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Lactic acid bacterial bacteriocins and their bioactive properties against food-associated antibiotic-resistant bacteria

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Abstract

Purpose: Incidence of foodborne 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 fewer 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. Cell-free supernatants were prepared from 18-24 h LAB culture grown on MRS broth. Effect of organic acid was eliminated by adjusting the pH of the supernatants to 7.0 with 1M NaOH while the effect of hydrogen peroxide was eliminated by treating with Catalase enzyme. 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 against foodborne antibiotic-resistant bacteria.

Result: A total of 162 isolates were obtained from the food samples. The antimicrobial sensitivity test yielded positive results for 45 LAB isolates against *Staphylococcus aureus* ATCC 25923 while 52 LAB isolates inhibited *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 against antibiotic-resistant foodborne pathogens using the cell-free supernatant (CFS) obtained from *Lactobacillus fermentum* strain NBRC15885 (*Limosilactobacillus fermentum*), *Lactobacillus fermentum* strain CIP102980 (*Limosilactobacillus fermentum*), *Lactobacillus plantarum* strain JCM1149 (*Lactiplantibacillus garii*), and *Lactobacillus natensis* strain LP33 (*Companilactobacillus nantensis*). The foodborne pathogens exhibited a notable level of resistance to antibiotics, with *B. cereus* exhibiting a resistance profile of 40%, *S. aureus* (50%), *K. pnuemoniae* (70%), *E. coli* (60%), and *S. typhi* (40%). The (CFS) was able to inhibit the growth of *B. cereus*, *Klebsiella pneumonia*, *S. typhimurium*, *S. aureus*, and *E. coli*.

Conclusion: Therefore, it 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.

Keywords: Drug-resistant microorganisms, Fermented food, Inhibitory activity, Probiotics

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Introduction

One of the concerns in the food industry is the contamination of food by pathogens, which are a 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; Akindolire et al. 2015). The 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 a quest for alternative means to surmount this impending 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. However, 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 fewer chemical additives and more 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 foodborne 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 are of interest since they are safe, active in a nanomolar range, heat stable, readily digested by gastric enzymes, and there are currently no reports of pathogenic bacteria developing antimicrobial resistance to them. Effective application in food preservation has been reported and to date no toxic effect has been attributed to their usage. Bacteriocins are multifunctional, ribosomally produced, proteinaceous substances produced by bacteria that are biologically active with antimicrobial action against other bacteria, principally closely related species. They are not usually called antibiotics to avoid confusion with therapeutic drugs, which can cause adverse responses in people with certain medical conditions (Deraz et al. 2005; Negash and Tsehai 2020). Bacteriocins are proteinaceous agents readily degraded by proteases in the human gastrointestinal tract, unlike most therapeutic antibiotics. Antibiotics are secondary metabolites, which are inhibitory agents in low concentrations, excluding inhibition induced by metabolic by-products like organic acids, ammonia, and hydrogen peroxide. Most, if not all, bacteria can create a diverse array of chemicals that may be inhibitory to themselves or to other bacteria during their growth in vitro (and presumably also in their natural habitats) (Ayivi et al. 2020). Bacteriocin synthesis could be advantageous for food and feed manufacturers since these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool when used in adequate levels (Bello et al. 2018). The fact that many bacteriocins have a narrow host range suggests that they are best effective against bacteria with similar nutritional demands for the same limited resources (Yang et al. 2018). Bacteriocins are frequently thought to be more natural because they are believed to have been present in many foods since antiquity. 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 (Negash and Tsehai 2020). Although antimicrobial peptides have a narrower inhibition spectrum than antibiotics, the bacteriocins produced by LAB have been reported to infiltrate Gram-negative bacteria's outer membrane and cause inactivation when combined with other antimicrobial environmental factors such as organic acid, low temperature, and detergents (Parada et al. 2007). Bacteriocins are named by the genus or species of the strain that produces them. *Lactobacillus plantarum* (plantaricin), *Lactococcus* spp. (lacticin, nisin), and *Carnobacterium* spp. (carnocin), *Enterococcus* spp. (enterocin) *Leuconostoc* spp. (leucocin), and *Pediococcus* spp. (pediocin) are only a few examples (Yusuf 2013). LAB are Gram-positive, non-spore-forming rods, non-aerobic but aero-tolerant cocci and cocco-bacilli that can ferment carbohydrates into energy and lactic acid (Jay 2000). Firmicutes is the phylum in which LAB is found. The different major genera of LAB include *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Weissella*, *Vagococcus*, and *Tetragenococcus*. Other genera include *Aerococcus*, *Propionibacterium*, *Microbacterium*, and *Bifidobacterium*. However, based on a recent study, reclassification of the genus Lactobacillus into 25 genera was proposed, these includes *Lactobacillus delbrueckii* group, Paralactobacillus, Acetilactobacillus, Agrilactobacillus, Amylolactobacillus, Apilactobacillus, *Bombilactobacillus*, *Companilactobacillus*, *Dellaglioa*, *Fructilactobacillus*, *Furfurilactobacillus*, *Holzapfelia*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lapidilactobacillus*, *Latilactobacillus*, *Lentilactobacillus*, *Levilactobacillus*, *Liquorilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Loigolactobacilus*, *Paucilactobacillus*, *Schleiferilactobacillus*, and *Secundilactobacillus* (Zheng et al. 2020). 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 (Gomez-Gallego et al., 2016; Bello et al. 2016). They are also readily available in local food condiments such as *fufu*, *gari*, *kunu*, *nono*, and *ogi* commonly consumed in Nigeria and some African countries (Nwaiwu et al. 2020). *Fufu* is a solid meal of soaked fermented cassava roots while *gari* is a granular starchy food made from the bitter variety of cassava root. Traditional *Ogi* preparation is done by soaking maize, millet, or sorghum in water for a day or two, followed by wet-milling, wet-sieving, and fermentation for 2-3 days. Similarly, Nono and kunu are drinks obtained from fermented cow milk and whole grains of millet, sorghum, or corn respectively (Egwim et al. 2013).

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 aimed to isolate and identify LAB and screen their bioactive properties against food-associated antibiotic-resistant bacteria.

Materials and methods

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 h under anaerobic conditions, discrete colonies that developed were counted and recorded (Putria et al. 2020; Vantsawa et al. 2017). 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 made using the standard morphological, physiological, biochemical, and molecular assays (Kim and Kim 2014; Islam et al. 2016).

Bacteriocin assay

The inhibitory activity of the selected LAB isolates 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) to get a concentration of 1.5×10^8 bacterial suspension per ml. This was then 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 cover the cultured agar plate surface entirely and left to solidify. After incubation at 32 °C for 24 h under anaerobic conditions, 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).

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

The strains of the selected LAB isolates, which showed the 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 producer strains' culture extract was 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 (Valgas et al. 2007). Nutrient agar was inoculated with 1 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).

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.

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

The 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 cultures 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 h of 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).

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).

Purification of bacteriocin

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

Molecular weight determination of purified bacteriocins

The molecular weight of purified bacteriocin was estimated using sodium dodecyl sulfate-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).

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 a minced pie. Nutrient agar was inoculated with 1 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 was read after 24 h of incubation at 35 °C and recorded (Balouiri et al. 2016).

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 foodborne 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 the 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 positive when the inhibition halo around the well was more than 2 mm (Amarantini et al. 2019).

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 shaken 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. Extract concentration was

Table 1 pH values and total viable LAB counts (cfu/ml) of fermented food samples

Food sample	pH	Mean LAB count ($\times 10^5$ cfu/g or cfu/ml)
Fufu	3.6^{ac}	3.19 ± 0.16^{a}
Gari	4.6^{b}	0.04 ± 0.02^{b}
Kunu	3.9^{ab}	55.90 ± 0.99^{c}
Nono	2.8^{c}	0.07 ± 0.02^{b}
Ogi	3.8^{ab}	55.50 ± 1.80^{c}

Key: Same alphabets in superscript across column indicate no significant difference ($p > 0.05$)

extrapolated from the standard curve prepared using bovine serum albumin (Lowry et al. 1951).

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.

The characterization at the molecular level was done using DNA extraction and PCR amplification with the aid of LAB specific primer, which included 10F (10F AGTTTGATCATGGCTCAGATTG) and 1507R (TACC TTGTTACGACTTCACCCCAG) as well as 27F (GAGAGTTTGATCCTGGCTCAG) and 1492R (GGTT ACCTTGTTACGACTT). DNA extraction was carried out on the cell suspensions with an OD_{600} of 2.0 in 100 μl (Prosekov et al. 2013).

Agarose gel electrophoresis

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

Sequencing of PCR amplicons

The remaining amplicons from PCR (after gel electrophoresis) were transferred from PCR tubes into appropriately labeled sterile Eppendorf tubes. The tubes were frozen at −20 °C in freezer boxes before transportation for sequencing. The amplicons were purified using a DNA purification kit and after that sequenced with the Sanger method by Inqaba Biotech Company using the 27F 16S rRNA primer.

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

Table 2 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	-	-	-	-	-
O_{1D}	++	++	++	+	++
O_{2E}	-	-	-	-	-
O_{3F}	-	-	-	-	-
N5	++	-	-	-	-
G5	++	-	-	-	-
F4	-	-	-	-	-
K10	++	++	++	++	++
K11	++	++	++	++	++
N14	++	++	++	++	++

K kunu, *O ogi*, *N* nono, *G gari*, *F* fufu
*++ >15 mm zone of inhibition
+ < 15 mm zone of inhibition
- Not sensitive

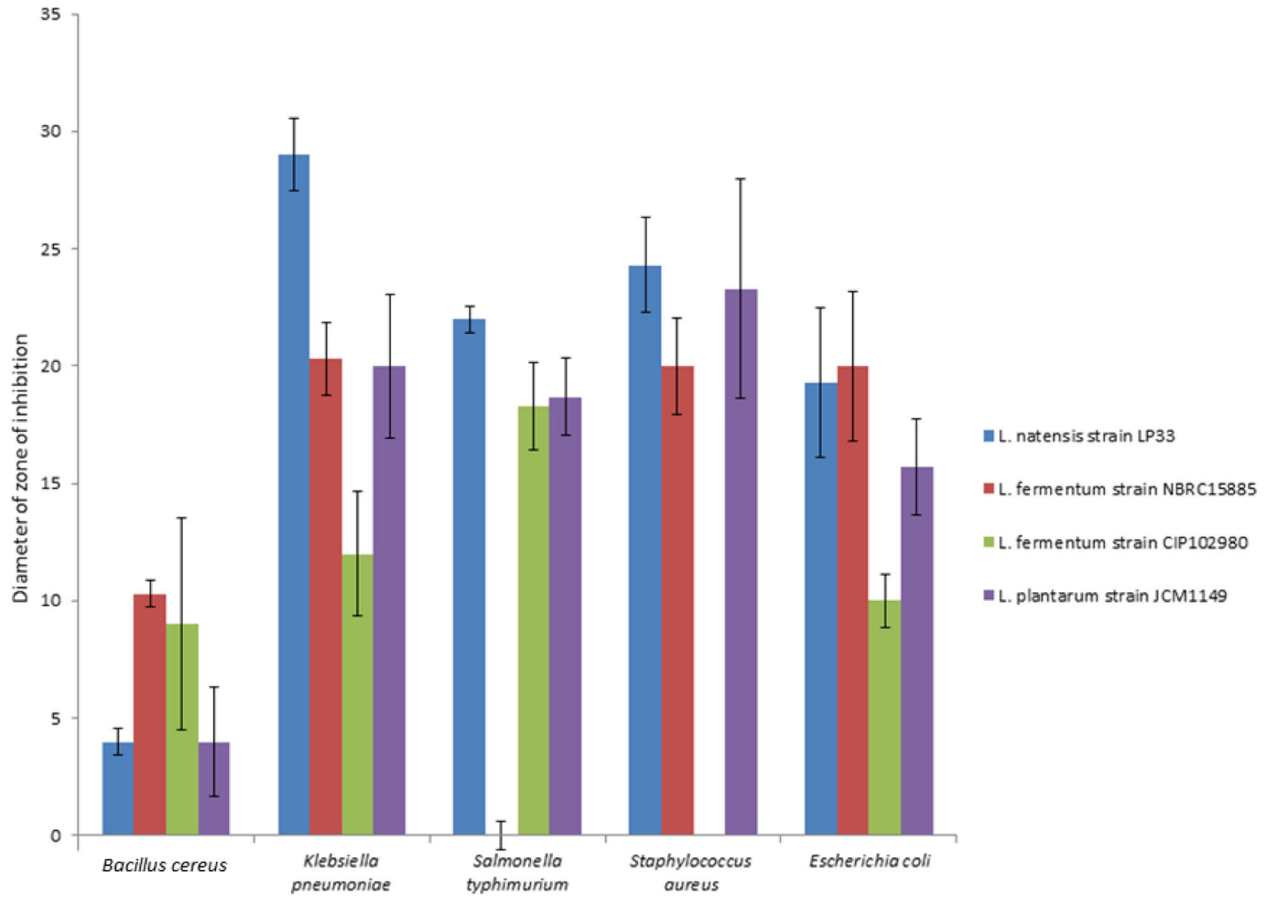

Fig. 1 Antibacterial susceptibility of foodborne pathogens to LAB bacteriocin. Values are the mean of three experiments and error bars represent standard deviation

(National Center for Biotechnology Information) database. The database employed was 16S rRNA sequences, while the program 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 occur at the beginning and end of sequence are deleted, was run in each case to confirm the identity of each sequence further. 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).

Multiple sequence alignment and phylogenetic analysis

The 16S rRNA gene sequence was compared with sequences from the NCBI database by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) as well as with sequences available at the EZTaxon database (www.ezbiocloud.net/) (Yoon et al. 2017). The 16S-based ID isolation of bacterial isolate using the 16S rRNA sequences option was selected. Comparison of the 16S rRNA gene sequence was done for each strain while the strains with the highest similarity were used for phylogenetic tree construction. The evolutionary history was inferred following the recommendation from the recent re-evaluation of the taxonomy of *Lactobacillaceae* and *Leuconostocaceae* (Zheng et al. 2020).

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 2005).

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

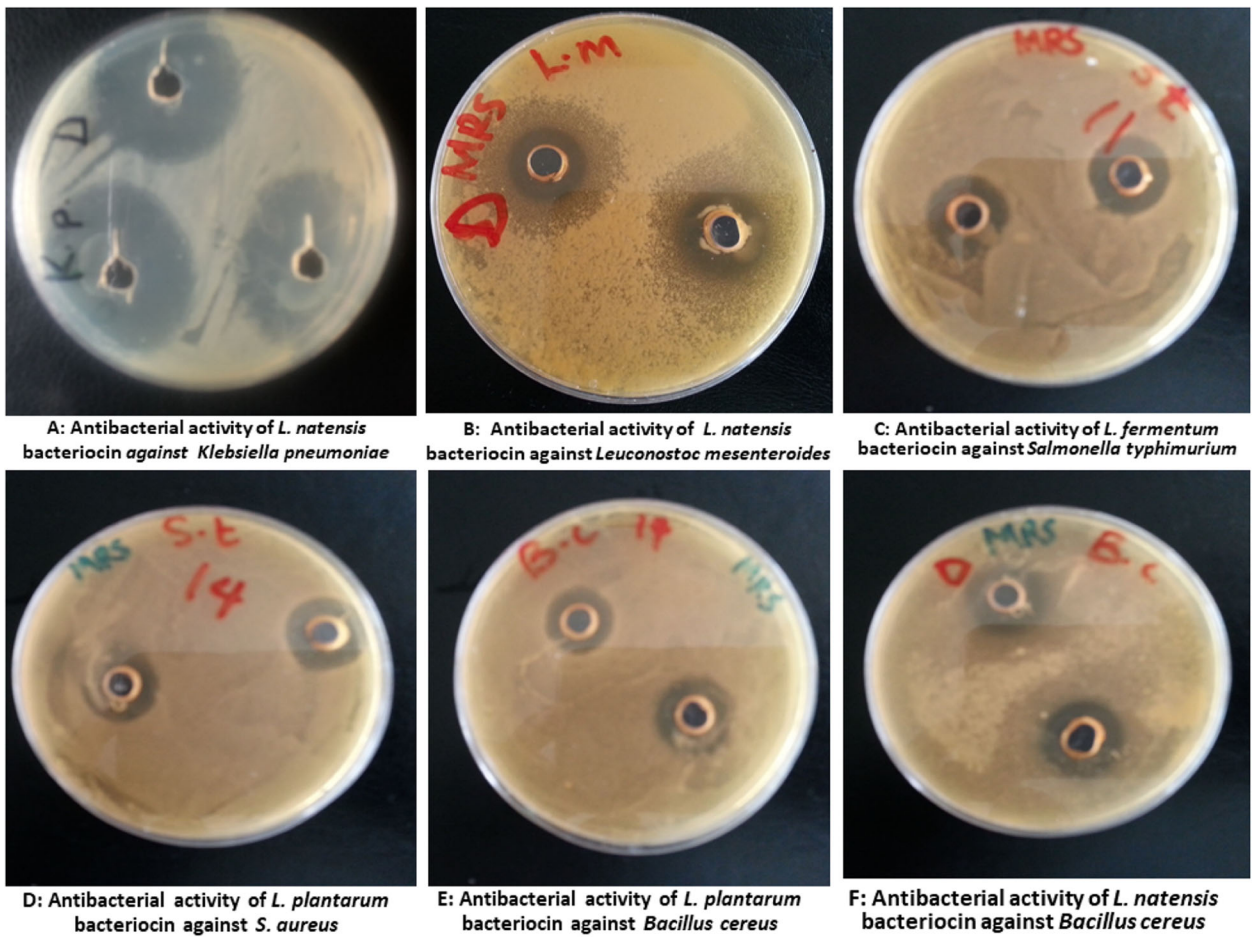

Plate 1 Antimicrobial activity of selected lactic acid bacteria bacteriocin against food associated antibiotic resistant bacteria

the fermented food samples is shown in Tables 1 and 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 dilutions. The first two dilutions for *kunu* had a growth that was 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 dilutions 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*.

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 *S. 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 *S. aureus* and 24 colonies were inhibiting the growth of *E. coli.* A total of 32 colonies were picked from *ogi*, with 8 inhibiting the growth of *S. aureus* and 14 inhibiting the growth of *E. coli. Nono* had 61 colonies, 5 inhibited the growth of *S. aureus* and 12 inhibited the growth of *E. coli.* Out of the 51 colonies picked from *fufu*, *S. 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 a zone of inhibition in overlayed spot agar containing *S. aureus* and 7 colonies showing zone of inhibition in overlayed spot agar containing *E. coli.* This is shown in Tables 1 and 2.

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 *S. aureus* ATCC 25923. Four of these isolates were also able to inhibit the growth of

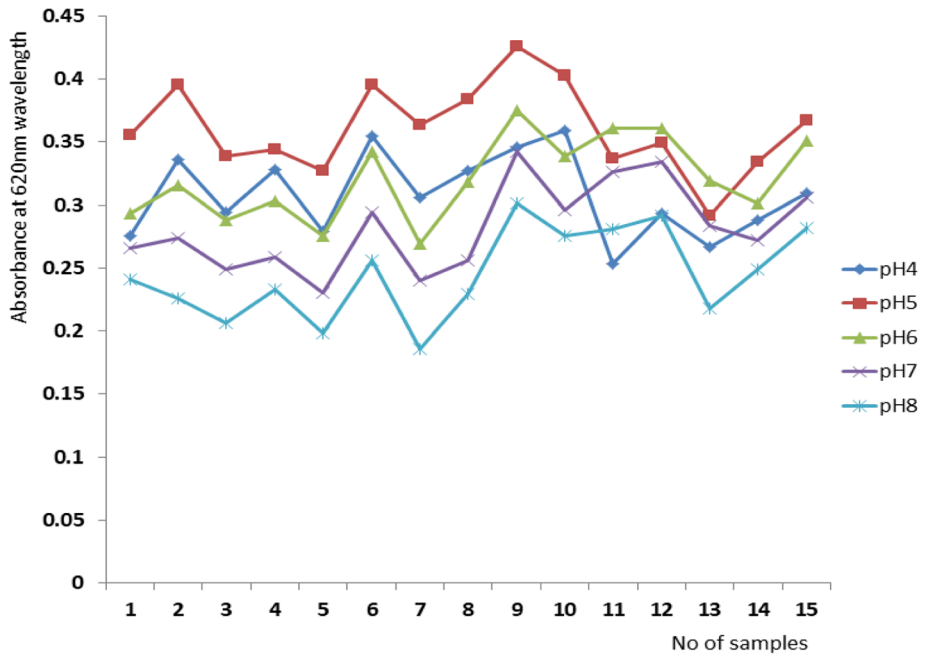

Fig. 2 Optimization assay for pH (absorbance at 620 nm wavelength)

pathogenic strains of *B. subtilis*, *Klebsiella pneumonia*, and *S. typhimurium* isolated from minced pie (Fig. 1 and Plate 1). These isolates were identified as *Lactobacillus fermentum* strain NBRC15885, now known as (*Limosilactobacillus fermentum* NBRC15885), *Lactobacillus fermentum* strain CIP102980 (*Limosilactobacillus fermentum* CIP102980), *Lactobacillus plantarum* strain JCM1149 (*Lactiplantibacillus garii* JCM1149), and *Lactobacillus natensis* strain LP33 (*Companilactobacillus nantensis* LP33).

Confirmation of the bacteriocinogenic nature of the inhibitory substance

The 9 isolates that displayed notable inhibitory effects against the growth of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were further tested against *Leuconostoc mesenteriodes*, *Salmonella typhimurium*, and *Bacillus cereus.* They exhibited a 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 more significant 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 (Tables 1 and 2).

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 foodborne pathogens

B. cereus was resistant to 40% of the antibiotics and sensitive to 30% of the antibiotics. The remaining 30% exhibited an 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 sulfate 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 fractionates inhibited the test organisms' growth.

Molecular phylogeny

The phylogenetic data were obtained using the EzTaxon database, which includes sequences from type strains and therefore ensured more precise identification of bacterial strains. The four isolates' 16S gene sequences were compared to 16S gene sequences deposited in the EZTaxon

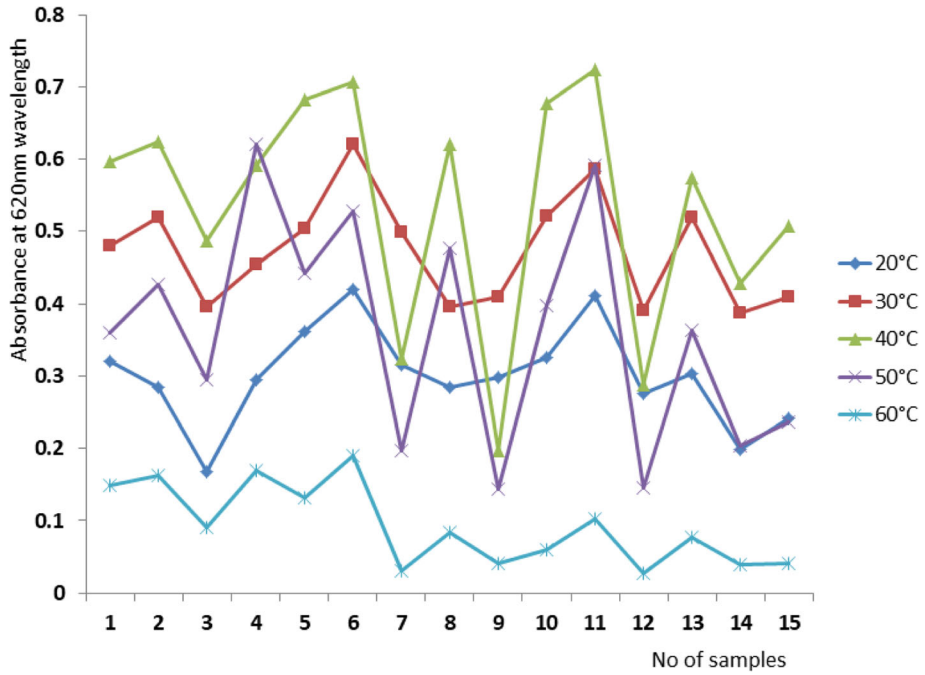

Fig. 3 Optimization assay for temperature (absorbance at 620 nm wavelength)

database (EZBioCloud.net). *Companilactobacillus nantensis* LP33, *Lactiplantibacillus garii* JCM 1149, *Limosilactobacillus fermentum* CIP 102980, and *Limosilactobacillus fermentum* NBRC 15885 were the isolates with the most similar 16S rRNA gene sequences. Figure 6 illustrates the evolutionary tree.

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 LAB strains were about 23 kDa. Vasilchenko et al. (2018) reported that

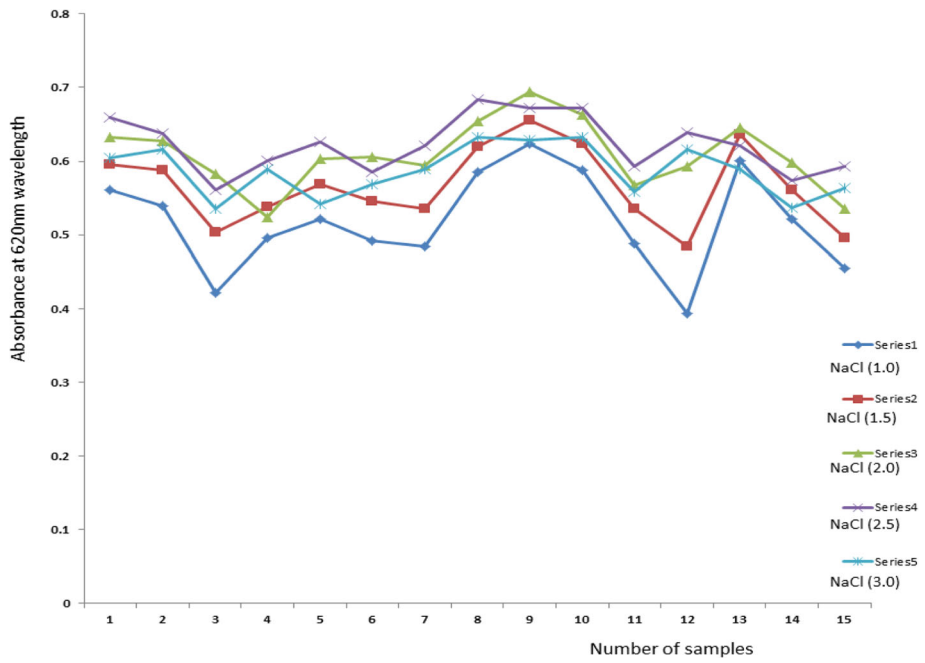

Fig. 4 Optimization assay for sodium chloride (absorbance at 620 nm wavelength)

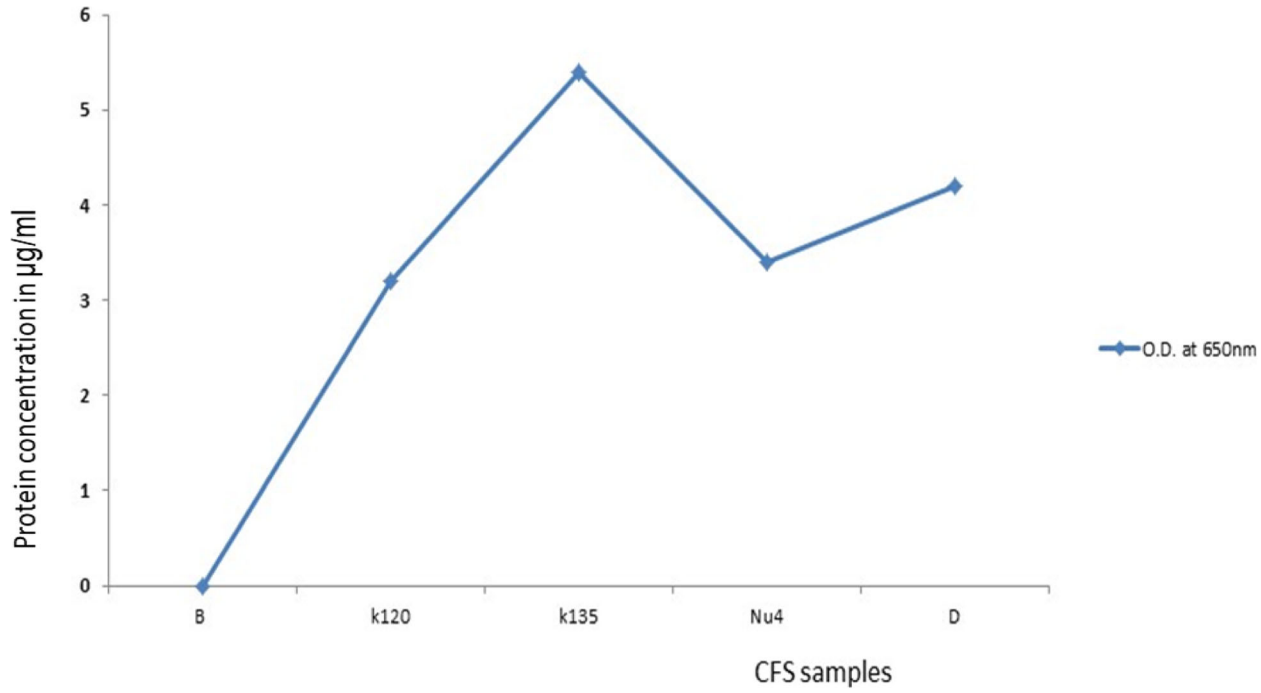

Fig. 5 Protein estimation in dialysate using Lowry's method

high SDS-PAGE molecular weight result indicates that they belong to class III in bacteriocin classification schemes (Fig. 7).

Discussion

Consumption of fermented food has become a part of the cultural and traditional norm among the indigenous communities in Nigeria. Different zones in the country have peculiar favorites 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 LAB's isolation, characterization, and identification 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). In addition, the pH of all the food samples was low; this is in line with the findings of Imade et al. (2013) that reported a reduction in pH as a result of fermentation. The organisms isolated from these fermented food samples are *Lactobacillus fermentum* strain NBRC15885 now known as (*Limosilactobacillus fermentum NBRC 15885*) and *Lactobacillus fermentum* strain CIP102980 (*Limosilactobacillus fermentum* CIP102980) from *kunu*, *Lactobacillus plantarum* strain JCM1149 (*Lactiplantibacillus garii* JCM1149) from *nono*, and *Lactobacillus nantensis* strain LP33 (*Companilactobacillus nantensis* LP33) from *ogi*. The genus *Lactobacillus* comprises species that are very important and beneficial to man. As at March 2020, about 261 species that are dissimilar at ecological, genotypic and phenotypic levels had been identified. This necessitated the re-evaluation of the taxonomy of *Lactobacillaceae* and *Leuconostocaceae* on the basis of whole-genome sequences. Based on the findings of the study, reclassification of the genus *Lactobacillus* into 25 genera was proposed; this is responsible for the change in the generic name from *Lactobacillus* to the respective new names represented above (Zheng et al. 2020).

CFS containing crude bacteriocin was obtained from the isolated LAB. This CFS was 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 pneumoniae*. 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 CFS from the broth culture

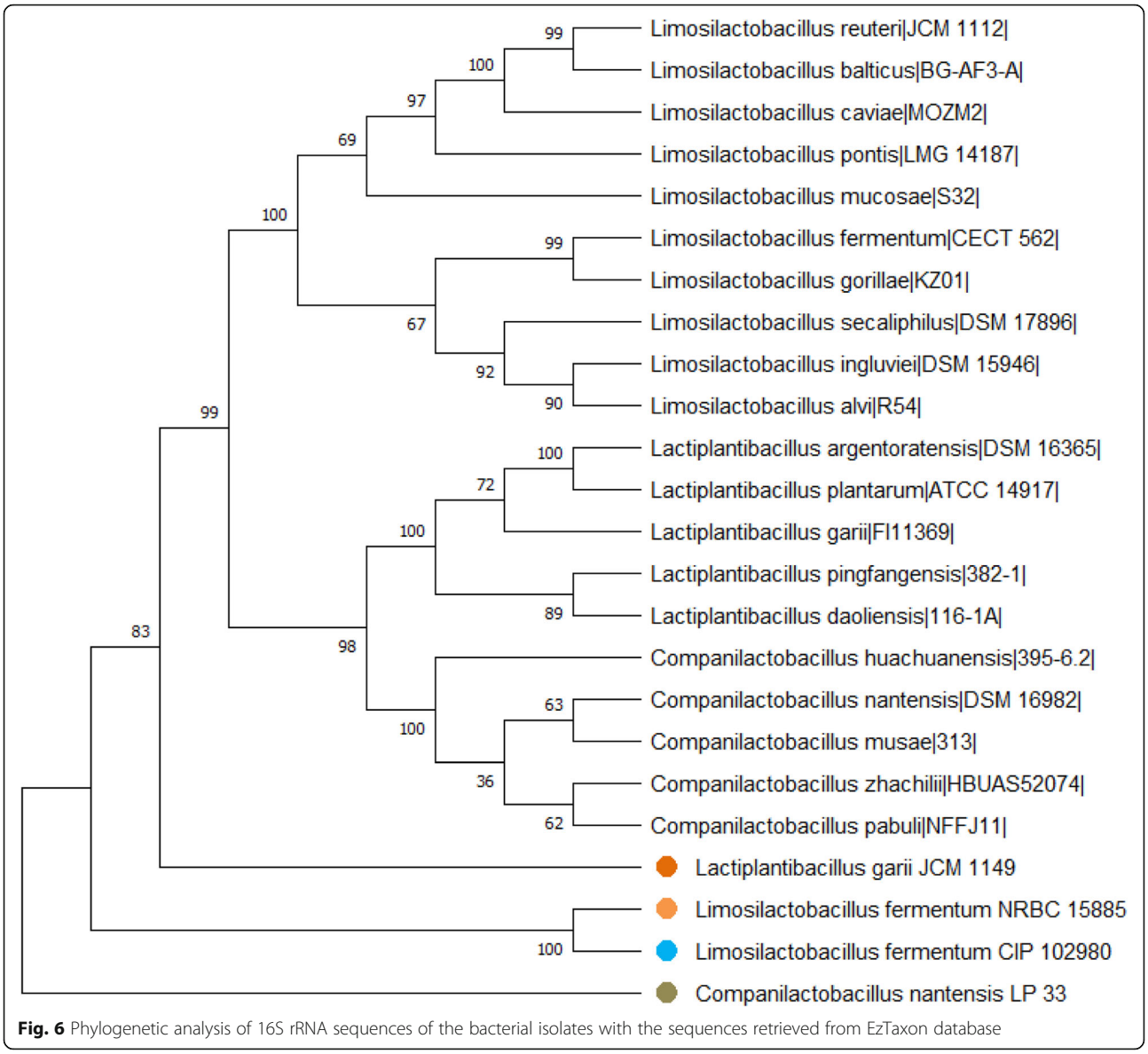

Fig. 6 Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates with the sequences retrieved from EzTaxon database

of these strains could inhibit the growth of *B. cereus*, *Klebsiella pneumonia*, *S. typhimurium*, *S. aureus*, and *E. coli*. The inhibitory activity demonstrated by crude bacteriocin against these organisms is a strong pointer to the presence of active bacteriocin in the test supernatant. Similar results have been observed in experiments related to the inhibitory effect of bacteriocin produced by other *Lactobacillus* species (Voidarou et al. 2020; Onipede 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 the production of bacteriocin (Nisin) from lactic acid bacteria isolated from selected fermented food sources, such as curd, mayonnaise, and jelly, in India. They obtained a crude extract of bacteriocin from the selected food samples which displayed antagonistic effect against *E. coli* and *Kleibshella* sp. using agar well diffusion assay.

Production of bacteriocin and optimal cell growth are complementary 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. This study observed optimal

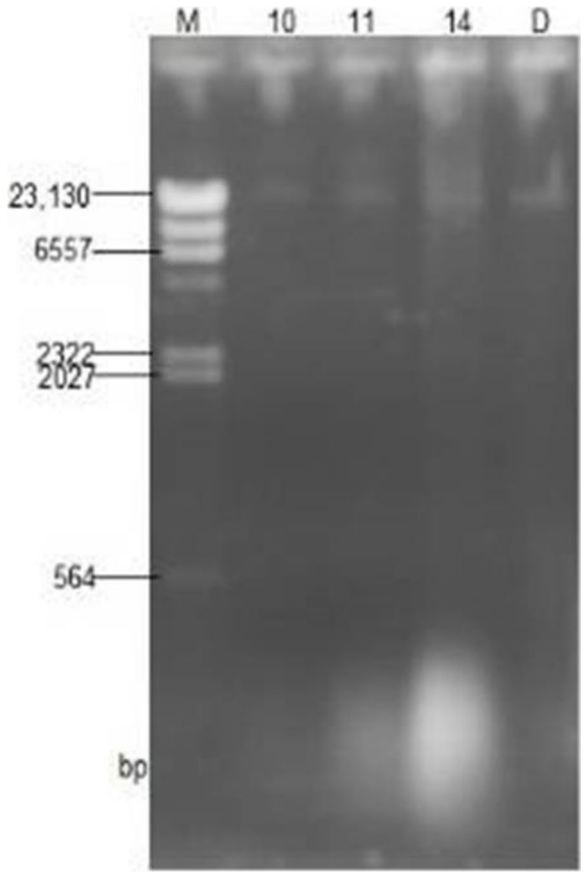

Fig. 7 Molecular weight bands of different purified bacteriocins on SDS PAGE gel

growth and bacteriocin production 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). However, Babalola (2007) observed optimum bacteriocin production by *Lactococcus lactis* at a pH value of 6.

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) evaluated 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 *Lactobacillus* 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. The combined application of different LAB-bacteriocins may effectively reduce the possible development of resistant bacterial populations and improve the safety/quality and shelf-life of food products. However, further research is required to gain insights into the molecular mechanisms involved in bacteriocin production, immunity, and mode of action.

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. cereus*, *Klebsiella pneumonia*, and *S. typhimurium* which were isolated from minced pie. The 4 isolates were identified as *Limosilactobacillus fermentum* NBRC15885, *Limosilactobacillus fermentum* CIP102980, *Companilactobacillus nantensis* LP33, and *Lactiplantibacillus garii* JCM1149. This research has demonstrated that bacteriocin-producing LAB with good primary probiotic properties can be isolated from *fufu*, *nono*, *ogi*, and *kunu*.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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