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Original Research Article

Antimicrobial profiles of probiotic lactic acid bacteria isolated from Nigerian fermented spice condiment Okpeye (*Prosopis africana*)

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Abstract

Purpose: *To investigate the antimicrobial profile of lactic acid bacteria (LAB), also called friendly bacteria or probiotics, found in Nigerian fermented spice condiment Okpeye (Prosopis africana).*
Methods: *The LAB was isolated by enrichment and serial dilution. Biochemical characterization of isolates was done using standard microbiological protocols and determined using 16S rDNA gene sequencing. The survival of LAB in low pH 2.5 and 0.3 % bile salt media were evaluated. Using the agar well diffusion assay, the cell-free supernatant (CFS) standardized to 0.5 McFarland was evaluated for antibacterial, hydrogen peroxide, and bacteriocin activities to combat multi-drug resistant and foodborne pathogens (MDR-FBPs). Antibiotic susceptibility profile of isolates was profiled against seventeen antibiotics comprising twelve different classes using disk diffusion method.*
Results: *Isolates identified were Pediococuss pentosaceus, Lactobacillus brevis, and Lactobacillus plantarum, with strong antibacterial activities against S. aureus, moderate activities against L. monocytogenes, and weak activities against P. aeruginosa, B. cereus, and E. coli. The isolate retained viability greater than 80 % at pH 2.5 and 0.3 % w/v bile condition after 4 h of exposure. The isolates showed a wide range of activities against MDR-FBPs. Regarding antibacterial activity, all isolates were effective against S. aureus, moderate against L. monocytogenes ATCC 13932, and weak against P. aeruginosa ATCC 27883, Bacillus cereus and E. coli.*
Conclusion: *Okpeye is not just a spice; it contains high probiotics, which have antibacterial properties against foodborne and multidrug-resistant organisms, making it a beneficial addition to any diet.*

Keywords: *Bacteriocin, Probiotics, Hydrogen peroxide, Multidrug resistant, Foodborne pathogens*

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INTRODUCTION

Foods are not sterile products, and the food chain is typically the main means through which multi-drug resistant and foodborne pathogens (MDR-FBPs) spread. Probiotics have drawn more attention as a natural biocontrol method to lower foodborne infections throughout the food continuum due to consumers' growing concerns about antimicrobial resistance (AMR) and their

increased desire for a safe food supply [1]. Antibiotic resistance, healthy living, and food security are all at risk from MDR-FBP consortiums. Several foodborne pathogens infiltrate food and develop to degrade and sicken it. Seasonings and fermented food are still significant sources of probiotics or good bacteria. Okpeye (*P. africana*), a famous fermented spice condiment produced locally in the Southeastern geopolitical zone of Nigeria, is great with stews and soups. According to microbiological studies, few LAB genera are present in fermented *Okpeye* product [2]. Lactic acid bacteria are beneficial bacteria and rich sources of probiotics. Probiotics give fermented foods various organoleptic qualities and neutralize harmful or fatal food pathogens. Probiotics are also useful to preserve food since they produce several antimicrobial compounds, such as ethanol, diacetyl, hydrogen peroxide (H_2O_2), carbon dioxide, and bacteriocins [3]. Gram-positive and Gram-negative bacteria produce bacteriocins, ribosomally manufactured antimicrobial peptides. (AMPs) that either inhibit or kill other related or unrelated microbes [4]. This study aims to evaluate the antimicrobial profile of beneficial bacteria from *Okpeye*, an inexpensive and popular locally fermented seasoning ingredient in Nigeria, against prevalent MDR-FBPs.

EXPERIMENTAL

The LABs utilized in this investigation were isolated in previous study [5].

Biochemical characterization of LAB

Using standard microbiological protocols, the study examined the biochemical characteristics of selected LAB isolates, such as hemolysis, oxidase, sodium chloride, and sugar fermentation patterns. The LAB growth and bile salt hydrolase (BSH) activities were assessed at different temperatures [6].

Indicator pathogens

The stock culture contained MDR-FBPs, *B. cereus, E. coli, P. aeruginosa ATCC 27883, S. typhimurium, L. monocytogenes ATCC 13932, and S. aureus.* Pathogens were supplemented with brain heart infusion (BHI) broth (HiMedia, India) and kept on BHI agar (HiMedia, India) for 18 h before use.

Viability of LAB in low acid and bile conditions

The viability of LAB to endure in a bile salt medium containing 0.3 % w/v and at a low pH of 2.5 was examined [7].

Lactobacillus identification

16S rDNA gene sequencing method was employed to identify LAB. The Lactobacillus isolates were subjected to the boiling technique for genomic DNA extraction. The extracted DNA was amplified in accordance with Kumar *et al* [8]. The LAB was sequenced to verify identification with the expected band size following electrophoresis. Neighbor-Joining method was used to create the dendrogram after 16S rDNA sequences were trimmed.

Preparation of LAB cell-free supernatant (CFS)

Using an anaerobic jar (DESCO™, India), each isolate was first inoculated into 20 mL of MRS broth and incubated at 37 °C for 24 to 48 h. Broth culture was corrected to 0.5 McFarland turbidity standard after being centrifuged for 15 min at 15,000 rpm. After filtration through a 0.45 µm filter (Millipore, India), the CFS was divided into 3 portions for antibacterial activity, bacteriocin, and H_2O_2 production.

Antimicrobial activities of LAB

Antibacterial (Antipathogen) activity

The LAB isolates were tested for antibacterial activity against indicator pathogens using agar well diffusion technique. Standard inoculum of LAB were evenly coated on the surface of MRS agar plate and the plate was left to dry at 37 °C. Each MRS agar plate was aseptically bored with 6 mm diameter wells. An aliquot (100 µL) of the CFS was added to each well, and it was let to diffuse into the agar plate for 4 h at ambient temperature. The plates were incubated for 24 h at 37 °C. A clean zone surrounding a test well indicated the presence of antibacterial action. Each LAB isolate's inhibition zone diameter (IZD) was measured and categorized as weak (+), moderate (+), or no inhibition (-). All tests were carried out in duplicate.

Hydrogen peroxide generated from LAB strains was tested for antibacterial activity [9] with modification. The second portion of 5 mL of CFS received 1 mg/mL of treatment with catalase (Sigma Aldrich, Germany) and adjusted to pH 6.5 - 7.0 using 1 M NaOH.

Using the procedure outlined [10] with modification, the antibacterial activity of bacteriocin produced by LAB strains was assessed. To determine the activity of bacteriocins, a third portion of 5 mL of supernatant was treated with 1 mg/mL catalase

(Sigma Aldrich, Germany), 1 M NaOH (pH 7.0 ± 0.1), and 1 mg/mL trypsin (CDH Pvt Ltd, Mumbai). A 100 µL of the bacteriocins were inoculated into an MRS agar plate previously inoculated with standard inoculum of the indicator pathogens and activity was measured using the inhibition zone diameter (IZD).

Antibiotic susceptibility profiles

Disk diffusion technique on Mueller Hinton Agar (Oxoid, Ltd, South Africa) was used in the investigation to identify antibiotic resistance in LAB isolates. Inhibition zones were measured and contrasted with CLSI [11] and EUCAST [12]. Isolates were recorded as Resistant (R) or Sensitive (S), as appropriate.

Statistical analysis

Every determination was carried out twice, and mean ± standard error of mean (SEM) was used to express the results. To identify significant differences at $p < 0.05$, the study employed statistical analysis using GraphPad Prism® version 7 and SPSS software, such as one-way ANOVA and Duncan's multiple range test.

RESULTS

Identification of LAB isolates

The isolated LAB from fermented Okpeye was identified using 16S rDNA gene sequencing. The bacteria are closely related to *P. pentosaceus, L. brevis*, and *L. plantarum*, with similarity levels of 80 - 95 %. The accession numbers are MW672184 (strain OK1), MW672185 (strain OK14), and MW672186 (strain OK15). Morphological, physiological, biochemical, and molecular characterizations confirmed the identity of the isolates (Table 1). Figure 1 displays the phylogenetic tree of LAB isolates.

Table 1: Morphological, physiological, biochemical characterization and molecular identification of the isolated LAB

Test	**LAB isolate**		
	OK1	**OK14**	**OK15**
Morphology	Coccus	Rod	Rod
Gram reaction	+	+	+
Catalase test	-	-	-
Oxidase test	-	-	-
Haemolysis	-	-	-
Growth at varied temperature (°C)			
10	+	+	+
37	+	+	+
Growth with varied concentrations of NaCl (%w/v)			
4.5	+	+	+
6.5	+	+	+
pH (2.5)	+	+	+
Bile salt hydrolysis (0.3 % oxgall)	+	+	+
Bile salt hydrolase (BSH) activity	-	-	-
Carbohydrate fermentation			
Gas from Glucose	-	-	-
Glucose	+	+	+
Galactose	+	-	+
Fructose	+	+	+
Lactose	+	-	+
Maltose	-	-	-
Mannitol	-	-	-
Sucrose	+	+	-
Sorbitol	-	-	-
Xylose	-	+	-
Molecular identification			
GenBank submission code given	SUB9155184 OK1	SUB9155184 OK14	SUB9155184 OK15
Accession numbers generated	MW672184	MW672185	MW672186
% Gene Similarity	80.0	84.0	95.0
Identity	*P. pentosaceus*	*L. brevis*	*L. plantarum*

OK = Okpeye

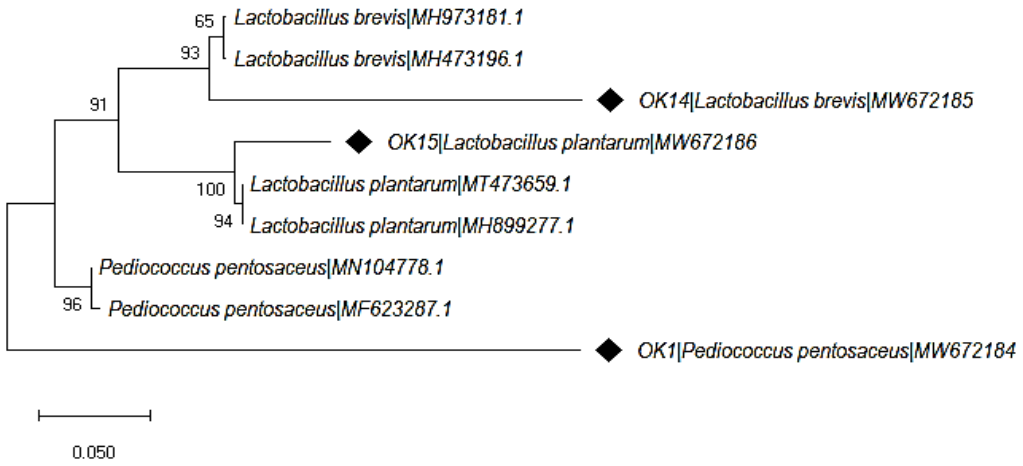

Figure 1: Phylogenetic tree of LAB isolates constructed by BLAST algorithm and neighbor-joining method

Viability of LAB in low acid and bile conditions

Viabilities of LAB to pH 2.5 and 0.3 %w/v bile concentration are shown in Figure 2 and Figure 3. Percentage viability of *P. pentosaceus, L. brevis and L. brevis* to pH stress were 95, 84 and 87 % after 4 h of exposure (Figure 2). Similarly, results in Figure 3 indicated that percentage viability of LAB to 0.3 % bile salt stress after 4 h of exposure was 95, 95 and 80 % for *P. pentosaceus, L. brevis and L. brevis*.

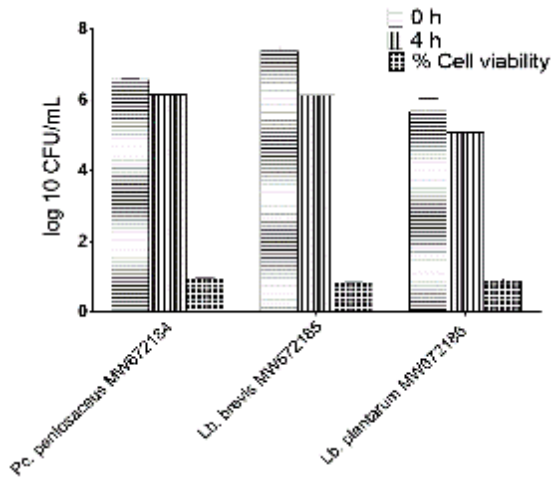

Figure 2: The viability of LAB isolates to pH 2.5

Antibacterial activities of LAB

Antibacterial activities measured as IZD of LAB against selected pathogens are summarized in Table 2. The result obtained indicated that *P. pentosaceus, L. brevis and L. plantarum* had strong antibacterial activities against *S. aureus* but moderate activities against *L. monocytogenes ATCC* 13932. However, weak activity was recorded against *B. cereus* in *P. pentosaceus*. Similarly, weak activity against *P. aeruginosa ATCC* 27883 was observed in *L. plantarum*. *L. brevis* showed minimal activity against *E. coli* and *B. cereus*, respectively.

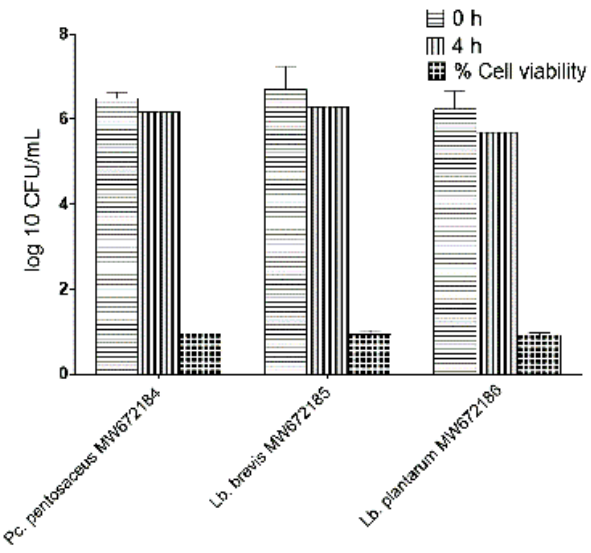

Figure 3: The viability of LAB isolates to 0.3 % w/v bile salt

Hydrogen peroxide (H_2O_2) and Bacteriocin activities of LAB isolates

The H_2O_2 and bacteriocin activities of LAB isolates are shown in Table 3 and Table 4. The H_2O_2 generated by *P. pentosaceus, L. brevis,* and *L. plantarum* strongly ($p < 0.05$) reduced the growth of *L. monocytogenes* ATCC 13932, *S. typhimurium,* and *S. aureus.* Similarly, *B. cereus* was significantly ($p < 0.05$) inhibited by H_2O_2 produced by *L. brevis and L. plantarum.* Regarding *E. coli*, only H_2O_2 generated by *P. pentosaceus* caused significant ($p < 0.05$) inhibitory effects. Both *L. brevis* and *P.*

pentosaceus produced H_2O_2 with anti-pseudomonas properties.

The bacteriocin produced by *L. brevis* and *L. plantarum* significantly ($p < 0.05$) inhibited *L. monocytogenes* ATCC 13932, *S. aureus*, and *S. typhimurium*. Apart from *L. plantarum*, a bacteriocin produced by *P. pentosaceus* and *L. brevis* was not statistically significant ($p > 0.05$) against *B.* cereus. However, *P. pentosaceus* and *L. brevis bacteriocins* significantly ($p < 0.05$) inhibited *P. aeruginosa*. Bacteriocins activities of the isolates were not inhibitory to *E. coli*.

Antibiotic susceptibility profiles of LAB isolates

Table 5 summarizes the safety evaluation of LAB isolates by examining their phenotypic resistance to different antibiotics. The present study revealed resistance of *P. pentosaceus, L. brevis* and *L. plantarum* to penicillin (ampicillin, amoxicillin, methicillin), vancomycin, methicillin, and chloramphenicol. In addition, *L. brevis* showed resistance to gentamycin, ceftriaxone**,** and clindamycin, while *L. plantarum* was resistant to rifampicin, erythromycin, and tetracycline. All the isolates were highly susceptible to erythromycin, streptomycin, kanamycin, cefuroxime, colistin, ciprofloxacin, and tetracycline.

DISCUSSION

This study has demonstrated the antimicrobial activity and *in vitro* potential of LAB previously isolated from Okpeye [11] using microbiological, biochemical, and genetic assays to find suitable probiotic candidates for a sustainable pharmaceutical formulation. A crucial factor in assessing a probiotic is its clear taxonomic identification. LAB was identified at the species level based on 16S rDNA analysis. The LAB showed good capability for withstanding bile acid release and gastric acidity, and it also supported the functions of the gut microbiota. The ability of *P. pentosaceus, L. brevis, and L. plantarum* to withstand the gastrointestinal tract assault necessary for probiotics meant for human or animal usage was demonstrated by their viability in the presence of acidic pH 2.5 and 0.3 % w/v bile salt. The capacity of LAB to withstand bile and an acidic environment differed according to the strain.

Table 2: Antipathogen (antibacterial) activities of LAB isolates

LAB		Indicator microorganisms (IZD, mm)					
Code	***Genbank Identity***	***L. monocytogenes ATCC 13932***	***P. aeruginosa ATCC 27883***	***E. coli***	***Bacillus cereus***	***S. typhimurium***	***S. aureus***
OK1	*P. pentosaceus*	++	++	++	+	+++	+++
OK14	*L. brevis*	++	++	+	+	+++	+++
OK15	*L. plantarum*	++	+	++	++	++	+++

IZD classification of antibacterial activity: strong (+++) for IZD ≥ 20 mm, moderate (++) for 20 mm > IZD > 10 mm, and weak (+) for IZD ≤ 10 mm

Table 3: Hydrogen peroxide (H_2O_2) activities of LAB isolates

LAB	Indicator pathogens (IZD, mm)					
	L. monocytogenes ATCC 13932	***P. aeruginosa ATCC 27883***	***E. coli***	***B. cereus***	***S. typhimurium***	***S. aureus***
P. pentosaceus	12.5±0.5^{a}	10.0±0.0^{a}	9.5±0.5^{a}	9.5±0.5^{a}	9.5±0.5^{a,b}	11.0 ± 2.0^{a}
L. brevis	10.0±0.0^{a}	12.5±1.5^{a}	9.0±0.0*	11.0±0.0^{a}	14.5±0.5^{a}	12.5 ± 1.5^{a}
L. plantarum	12.5±0.0^{a}	9.0±0.0*	9.0±0.0*	10.0±0.0^{a}	21.5±1.5^{a,b}	22.5 ± 1.0^{a}

*No result for Duncan statistic; Mean values of the columns with distinct letters differ considerably ($p < 0.05$), n = 3

Table 4: Bacteriocin activity of LAB isolates

LAB	Indicator pathogens (IZD, mm)					
	L. monocytogenes ATCC 13932	***P. aeruginosa ATCC 27883***	***E. coli***	***B. cereus***	***S. typhimurium***	***S. aureus***
P. pentosaceus	11.5±0.0^{a}	11.0±1.0^{a}	10.0±0.0^{a}	10.5±0.5^{a}	14.5±1.5^{a}	12.5±0.5^{a}
L. brevis	10.5±0.5^{a}	15.0±1.0^{a}	9.0±0.0*	9.5±0.5^{a}	12.5±1.5^{a}	20.0±1.0^{a}
L. plantarum	14.5±0.5^{b}	9.0±0.0*	9.0±0.0*	9.5±0.5^{a}	24.5±1.5^{b}	14.5±1.5^{a}

*No result for Duncan statistic; Mean values of the columns with distinct letters differ considerably ($p < 0.05$), n = 3

Table 5: Antibiotic susceptibility profiles of LAB isolates

Class of antibiotic	Antibiotics/concentration (µg)	Probiont		
		P. pentosaceus MW672184	***L. brevis MW672185***	***L. plantarum MW672186***
		IZD (mm)/(Breakpoints as per CLSI and EUCAST)		
Aminoglycosides	Gentamycin (30)	24.5±0.5^{e} (S)	14.5±0.5^{c} (R)	26.0±0.0^{e} (S)
	Streptomycin (30)	28.5±0.5^{b} (S)	24.5±0.5^{e} (S)	26.0±0.0^{e} (S)
	Kanamycin (30)	22.5±0.5^{d} (S)	12.5±0.5^{c} (S)	14.5±0.5^{c} (S)
Antimycobacterials (Macrolactams)	Rifampicin (5)	26.5±0.5^{f} (S)	26.5±0.5^{f} (S)	24.5±0.5^{d} (R)
Carbapenems	Meropenem (10)	36.5±0.5^{i}(S)	16.0±0.5^{d} (ND)	26.0±0.0^{e} (S)
Cephalosporins	Cefuroxime (30)	26.0±0.5^{g}(S)	24.5±0.5^{f} (S)	26.5±0.5^{e} (S)
	Ceftriaxone (30)	24.5±0.5^{e} (S)	20.5±0.5^{e} (R)	26.5±0.5^{e} (S)
Glycopeptides	Vancomycin (30)	0.0±0.0 ^{a} (R)	7.5±0.5 ^{b} (R)	0.0±0.0^{a} (R)
Lincosamides	Clindamycin (30)	30.5±0.5^{h}(S)	16.0±0.5^{d} (R)	26.5±0.5^{e} (S)
Macrolides	Erythromycin (15)	26.0±0.0^{f} (S)	28.5±0.5^{c} (S)	28.5±0.0^{f} (S)
Penicillin	Ampicillin (10) Amoxicillin (10)	0.0±0.0^{a} (R)	0.0±0.5^{a} (R)	0.0±0.0^{a} (R)
		24.0±0.0 ^{e}(R)	0.0±0.0 ^{a} (R)	0.0±0.0^{a} (R)
	Methicillin (5)	0.0±0.0 ^{a} (R)	6.5±0.5^{b} (R)	7.5±0.5^{b} (R)
Nitrobenzenes	Chloramphenicol (30)	8.5±0.5^{a} (R)	15.5±0.5^{c} (R)	0.0±0.5^{a} (R)
Polypeptides	Colistin (25)	28.5±0.5^{g} (S)	26.0±0.5^{g} (S)	24.5±0.5^{d} (S)
Quinolones	Ciprofloxacin (5)	28.0±0.0^{g} (S)	30.5±0.5^{h} (S)	37.5±0.5^{g} (S)
Tetracycline	Tetracycline (30)	30.5±0.5^{i} (S)	30.5±0.5^{h} (S)	26.5±0.5^{e} (R)

Interpretive IZD breakpoints inferred from CLSI, 2020 and EUCAST, 2020; R = Resistance, I = intermediate (moderately susceptible), and S = susceptible. Results are expressed as Mean ± SEM. Mean values of the columns with distinct letters differ considerably ($p < 0.05$). n = 3. Not determined (ND)

The results corroborate those of other authors who demonstrated that *L. brevis* and *L. plantarum* produced significant H_2O_2 [13]. Zalán *et al* [14] found in a related investigation that although *L. plantarum* repressed the development of *B. cereus* and *L. monocytogenes*, it did not show any inhibitory effects against *E. coli*. Instead, the strain produced huge amounts of H_2O_2. Certain strains of these LAB isolates produced hydrogen peroxide that was beyond toxicity standards, killing sensitive bacteria by the oxidation of cellular components or destroying basic molecular structures of cell protein [13]. Hydrogen peroxide has an antimicrobial impact by increasing membrane permeability and causing oxidative damage to proteins. Depending on quantities and environmental variables such as pH and temperature, the H_2O_2 generated by LAB is bactericidal to pathogenic bacteria lacking catalase [15]. Some studies have reported the production of organic acids and H_2O_2 facilitates the antagonistic actions of LAB against Gram-negative bacteria. According to a previous study, LAB's hostility toward Gram-negative pathogens stems from the synthesis of organic acids and H_2O_2 [16].

These isolates exhibited anti-Salmonella activities and may effectively mitigate Salmonellosis in probiotic food contaminated with Salmonella spp. The inhibitory activities of the isolates suggest their enormous benefits as probiotics and indirect strategies to lessen the risk of *P. aeruginosa* infection in local functional food users. This result corroborates other authors. The inhibitory actions of LAB isolate indicated that they have significant advantages as probiotics and might be used as a covert and biocontrol strategy to lessen the risk of *P. aeruginosa* infection in those who consume local functional foods. The LAB species such as *L. paracasei and L. plantarum* were shown to have *in vitro* bacteriocin activities against *S. aureus*. In agreement with this study, LAB species like *L. paracasei* and *L. plantarum* have demonstrated *in vitro* activity against mastitis pathogens like *S. aureus, S. epidermidis, and S. uberis* [17].

The antibiotic susceptibility result shows a preponderance of resistance of all the isolates to penicillin glycopeptides and nitrobenzenes classes of antibiotics. Similarly, all the lactobacillus strains were susceptible to macrolides, aminoglycosides, polypeptides, some cephalosporins, quinolones, and tetracycline. Since the typical dipeptide D-alanyl-D-alanine in the peptidoglycan of Lactobacillus species is irreversibly replaced by D-alanyl-D-lactate, the bacteria exhibited intrinsic resistance to vancomycin [18]. This study demonstrated that varied responses in terms of susceptibility and resistance towards antibiotics were strain-specific and common among the Lactobacillus spp.

CONCLUSION

The study has substantiated the nutraceutical and pharmaceutical importance of Okpeye beyond its use as a local seasoning agent. This work demonstrates the antibacterial, bacteriocin, and hydrogen peroxides properties of the isolated *P. pentosaceus, L. brevis, and L. plantarum* from Okpeye demonstrating a broad spectrum of activity against MDR-FBPs. Further studies are required to determine if the antagonist potentials of the strains may be increased by combining them with other stressors.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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