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Antibacterial Activity of Bacteriocin extracted from *Lactobacillus plantarum* isolated from Fermented Rice-Water Milk Extract against selected Pathogenic Bacteria.

Olisaka, F. N.[1], Mgbemene, A, O.[1*] Onyia, C. F.[2], Iwu, V.[3]. and Onyemaechi, P.[1]

1 Department of Biological Sciences, Faculty of Natural Science and Environmental Studies, Godfrey Okoye University, Enugu, Enugu State, Nigeria
2 Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Federal University Oye-Ekiti, Ekiti State, Nigeria
3 Bioscience Centre, International Institute of Tropical Agriculture, Ibadan, Oyo State

Abstract
Bacteriocin are produced by lactic acid bacteria during their primary growth phase as natural antimicrobial agents against closely or distantly related microorganisms in their natural environment. This study aimed at ascertaining the inhibitory effect of bacteriocin extracted from *Lactobacillus* strains, against *Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli. Lactobacillus* sp. was isolated from rice water samples utilizing the de Mann, Rogosa and Sharpe (MRS) agar, and subsequently, the MRS broth. The isolates were morphologically and biochemically identified, then further confirmed to be *Lactobacillus plantarum* strains using the 16S rRNA gene sequence analysis. Chloroform solvent precipitation was carried out to extract the bacteriocin from the MRS broth culture. The bacteriocin from *Lactobacillus plantarum* 1 (RW1) displayed high inhibitory activity against the Gram-negative bacteria at 5-13mm, as compared to 9mm for the Gram-positive bacteria. The *Lactobacillus plantarum* 2 (RW2) bacteriocin displayed the highest activity against both the Gram-negative bacteria and the Gram-positive bacteria at 14mm and 10mm respectively. Characterization of the bacterial isolates using the 16SrRNA gene sequence analysis revealed the isolates to be *Lactobacillus plantarum* strain LL441 and *Lactobacillus plantarum* strain C11. In conclusion, the chloroform extracted bacteriocin produced from *Lactobacillus plantarum* isolated from new sources, displayed antibacterial activity and could serve as bio-preservatives against pathogens in food and the food industries.

Keywords: Bacteriocin, Chloroform precipitation, Lactic acid bacteria, *Lactobacillus plantarum,* Gram-positive bacteria, Gram-negative bacteria.
Corresponding Author Email: amgbemene19@gmail.com

Introduction

Bacteriocin are short polypeptides or small protein molecules that are synthesized in the ribosome. They are antagonistic against bacteria and are also closely related to the strain of bacteria that produce them. The interaction and destruction of microbial cells with cell receptors is how these bacteriocin basically function (Sugrue *et al.,* 2020). Some cells in any community of microorganisms may be bacteriocinogenic (they produce bacteriocin), sensitive to different bacteriocin, or even resistant to them. Only a few of the bacteriocin-producing microbial cells will be induced to produce and release bacteriocin to limit the growth of competing cells if these three types of cells are present in a microbial community and must compete for limited resources to ensure survival (Bindiya and Bhat, 2016). Bacteriocin would kill some of the sensitive cells in that microbial community,

while other cells might develop resistance because of mutations they might have. Due to the "cost" of producing bacteriocin in their environment, the remaining resistant cells may eventually destroy the bacteriocinogenic cells (Yongkiettraul *et al.,* 2019). These lactic acid bacteria either become bactericidal or bacteriostatic to eliminate or prevent the growth and development of other microorganisms as a means of competition and survival in the community of various microorganisms (Darbandi *et al.,* 2021). In different fermented food products like vegetables, grains and dairy, Lactic acid bacteria can produce numerous metabolites having antibacterial, antifungal, anticancer and even probiotic properties, hence, reputable members of the antimicrobial producing Lactic acid bacteria genera can be extracted from a variety of fermented plant based and dairy products under various environmental conditions (Esayas *et al.,* 2008). Bacteriocin typically produced by *Lactobacillus plantarum* are of the Class II bacteriocin group commonly called Plantaricin. They have characteristics like being small non-lantibiotic (<10kDa) two peptide molecules which are hydrophobic, cationic, unmodified and mostly stable to heat (Yilmaz *et al.,* 2022). These Plantaricin could be chromosomally or plasmid encoded, and are mostly arranged within operon clusters (Fidanza *et al.,* 2021). The ability of lactic acid bacteria (LAB), a group of Gram-positive rod or cocci-shaped facultative anaerobes, to produce inhibitory substances (bacteriocin) to prevent the growth of other bacteria in that very environment is the focus of this current research. In recent times, many studies have given attention to the promising probiotic potential of *Lactobacillus plantarum* and on the cell free supernatant (CFS) as well as the bacteriocin produced by the different strains of *Lactobacillus plantarum* as bio-preservatives to prevent food spoilage caused by foodborne pathogens (Wang *et al.,* 2023). Wang *et al.,* (2023) recently demonstrated the usefulness of the cell free supernatant of *Lactobacillus plantarum* 90 as a potent antibacterial agent in improving the shelf life of ground meat gel (Wang *et al.,* 2023). Additionally, Li *et al.,* (2024) in their study described a decrease of *Staphylococcus aureus* counts in sausages inoculated with both the whole bacterium *Lactobacillus plantarum* SL47 and it's derived bacteriocin SL47 (Li *et al.,* 2024). *Lactobacillus plantarum* has been widely recongized as an enhancement to the saftey of food products during fermentation process (Zapásnik *et al.,* 2022) which makes it a highly valuable species for use in developing probiotics (Seddik *et al.,* 2017; Liu *et al.,* 2018). The extraction of bacteriocin involves the use of precipitating agents that bind to the peptide chains to permit the dissociation of the bacteriocin for easy recovery after centrifugation. The current study aimed at extracting bacteriocin from the identified *Lactobacillus plantarum* strains in fermented rice water-milk extract using chloroform solvent. The extracted and semi purified bacteriocin was tested as an antibacterial agent against three selected pathogenic bacteria strains. The specific objectives were to identify the two *Lactobacillus plantarum* strains via DNA extraction and nucleotide sequencing and to determine their bacteriocin antibacterial activity against the selected pathogenic bacteria (*Escherichia coli, Staphylococcus aureus*, and *Klebsiella pneumoniae*) isolated from spoiled mango puree and keypads for automated teller machines. These two sources were selected because these Bacteria species such as *Staphylococcus aureus* and *Escherichia coli* have very high prevalence as food contaminants and are considered as "One Health" threats (Hotiet *et al.,* 2022). The second reason being that the effects of these harmful bacteria from such sources could potentially be neutralized by the antibacterial action of bacteriocin. The significance of this study is centered on the easy technique of isolating *Lactobacillus plantarum* with bacteriocinogenic potential from rice by directly fermenting the first sterile water used in washing the raw rice to obtain bacteria colonies for about 48h. Secondly, on the extraction and recovery of semi purified bacteriocin using chloroform by physically shaking the mixture of the cell free supernatant and chloroform to obtain a homogeneous solution which separates and concentrates after settling for a while.

Materials and Methods

Location of the study (Study Area)

This research was conducted at the Microbiology Laboratory of Godfrey Okoye University, Thinkers Corner, Enugu State, Nigeria. The fermented rice water samples in this study were made from scratch. The sample isolates were then prepared and utilized for the DNA extraction and downstream nucleotide sequence analyses.

Preparation of the Fermented Rice Water Milk Extract

An initial volume of 500 ml of distilled water was poured into a sterile container containing 250 grams of raw rice grains and was covered with a lid and set aside to ferment for 48h at room temperature. An additional volume of 250 ml of store bought liquid milk was added to the fermented rice water and was further fermented for 48h. This was done to establish the presence of possibly diverse lactic acid bacteria in the rice-water samples. The resultant cloudy liquid sediment were separated and used as the source sample material.

Sample Collection

Two samples of the prepared fermented rice water-milk extract were collected into sterile 50ml beakers for the subsequent Ten-fold serial dilutions in the Laboratory to isolate the bacteria present in both samples.

Collection of the Test Organisms

The pathogenic bacteria to be tested on (*Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus*) were isolated from spoiled fruit and environmental samples by research student understudies in Godfrey Okoye University Microbiology Laboratory, Thinkers Corner Enugu State. They were streaked on Eosin Methylene Blue agar (EMB) for *Escherichia coli* and *Klebsiella pneumoniae,* and Manitol Salt agar (MSA) for *Staphylococcus aureus*, and incubated for 24h at 37°C.

Isolation and Enumeration of Bacteriocin Producing Lactic Acid Bacteria

Ten-fold serial dilution was done for the two samples, after which 0.1ml aliquot of dilutions 10^{-2} and 10^{-4} were inoculated onto the surface of prepared 20 ml sterile, solidified de Man, Rogosa and Sharpe (MRS) agar utilizing a sterile hockey stick spreader. These were left to diffuse for 1 hour prior to being inverted and incubated anaerobically in an anaerobic jar for 24h at 37°C. All counts were expressed in cfu/ml. Pure colonies were observed by sub-culturing onto MRS agar slants and preserved appropriately in a 4°C refrigerator (Abd *et al.,* 2010).

Identification of Lactic Acid Bacteria Strains

Morphological and biochemical tests such as Gram stain reaction, catalase test, and oxidase test (Cheesbrough, 2009), followed by sugar fermentation test and salt susceptibility test (Ngene *et al.,* 2019) to predetermine the identity of the isolates, and then confirmatory molecular assay (DNA extraction, PCR amplification, Agarose gel Electrophoresis and Nucleotide sequencing) were employed here.

Production of Bacteriocin

The bacteria isolates were inoculated into MRS broth from culture plates and incubated anaerobically for 24h at 37°C. After 24h, the resulting growth of bacteria in the culture broth was clarified to release the extracellular metabolite (bacteriocin) into the culture broth by centrifugation at 4,000 rpm (revolutions per minute) at room temperature for 20 minutes for the 2 isolates obtained. The resultant crude supernatant fluid or the cell-free supernatant was filter sterilized using sterile 0.47µm filter membrane. The bacteriocin activity unit, measured in AU/ml was obtained by a two-fold serial dilution (1:1ml) of the filtered supernatant with equal volume of 0.9% saline solution. The corresponding solution was used for further analysis (Todorov and Dicks, 2005).

Solvent Extraction of Crude Bacteriocin

Chloroform solvent was used to completely precipitate the peptide subunits from the crude supernatant fluid (CSF) of the bacteriocin to obtain a semi-purified form, expected to have high inhibitory activity against the indicator bacteria. Exactly (15 ml) of CSF was transferred into 2 sterile bottles with a screw cap. A 15ml volume of chloroform was added into the bottle. The solution was vigorously shook for about 10 minutes to ensure homogeneous mixing, and was stored in a 4°C refrigerator for 24h. The top layer of the mixture (the chloroform) was properly decanted while the bottom layer (whitish in color) was transferred into sterile 50ml beaker and allowed to settle until a floating white interfacial layer was observed inside the vessel. The white interfacial layer was finally transferred into a beaker for the susceptibility test by agar well diffusion (Cheng *et al.,* 2020).

Agar Well Diffusion Assay

Muller Hinton Agar was prepared for the susceptibility test and transferred into petri dishes in accordance with the manufacturer's manual. A sterile cork borer (5mm) was used to make wells on the agar surface after it had solidified, and 40µl aliquots of the bacteriocin extract were seeded into the wells. They were left for an hour on the bench top for pre-diffusion and incubated aerobically at 37°c for 18 to 24h. Positive control antibiotic discs were

used to create a synergy. The inhibition zone diameter (IZD) of the plates was measured to determine the degree of susceptibility of the test organisms to the bacteriocin extracts using a meter rule (Onyia *et al.,* 2019).

Molecular Identification of Bacteriocin-Producing Strains

Pure DNA was extracted from the two biochemically confirmed *Lactobacillus plantarum* strains using the ZR FUNGAL/BACTERIAL DNA MINIPREP KIT according to the manual (Manufactured by Zymo Research).

Lactobacillus plantarum specific primer (F: *GCT GGC AAT GCC ATC GTG CT* and R: *TCT CAA CGG TTG CTG TAT CG*) (Kim *et al.,* 2020) was used alongside the 16SrRNA gene primer (27F: *AGAGTTTGATCMTGGCTCAG*) and reverse (1492R: *AAGGAGGTGWTCCARCCGCA*) to identify target genes present in the template DNA. The PCR reaction mixture consisted of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270), 1µL each of 10µM forward and reverse primers, 2µL of DNA template, and 8.5µL of Nuclease free water. After the PCR amplification, gel electrophoresis was performed to visualize and analyze the amplified DNA fragments. The PCR amplification protocol included an initial denaturation step at 94˚C for 5 minutes, followed by 36 cycles of denaturation at 94˚C for 30 seconds, annealing at 56˚C for 30 seconds, and extension at 72˚C for 45 seconds. At last, a final elongation step at 72˚C for 7 minutes, and then an indefinite temperature hold at 10°c.

The amplified PCR products were visualized by electrophoresis in 2% agarose gel stained with 10µl EZ vision DNA stain. The EZ vision binds to the DNA and permits the DNA to be visualized under bright (UV) light. The separated DNA fragments were visualized under UV transilluminator.

In order to accurately identify the *Lactobacilli* under examination, 16SrRNA gene sequencing was done. The fragments that were amplified underwent sequencing using a Genetic Analyzer 3130xl sequencer manufactured by Applied Biosystems, following the instructions provided by the manufacturer. The sequencing kit employed for this process was the BigDye terminator v3.1 cycle sequencing kit. Subsequent genetic analysis was carried out using NCBI Nucleotide BLAST.

Results

Isolation and characterization of Lactic Acid Bacteria from Rice water samples

The total lactic acid bacteria count using MRS agar is presented in Table 1. The first rice water sample had a higher bacterial count of 1.30×10^{6} cfu/ml, while the second had a lower bacterial count of 1.15×10^{6} cfu/ml. Table 2 shows the morphological and biochemical characteristics of the two bacteria isolates.

Bacteriocin Antibacterial Activity via Inhibition Zone Diameter (IZD)

Figure 1 shows the semi-purified bacteriocin extracts investigated for antibacterial activity. The results in Table 3 indicates that *Escherichia coli* and *Klebsiella pneumoniae* both had the highest inhibition zone diameter at 14mm, whereas *Staphylococcus aureus* had the least inhibition zone diameter at 0mm, 9mm and 10mm respectively.

Estimation of bacteriocin activity (Arbitrary Units per milliliter (AU/ml)

The bacteriocin activity of the extracts is based on the highest dilution of bacteriocin showing the most inhibition against the test bacteria as indicated in Table 4. The results show that bacteriocin from *Lactobacillus plantarum* C11 (RW_4^{01-04}) at the 16th dilution had the highest activity against *Escherichia coli* and *Klebsiella pneumoniae* at 400AU/ml respectively. On the other hand, bacteriocin from the same bacteria at the 2nd dilution recorded the least activity against *Staphylococcus aureus* at 50AU/ml. Only bacteriocin from *Lactobacillus plantarum* LL441 (RW_4^{1-4}) at the 16th dilution had the most activity against *Staphylococcus aureus* at 400AU/ml.

Molecular Identification and Sequence Analysis

The sequence result revealed *Lactobacillus plantarum* 1 has 96.95% pairwise similarity with *Lactobacillus plantarum* strain LL441 with NCBI accession number CP114874.1 (Figure 4). The second isolate, *Lactobacillus plantarum* 2 comprise a 92.90% pairwise similarity with *Lactobacillus plantarum* strain C11 with NCBI accession number MN704589.1 (Figure 5).

Table 1: Sample type, sample ID and lactic acid bacteria count (cfu/ml)

Sample type	Sample ID	Lactic acid bacteria colony forming unit (cfu/ml) $\times10^{6}$

Rice water 1	RW1	$1.30 \pm 1.06 \times 10^6$
Rice water 2	RW2	$1.15 \pm 1.06 \times 10^6$

Key: (RW1): Rice water sample 1; **(RW2)**: Rice water sample 2.

Table 2: The Morphological and Biochemical Characteristics of bacteria isolates from food samples

Bacteria Isolates ID	RW1	RW2
Isolate colors	Cream colored colonies with uneven edges	Pale colored colonies with smooth edges
Appearance	Opaque colonies observed	Shiny large colonies observed
Elevation	Colonies with flat centers	Raised center colonies
Texture	Slimy and smooth surface	Dry and rough surface
Growth formation	Moderate growth observed	Thick lump like growth observed
Gram stain	**+**	**+**
Cell arrangement	Short curved rods	Short curved and clustered rods
Catalase	**-**	**-**
Oxidase	**-**	**-**
Growth in 4.5% NaCl	**+**	**+**
Growth in 6.5% NaCl	**+**	**+**
Glucose	**+AG**	**+AG**
Sucrose	**+A**	**+A**
Maltose	**+A**	**+AG**
Likely Organism	***Lactobacillus plantarum* LL441**	***Lactobacillus plantarum* C11**

Key: (RW1): Rice water sample 1; **(RW2)**: Rice water sample 2; **(-)**: negative; **(+)**: positive; **(+AG)**: positive for acid and gas; **(+A)**: positive for acid.

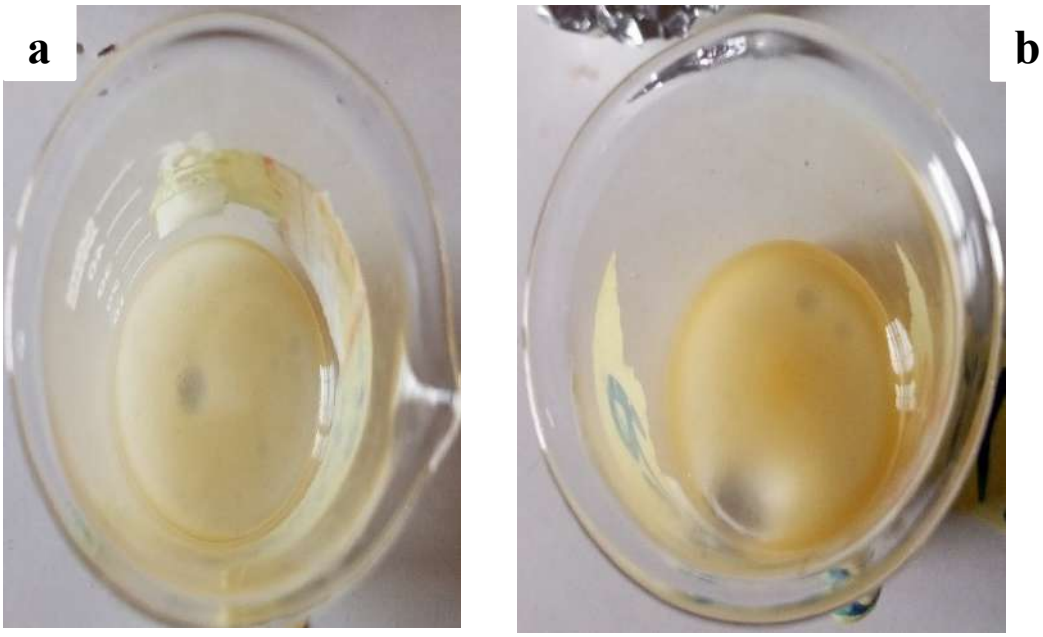

Fig 1. Top view (a & b) and Side view (c & d) of Interfacial Floating Bacteriocin precipitates (whitish colored substance) in chloroform solvent (yellowish substance)

Table 3: Bacteriocin Antibacterial activity via Inhibition Zone Diameter (IZD)

Bacteriocin extract ID	Bacteriocin Inhibition Zone Diameter (mm) *Escherichia coli*	Bacteriocin Inhibition Zone Diameter (mm) *Klebsiella pneumoniae*	Bacteriocin Inhibition Zone Diameter (mm) *Staphylococcus aureus*
RW_4^{01-04} No. 2	5.0	10.0	10.0
RW_4^{01-04} No. 4	7.0	11.0	ND (0)
RW_4^{01-04} No. 8	13.0	13.0	ND (0)
RW_4^{01-04}No. 16	14.0	14.0	ND (0)
RW_4^{1-4} No. 2	NT	9.0	ND (0)
RW_4^{1-4} No. 4	12.0	11.0	9.0
RW_4^{1-4} No. 8	12.0	13.0	9.0
RW_4^{1-4} No. 16	11.0	12.0	9.0

Key: (NT): Not tested using bacteriocin extract; **(ND)**: No inhibition detected.

Table 4: Bacteriocin Activity Unit (AU/ml) of the bacteriocin extracts

Bacteriocin dilutions	Activity against *Escherichia coli* (AU/ml)	Activity against *Klebsiella pneumoniae* (AU/ml)	Activity against *Staphylococcus aureus* (AU/ml)
RW_4^{1-4} NO. 8	200	200	-
RW_4^{1-4} NO. 16	-	-	400
RW_4^{01-04} NO. 2	-	-	50
RW_4^{01-04} NO. 16	400	400	-

Key: (-): Minimum bacteriocin activity detected. **(AU/ml):** Arbitrary units per milliliter. Bacteriocin activity unit (AU/ml)= $\frac{\text{Concentration of the highest dilutionshowing inhubitory activity} \times 1000\text{ml}}{\text{Volume of bacteriocin extract in the well}}$

Volume of bacteriocin used in well= 40µl.

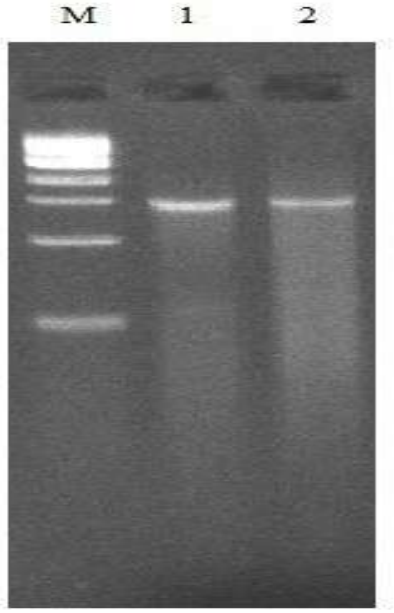

Fig 2. Gel image of the 16SrRNA gene at about 1500bp.
M (Molecular ladder) is a 1kbp DNA ladder

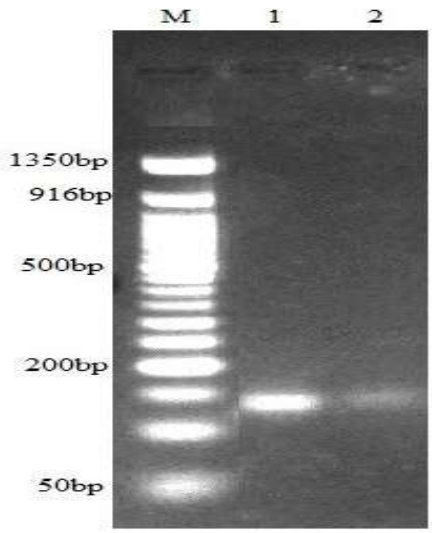

Fig 3. Gel image showing amplification of *Lactobacillus plantarum* at about 147bp
M (Molecular ladder) is a 50bp DNA ladder

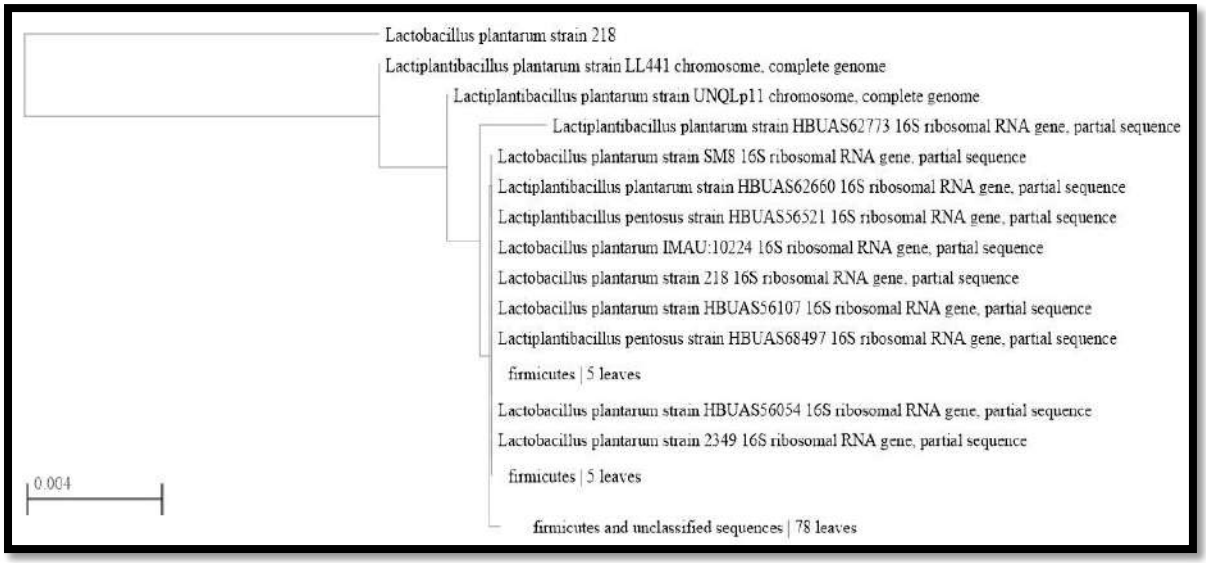

Fig 4. Phylogenetic tree showing the phylogenetic placement of *Lactiplantibacillus plantarum* strain LL441 being most related to *Lactobacillus plantarum* strain 218, as inferred by the NCBI Distance tree results Neighbor-joining method based on 16SrRNA genetic sequence analyses.

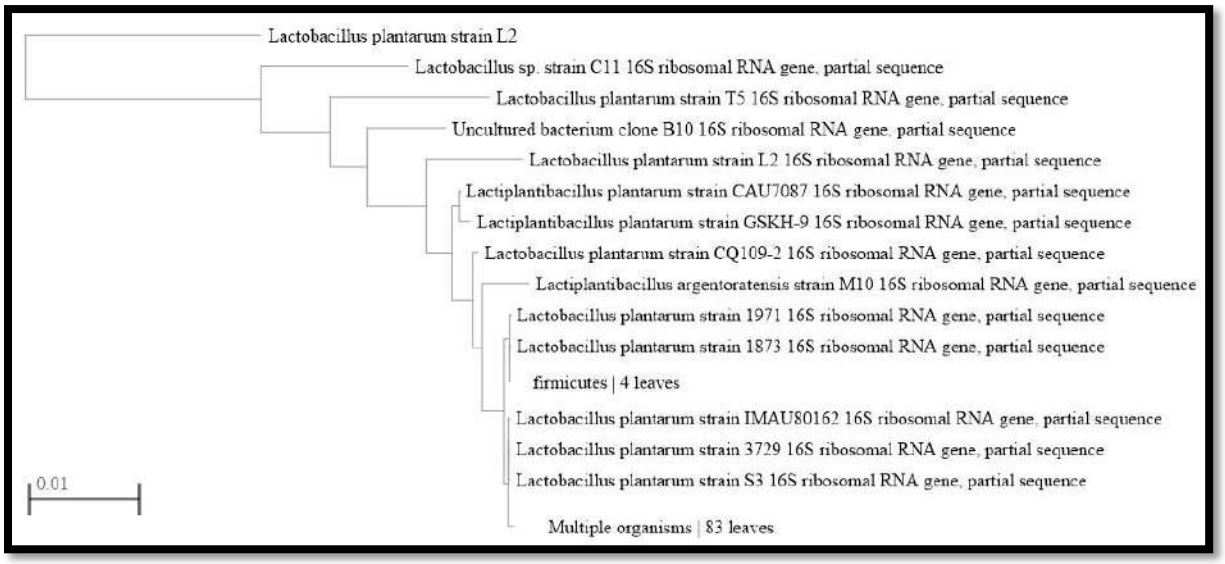

Fig 5. Phylogenetic tree showing the phylogenetic placement of *Lactobacillus plantarum* strain C11 being in closest relationship with *Lactobacillus plantarum* strain L2, as inferred by the NCBI Distance tree results Neighbor-joining method based on 16SrRNA genetic sequence analyses**.**

Discussion and Conclusion

The present study aimed at extracting bacteriocin from *Lactobacillus plantarum* in fermented rice water-milk extract to determine its antagonistic ability against selected pathogens. The two *Lactobacillus* strains were identified based on their morphology and biochemical characteristics like being catalase negative as they were unable to produce catalase to degrade the reagent hydrogen peroxide; their ability to ferment sugar substrates (maltose, sucrose and glucose) and particularly produce CO_2 (Carbon IV oxide) on fermenting glucose, and their gram stain reaction (Gram-positive), amongst others. These characteristics therefore served as the criteria for determining the probable organism present in the sample as *Lactobacillus plantarum. Lactobacillus* rods are known to be present in a variety of fermented food products, and as such, were found in the fermented rice water samples. This result was consistent with the findings of other studies of a similar nature, which indicated that *Lactobacillus* rods are also prevalent in fermented foods (Sharma *et al.*, 2020).

The bacteriocin from the sample isolates displayed antibacterial activity against the 3 test bacteria used in this study. Lactic acid bacteria are known to produce several antimicrobial compounds asides bacteriocin which could primarily inhibit the growth of other bacteria in that environment, like hydrogen peroxide and organic acids. For this reason, a particular measure to ensure that the growth inhibition of the test bacteria was caused only by the bacteriocin in this study, was to exclude the effect of hydrogen peroxide through the process of anaerobic incubation of the bacteriocin producer strains, which eventually reduces the potent effect of hydrogen peroxide (H_2O_2) on the indicator bacteria. This is a significant method still in practice today, and was established by Schillinger and Lucke, 1989. After the elimination of the possible antibacterial factor on the indicator bacteria strains, the two *Lactobacillus plantarum* strains showed antagonistic activity against the 3 indicator bacteria strains, although some to a higher degree than others after chloroform extraction was conducted. A preliminary antibacterial activity test was done using just the cell free supernatant (CFS) of the two isolates. The results (not included in this study) were negative as there were no observable inhibition zones after heat treatment at 60, 80 100, and 121°C for 20 minutes respectively. This could indicate that the bacteriocin-like substances obtained after centrifugation were not thermo stable, which is contrary to the work of (Okpara *et al.,* 2013) and (Zhou *et al.,* 2014), who reported in their various studies that bacteriocin-like substances were stable and effective when treated at 100°C for 60 minutes and 121°C for 15 minutes respectively. The antimicrobial peptide thermo stable property of bacteriocin compounds is a very useful and significant property for use as a biomedical

product, making it effective in food preservation.

The chloroform solvent extraction technique used here proved to be both more efficient and less labor-intensive in terms of precipitating and recovering bacteriocin from the cell free supernatant. Based on the early work of Muriana and Klaenhammer (1991), chloroform reagent has been shown to have a higher recovery rate from the supernatant fluid when used with a variety of bacteriocin types like subtilin, nisin, pediocin, and lacidin. The total bacteriocin activity of the chloroform harvest was significantly higher than that of the non-precipitated cell free supernatant fluid in this method. This could be due to the chloroform increasing the rate of bacteriocin recovery from the culture medium by dispersing various bacteriocin aggregates into the chloroform for a higher recovery. As per Breukink and Kruijff (1999), bacteriocin are essentially amphiphilic peptides that have a high affinity for the lipid membrane. Amphiphilic compounds like bacteriocin can be concentrated in an environment that is created by the interaction between chloroform and the aqueous media solution. In this study, the vigorous shaking of the vessel containing bacteriocin allowed the compounds to easily migrate from the aqueous medium to the interfacial layer, where they concentrated at, and were recovered. After this was done, the bacteriocin displayed antibacterial activity against the test organism within a range of 5 to 14mm in diameter, demonstrating the effectiveness of the chloroform in bacteriocin extraction. As stated by Savadogo *et al.,* 2004; Esayas *et al.,* 2008 and Ren *et al.,* 2018, the observed known inhibition zones of the cell free supernatant of lactic acid bacteria against known human pathogens are within the ranges of 8 to 10mm, 9 to 11mm and 7 to 9mm respectively. This indicates that the result of this study displayed similar inhibition zone diameters within the ranges of 5 to 14 mm, which is in line with the findings of a comparable study by Abayeneh and Aleka (2021) who detailed an inhibition zone diameter within the range of 5 to 16 mm in measurement of the cell free supernatant of lactic acid bacteria isolated from traditional Ethiopian Dairy products.

The identified *Lactobacillus plantarum* strain LL441 is a known lactic acid bacteria strain mostly utilized as starter cultures in most dairy creation like cheese from raw milk (Mayo *et al.,* 1989). It is known to produce a plasmid-encoded lantibiotic (plantaricin C) with the capacity to inhibit a few Gram-positive pathogenic and spoilage bacteria respectively (Gonález *et al.,* 1994). This specific strain's bacteriocin extract exhibited a higher antagonistic capacity against the Gram-positive bacteria (*Staphylococcus aureus*) when contrasted with the other identified strain as noted in the current study. This might be attributed to the mechanism of action of the Plantaricin C bacteriocin of hindering cell wall biosynthesis by forming a complex with the peptidoglycan precursor lipid II (Flórez and Mayo, 2018). The presence of this specific strain in the fermented rice water might be far-fetched, as it is known to be transcendent in dairy based fermented food products as either a starter culture lactic acid bacteria or a non-starter culture lactic acid bacteria, yet might be proposed as being present as a result of the addition of the fermented rice water extract into the liquid milk for a second fermentation for 48 hours, subsequently advancing the proliferation of this specific strain. This eventually caused a physical separation of the milk solids from the fluids in the fermentation vessel, forming two layers; a top layer containing the milk solids and the bottom layer the liquid phase concentrated with live actively dividing bacteria cells.

The subsequently identified *Lactobacillus plantarum* strain C11 is majorly isolated from plant based fermented food products like fermented vegetables, for example, cucumber, cereals, grains and known to produce a variety of Plantaricin bacteriocin which belong to the class of *pln* structural gene loci such as the two peptide bacteriocin like Plantaricin EF and Plantaricin JK etc. The Plantaricin EF is a two peptide bacteriocin comprising Plantaricin E and F (plnE and plnF) whose functionality is subject to the synergistic and complementary activity of the two component peptides (Ekbald *et al.,* 2016). As per Ekbald *et al.,* (2016), the two peptides bind to specific membrane proteins or bacteriocin receptors, prompting membrane leakage and cell death of the bacterial cell. This specific bacteriocin is known to be active against both Gram-negative and Gram-positive microorganisms the same, making cell membrane permeation cause cell internal content outflow and death (Pal and Srivastava, 2014). The bacteriocin extracted from this bacteria strain was able to repress a greater amount of the Gram-negative test bacteria and is suspected to be the Plantaricin EF due to their similar mechanism of action. The Plantaricin EF bacteriocin in question is known to also have a higher antimicrobial potential against Gram-

negative bacteria because of the combining of single peptides E and F into a twofold peptide, thereby eliciting greater bactericidal effect on Gram-negative bacteria species.

A conclusion to this study could be given that the bacteriocin extracted from the respectively identified *Lactobacillus plantarum* strain LL441 and *Lactobacillus plantarum* strain C11 were able to inhibit the growth of pathogenic Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) isolated from spoiled fruit (mango) and environmental samples (keypads for Automated Teller Machines) respectively. The strains therefore produced structural bacteriocin with potent antibacterial effect.

Conflict of interests

The authors declare the research was conducted with no personal or financial relationships resulting in conflicts of interests.

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