin activity is observed after a span of 10 hours, in harmony with the findings of Kormin *et al*. (2001), who also reported optimal bacteriocin synthesis during same 10 hours incubation periods. The impact of temperature on bacteriocin production, as delineated in Table 2, highlights peak activity at 37°C. This concurs with the insights from Yang *et al*. (2018), underscoring the pivotal role of temperature in bacteriocin generation. Similarly, Table 3 presents the influence of pH, indicating an optimum pH of 6.5 for bacteriocin production, also in accordance with the optimal pH range advocated by Yang *et al*. (2016).

Table 4 underscores the augmented antagonistic efficacy of partially purified Plantaricin Y11 against foodborne pathogens in contrast to its crude form. This is in contrast with the studies conducted by Muhammad *et al*. (2021) who observed declined spectrum of bacteriocin activity post-purification. Tables 5 and 6 delve into the impacts of temperature and pH, respectively, revealing the ideal conditions for optimized purified bacteriocin activity. Notably, exposure to higher temperatures than the established optimum adversely affects activity, underscoring the imperative of meticulous temperature control in antimicrobial production. The pH data at this level still aligns with the research by Yang *et al*. (2016), which underscores the significant influence of pH on bacteriocin efficacy.

The phylogenetic tree depicted in Figure 4 provides insights into the genetic lineage of *L. plantarum* strains Y11, elucidating its genetic relatedness with other species. Our strain, *Lactiplantibacillus plantarum* strains Y11 (MW642245.1) has about 99.32% relatedness to *Lactobacillus plantarum* strain BM4 (KP976095.1). This genetic analysis complements the exploratory work of Rodriguez *et al*. (2022), thereby enhancing our understanding of the evolutionary connections inherent among *L. plantarum* strains.

Furthermore, the successful amplification of virulence determinant genes (*HblC* and *CytK*), as demonstrated through molecular detection, offers insights into potential pathogenic attributes of *L. plantarum* Y11. This observation is consistent with the study conducted by Martinez *et al*. (2019), which delved into similar virulence factors within the domain of LAB. Martin-Platero *et al*. (2009) discovered that the virulent genes *gelE, esp, asa1, efaA*, and *ace* were found at various levels in *Enterococcus* spp. Similarly, Todorov *et al*. (2017) screened and found a few virulence genes in 21 lactic acid bacteria. Presence of such virulence genes might only suggest need for cushions and additional step in safety assessments of the products.

Plantaricin Y11 showed a fairly comparative preservative capacity to sodium benzoate by checking bacterial counts compare to the control. Bacteriocins synthesized by other lactic acid bacteria have been proven to have utility in improving the quality and safety of milk. In a study conducted by Yildirim *et al*. (2016), it was observed that lactococcin BZ exhibited potent anti-Listeria activity, effectively reducing the viable L. monocytogenes cell count to undetectable levels in milk samples throughout storage periods at both 4°C and 20°C. Verma *et al*. (2017) noted that the utilization of partially purified pediocin PA-1 enriched fermented cheese whey (PCFCW) as a natural preservative was successful in reducing the overall microbial load in raw buffalo milk, consequently enhancing its shelf life. Research examining the efficacy of Reuterin, produced by *Lactobacillus reuteri* strain LR47 in combination with Nisin and Pediocin, demonstrated an extension of the shelf life of raw dairy farm milk by six hours at 37°C while preserving its initial microbial quality (Kumar *et al*., 2020). In findings by Vandera *et al*. (2018), it was revealed that raw milk and sterile raw milk samples inoculated with *Enterococcus faecium* KE82 displayed robust in situ inhibitory activity against *L. monocytogenes* in well diffusion bioassays.

Having a bacteriocin with a total of 16 distinct amino acid types holds significant importance in various aspects of biopreservation and food safety. Bacteriocins are antimicrobial peptides produced by bacteria, primarily lactic acid bacteria, to inhibit the growth of closely related or competing microorganisms. Their potential applications range from extending the shelf life of food products to preventing the growth of pathogens, which is crucial for ensuring food safety. The amino acid makeup of NSHGTADYCVMWLIXK, consisting of 16 different amino acids, contributes to the bacteriocin's diverse structure (Pérez-Ramos *et al*., 2021). This diversity might enable the bacteriocin to interact with a wider range of microbial targets, potentially enhancing its antimicrobial effectiveness. The molecular weight of 1540 Da is relatively low, which can facilitate its diffusion and interaction with microbial membranes, further underscoring its potential as an effective antimicrobial agent.

Several studies have highlighted the significance of amino acid composition and diversity in bacteriocins for their functionality (Yang *et al*., 2014; Simon *et al*., 2020; Pérez-Ramos *et al*., 2021). For example, in a study by Yang *et al*. (2014), the researchers emphasized the importance of specific amino acid residues in the structure of a bacteriocin, which determined its target specificity and antimicrobial activity. This resonates with the notion that the diverse amino acid composition of the given bacteriocin (NSHGTADYCVMWLIXK) could contribute to its ability to target a range of microorganisms. Moreover, the work of Simon *et al*. (2020) highlighted that the size and molecular weight of bacteriocins play a role in their mode of action. Bacteriocins with lower molecular weights, similar to the 1540 Da bacteriocin in question, might have an advantage in penetrating microbial membranes and disrupting their integrity (Pérez-Ramos *et al*., 2021).

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