Lactic Acid Bacterial Bacteriocins and Their Bioactive Properties Against Food Associated Antibiotic Resistant Bacteria

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Abstract

Purpose: Incidence of food borne diseases and growing resistance of pathogens to classical antibiotics is a major concern in the food industry. Consequently, there is increasing demand for safe foods with less chemical additives but natural products which are not harmful to the consumers. Bacteriocins produced by lactic acid bacteria (LAB), is of interest because they are active in a nanomolar range, do not have toxic effects and are readily available in fermented food products.

Methods: In this research, LAB were isolated from *fufu, gari, kunu, nono* and *ogi* using De Mann, Rogosa and Sharpe agar.

Result: A total of 162 isolates were obtained from the food samples. Antimicrobial sensitivity test yielded positive result for 45 LAB isolates against *Staphylococcus aureus* ATCC 25923 while 52 LAB isolates inhibited the growth of *Escherichia coli* ATCC 25922*.* On confirmation of the bacteriocinogenic nature of the inhibitory substance, 4 of the LAB isolates displayed a remarkable degree of inhibition to *Leuconostoc mesenteroides, Salmonella typhimurium* and *Bacillus cereus.* Agar well diffusion assay was also performed using the cell-free supernatant (CFS) obtained from *Lactobacillus fermentum* strain NBRC15885, *Lactobacillus fermentum* strain CIP102980, *Lactobacillus plantarum* strain JCM1149 and *Lactobacillus natensis* strain LP33. The (CFS) was able to inhibit the growth of *B. subtilis, Klebsiella pneumonia, S. typhimurium, S. aureus* and *E. coli* which are foodborne pathogens*.*

Conclusion*:* It therefore portends that the bacteriocins produced by the LAB isolated from these food products could act as probiotics for effective inhibition of the growth of antibiotic resistant foodborne pathogens.

1.0 Introduction

One of the concerns in food industry is the contamination of food by pathogens, which are frequent cause of foodborne diseases. Recurrent outbreaks of diarrhea and other foodborne illnesses combined with the natural resistance of the causative agents, is a huge risk to global health, food security and development (Caniça et al., 2019). Use of antibiotics in the control of such infections is faced with the challenge of resistance of pathogens to antibiotics as a result of misuse/overuse of antibiotics, incorrect dosing, low potency, poor solubility and lack of quick or accurate tests to diagnose infection (Castro-Sanchez, et al., 2016). Consequently, there is quest for alternative means to surmount this impeding danger. Nutrition was an essential component in many traditional forms of medicine (Georgiou et al., 2011), until the last century when its role in curative medicine started to decline. Following the increased awareness of the importance of lifestyle for disease prevention, we are now facing a reawakening of nutrition or lifestyle in general, for disease management and control (Witkamp and Norren, 2018).

A viable option is to opt for safe foods with less chemical additives and more of natural products which do not deter the organoleptic quality of the food or harm the consumers (Soltani et al., 2021). Biotechnology in the food-processing sector targets the selection, production and improvement of useful

microorganisms and their products, as well as their technical application in food quality and control of food borne diseases. Generally, food with no additives is more desirable, but if not available, consumers will choose foods containing natural additives over synthetic equivalents (Coderoni and Perito, 2020; Perito et al., 2020). Bacteriocins produced by lactic acid bacteria, is of interest since they are safe, active in a nanomolar range, heat stable, readily digested by gastric enzymes and there is currently no reports of pathogenic bacteria developing antimicrobial resistance to them. Effective application in food preservation has been reported and till date no toxic effect has been attributed to their usage. Bacteriocins are multifunctional, ribosomally produced, proteinaceous substances produced by bacteria which are biologically active with antimicrobial action against other bacteria principally closely related species. They are normally not termed antibiotics in order to avoid confusion with therapeutic antibiotics, which can potentially elicit allergic reactions in humans with related medical problems (Deraz et al., 2005; Negash and Tsehai 2020). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents and as such rapidly digested by proteases in the human digestive tract. Antibiotics are generally considered to be secondary metabolites that are inhibitory substances in small concentration, excluding the inhibition caused by metabolic by-products like ammonia, organic acids and hydrogen peroxide. It is likely that most if not all bacteria are capable of producing a heterogeneous array of molecules in the course of their growth *in vitro* (and presumably also in their natural habitats) that may be inhibitory either to themselves or to other bacteria (Ayivi et al., 2020). Bacteriocin production could be considered as an advantage for food and feed producers since, in sufficient amounts, these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources (Yang et al., 2018). Bacteriocins are often considered more natural because they are believed to have been present in many of the foods consumed since ancient times. Bacteriocins are inactivated by enzymes, such as trypsin and pepsin, found in the gastrointestinal tract and therefore do not alter the microbiota of the digestive tract (Balciunas et al., 2013; Negash and Tsehai 2020).

Despite the fact that antimicrobial peptides have an inhibition spectrum narrower than that of antibiotics, the bacteriocins produced by LAB have been reported to infiltrate the outer membrane of Gram-negative bacteria and lead to the inactivation of Gram-negative bacteria in combination with other enhancing antimicrobial environmental factors, such as organic acid, low temperature and detergents materials (Parada et al., 2007). Bacteriocins are generally named based on the genus or species of the strain producing it. For example, *Lactobacillus plantarum* produce plantaricin, *Lactococcus* spp. (lacticin, nisin), and *Carnobacterium* spp. (carnocin), *Enterococcus* spp. (enterocin) *Leuconostoc* spp. (leucocin) and *Pediococcus* spp. (pediocin) (Yusuf, 2013).

LAB are known to be Gram-positive, non-spore-forming rods, cocci and cocco-bacilli non-aerobic but aero-tolerant, able to ferment carbohydrates into energy and lactic acid (Jay, 2000). LAB belong to the phylum Firmicutes. The different major genera of LAB include: *Carnobacterium, Lactobacillus, Lactococcus, Enterococcus, Melissococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Weissella, Vagococcus*, and *Tetragenococcus*. Other genera include: *Aerococcus, Propionibacterium,*

Microbacterium, and *Bifidobacterium*. They are known to constitute the highest percentage of bacteria that display probiotic properties. Among compounds produced by LAB during lactic acid fermentations are: organic acids, diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins (Yusuf, 2013).

These microorganisms are ubiquitous in nature, they were first discovered in milk (Carr et al., 2002). They are also found in meat, fermented products, fermented vegetables and beverages (Gallego and Salminen 2016). Humans and some other animals also harbor LAB (Amarantini et al., 2019) without causing disease in them. Thus, because of the incidence of foodborne diseases in humans and growing resistance of pathogens to most antibiotics, this study was designed to isolate and identify LAB and screen their bioactive properties against food associated antibiotic resistant bacteria.

2.0 Materials And Methods

2.1 Isolation and Identification of Lactic Acid Bacteria

Fermented food samples (*fufu, gari, kunu, nono* and *ogi*) were purchased from local vendors for this study. Serial dilution and pour plate procedures were utilized in the evaluation of the culturable LAB flora of these fermented food samples using commercially available De Mann, Rogosa and Sharpe (MRS, Oxoid, Fisher Scientific) agar. Six blanks were prepared by pipetting 9 ml of distilled water in 6 macCartney bottles, corking them and sterilizing for serial dilution from 10^{-1} to 10^{-6}. Pour plate technique was carried out in plating 0.1 ml of dilution 10^{-3}, 10^{-5} and 10^{-6} on each of De Mann Rogosa Sharpe (MRS) agar. After incubation at 37°C for 48 hours under anaerobic condition, discrete colonies that developed were counted and recorded (Aneja, 2003; Karthikeyan and Santosh, 2009; Sharma, 2009). Isolated colonies with cultural characteristics namely raised, off white, spherical small with entire margins were picked from each plate and transferred to MRS broth for further analysis. The strain identification was done using the standard morphological, physiological, biochemical and molecular assays (Kim and Kim, 2014; Islam *et al*., 2016).

2.2 Bacteriocin Assay

The inhibitory activity of the selected LAB isolate against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, which were used as indicator organisms, was assayed by the agar spot test. The LAB isolates were spotted onto the surface of the MRS agar and incubated at 32°C for 24 h to allow colonies to develop. Sterile water (5 ml) was seeded with the indicator organism and poured into 50 ml of soft nutrient agar (0.9 % agar) which was cooled to about 45°C to prevent cell death. This was mixed by rotating the flask between two hands, the contents were gently overlaid onto each plate on which the LAB isolates were grown and allowed to completely cover the cultured agar plate surface and left to solidify. After incubation at 32°C for 24 h under anaerobic condition, the plates were examined for the presence of inhibition zones. Inhibition was considered positive when the inhibition halo of the indicator strain above the LAB colonies was more than 2 mm (Hockett and Baltrus, 2017).

2.3 Production and antimicrobial assay of the cell free supernatant (CFS) obtained from LAB broth culture

The strains of the selected LAB isolates which showed inhibition zone were used for further studies. The cell-free supernatants were prepared based on methods described by Mariam *et al.* (2014) with a little modification. The culture extract of the producer strains were obtained from 18–24 h culture grown on MRS broth. The cultures were then centrifuged at 10,000 rpm for 15 min (Hettich EBA 85 Tutthugen, Made in Germany). The inhibitory activity against the indicator organisms was assayed by the agar well-diffusion test (Cintas *et al*., 1998). Nutrient agar was inoculated with I ml of sterile water seeded with 18 h culture of indicator bacteria (*E. coli* or *S. aureus*). This was spread using a hockey stick to cover the surface of the agar and allowed to diffuse. Wells (5 mm in diameter) were cut into the agar using a sterile cork borer and filled with the cell-free supernatant (CFS) obtained from each LAB isolate. After incubation at 32°C for 24 h, the plates were examined for the presence of inhibition zones. Inhibition was considered positive when the width of the clear zone around the wells was 0.5 mm or larger (Balouiri *et al*., 2016).

2.4 Confirmation of the bacteriocinogenic nature of the inhibitory substances

To confirm the bacteriocinogenic nature of the inhibitory substances produced by the putative bacteriocin-producing strains, additional tests were performed to exclude the effect of organic acids, hydrogen peroxide and to confirm the proteinaceous nature of the inhibitory substance and its bactericidal mode of action according to the techniques described below.

2.5 Elimination of the effect of organic acids and hydrogen peroxide as inhibitory agents

Effect of organic acid was eliminated by adjusting the pH of the supernatants to 7.0 with 1M NaOH. The supernatant was then filter-sterilized using a membrane filtration unit with a 0.2 µm pore size millipore filter and subjected to agar well diffusion assay.

To exclude the action of hydrogen peroxide, 18 h cultures of strains showing antimicrobial activity after acid neutralization was diluted at a ratio of 1:10 mM Tris HCl (pH 7.0) and 2 µl of the suspension (about 10^6 cells/ml) was inoculated on Rogosa SL agar in culture plate and incubated. Eight hour growing culture of indicator organisms were diluted at a 1:10 ratio in 10 mM Tris HCl (pH 7.0) and mixed with Rogosa SL soft agar (48°C). Catalase enzyme was added at a final concentration of 0.5 mg/ml. The mixture was poured onto the culture plate wells, one well having no catalase enzyme served as the control. The final culture plates were examined after 18–24 hours incubation. The presence of an inhibition zone around wells both with and without catalase was observed and was determined to be the effect of bacteriocin (Tatsinkou *et al*., 2017; Voidarou et al., 2020).

2.6 Optimization assay

The selected strain of LAB was subjected to different culture conditions to derive the optimum conditions for bacteriocin production in MRS broth (Todorov and Dicks, 2004). Growth and bacteriocin production were estimated at temperatures 20°C, 30°C, 40°C, 50°C and 60°C, pH 4.0, 5.0, 6.0, 7.0 and 8.0 and NaCl concentrations 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. The absorbance of the broth culture was taken at a wavelength of 620 nm using Jenway Spectrophotometer (Todorov and Dicks 2004; Benmouna *et al.*, 2018).

2.7 Purification of Bacteriocin

The cell free supernatant was subjected to ammonium sulphate fractionation. On centrifugation at 10,000 rpm and temperature of 4°C for 10 mins., the pellets were collected and resuspended in a minimal volume of 0.2 ml Tris-HCl buffer pH 7.0 (Karthikeyan and Santosh, 2009). Dialysis tube was treated to remove protectants such as sulphur or glycerin compounds present in it. The protein content of the CFS was determined following the protocol described by Lowry et al., 1951.

2.8 Molecular weight determination of purified bacteriocins

The molecular weight of purified bacteriocin was estimated using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to characterize the bacteriocins. Gels were stained with coomassie brilliant blue R-250 after electrophoresis. The molecular weights of the bacteriocins were then estimated using a protein ladder of 170–10 kDa (Hassan et al., 2020).

3.0 Antibiotics Sensitivity Pattern Of Isolated Foodborne Pathogens

Antibiotics sensitivity test was conducted on pathogenic strains of *Bacillus subtilis, Klebsiella pneumonia and Salmonella typhimurium* obtained from minced pie. Nutrient agar was inoculated with I ml of sterile water seeded with 18 h culture of isolated foodborne pathogens. This was spread using a hockey stick to cover the surface of the agar and allowed to diffuse. Antibiotics disc containing Ampicillin (10 µg), Ampiclox (30 µg), Augmentin (30 µg), Amoxycilin (25 µg), Cefuroxime (30 µg), Ceftriaxone, Chloramphenicol (30 µg), Ciprofloxacin (5 µg) Cotrimoxazole (25 µg), Erytromycin (5 µg) Gentamicin (10 µg), Ofloxacin (5 µg), Pefloxacin (5 µg) and Streptomycin (10 µg), were placed on the agar surface using sterile forceps. Zone of inhibition were read after 24 h of incubation at 35°C and recorded (Balouiri *et al.*, 2016).

3.1 Determination of antibacterial susceptibility and mode of inhibitory action of the cell free supernatant (CFS) against the antibiotics resistant foodborne pathogens

Eight hour growing culture of antibiotic resistant food-borne pathogenic organisms were diluted at a 1:10 ratio in 10 mM Tris HCl (pH 7.0) and mixed with molten nutrient agar (48°C). Catalase enzyme was added at a final concentration of 0.5 mg/ml. The mixture was swirled to ensure even distribution and poured onto culture plate and allowed to solidify. A well with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer, and a volume (1 ml) of the neutralized cell free supernatant (NCFS) was introduced into the well using sterile pipette tips. The plates were incubated at 37°C for 24 h after which

they were observed for zones of inhibition and the diameter was noted. Inhibition was indicated as positive when the inhibition halo around the well was more than 2 mm (Amarantini et al., 2019).

3.2 Estimation of bacteriocin concentration

The concentration of bacteriocin in the CFS was quantified using Lowry's method. One mL of the bacteriocin solution was mixed with 1.4 mL of Lowry solution. This was shakened and incubated in the dark at room temperature. After 20 min of incubation, 1.3 mL of the suspension was collected while 0.1 mL diluted Folin reagent was added, mixed thoroughly and incubated under the same condition for 30 min. The absorbance reading was taken at 650 nm. From the standard curve prepared using bovine serum albumin, extract concentration was extrapolated (Lowry et al., 1951).

4.0 Molecular Identification Of The Bacteriocin Producing Lactic Acid Bacteria

Chromosomal DNA extraction was done using MP Biomedicals fast spin kit for soil following the manufacturer's protocol. Microcentrifuge tubes containing 2 mL of overnight grown LAB culture were spinned in a centrifuge at 5000 rpm for 5 mins to pellet the cells. The supernatant were discarded and 978 µL of MT Sodium Phosphate Buffer was added to the tubes. The mixture was transferred to a lysing matrix E tube followed by addition of 122 µL MT Buffer. Homogenization of the lysing matrix tube was done in a FastPrep instrument for 40 seconds with speed setting of 6.0. The Lysing matrix tubes were then centrifuged at 14,000 x g for 5–10 mins to pellet the debris. The supernatant was transferred to a clean 2.0 mL microcentrifuge tube while 250 µL protein precipitation solution was added. The tubes were inverted 10 times to mix the content and centrifuged at 14,000 x g for 5 minutes with the supernatant transferred to a clean 15 mL tube. Binding Matrix Solution was vortexed before taking 1 mL which was added to the 15 mL tubes. Using hands, the content of the 15 mL tubes were inverted for 2 minutes to allow binding of DNA and left on a rack for 3 minutes to allow settling of silica matrix before carefully discarding 500 µL of supernatant. The content of the 15 mL tube was resuspended by pipetting it up and down after which 700 µL of the tube content was transferred to a spin filter and centrifuged at 14,000 x g for 1 min. This step was repeated for the remaining mixture in the 15 mL tube while emptying the catch tubes every time. Thereafter, 500 µL of SEWS-M was added (mixed with 100 ml of 100% ethanol) and centrifuged at 14,000 x g for 1 minute. The content of the catch tubes was emptied and replaced while the Spin Filter centrifuged again to "dry" the matrix of residual wash solution with the catch tube discarded and replaced with a new one. After air drying the Spin filter for 5 mins at room temp, the binding matrix was carefully resuspended with 100 µL DES (DNase/Pyrogen-Free Water). The spin filters were placed into a new 1.5 mL microcentrifuge tube and centrifuged at 14,000 x rpm for 1minute to bring eluted DNA into the new tubes and stored at -20°C until use.

4.1 Polymerase chain reaction (PCR)

PCR was conducted for the isolates following the protocol of Mateos *et al*. (2006). Two sets of PCR were run using two sets of primers; 10F (10F AGTTTGATCATGGCTCAGATTG) and 1507R (TACCTTGTTACGACTTCACCCCAG) as well as 27F (GAGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The PCRs were run with (illustra TM) puRe Taq Ready-To-Go PCR beads. Each PCR bead when reconstituted to 25 µl contains; 200 µM of each deoxy-ribonucleotide triphosphate (dNTPs), recombinant puRe Taq DNA polymerase, reaction buffer, stabilizers, magnesium chloride and potassium chloride. The concentrations of each primer used were 1 µl per 100 ml reaction. The final PCR protocols are as follows:

For 10F and 1507R primer set: Initial denaturing temperature 94°C for 2 minutes, denaturing temperature 94°C for 30 seconds, annealing temperature 59°C for 30 seconds, Extension temperature 72°C for 1 minute and 30 seconds, and Final step 72°C for 5 minutes. A total of 36 cycles were run. For 27F and 1492R primer set: Initial denaturing temperature 94°C for 2 minutes, denaturing temperature 94°C for 30 seconds, Annealing temperature 53°C for 30 seconds, Extension temperature 72°C for 1 minute and 30 seconds, and Final step 72°C for 5 minutes. A total of 36 cycles were run.

An optimized PCR was run for isolates which produced nonspecific products in addition to that expected. This occurred only with the 10F and 1507R primer s*et.* The protocol for the optimized PCR was: Initial denaturing temperature 94°C for 2 minutes, denaturing temperature 94°C for 30 seconds, Annealing temperature 61°C for 30 seconds, extension temperature 72°C for 1 minute and 30 seconds, and Final step 72°C for 5 minutes. A total of 36 cycles were run.

4.2 Agarose gel electrophoresis

Five 5 µl of each PCR products were electrophoresed on 1.5% (w/v) agarose gel at 110 volts for 45 minutes. The gels were thereafter stained in ethidium bromide for 10 minutes and de-stained in clean water for 20 minutes after which they were viewed under ultraviolet (UV) light with the aid of a transilluminator.

4.3 Sequencing of PCR amplicons

The remaining amplicons from PCR (after gel electrophoresis) were transferred from PCR tubes into appropriately labelled sterile eppendorf tubes. The tubes were frozen at -20°C in freezer boxes prior to transportation for sequencing. The amplicons were purified using a DNA purification kit and thereafter sequenced with the Sanger method by Inqaba biotech company using the 27F 16S rRNA primer.

4.4 Sequence analysis and identification of isolates

The obtained 16S rRNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) (Zhang et al., 2000) version 2.6.0 + tool of the NCBI (National Center for Biotechnology Information) database. The database employed was 16S rRNA sequences while the programme selection was set to optimize for highly similar sequences (megaBLAST). The BLAST was run for each 16S rRNA sequence. An optimized BLAST, in which low quality nucleotide bases which usually occurs at the beginning and end of sequence are deleted, was run in each case to further confirm the identity of each sequence.

Strains with sequences that were 97% identical to the database match were presumed to belong to the same species as the matching organism in the database and a 95% cut-off was used to define genera (Kim *et al*., 2014).

4.5 Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignments and phylogenetic tree construction were performed using the MEGA tool (http://www.megasoftware.net/) following directions and recommendations by Hall, (2013). The alignment method chosen was MUSCLE (Edgar, 2004). The default settings for DNA were accepted and subsequently used to perform the alignment. The Parameters were: Gap penalties- Gap open – 400, Gap extend – 0; Memory/Iterations- Max memory in MB 1685, Max Iterations 8; More advanced options- Clustering method (Iteration 1,2) UPGMB; Clustering method (other iterations) UPGMB; Min Diag Length (lambda) 24. All sequences were trimmed to the same size prior to the analysis, the alignment was then executed and the session saved. The data obtained from the alignment was exported and saved as a mega file after which it was used to build a phylogenetic tree from the MEGA platform. The tree building was then executed using the phylogeny menu from which 'Construct/Test Maximum Likelihood Tree' was chosen.

4.6 Statistical analysis

All the assays were done in triplicates. Analysis of variance and Descriptive statistics were employed to examine the data gotten from the study using Statistical Package for the Social Sciences ® version 21, PAST version 2.17c and Microsoft Excel version 2010 (Ogbeibu, 2014).

5.0 Results

Determination of the pH of the fermented food samples and isolation of LAB

Table 1 shows the pH values of the fermented food samples, the highest pH value was recorded for *gari* with a pH of 4.2 while *kunu* had the lowest pH with a value of 3.1. *Fufu* had pH values of 3.8 while *nono* and *ogi* had a pH of 3.6. The colony count of the LAB isolated from the fermented food samples is shown in Table 2. The total viable count varied for the different food samples. The highest LAB count was observed in *kunu* samples followed by *ogi, fufu* and *nono* while *gari* had the lowest count. The *kunu* samples had an average count of $5.60 \pm 0.10 \times 10^6$ cfu/ml and $3.38 \pm 0.21 \times 10^7$ cfu/ml for the third and fourth dilution respectively. *Ogi* had a count of $5.55 \pm 0.18 \times 10^6$ cfu/g and $3.43 \pm 0.11 \times 16^6$ cfu/g for the second and third diutions. The first two dilution for *kunu* had growth that were too numerous to count. For *fufu*, the total plate counts for the second and third dilutions were $3.19 \pm 0.16 \times 10^5$ cfu/g and $7.07 \pm 0.75 \times 16^5$ cfu/g. No growth was observed in the third and fourth dilutions of *nono* and *gari*, however the first and second dilution had an average count of $2.23 \pm 0.6 \times 10^3$ cfu/ml and $6.50 \pm 2.5 \times 10^3$ cfu/ml for *nono* and $1.30 \pm 0.6 \times 10^3$ cfu/ml and $2.30 \pm 1.86 \times 10^3$ cfu/g for *gari.*

Table 1
pH values of the fermented food samples

Food Sample	pH
Kunu	3.1
Fufu	3.8
Ogi	3.6
Nono	3.6
Gari	4.2

Table 2
Total aerobic bacterial counts (cfu/ml) of fermented food samples

Food Sample		Mean microbial count(cfu/ml)		
Kunu	TNC	TNC	$5.60 \pm 0.10 \times 10^6$	$3.38 \pm 0.21 \times 10^7$
Fufu	TNC	$3.19 \pm 0.16 \times 10^5$	$7.07 \pm 0.75 \times 16^5$	-
Ogi	TNC	$5.55 \pm 0.18 \times 10^6$	$3.43 \pm 0.11 \times 16^6$	-
Nono	$2.23 \pm 0.6 \times 10^3$	$6.50 \pm 2.5 \times 10^3$	-	-
Gari	$1.30 \pm 0.6 \times 10^3$	$2.30 \pm 1.86 \times 10^3$	-	-
Key: TNC – Too Numerous to Count, - No growth				

Antimicrobial activity of bacteriocin-producing LAB

A total of 194 colonies that were gram-positive and catalase negative were subjected to overlayed spot agar test to assay for bacteriocin producing properties using *Staph. aureus* and *E. coli* as test organisms for the primary bacteriocin screening process. The number of colonies picked from *kunu* was 52 with 17 inhibiting the growth of *Staph. aureus* and 24 colonies inhibiting the growth of *E. coli.* A total of 32 colonies were picked from *ogi* with 8 inhibiting the growth of *Staph. aureus* and 14 inhibiting the growth of *E. coli. Nono* had 61 colonies, 5 inhibited the growth of *Staph. aureus* and 12 inhibited the growth of *E. coli.* Out of the 51 colonies picked from *fufu*, *Staph. aureus* was sensitive to 30 colonies while *E. coli* was sensitive to 20 colonies. A total of 17 colonies were picked from *gari* sample with 10 colonies showing zone of inhibition in overlayed spot agar containing *Staph. aureus* and 7 colonies showing zone of inhibition in overlayed spot agar containing *E. coli.* This is shown in Table 3.

Table 3
Inhibitory activity of selected lactic acid bacteria strains against bacterial indicator strains after eliminating effect of organic acid and H_2O_2 by using agar well diffusion method

Test Isolate	Sensitivity				
	Staphylococcus aureus	*Escherichia coli*	*Leuconostoc mesenteroides*	*Salmonella typhimurium*	*Bacillus cereus*
$K_2$45	-	-	-	-	-
$K_2$5	++	++	++	++	++
$K_1$7	++	++	+	+	++
$K_1$20	++	++	++	+	++
$K_1$35	++	++	++	++	++
N4	++	++	++	++	++
$K_2$46	-	-	-	-	-
$K_2$28	-	-	-	-	-
D	++	++	++	+	++
E	-	-	-	-	-
F	-	-	-	-	-
N5	++	-	-	-	-
G5	++	-	-	-	-
F4	-	-	-	-	-
K10	++	++	++	++	++
K11	++	++	++	++	++
N14	++	++	++	++	++
*++ >15 mm zone of inhibition					
+ < 15 mm zone of inhibition					
- Not sensitive					

On neutralization and treatment of the CFS with catalase to exclude the effect of lactic acid and hydrogen peroxide, only 9 isolates could inhibit the growth of *E. coli* ATCC 25922 and *Staph. aureus* ATCC 25923. Four of these isolates, which were identified as *Lactobacillus fermentum* strain NBRC15885, *Lactobacillus fermentum* strain CIP102980, *Lactobacillus plantarum* strain JCM1149 and *Lactobacillus*

natensis strain LP33, were also able to inhibit the growth of pathogenic strains of *B. subtilis, Klebsiella pneumonia and S. typhimurium* which were isolated from minced pie (Fig. 1 and Plate 1).

Confirmation of the bacteriocinogenic nature of the inhibitory substance

The 9 isolates that displayed notable inhibitory effect against the growth of *E. coli* ATCC 25922 and *Staph. aureus* ATCC 25923 were further tested against *Leuconostoc mesenteriodes*, *Salmonella typhimurium and Bacillus cereus.* They exhibited varied level of inhibition with $K_2$5,$K_1$25, K10 and K11 from *kunu* and samples N4 and N14 from *nono* displaying inhibition zone greater than 15 mm. Zone of inhibition greater than 15 mm was only observed against *Bacillus cereus* for sample $K_1$7 while sample D inhibited the growth of *Leuconostoc mesenteriodes* and *Bacillus cereus* to a level greater than 15 mm (Table 3).

Optimization assay for bacteriocin production

The selected pure LAB culture showed optimal growth in MRS broth with an optimal bactericidal protein production observed at pH 5.0 and 2.5% NaCl when cultures were incubated at 40°C (Figs. 2, 3 and 4).

Antibiotic sensitivity pattern of test food-borne pathogens

B. subtilis was resistant to 40% of the antibiotics and sensitive to 30% of the antibiotics. The remaining 30% exhibited intermediate zone of inhibition. *S. aureus* exhibited a higher level of resistance of 50% to the antibiotics, 30% susceptibility and intermediate inhibition of 30%. Among the Gram negative organisms, *K. pnuemoniae* had the highest resistance of 70% followed by *E. coli* (60%) and then *S. typhi* (40%). Susceptibility of 30% was observed for *S.typhi* while *K.pnuemoniae* and *E.coli* had susceptibility of 20% each.

Protein estimation and Screening for bacteriocin activity

Protein estimation in the dialysate obtained by ammonium sulphate precipitation and dialysis is represented in Fig. 5. When agar well diffusion assay was employed to qualitatively evaluate the fractionate containing the purified bacteriocin, four of the fractionates inhibited the growth of the test organisms.

Molecular phylogeny

The resulted 16 rRNA sequences were aligned with available, almost complete sequence of strains of Lactobacilli family. Then, corresponding sequences of representative *Lactobacillus* spp., in each case, the reference sequences were retrieved from the Gene Bank Databases. The phylogenetic tree is presented in Fig. 6. The phylogenetic data described were obtained by using MEGA4 package using neighbour-joining, minimum evolution, maximum parsimony and bootstrapping methods. The evolutionary history was inferred using the UPGMA method (Tamura et al., 2007).

Characterization of bacteriocin

SDS-PAGE is the ideal electrophoretic system for the resolution of proteins smaller than 30 kDa. With the aid of this approach, we observed that the molecular weights of the bacteriocins produced by these four LAB strains ranged from 4.5–6 kDa. Upendra et al. (2016) reported that SDS-PAGE molecular weight result of less than 14 kDa can serve as confirmation for the presence of bacteriocins (Fig. 7).

6.0 Discussion

Consumption of fermented food has over time become a part of the cultural and traditional norm among the indigenous communities in Nigeria. Different zones in the country have peculiar favourites that have evolved over centuries, depending on the customs, tradition and religion of the people. Worthy of note is that these fermented foods are rich in LAB which possess probiotic properties. Interestingly, local non-alcoholic beverages like *nono* and *kunu* are preferred to carbonated drinks by consumers, however, the frequency of consumption appeared to be low (Dada and Awotunde, 2017).

The present investigation highlights the isolation, characterization and identification of LAB from *fufu, kunu, nono* and *gari*. The total LAB count was in the following order: *kunu* > *fufu* > *nono* > *gari*. The lowest count was observed for *gari* due to the low water activity and the production process; this is similar to the findings of Ayodeji et al. (2017). The pH of all the food samples were low, this is in line with the findings of Imade et al. (2013) that reported reduction in pH as a result of fermentation. The organisms isolated from these fermented food samples are *Lactobacillus fermentum* strain NBRC15885 and *Lactobacillus fermentum* strain CIP102980 from *kunu*, *Lactobacillus plantarum* strain JCM1149 from *nono* and *Lactobacillus natensis* strain LP33 from *ogi.*

CFS containing crude bacteriocin were obtained from the isolated LAB. This CFS were tested for antimicrobial susceptibility to a spectrum of foodborne antibiotic resistant Gram-positive and Gram-negative bacteria commonly known to be associated with various clinical manifestations by agar well diffusion method. The highest inhibitory activity was demonstrated by bacteriocins produced by *Lactobacillus natensis* strain LP33 against *Klebsiella pneumonia.* Also worthy of note is the inhibitory effect of the bacteriocins produced by *Lactobacillus fermentum* strain NBRC15885, *Lactobacillus fermentum* strain CIP102980 and *Lactobacillus plantarum* strain JCM1149. The CFSfrom the broth culture of these strains was able to inhibit the growth of *B. subtilis, Klebsiella pneumonia, S. typhimurium, S. aureus* and *E. coli*. The inhibitory activity demonstrated by crude bacteriocin against these organisms is a strong pointer of the presence of active bacteriocin in the test supernatant. Similar results have been observed in experiments related to inhibitory effect of bacteriocin produced by other *Lactobacillus* species (Voidarou et al., 2020). Jena et al., (2013) reported that bacteriocin PJ4, produced by *Lactobacillus helveticus*, was active against some Gram-positive and Gram-negative pathogens such as *Enterococcus faecalis*, *S. aureus P. aeruginosa* and *E. coli.* Furthermore, bacteriocin produced by *Lactococcus lactis* has been reported to inhibit the growth of methicillin-resistant *Staphylococcus aureus* (Simons et al., 2020). It was observed that fermented foods are rich in LAB that can produce bacteriocin

with creditable inhibitory ability. This is in concordance with the findings of Upendra et al. (2016) on production of bacteriocin (Nisin) from lactic acid bacteria isolated from selected fermented food sources, such as Curd, Mayonnaise and Jelly, in India.

Production of bacteriocin and optimal cell growth are complimentary to each other (Oshoma et al., 2020). Similarly, Ashokkumar et al. (2011) reported that bacteriocin production is greatly influenced by the pH, temperature and nutrient levels of the culture environment. In this study, optimal growth and bacteriocin production was observed at pH 5.0 and 2.5% NaCl concentration when cultures were incubated at 40°C. This is similar to the findings of the studies conducted by Yang et al. (2018).

The biochemical and phylogenetic analyses of the characterized LAB revealed that all the bacteriocinogenic LAB belong to the genera *Lactobacillus*. The LAB species identified in this study were common inhabitants of a variety of fermented food products. This finding was consistent with the results of other studies, which disclosed that *Lactococcus* spp. are predominant in fermented food samples (Sharma et al., 2020). In a similar study, Vantsawa et al., 2017 evaluate the lactic acid bacteria with probiotic potential from fermented cow milk (nono) in Unguwa Rimi, Kaduna State, Nigeria. They obtained 6 pure colonies which all turned out to be *Lacobacillus* strains on characterization using morphological, biochemical and carbohydrate fermentation tests.

Bacteriocins can be used as antimicrobial agents either as powdered food ingredients, purified-or partially purified-peptides or through bacteriocinogenic LAB cultures. Combined application of different LAB-bacteriocins may effectively reduce possible development of resistant bacterial populations and improve the safety/quality and shelf-life of food products. Further research is required to gain insights into the molecular mechanisms involved in bacteriocin production, immunity and mode of action.

7.0 Conclusion

The results show that 9 LAB strains out of 194 isolates showed bacteriocin activity. The bacteriocins produced from 4 out of the 9 LAB were able to inhibit the growth of pathogenic strains of *B. subtilis, Klebsiella pneumonia and S. typhimurium* which were isolated from minced pie. The 4 isolates were identified as *Lactobacillus fermentum* strain NBRC15885, *Lactobacillus fermentum* strain CIP102980, *Lactobacillus plantarum* strain JCM1149 and *Lactobacillus natensis* strain LP33. This research has demonstrated that bacteriocin-producing LAB with good primary probiotic properties can be isolated from *fufu*, *nono, ogi* and *kunu*.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

EEI performed the research, analysed and interpreted. SEO critically revised the manuscript, helped structure and edit it, and approved its final version for publication.

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Tables

Table 1: pH values of the fermented food samples

Food Sample	pH
Kunu	3.1
Fufu	3.8
Ogi	3.6
Nono	3.6
Gari	4.2

Table 2: Total aerobic bacterial counts (cfu/ml) of fermented food samples

Food Sample		Mean microbial count(cfu/ml)		
Kunu	TNC	TNC	$5.60 \pm 0.10 \times 10^6$	$3.38 \pm 0.21 \times 10^7$
Fufu	TNC	$3.19 \pm 0.16 \times 10^5$	$7.07 \pm 0.75 \times 16^5$	-
Ogi	TNC	$5.55 \pm 0.18 \times 10^6$	$3.43 \pm 0.11 \times 16^6$	-
Nono	$2.23 \pm 0.6 \times 10^3$	$6.50 \pm 2.5 \times 10^3$	-	-
Gari	$1.30 \pm 0.6 \times 10^3$	$2.30 \pm 1.86 \times 10^3$	-	-

Key: TNC – Too Numerous to Count, - No growth

Table 3: Inhibitory activity of selected lactic acid bacteria strains against bacterial indicator strains after eliminating effect of organic acid and H_2O_2 by using agar well diffusion method

Test Isolate	Sensitivity				
	Staphylococcus aureus	*Escherichia coli*	*Leuconostoc mesenteroides*	*Salmonella typhimurium*	*Bacillus cereus*
$K_2$45	-	-	-	-	-
$K_2$5	++	++	++	++	++
$K_1$7	++	++	+	+	++
$K_1$20	++	++	++	+	++
$K_1$35	++	++	++	++	++
N4	++	++	++	++	++
$K_2$46	-	-	-	-	-
$K_2$28	-	-	-	-	-
D	++	++	++	+	++
E	-	-	-	-	-
F	-	-	-	-	-
N5	++	-	-	-	-
G5	++	-	-	-	-
F4	-	-	-	-	-
K10	++	++	++	++	++
K11	++	++	++	++	++
N14	++	++	++	++	++

*++ >15 mm zone of inhibition

+ < 15 mm zone of inhibition

- Not sensitive

Figures

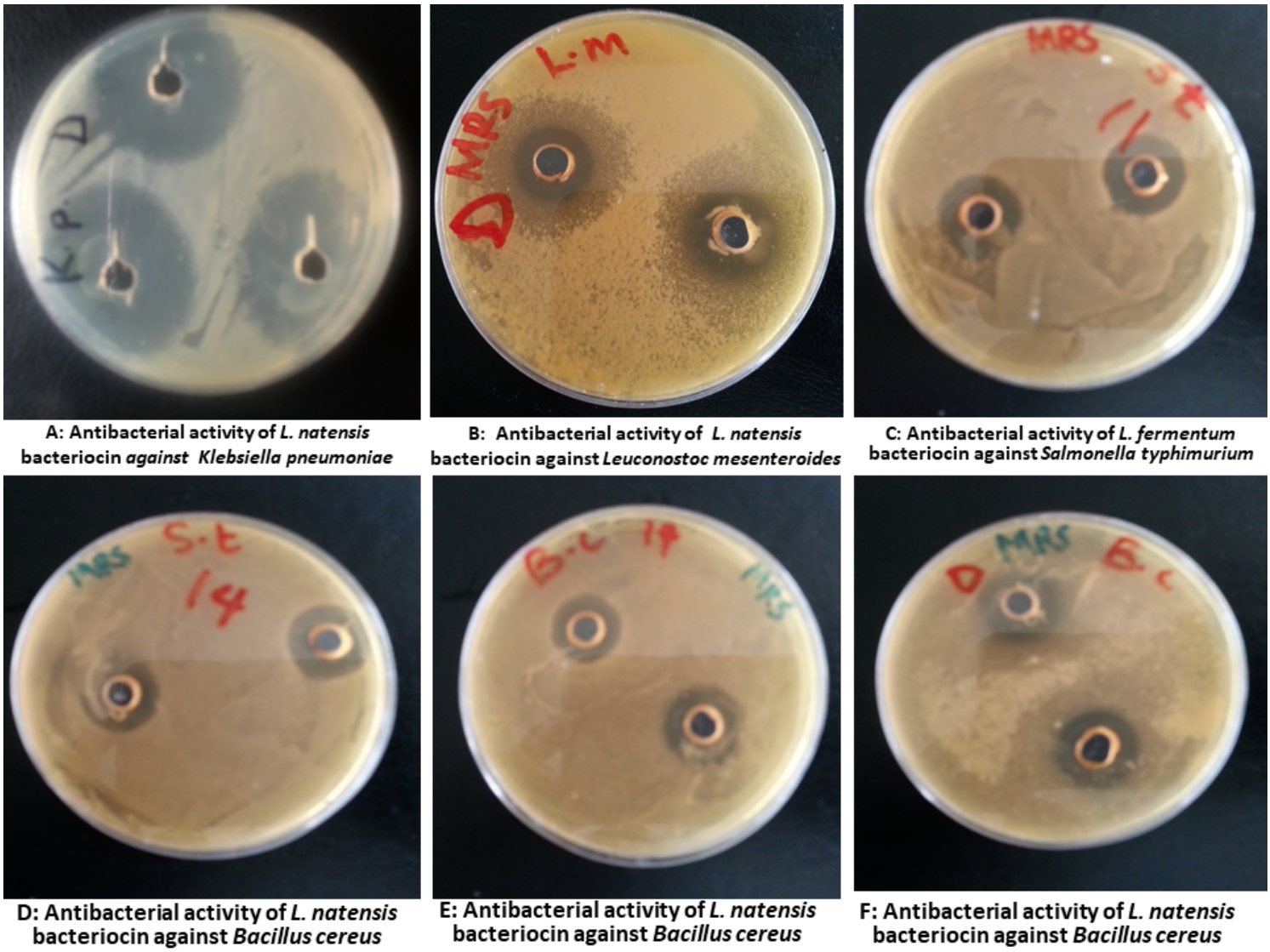

Figure 1

Antimicrobial activity of selected lactic acid bacteria bacteriocin against food associated antibiotic resistant bacteria

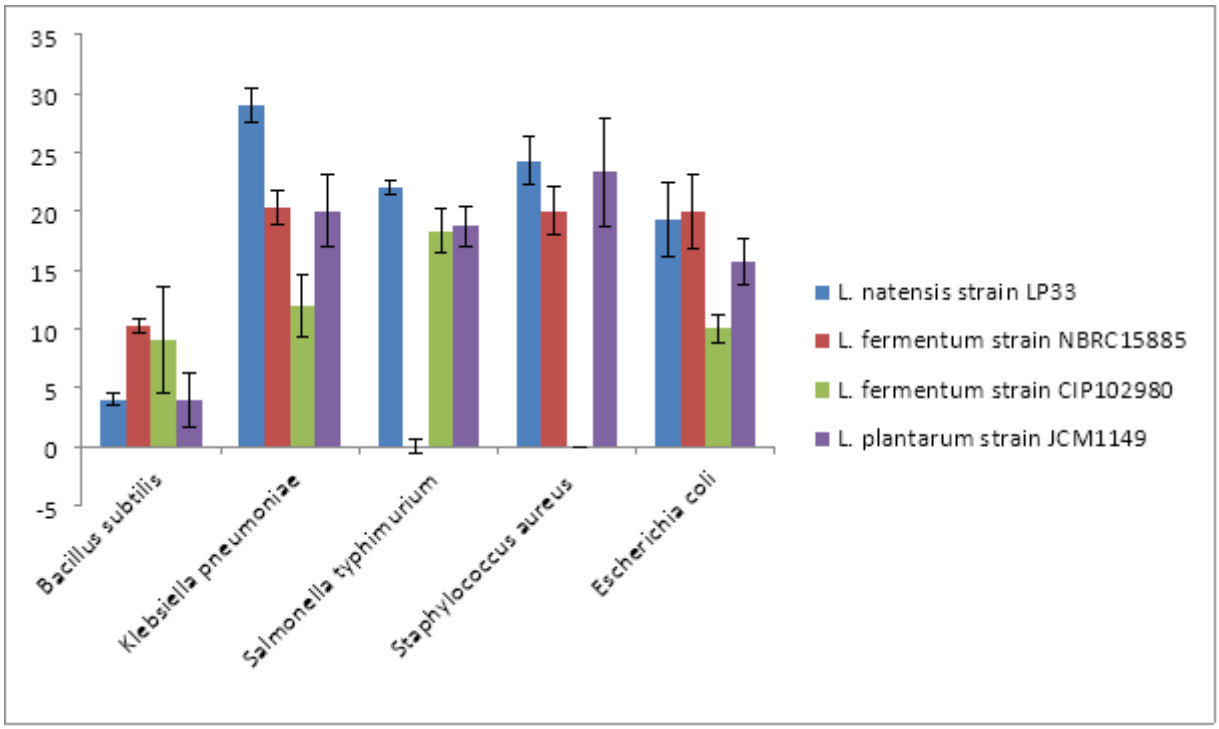

Figure 2

Antibacterial susceptibility of foodborne pathogens to LAB bacteriocin Values are the mean of three experiments and error bars represent standard deviation

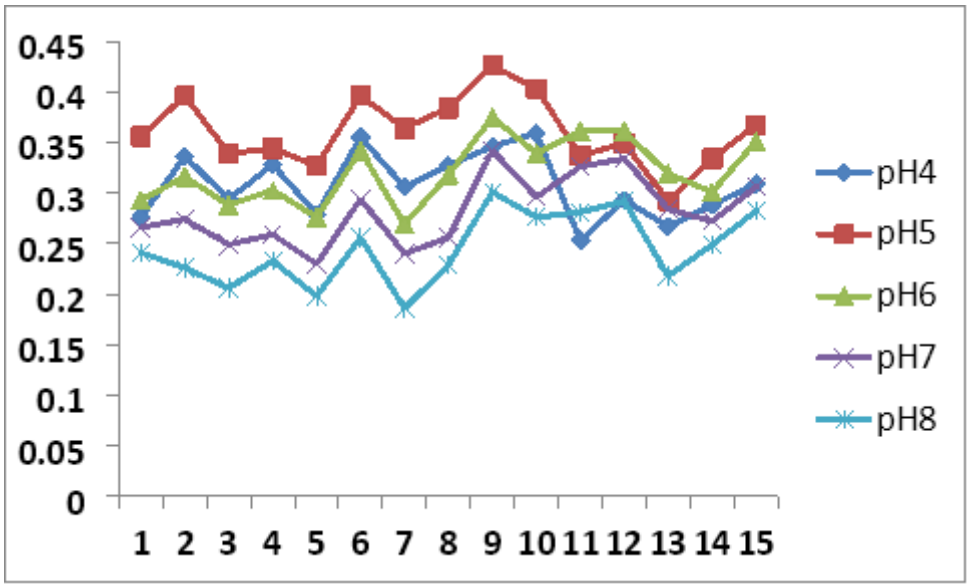

Figure 3

Optimization assay for pH (Absorbance at 620nm wavelength)

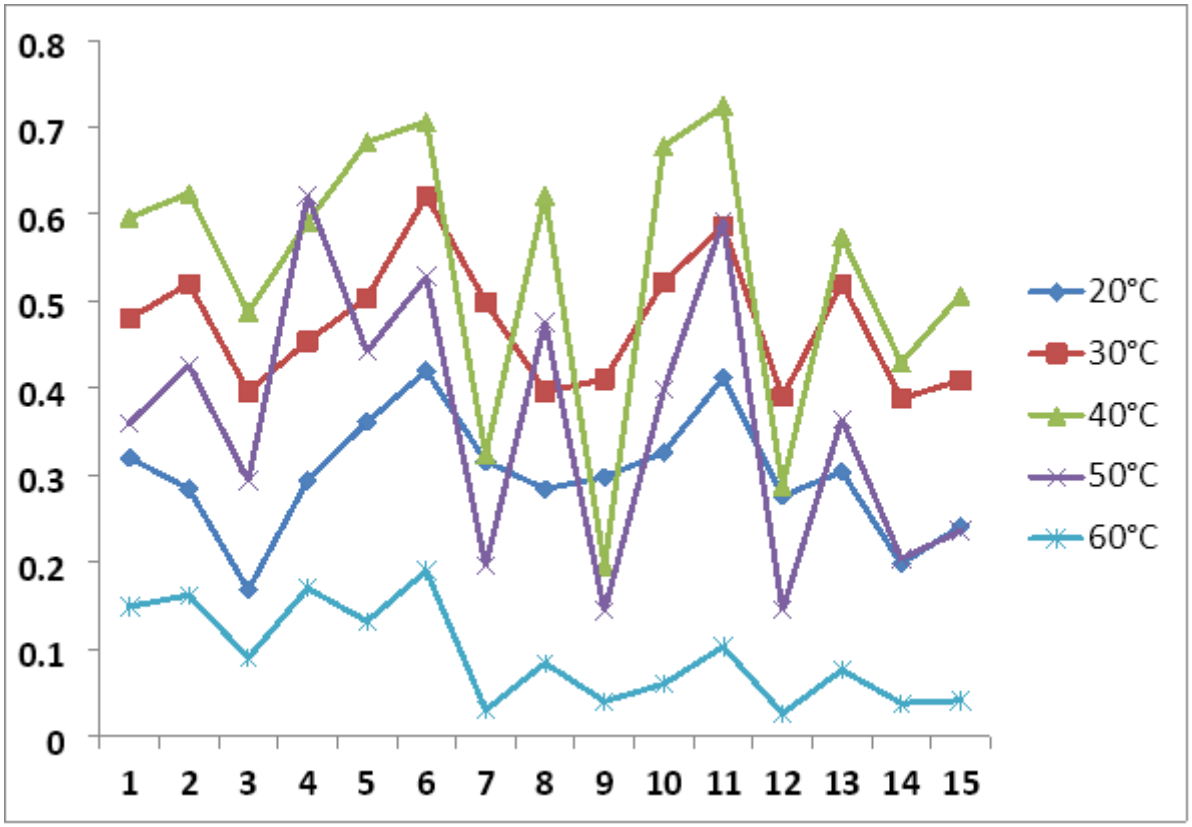

Figure 4

Optimization assay for temperature (Absorbance at 620nm wavelength)

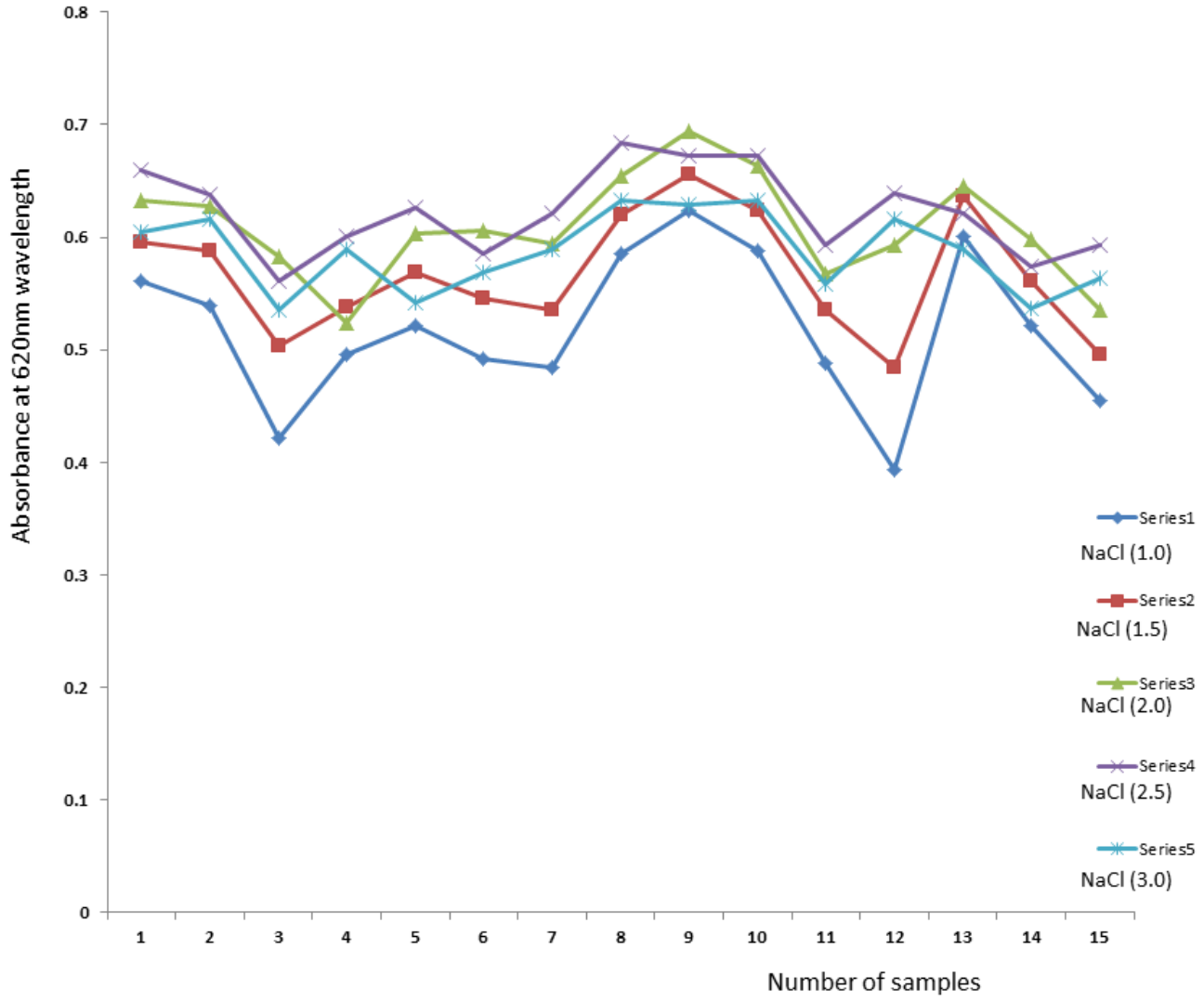

Figure 5

Optimization assay for Sodium chloride (Absorbance at 620nm wavelength)

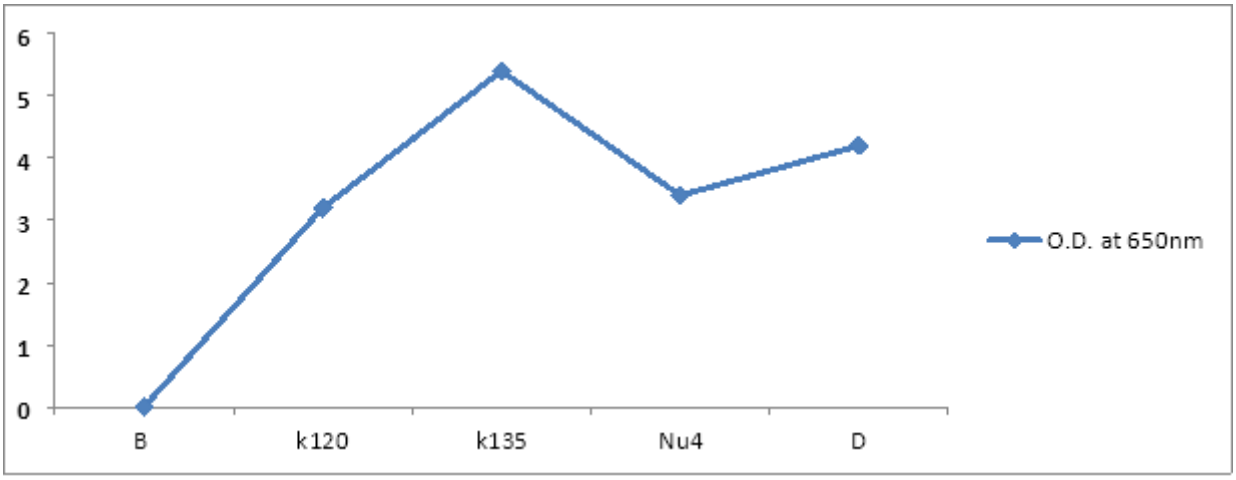

Figure 6

Protein estimation in dialysate using lowry's method

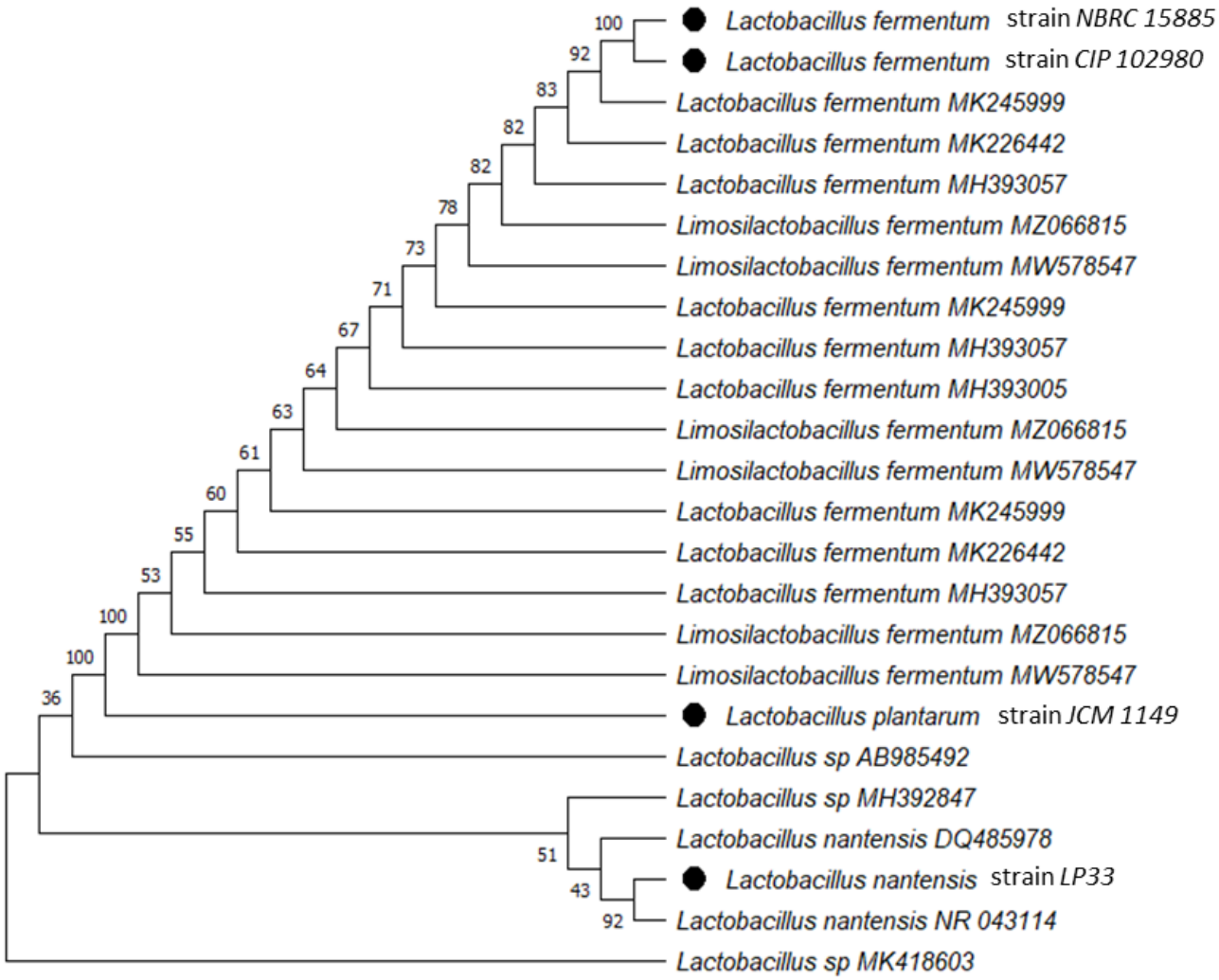

Figure 7

Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates with the sequences retrieved from NCBI (National Center for Biotechnology Information).

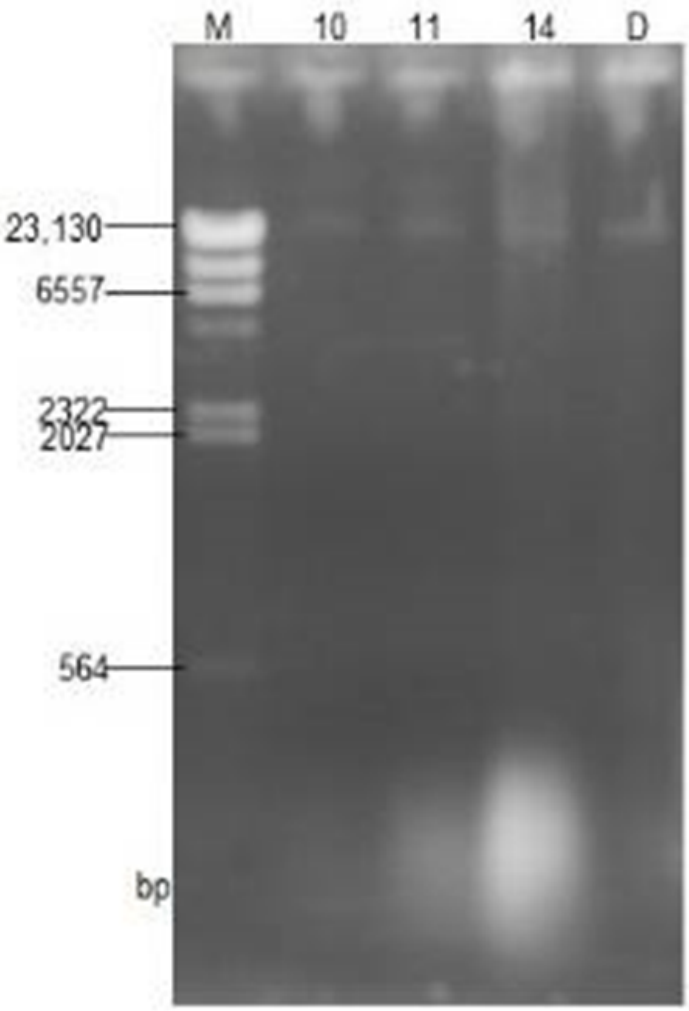

Figure 8

Molecular weight bands of different purified bacteriocins on SDS PAGE gel