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Research Article

Molecular Characterization of Lactic Acid Bacteria Isolated from Food Samples with Potentials for Bacteriocin Production

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ABSTRACT

Lactic acid bacteria (LAB) are naturally found as part of fermented food products and other foodstuff. These organisms are used in the fermentation of vegetables to improve their nutritional value, acceptability, palatability, as well as microbiological quality and shelf life. Standard microbiological techniques were used to examine 85 different food products for the presence of LAB. The crowded plate approach was used to screen distinct isolates for bacteriocin producing capacity. PCR-based techniques (16S rRNA) were used to identify and characterize lactic acid bacteria associated with food products that have the potential to produce bacteriocins. A total of five hundred and sixty-one (561) lactic acid bacteria were isolated from the food samples of which only 8 (1.42 %) were positive for bacteriocin production. The best five isolates from the 14 with an inhibition zone of 20 mm and more were identified as Lactobacillus plantarum strain PMM09-2528L, Lactobacillus acidophilus strain NK-S10, Lactococcus lactis subsp. lactis strain IFM 63517, Leuconostoc mesenteroides strain M6 and Pediococcus cellicola strain PMM-25. These bacteriocinogenic LAB can be used in the formulation of starter cultures and for commercial use in the biopreservation of vegetables and other food products.

Keywords: *Bacteriocinogenic; Fermented products; Lactic acid bacteria; 16S rRNA*

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INTRODUCTION

Lactic acid bacteria (LAB) are common microorganisms found in foods and also constitute the natural flora in the intestinal microbiota of humans and most animals (Rojo-Bezares *et al*., 2006). According to John and Lennox (2018), bacteriocin-producing bacteria are found to be isolated from foods that normally contain LAB such as vegetables and dairy products. These food products are consumed for a long time (John and Lennox, 2018). Bacteriocins produced by LAB are defined as 'extracellularly produced primary or modified products of the bacterial ribosome, which are characterized by a narrow spectrum of bactericidal activity' (Caplice and Fitzgerald, 1999). In order to improve the safety and quality of fermented foods and other food products, bacteriocin-producing strains of LAB can be applied as part of or adjuncts to starter cultures. Recently, the use of PCR based techniques for the genomic identification and phylogenetic analysis of different microbial strains has been the most generally accepted method. PCR based techniques make use of pure PCR product of the 16S gene that was obtained and sequenced, to effectively utilized them for the identification and detection of various microorganisms in the soil, digestive tract, food products, and clinical samples (Barry *et al*., 1990). Bacteriocins production have been attributed to LAB where they have the potential to be applied in several industries including food and feed industry where they are used as a substitute for chemical preservatives (Gao *et al*., 2010; John and Lennox, 2018; Angmo *et al*., 2016). Bacteriocins produced by LAB have sparked particular interest as a possible safe alternative for food preservation. Over the years, the application of LAB as feed and food preservatives have been reported. The possibility of replacing chemical preservatives using bacteriocin-producing LAB to prevent bacterial deterioration and outgrowth of pathogenic bacterial in food products is highly possible (Daeschel, 1989).

LAB has been discovered to improve the nutritional value of fermented foods. As a result, there has been a surge in interest in LAB, which can be found in naturally fermented milk products (Li *et al*., 2016; Holzapfel, 2012). Although there has been a significant amount of research on commercially used LAB, the majority of these studies have been based on morphological, cultural, and phenotypic features (Feresu and Muzondo, 2010). Thus, there is paucity of information on the advanced techniques about the identity of biotechnologically important LAB such as those in other food products other than in fermented foods. (Abdelgadir *et*

al., 2011; Beukes *et al*., 2014; Lane, 2011; Obodai and Dodd, 2015; Saleh, 2013).

Nevertheless, conventional methods of identification of LAB have some certain drawbacks, ranging from time-consumption to potential inaccuracies in their profiling. Bearing in mind the biotechnological importance of these LAB, there is need to implore the molecular techniques which enable genetic identification of the microorganisms involved in these fermentation processes. With the recent development and advancement of PCR-based methods using random amplification of polymorphic DNA (RAPD), analysis of 16S rRNA gene homology, amplified and species-specific primers; there have been rapid improvement in the identification of different species of microorganisms. These methods have proved useful for the identification of various important species of LAB (Federici *et al*., 2014). This study was therefore undertaken to isolate, identify and characterize lactic acid bacteria found in different food products with the capability of producing bacteriocin through PCR-based molecular methods. This may help in the formulation of starter culture which can be used for the biological preservation of foods.

MATERIALS AND METHODS

Collection of food samples: Several food samples (85) were obtained at random from various sites in Calabar. The collected food samples were cucumber, ogi, meat, yoghurt, fufu and raw milk (nunu). The samples were collected in an aseptic manner and transported to the laboratory for microbial analysis.

Sample Analysis

Isolation of lactic acid bacteria: With modest modifications in sample size, quantity and type, the procedures of Downes and Ito (2001) and Von Schelhorn (1980) were utilized to isolate LAB. Each food sample was weighed precisely 100g and placed in a sterile blender that had earlier been sterilized with 70% alcohol and rinsed with deionized water. After that, twenty-five grams of the product were enriched in 225 mL of sterile peptone water in a sterile flask. After agitating the homogenates for 2 to 3 minutes, they were serially diluted using ten-fold dilutions of up to 10^{-4}. Precisely 0.1ml of the 10^{-4} dilution was pipette onto de Mann Rogosa Sharpe (MRS) agar plates in triplicate and incubated anaerobically as reported by John and Lennox, (2018).

Preparation of pure cultures: To obtain pure culture of LAB, discrete colonies were selected and repeatedly subcultured on MRS agar, as described by John and Lennox (2018).
Pure cultures were then kept for subsequent examination on nutrient agar slants covered with paraffin oil and stored at -4ºC.

Screening of lactic acid bacteria for bacteriocin production: The test organisms were evaluated against 45 distinct LAB colonies obtained from the food samples analysed (Willey *et al.,* 2009*). Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumoniae* were used as test organisms. Each of the test organism's 18-hour broth culture was disseminated in duplicate on nutrient agar Petri dishes. The agar disk diffusion method was used to inoculate the plates. The plates were then incubated for 24 to 48 hours at 28°C. Colonies with clean zones around them were selected. Potential bacteriocin generating isolates were chosen from LAB isolates with apparent zones of inhibition.

Secondary screening of the isolates for bacteriocin activity after elimination of other inhibitors: After eliminating organic acid and hydrogen peroxide, the selected LAB isolates that were positive for bacteriocin synthesis were evaluated for antimicrobial activity against target pathogens using an agar disk diffusion assay. The presence of other inhibitors such as lactic acid and hydrogen peroxide were eliminated using the following assays. According to Moreno *et al.,* (2006), the presence of lytic bacteriophages was eliminated using the reverse side approach, organic acids were removed using dialysis, and the effects of hydrogen peroxide were eliminated by adding catalase at a concentration of 1 mg/ml (John and Lennox, 2019).

The susceptibility test was conducted using Bauer *et al* (1996) disc diffusion method. About 3-5 colonies of the test organisms were picked and suspended in normal saline using a sterile inoculating loop, after which the inoculums were adjusted to a turbidity comparable to a 0.5 McFarland standard (about 1.5×10^8 CFU/ml). The suspension was then vortexed to ensure that it was completely mixed. A sterile swab stick was dip into the solution and excess liquid was removed by pushing the swab against the tube's side. The swab was then evenly inoculated onto a plate containing newly prepared Mueller Hinton Agar (MHA). The disc containing soaked filtered supernatant of lactic acid bacteria isolates was inserted with a sterile set of forceps and aggressively pressed down to produce a firm, levelled contact with the agar within 15 minutes of inoculating the MHA plate. The plate was inverted and incubated at 35°C for 16-18 hours after being inverted. After incubation, the clear zone around each disc was measured. All of the selected LAB bacterial isolates were subjected to this technique.

Molecular Identification: Five milliliters of the bacterial isolate's overnight broth culture in Luria Bertani (LB) broth were spun at 14000 rpm for three minutes. The cells were re-suspended in 500 liters of normal saline and heated for 20 minutes at 95 degrees Celsius. The heated bacterial suspension was spun for 3 minutes at 14000 rpm after cooling on ice. The DNA-containing supernatant was transferred to a 1.5 mL microcentrifuge tube and kept at -20ºC for use in subsequent analysis. The extracted genomic DNA was quantified using the Nano Drop 1000 spectrophotometer (Reyes-Escogido *et al.,* 2010). The 16S rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mix included: X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M, and the extracted DNA as template. The PCR

conditions were as follows: Initial denaturation, 95 ºC for 5 min; denaturation, 95 ºC for 30 s; annealing, 52 ºC for 30 s extension, 72 ºC for 30 s for 35 cycles and final extension, 72 ºC for 5 min. The product was resolved on a 1% agarose gel at 120 V for 15 min and visualized on a UV transilluminator.

The product of the PCR amplification was resolved on a 1% agarose gel at 120 V for 15 min and visualized on a UV transilluminator (Reyes-Escogido *et al.*, 2010). Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa (Reyes-Escogido *et al.*, 2010).

RESULTS

Results from Table 1 present the primary screening of isolates for bacteriocin production. The result showed that only 8 isolates (1.42%) of the 561 LAB colonies examined were positive for bacteriocin production, suggesting their capability to manufacture bacteriocin. This indicates bacteriocin production potential is not attributed to all lactic acid bacteria.

Table 2 shows the results of antimicrobial testing using the disc diffusion method on the isolates' broth cultures after additional inhibitors were removed. The Table shows five of the best isolates with inhibition zones of 20mm or more. The isolate F3 *(Lactobacillus acidophilus*) had the maximum inhibition zone of 42.5 mm against *S. aureus*, whereas *Pediococcus cellicola* (F2) had the lowest inhibition zone of 20 mm *against K. pneumoniae*. All of the test organisms were found to be inhibited by isolate F1 (*Lactobacillus plantarum*). However, isolate F3 (*Lactobacillus acidophilus*) was only effective against *S. aureus* and *S. pyogenes*, while *Leuconostoc mesenteroides* (B1) was effective against *S. typhi*, *K. pneumoniae*, and *P. aeruginosa*.

Table 1
Primary screening of isolates for bacteriocin production

S.N.	Food samples	No. of distinct colonies.	No. of bacteriocin-positive isolates.
1	Fufu	80	2
2	Yoghurt	185	1
3	Meat	44	0
4	Cucumber	65	3
5	Nunu	104	2
6	Ogi	83	0
	TOTAL	561	8

Table 2
Antibacterial activity of bacteriocin positive bacteria against test isolates using disc diffusion method

	Zone of clearance in mm					
Isolate code	*S. aureus*	*E. coli*	*S. typhi*	*K. pneumoniae*	*S. pyogenes*	*P. aeruginosa*
F3	42.5	-	-	-	42	-
B1	-	28	34.5	27.6	-	29.5
F1	35.5	38	36.5	31	36.5	32.6
F4	41	-	-	-	41.5	-
F2	-	27.5	34	20	-	29.5

Key: *- : No activity*

Table 3
Nanodrop quantified bacterial DNA

S/N	Isolate code	Quantity (ng/µl) of bacterial DNA
1	F3	12.7
2	B1	21.0
3	F1	8.2
4	F4	15.9
5	F2	5.6

The identification of the five best bacteriocin-positive LAB to determine the strain was carried out using molecular techniques. Table 3 below presents both the quantity and purity of genomic DNA that was extracted from the selected lactic acid bacterial isolates for genomic identification. The varying quantities of genomic DNA extracted from the selected isolates to be used for molecular analysis ranged from 5.6 ng/µl to 21.0 ng/µl.

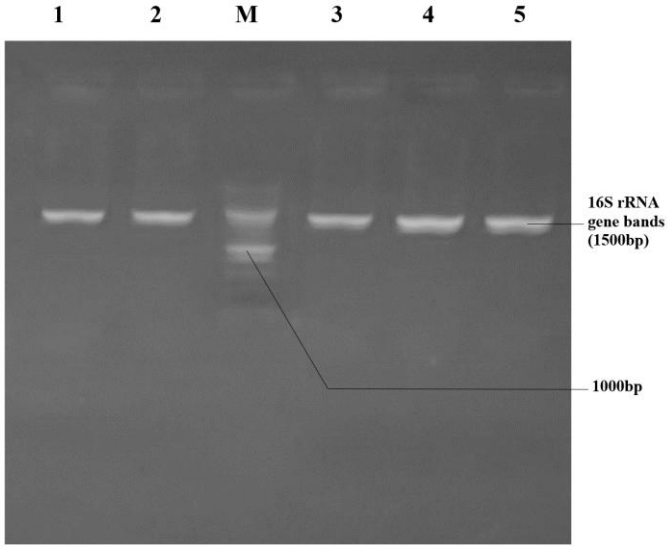

Figure 1:
Agarose gel electrophoresis of the 16S rRNA (1500bp) gene of the bacterial isolates. Lane M represents a 1000bp molecular ladder.

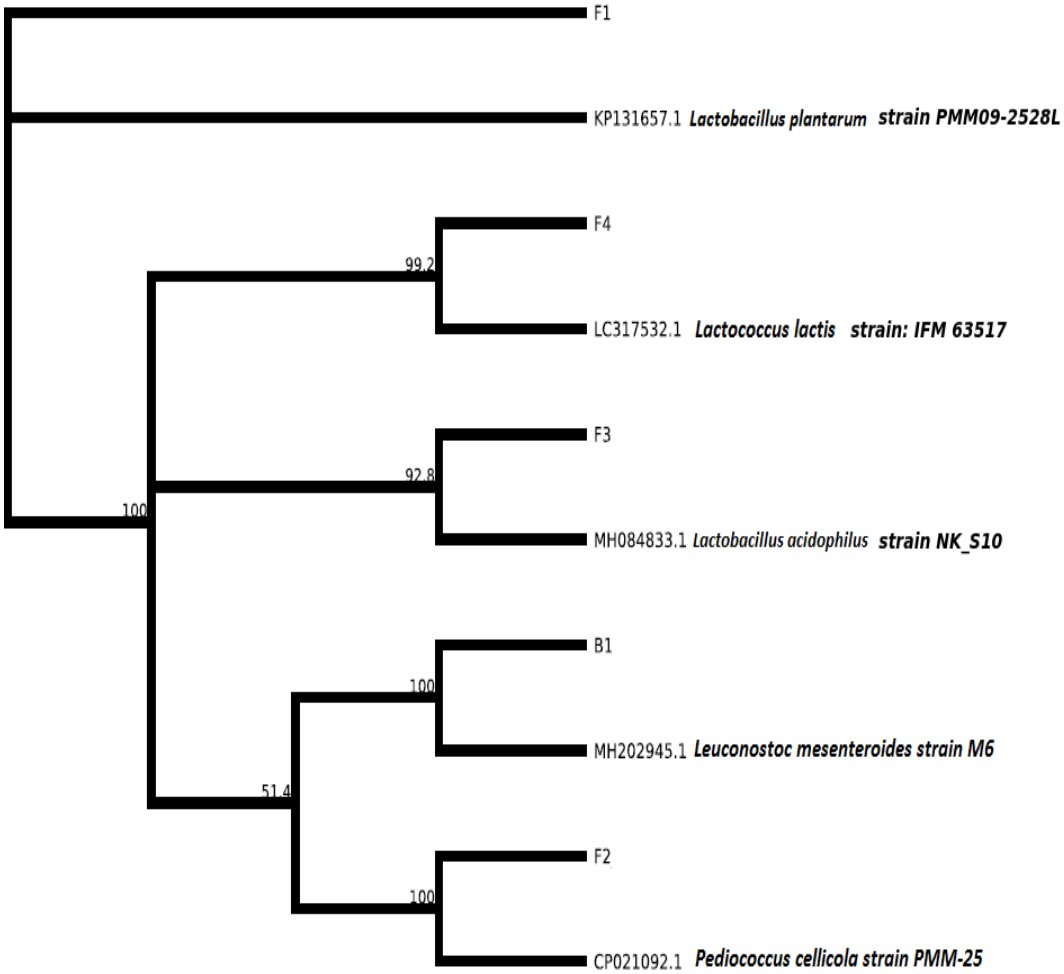

Figure 2:
Phylogenetic tree of the bacteriocin producing bacterial isolates

TABLE 4
Identification of the isolates by 16S rRNA genes amplification showing percentage similarities

S/N	Gene Bank isolates	Accession number	Bacteriocin positive isolates	% Similarity
1	*Lactobacillus acidophilus* strain NK-S10	MH084833.1	F3	96.8
2	*Lactobacillus plantarum* strain PMM09-2528L	KP 131657.1	F1	99.8
3	*Lactococcus lactis* subsp. *lactis* strain IFM 63517	LC317532.1	F4	97.5
4	*Leuconostoc mesenteroides* strain M6	MH202945.1	B1	95.6
5	*Pediococcus cellicola* strain: PMM-25	CP021092.1	F2	98.2

DISCUSSION

In the food samples studied, the actual prevalence of bacteriocin-positive bacteria is extremely lower than that reported by other researchers. Sharpe (2009) and Aruna and Mounica (2016) found 8.7% bacteriocin-producing bacteria among 92 isolates from fresh-cut vegetable items, but Sezer and Güven (2009) screened 12,700 LAB isolates from meat and milk products and discovered just 35 of them produced bacteriocin. Also, 3000 lactic acid bacterial isolates from foods traditionally fermented were screened by Salasiah *et al.* (2016) and discovered that only one colony produced an inhibitory zone. The variation can be linked to the type of food samples chosen as well as the production processes used.

When compared to the other test organisms, all of the bacteriocin-positive isolates had high antibacterial activity against *S. aureus* and *S. typhi*. The results showed that the cell free supernatant from the 5 best isolates had inhibitory effects on all the test organisms after the presence of organic acid and hydrogen peroxide were eliminated. As a result, it was discovered that removing acid from the cell-free supernatant had no influence on the inhibitory activity of the test isolates. As a result, the presence of bacteriocin is thought to be the reason of the inhibitory effect. This is in contrast to Yang *et*

al. (2012), who found that after pH neutralization and H_2O_2 removal, the CFS from the LAB isolates had inhibitory effects only on *L. innocua* but not on the other test bacteria, implying that the isolates' organic acids and/or H_2O_2 had strong antimicrobial effects on the bacteria tested.

The identification of the five best bacteriocin-positive LAB to determine the strain was carried out using molecular techniques. Table 3 below presents both the quantity and purity of genomic DNA that was extracted from the selected lactic acid bacterial isolates for genomic identification. The varying quantities of genomic DNA extracted from the selected isolates to be used for molecular analysis ranged from 5.6 ng/µl to 21.0 ng/µl. The PCR reaction of the extracted genomic DNA samples revealed positive amplification of the 16S rRNA gene in all the multidrug resistant isolates when ran on 1.5% agarose gel at 120V for 20 min. Clear bands observed in lane 1 to 5 shows positive amplification of the extracted DNA samples with Lane M representing a 1000 base pair molecular ladder.

In this study using the p-distance method, phylogenetic tree was drew to calculate the evolutionary distances between the identified isolates. DNA sequencing results were analyzed using NCBI BLAST and the similarity of the isolates to those already available in Gene Bank was determined. The 16S rRNA gene sequencing produced an exact match during the mega blast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolates showed a percentage similarity to other species ranging from 95%-100%. This also agrees with the work of Kawthar *et al.* (2018) and Negussie *et al.* (2016).

The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates as follows: B1 revealed a close relatedness to Leuconostoc mesenteroides strain M6 (gb: MH202945.1). F1 to Lactobacillus plantarum strain PMM09-2528L (gb: KP 131657.1). F2 to Pediococcus cellicola strain PMM-25 (gb: CP021092.1). F3 to Lactobacillus acidophilus strain NK-S10 (gb: MH084833.1) and F4 to Lactococcus lactis subsp. lactis strain IFM 63517 (gb: LC317532.1) as presented in Table 4. This result agreed with the work of Lili *et al.* (2019) where Pediococcus acidilactici was identified using a molecular technique with 99% similarity coverage to Pediococcus acidilactici strain DSM 20284. Lactobacillus plantarum, Pediococcus spp. and Lactococcus lactis are widely described as probiotics (Porto *et al*., 2017). Abbasiliasi *et al*. (2012) also found Pediococcus acidilactici and Lactobacillus acidophilus in fermented milk products. Lactobacillus acidophilus, Pediococcus cellicola and Lactococcus lactis are major LAB that have been employed as starter cultures in meat, vegetable, and dairy fermentation creating characteristic flavor changes, increasing hygiene, and extending the shelf life of these products (Mora *et al*., 2016; Porto *et al*., 2017).

In conclusion, the PCR based technique of the 16S rRNA gene sequence gave an approximately 1500 bp amplicon for all the selected bacteriocin positive LAB isolated from food samples. Phylogenetic analysis showed that all the identified isolates showed a percentage similarity to other species ranging from 95%-100%. The results presented in this work provide insight into the LAB population associated with food products consumed in Calabar, Nigeria. The LAB with bacteriocin-producing capability microflora associated with these food products belongs to *Lactobacillus, Leuconostoc, Lactococcus,* and *Pediococcus* group as in many other environmental samples. This technique of utilizing PCR and 16S sequencing to identify the LAB strains participating in a fermentation process, then confirming them with a species-specific PCR, would unambiguously permit their identification. Aside from identification, the ability of REP-PCR to reveal strain level variation could aid in the selection of bacterial strains with desirable characteristics for controlled fermentation and preservation, or for use in the manufacture of innovative functional foods.

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