Probiotic Activities of Lactic Acid Bacteria Isolated from Different Types of Pap Against Selected Bacterial Strains

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ABSTRACT

Probiotics are different kinds of living organisms that have beneficial health effects on humans. Their first origin was from dairy products, but today most probiotic organisms are sources from fermenting fruits and other agricultural products. Lactic acid bacteria (LAB) majorly used as probiotics are groups of Gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of the main fermentation products of metabolism from carbohydrates sources. This study investigated the probiotic potential of lactic acid bacteria (LAB) isolated from different types of *ogi* (pap), a traditional fermented cereal porridge made from maize (*Zea mays*), millet (*Pennisetum typhoideum*), and guinea corn (*Sorghum bicolor*). A total of fifteen *ogi* samples were collected from Eke Awka market, Nigeria. Using pour-plate method, total bacterial count was done using nutrient agar while total LAB count was done using deMan Rogosa and Sharpe (MRS) agar. Isolates that developed on the plates were characterized and identified using standard biochemical methods. Molecular identification of the isolates were also done using 16s rRNA sequencing. The isolates were evaluated for probiotic properties, including tolerance to NaCl (2–8%), bile salts (0.3%), and phenol (0.1–0.4%). The antimicrobial activity of crude bacteriocins from the LAB isolates was tested against multidrug-resistant clinical specimen namely; *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*. Results indicated that the total bacterial counts ranged from 3.50×10^4 to 2.30×10^5 CFU/ml, while LAB counts ranged from 1.80×10^4 to 8.90×10^4 CFU/ml in seven of the samples. Five LAB isolates were phenotypically identified as *Pediococcus acidilactici*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Leuconostoc mesenteroides*. Molecular identification via 16S rRNA sequencing confirmed the isolates to be *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus*. All LAB isolates exhibited inhibitory effects against at least three pathogens, with *Lactiplantibacillus plantarum* (J3) showing broad-spectrum activity against all test organisms. The findings showed that traditionally fermented *ogi* is a valuable source of probiotic LAB which can be harnessed and purified and used against some multidrug resistant organisms to achieve healthy conditions.

Keywords: Probiotics, Lactic Acid Bacteria, Pap, Antimicrobial

INTRODUCTION

The growing concern of consumers for food safety due to the extensive abuse of chemical synthetic additives has prompted the search for green and secure food additives to replace the chemical synthetic ones. Increased attention has been paid to the development of probiotic foods because they could prevent diseases. Probiotics are different kinds of living organisms that have beneficial health effects on humans (Speranza *et al*., 2013). At present, probiotics are not only used as antibiotic substitutes to prevent or heal several diseases but also applied as additives in food products to inhibit the growth of pathogenic microorganisms (Wang *et al*., 2016). Most bacteria considered as probiotics belong to lactic acid bacteria (LAB), such as *Bifidobacterium* and *Lactiplantibacillus*. LABs could produce many kinds of active metabolites, and they are widely used in health care, food fermentation, and industrial additives (Kanmari *et al*., 2013). Dairy products are the most convenient and suitable carriers for probiotics to provide consumers with health benefits. However, high sugar, cholesterol and lactose in tolerance in dairy products are the major bottlenecks that restrict the development of dairy-derived probiotic products. Screening of probiotic LAB strains from plant-derived foods is a potential method to

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overcome these draw backs. Therefore, new probiotics are worth isolating from the relatively undeveloped niche (Cebeci and Gürakan, 2016).

Lactic acid bacteria (LAB) are groups of Gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of the main fermentation products of the metabolism of carbohydrates (Hayek and Ibrahim, 2013). Apart from producing the lactic acid, LAB contributes to the flavour, texture and nutritional value of the fermented foods by the production of aroma components, modification or production of proteins and exopolysaccharides, and the production of nutritional components such as vitamins (LeBlanc *et al*., 2011). Some lactic acid bacteria like *Streptococcus* and *Lactobacillus* strain are mostly used in food and pharmaceutical industries due to their healthful properties. Many strains of LAB have the ability to synthesize some B vitamins such as riboflavin and niacin including folic acid in dairy products. The use of vitamin-producing microorganisms is thus a more natural and economically viable alternative than fortification with chemically synthesized pseudo-vitamins, and it would allow the production of foods with elevated concentrations of vitamins that are less likely to cause undesirable side effects. Lactic acid bacteria (LAB) are naturally present in a broad range of ecological niches such as foods and in the gastrointestinal and urogenital tract of animals, including humans. In addition to their technological important properties in food production, several studies have shown that LAB can confer beneficial properties to their hosts, in specific members of the genus *Lactobacillus*, reason for which these bacteria are the most commonly used probiotic micro-organisms (Soccol *et al*., 2011).

Pap (*ogi*) is one of the popular indigenous fermented foods in Nigeria. It is a fermented cereal porridge made from maize (*Zea mays*), sorghum (*Sorghum vulgare*) or millet (*Pennisetum typoideum*). The *ogi* porridge is very smooth in texture, and has a sour taste reminiscent of that of yoghurt. Traditional fermentation processes of *ogi* production are usually spontaneous and uncontrolled. *Ogi* is popularly produced by grinding cereals such as maize, sorghum or millet, sieving it wet and then allowing the starch to sediment and ferment in a liquid menstruum (Onyeze *et al*., 2013). In Nigeria, *ogi* is either prepared into a smooth porridge called *pap* or a solid gel known as *eko* or *agidi*. The consistency of *pap* varies from thick to watery according to choice. *Pap* can be sweetened with sugar and milk; it is then eaten with bean cake. It is used as the first native food for weaning babies. It also serves as breakfast meal for pre-school, school children and adults. In a more concentrated form, it is boiled into a thick gel and then allowed to set stiff in leaf moulds as *eko* or *agidi*. In either form, it is usually preferred to many other indigenous foods by the aged and the convalescence (Salami *et al*., 2015).

LAB-probiotics can reduce the spread of pathogenic bacteria by mechanisms involving production of inhibitory compounds and competitive exclusion (Vieco-Saiz *et al*., 2019). The antimicrobial effect of LAB may be due to the production of antimicrobial peptides (AMPs) or small organic molecules such as organic acids, ethanol, diacetyl, carbon dioxide, hydrogen peroxide and smaller peptides, that is, bacteriocins (Liao and Nyachoti, 2017). Several bacteriocins have been shown to act in synergy with conventional antibiotics (Cavera *et al*., 2015, Wolska *et al*., 2012), thus reducing bactericidal concentrations and reduction in their undesirable side-effects while some produced by Gram positive bacteria are active against viruses (Ben-Lagha *et al*., 2017). Hence this research delves into the search for lactic acid bacterial from fermented cereals and the probiotics activities of the isolated LABs.

MATERIALS AND METHOD

Sample Collection

Fifteen different *ogi* samples (five each from guinea corn [*Sorghum bicolor],* maize [*Zea mays*] and millet [*Pennisetum typhoideum*]) were obtained from different retailers from Eke Awka market, Awka, Anambra State, Nigeria, using different sterile zip lock polythene bags. They were transported to the Laboratory of the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka for analysis. The samples were stored in a fridge and used 48 hours after collection. Clinical isolates were obtained from the stock collections in Glanson Medical and Diagnostic Laboratory, Awka. Media, Chemicals and Reagents were obtained from the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka and were of analytical grade.

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Media Preparation

According to manufacturers' instruction, 14.3g of de Man Rogosa Sharpe (MRS) agar was dissolved in 250ml distilled water contained in a 250ml conical flask. It was heated gently over a Bunsen flame to dissolve. The mouth of the flask was plugged with cotton wool and wrapped with foil and the media sterilized by autoclaving at 15psi (121^{o}C) for 15 minutes. After sterilization, the media was left on the bench and allowed to cool to 45^{o}C. MRS broth was prepared by dissolving 14.3g of de Man Rogosa Sharpe (MRS) agar into 250ml of sterile distilled water and filtered to reduce the agar concentration and also sterilized and cooled. Nutrient agar was also prepared according to manufacturers' instruction and sterilized by autoclaving at 15psi (121^{o}C) for 15 minutes. It was also cooled to 45^{o}C.

The *ogi* samples were designated as SB for *Sorghum bicolor*, ZM for *Zea mays* and PT for *Pennisetum typhoideum* samples. Stock solutions of the *ogi* samples were prepared by dissolving 10g of each sample in 90ml of sterile water to form slurries. The mixture was shaken and allowed to stay on a bench for 1hr to settle. The settled liquid was filtered out and used for inoculation.

Determination of Total Heterotrophic Bacterial Count

This was done using the pour plate method of Berebon *et al*. (2019). A 0.1ml of the supernatant was added into an empty Petri dish. Twenty milliliters (20ml) of molten nutrient agar was poured into the Petri dish, swiveled to mix and allowed to set on a flat bench. The plates were then inverted and incubated aerobically at 37^{o}C for 24 hours. Developed colonies were counted and recorded. The process was repeated three times and the mean colony count were taken which represents the mean heterotrophic bacterial count. The count was calculated as colony forming unit (CFU/ml) using the equation:

$CFU/ml = (V \times N)/D$

Where:

V = volume of sample inoculated,

N = Number of colonies on the plate,

D = dilution factor used.

Determination of Total Lactic Acid Bacterial count

Using the pour plate method of Berebon *et al*. (2019), 0.1ml of the supernatant from the stock solution was introduced into different sterile Petri dishes and 20ml of molten MRS agar was poured into the Petri dishes, swiveled to mix and allowed to set on a flat bench. The plates were then inverted and incubated anaerobically at 37^{o}C for 48 hours. These were repeated thrice and mean developed colonies were recorded. The colony-forming unit which represents the LAB counts was calculated using the equation:

$CFU/ml = (V \times N)/D$

Where:

V = volume of sample inoculated,

N = Number of colonies on the plate,

D = dilution factor used.

Distinct colonies from the Nutrient agar plates and MRS agar plates were sub-cultured severally on fresh respective media and incubated at their respective conditions. They isolates were stored on their respective agar slants in bijou bottles in a refrigerator at 4^{o}C for further use.

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Characterization and Identification of the Lactic Acid Bacteria and the clinical specimen

This was done using the method of Cheesebrough (2010). Discrete colonies were subcultured, examined and recorded based on the shape, colour, border, texture, and general appearance of individual bacterial colonies on each plate and Gram stained. Gram staining was done to reveal the characteristic group and arrangement of the cells. Biochemical tests (indole test, methyl red test, Voges-Proskauer (VP) test, citrate test, oxidase test, coagulase test, and sugar fermentation test) were also carried out for the identification of bacterial isolates.

The six clinical isolates (*Staphylococcus aureus, Enterococcus Feacalis, Bacillus cereus, Escherichia coli, Salmonella enteric* and *Pseudomonas aeruginosa*) were reconstituted by sub culturing them on fresh Nutrient agar. They were also characterized using the methods as enumerated above as described below according to Cheesbrough (2010), to confirm their identity.

- **Gram staining**

An evenly spread smear of the isolates were made on different clean grease free, dry slides using sterile normal saline and the smears allowed to air-dry in a safe place. The smears were fixed by passing three times over Bunsen flame and stained by the Gram technique.

Each smear was covered with crystal violet stain for 60 seconds. After that, it was rapidly washed off with clean water. All the water was tipped out, and then Lugol's iodine was added for 60 seconds. The iodine was washed off with clean water. Then, the smear was decolorized rapidly with acetone – alcohol and washed immediately with clean water. After that, the smear was covered with neutral red stain for 2 minutes. The stain was washed off with clean water. The back of the slide was wiped clean, and the slide was placed in a draining rack for the smear to air-dry. The smear was examined microscopically, first with the x100 objective to check the staining and to see the distribution of material and then with the oil immersion objective.

- **Catalase test**

This test is used to differentiate those bacteria that produce the enzyme catalase, such as *Staphylococci*, from non-catalase producing bacteria such as *Streptococci*. A 2 ml of 3% hydrogen peroxide were poured into a test tube, by means of a sterile wooden stick, a loopful of the test organism was immersed in the hydrogen peroxide solution. Immediate bubbling in the solution indicates positive test.

- **Coagulase test**

This test is used to differentiate *Staphylococcus aureus* (*S. aureus)* which produces the enzyme coagulase, from *S. epidermidis* and *S. saprophyticus* which do not produce coagulase. A drop of physiological saline was placed on a slide. A colony of the test organism was emulsified in the drop to make a thick suspension. A drop of plasma was added to the suspension and gently mixed. Clumping of the organisms within 10 seconds indicates positive test.

- **Fermentation of Sugars**

This test was performed according to Cheesbrough (2010) in a sugar broth medium to test an organism's ability to ferment different sugars as well as its ability to produce gas and H_2S. The medium contains PH indicator, bromothymol blue which converts to green color in the acidic PH indicating sugar fermentation. The sugars used were fructose, glucose, mannitol, lactose and sucrose.

A suspension of the tested isolate was prepared using a sterile normal saline. Then 10ml of each sugar broth was introduced in series of test tubes (containing an inverted Durham tube) and inoculated separately with 5ml of each prepared suspension using sterile pipettes. The tubes were incubated at 37ºC for 24 hours for bacteria and anaerobically at 37ºC for 48hr for LABs. A change in the color from blue to green will indicate fermentation of the sugar.

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Urease test

Testing for enzyme activity is important in differentiating enterobacteria. The test organism was cultured on a medium containing urea and the indicator phenol red. If the strain is urease-producing, enzyme will break down urea (by hydrolysis) to give ammonia and carbondioxide. With the release of ammonia, the medium becomes alkaline shown by change in colour of indicator to red-pink (Cheesbrough, 2010). Using a sterile straight wire, tubes containing the medium were inoculated with test organism and then incubated for 24 hours at 37ºC. If the strain is urease-producing, the media become alkaline and change the colour of indicator to red-pink.

- **Citrate Utilization Test**

This test is one of several techniques used to assist in identification of enterobacteria. The test is based on the ability of an organism to use citrate as its only source of carbon and ammonia as its only source of nitrogen. The test organism was cultured on slope media containing sodium citrate and then incubated for 24 hours at 37ºC. Growth in media is shown by turbidity and change in colour of indicator (bromothymol blue) from light green to blue (Cheesbrough, 2010).

- **Indole Test**

Testing for indole production is important in identification of Enterobacteria. This test is based on the ability of organisms to break down the amino acid tryptophan with the release of indole by producing tryptophanase enzyme. A loopfull of the test organism is cultured on 5 ml peptone water containing tryptophan or tryptone water and then incubated for 24 hours at 37ºC. Indole production is detected by Kovac's reagents which contain 4(p)-dimethylaminobenzaldehyde. This reacts with indole to produce red color ring (Cheesbrough, 2010).

- **Motility Test**

This test is used to differentiate motile bacteria from non-motile. A loopful of the test organism was inoculated on semi-solid medium and incubated for 24 hours at 37ºC and a positive result shown as a moving of bacteria away from line of inoculation (Cheesbrough, 2010).

Molecular Identification of the Isolates

Molecular characterization of two (2) selected LAB strains was based on 16S rRNA conserved gene sequences using universal bacterial primers. The targeted gene sequence was amplified using the conventional PCR method, and the size of the amplified fragments was confirmed by running the final product by 1% gel electrophoresis. The amplified samples and appropriate sequencing fragments were sent for sequencing and the retrieved nucleotide sequences were phylogenetically studied using MEGA software (MEGA-11). Bacterial isolates were further verified/classified at the species level by BLAST search using GenBank NCBI (National Center for Biotechnology Information) (Sadiqi *et al*., 2022)

Preparation of Standard Inoculum

Each potential probiotic isolate was inoculated into 10ml MRS broth in test tubes. The cultures were incubated anaerobically for 48 hours at 37ºC. These cultures were used as the standard inoculum for further experiments.

Probiotic Evaluation of the LAB Strains

NaCl and phenol tolerance by the LAB isolates

The tolerance of LAB strains to NaCl were determined according to the method of Hoque (2010) by inoculating 0.5ml overnight cultures of LAB strains (1% v/v) into 10ml MRS broth containing different concentrations, 2% to 8% NaCl. Following incubation at 37 °C for 6h, the optical density of the grown LAB cultures at a wavelength of 600 nm (OD_{600nm}) were measured using a spectrophotometer (Metash UV-800, Shanghai, China) to assess their viability.

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Similarly, the ability of LAB strains to tolerate high concentrations of phenol was assessed. A 0.5ml overnight culture of LAB strains (1% v/v) were inoculated into 10ml MRS broth containing increasing concentrations of phenol (0.1–0.4%). The inoculated MRS broth tubes were appropriately incubated at 37°C for 24 h. The viability of the strains was determined by measuring the OD600nm of grown LAB strains using a spectrophotometer.

Bile salt tolerance by the isolates

This was done using the method described by Hoque (2010). The ability of the LAB strains to survive in the presence of 0.3% bile salt for 6hr was assessed. A loopfull of overnight LAB cultures (1% v/v) were inoculated into 10ml MRS broth supplemented with 0.3% bile salt, followed by subsequent incubation. The viability of LAB strains was recorded at 0 to 6h of incubation by determining the colony-forming unit (CFU) counts of strains on MRS agar plates.

Antimicrobial Activities of the LAB on the selected Bacterial Strains

Bacteriocin production by LAB

A 5ml LAB culture broth was grown in 50ml tryptic soy broth with 1% yeast extract and incubated anaerobically for 48 hours at 27ºC. The medium was then centrifuged at 10,000 rpm for 15 mins at 40ºC. The supernatant (crude bacteriocin) was filtered with 0.22µm size membrane filter and the pH of the supernatant adjusted to 6.5. The supernatant was stored in the refrigerator at 4ºC until needed.

Antibacterial activity assay

The antibacterial activities of the LAB isolates against the 6 clinical isolates were assessed by using the Agar well diffusion technique as described by Odetokun *et al*. (2019). A 100µl of each clinical isolate (adjusted to 0.5 McFarland standards using sterile saline) were spread on Mueller Hinton Agar plates and allowed to stand and diffuse for 20 min. With sterile cork borers wells were made on the agar plates. A 100µl of crude LAB bacteriocin were added into the wells and allowed to diffuse for 30 minutes at 27ºC into the agar. Subsequently, the inoculated plates were incubated anaerobically at 37°C for 24h. The antimicrobial activity of each probiotic strains was evaluated by measuring the inhibition zone diameter (IZD) around the crude bacteriocin wells.

The clinical isolates were tested for Multi drug Resistance (MDR) using Gram positive antibiotic disks containing Ciprofloxacin, Norfloxacin, Gentamycin, Amoxil, Rifampcin, Erythromycin, Streptomycin, Chloramphenicol, Levoflocaxin, Ampiclox and Gram-negative disks containing Augmentin, Gentamycin, Cefixime, Streptomycin, Terafid, Ciprofloxacin, Septrin, Cefurroxime, Ceftrazon and Penicillin using the above method. This was done to compare the antibiotic effects and the produced crude bacteriocin.

Statistical Analysis

The analyses were done in triplicates; data were recorded as mean ± SD. The data obtained were subjected to one-way ANOVA and correlation analysis using Statistical Package for Service Solution (SPSS) version 23.

RESULTS

Total Bacterial Count and Total Lactic Acid Bacteria Count

Bacterial colonies were seen on the pour plates ranging from $1.120 \pm 2.1 \times 10^4$ to $9.00 \pm 0.2 \times 10^4$ CFU/ml while total lactic acid (LAB) counts ranged from $1.80 \pm 2.1 \times 10^4$ to $8.90 \pm 0.2 \times 10^4$. Seven samples showed the presence of growth colonies on the MRS plate as presented in Table 1.

Identification of Lactic Acid Bacteria Isolates

Five different Lactic Acid Bacteria were identified presumptively as *Pediococcus acidilactici, Lactobacillus delbrueckii, Lactobacillus plantarum, Lactobacillus fermentum* and *Leuconostoc mesenteroides*. All the isolates were Gram-positive, non-motile, catalase-negative, oxidase-negative, urease-negative and indole-negative

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(Table 2). Three of the isolated presumptively identified as *Lactobacillus delbrueckii*, *Lactobacillus plantarum* and *Lactobacillus fermentum* were sent for molecular identification and two of them *Lactobacillus plantarum* and *Lactobacillus fermentum* were confirmed to be one as *Lactiplantibacillus plantarum* while the *Lactobacillus delbrueckii* was molecularly identified as *Lacticaseibacillus rhamnosus*. Both had 100% matches.

Table 1: Total Bacterial Count (CFUml-^{1}) and Total lactic acid bacteriacount (CFUml^{-1})

Sample	**Total Bacterial Count (CFUml^{-1})**	**Total Lactic Acid Bacteria count (CFUml^{-1})**
SB 1	2.30±0.2 x 10^4	6.10 ± 1.2 x 10^4
SB 2	3.50 ±0.15 x 10^4	NG
SB 3	4.10 ±0.2 x 10^4	NG
SB 4	2.29±1.2 x 10^4	NG
SB 5	1.88±1.0 x 10^4	8.70 ± 0.2 x 10^4
ZM 1	9.00 ±0.2 x 10^4	NG
ZM 2	1.32±2.0 x 10^4	5.20 ± 2.0 x 10^4
ZM 3	5.40 ±1.2x 10^4	NG
ZM 4	1.24±2.0 x 10^4	8.90 ± 0.2 x 10^4
ZM 5	4.40±1.3 x 10^4	1.80 ± 2.1 x 10^4
PT 1	1.68±2.1 x 10^4	4.50 ±1.2 x 10^4
PT 2	1.24±2.0 x 10^4	NG
PT 3	7.00±1.2 x 10^4	NG
PT 4	1.12±2.1 x 10^4	4.40 ± 0,2 x 10^4
PT 5	8.80±1.2 x 10^4	NG

Key:

1. Guinea corn (*Sorghum bicolor*) pap:**SB**

2. Maize (*Zea mays*) pap:**ZM**

3. Millet (*Pennisetum typhoideum*) pap:**PT**

Table 2: Morphological and Biochemical Identifications of the Various Lactic Acid Bacterial Isolates presumptively.

Isolate	**Form**	**Surface**	**Colour**	**Margin**	**Elevation**	**Opacity**	**Gram**	**Cat**	**Mot**	**Ind**	**MR**	**VP**	**Cit**	**Lac**	**Glu**	**Suc**	**Fru**	**Mal**	**Oxi**	**Ure**	**Probable Identity**
J1	Circular	Smooth	Cream	Entire	Flat	Translucent	+Cocci	-	-	-	+	+	-	+	+	+	+	+	-	-	*Pediococcus*

																				acidilactici
J2	Circular	Smooth	White	Entire	Convex	Moist	+Rod	-	-	-	-	+	+	+	+	+	+	+	-	-	*Lactobacillus delbrueckii*
J3	Circular	Smooth	White	Entire	Convex	Translucent	+Rod	-	-	-	-	-	+	+	+	+	+	+	-	-	*Lactobacillus plantarum*
J4	Circular	Smooth	White	Entire	Convex	Translucent	+Rod	-	-	-	-	-	+	+	+	+	+	+	-	-	*Lactobacillus fermentum*
J5	Circular	Smooth	White	Entire	Flat	Translucent	+Cocci	-	-	-	-	+	-	+	+	+	+	+	-	-	*Leuconostoc mesenteroides*

Key:

Gram: Gram reaction

Cat: Catalase test

Mot: Motility test

Ind: Indole test

MR: Methyl-red test

VP: Voges-Proskauer test

Cit: Citrate Utilization test

+: Positive

-: negative

Sugars Fermented:

Lac: Lactose Fermentation

Glu: Glucose Fermentation

Suc: Sucrose Fermentation

Fru: Fructose Fermentation

Mal: Maltose Fermentation

Oxi: Oxidase

Ure: Urease

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Molecular Identification of Isolates

>Lactiplantibacillus plantarum

GGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTT
GGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTAT
TAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATC
GGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCAC
AATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTC
TGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTG
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GTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGA
AATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTG
AGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGA
ATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGG
GGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGA
GATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
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ACTCTGGTGAGACTGCCGGTGACAAA

Sequence ID: **CP170649.1**

Length: 3238827

Match: 1056/1056

Percentage: 100%

E-value: 0.0

Start: **1296245**

End **1297300**

Max score 1951

>Lacticaseibacillus rhamnosus

GAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTG
GAAACAGATGCTAATACCGCATAAATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGCGTAA
GCTATCGCTTTTGGATGGACCCCGGCGTATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAA
TGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCTA
CGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAG
TNAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTG
TCGGCGTGACGGTATCCAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGA
TGTGAAAGCCCTCGGCTTAACCGAGGAAGTGCATCGGAAACTGGGAAACTTGAGTNCAGAAGAG
GACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAG
GCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATA
CCCTGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCG
CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGTCTTGACATCTTTTGATCACCTGAGAGATCAGGTTTCCCCTTCGGGGGCAAAA

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Sequence ID: **MK560053.1**

Length: **1431**

Match: 956/956

Percentage: 100%

E-value: 0.0

Start: 77

End 1032

Max Score: 1759

Confirmation of the Clinical Isolates

The six clinical isolates were confirmed based on their morphological and biochemical characteristics. The Gram-positive isolates are *Staphylococcus aureus, Enterococcus faecalis* and *Bacillus cereus* while the Gram-negative isolates are *Escherichia coli, Salmonella enterica* and *Pseudomonas aeruginosa* as shown in Table 3.

Probiotic Evaluation of the LAB Strains

The 5 isolates (*Pediococcus acidilactici, Lactobacillus delbrueckii, Lactobacillus plantarum, Lactobacillus fermentum* and *Leuconostoc mesenteroides*) designated as J1, J2, J3, J4 and J5 respectively exhibited different growth densities in different NaCl concentrations (Table 4). At 2% salt concentration, isolate J1 had the least growth (1.366) while J3 had the highest growth concentration (1.895). At 4% salt concentration, isolate J1 had the least growth (1.322) while J5 had the highest growth concentration (1.660). At 6% salt concentration, isolate J2 had the least growth (0.671) while J3 had the highest growth concentration (1.247). At 8% salt concentration, isolate J2 had the least growth (0.550) while J3 had the highest growth concentration (1.094).

In the presence of bile salt, isolates J1 and J5 was only able to survive for 3hrs, isolate J2 was able to survive for 5hrs, isolate J3 was able to tolerate the bile salt for the entire 6hrs while isolate J4 was only able to survive for 4 hours (Table 5).

All the isolates showed similar growth reaction to different phenol concentrations (Table 6).

Antibiotics Sensitivity of the Test Isolates

The organisms showed varying degrees of multidrug resistance (MDR). Among the Gram-positive isolates, *S. aureus* and *E. faecalis* showed MDR ratio of 0.6 while *Bacillus cereus* showed MDR ratio of 0.7. Ciprofloxacin showed highest activity against the 3 Gram-positive isolates while they were all resistant to Norfloxacin, Streptomycin, Chloramphenicol and Ampiclox (Table 7).

Among the Gram-negative isolates, *E. coli* and *Salmonella enterica* had MDR ratio of 0.7 while *Pseudomonas aeruginosa* had MDR ratio of 0.6. All the 3 isolates were resistant to Cefixime, Streptomycin, Cefurroxime and Penicillin (Table 8).

Antimicrobial Activities of the LAB on the selected Bacterial Strains

Following the agar well diffusion assay, all the isolates were found to be active against at least 3 of the 6 test bacteria (Table 9). Sample J3 was active against all the test bacteria giving a mean diameter zone of inhibition of 9.33mm to 14.50mm. Sample J5 showed activity only against *S. aureus, E. coli* and *Salmonella enterica* among the 6 isolates. *Enterococcus faecalis* and *Bacillus cereus* were the most resistant organisms, showing resistant to 2 samples (Table 9).

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Table 3: Morphological and Biochemical Identifications of the Various Pathogenic Bacterial Isolates.

Isolate	**Form**	**Surface**	**Colour**	**Margin**	**Elevation**	**Opacity**	**Gram**	**Cat**	**Mot**	**Ind**	**MR**	**VP**	**Cit**	**Lac**	**Glu**	**Suc**	**Fru**	**Mal**	**Oxi**	**Ure**	**Identity**
1	Circular	Smooth	Yellowish	Entire	Raised	Opaque	+ cocci	+	+	-	+	-	-	AG	AG	A	A	AG	-	+	*Staphylococcus aureus*
2	Circular	Smooth	cream	Entire	convex	Opaque	+coccus	-	-	-	-	+	-	+	+	+	-	+	-	-	*Enterococcus faecalis*
3	Circular	Rough	whitish	fimbriate	Raised	Opaque	+Rod	+	+	-	-	+	+	+	+	+	+	+	-	-	*Bacillus cereus*
4	Circular	Smooth	Whitish	Entire	Convex	Translucent	- Rod	+	+	+	+	-	-	+	+	-	-	-	-	-	*Escherichia coli*
5	Circular	Smooth	Greyish/white	Lobate	Low convex	Translucent	- Rod	+	+	-	+	-	+	-	+	-	+	+	-	-	*Salmonella enterica*
6	Circular	Shiny	White	Entire	Convex	Moist	- Rod	+	+	-	-	+	+	-	+	-	+	-	+	-	*Pseudomonas aeruginosa*

Key:

Gram: Gram reaction

Cat: Catalase test

Mot: Motility test

Ind: Indole test

MR: Methyl-red test

VP: Voges-Proskauer test

Cit: Citrate Utilization test

Sugar Fermentation Tests:

Lac: Lactose Fermentation

Glu: Glucose Fermentation

Suc: Sucrose Fermentation

Fru: Fructose Fermentation

Mal: Maltose Fermentation

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Oxi: Oxidase

Ure: Urease

Table 4: Tolerance of the Isolates in NaCl Concentration (Optical Density 600nm)

Isolate	**2%**	**4%**	**6%**	**8%**	**CONTROL (0%)**
J1	1.366 ± 0.00	1.322 ± 0.00	0.984 ± 0.00	0.896 ± 0.00	0.601 ± 0.00
J2	1.569 ± 0.00	1.437 ± 0.00	0.671 ± 0.00	0.550 ± 0.00	0.615 ± 0.01
J3	1.895 ± 0.00	1.640 ± 0.00	1.247 ± 0.00	1.094 ± 0.00	0.600 ± 0.00
J4	1.454 ± 0.00	1.384 ± 0.00	0.790 ± 0.00	0.640 ± 0.00	0.620 ± 0.00
J5	1.707 ± 0.00	1.660 ± 0.00	0.851 ± 0.00	0.810 ± 0.00	0.598 ± 0.01

Table 5: Bile Tolerance for 6hrs

Sample	**Bacterial Count (CFU/ml)**						
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
J1	1.05×10^3	9.4×10^2	6.9×10^2	3.9×10^2	NG	NG	NG
J2	2.04×10^3	1.29×10^3	1.01×10^3	7.1×10^2	4.9×10^2	3.2×10^2	NG
J3	2.37×10^3	2.18×10^3	1.86×10^3	1.33×10^3	5.4×10^2	3.8×10^2	3.1×10^2
J4	1.25×10^3	1.08×10^3	9.5×10^2	5.6×10^2	4.1×10^2	NG	NG
J5	2.22×10^3	1.12×10^3	4.2×10^2	3.2×10^2	NG	NG	NG

Key:

NG: No Growth

Table 6: Phenol Tolerance Assay (Optical Density 600nm)

Sample	**0.1%**	**0.2%**	**0.3%**	**0.4%**
J1	0.097 ± 0.00	0.102 ± 0.00	0.183 ± 0.00	1.104 ± 0.00
J2	0.117 ± 0.00	0.212 ± 0.00	0.446 ± 0.00	1.237 ± 0.00
J3	0.094 ± 0.00	0.227 ± 0.00	0.681 ± 0.00	1.360 ± 0.01
J4	0.099 ± 0.00	0.304 ± 0.00	0.544 ± 0.00	1.260 ± 0.01
J5	0.102 ± 0.01	0.212 ± 0.07	0.523 ± 0.20	1.242 ± 0.09

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Table 7: Antibiotics sensitivity of the Gram-Positive Isolates

Isolate	**Antibiotics**										**MDR Ratio**
	CPX	**NB**	**CN**	**AML**	**S**	**RD**	**E**	**CH**	**APX**	**LEV**	
Staphylococcus aureus	14.60 ± 0.36	0.00 ± 0.00	0.00 ± 0.00	14.03 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	15.97 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	14.13 ± 0.0.21	0.6
Enterococcus faecalis	15.67 ± 0.15	0.00 ± 0.00	14.33 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	13.27 ± 0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.07 ± 0.35	0.6
Bacillus cereus	14.03 ± 0.21	0.00 ± 0.00	14.97 ± 0.25	15.23 ± 0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.7

Key:

Ciproflox (CPX) 10µg

Norfloxicin (NB) 10µg

Gentamycin (CN) 10µg

Amoxil (AML) 20µg

Streptomycin (S) 30µg

Rifampicin (RD) 20µg

Erythromycin (E) 30µg

Chloramphenicol (CH) 30µg

Ampiclox (APX) 20µg

Levoflocaxin (LEV) 20µg

Table 8: Antibiotics Sensitivity of the Gram-negative isolates

Isolate	**Antibiotics**										**MDR Ratio**
	AU	**CN**	**CFX**	**S**	**OFX**	**CPX**	**SXT**	**CH**	**CIP**	**PN**	
Escherichia coli	16.47 ± 0.35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	15.50 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	15.20 ± 0.40	0.00 ± 0.00	**0.7**
Salmonella enterica	0.00 ± 0.00	16.50 ± 0.30	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	14.87 ± 0.25	0.00 ± 0.00	0.00 ± 0.00	14.47 ± 0.25	0.00 ± 0.00	**0.7**
Pseudomonas aeruginosa	14.46 ± 0.25	13.17 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	15.87 ± 0.15	15.13 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	**0.6**

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Key:

Augmentin (AU) 10µg

GENTAMYCIN (CN) 10µg

Cefixime (CFX) 5µg

Streptomycin (S) 30µg

Trafid (OFX) 10µg

CIPROFLOXACIN (CPX) 10µg

SEPTRIN (SXT) 30µg

CEFUROXIME (CH) 30µg

CEFTRAZON (CIP) 30µg

PENICILLIN (PN) 30µg

Table 9: Antimicrobial Sensitivity Screening of the Crude Bacteriocin (20µL) on various Pathogenic Gram-positive and Gram-negative Isolates

LAB Isolate	**Mean Diameter of Zone of Inhibition (mm)**					
	Staphylococcus aureus	***Enterococcus faecalis***	***Bacillus cereus***	***Escherichia coli***	***Salmonella enterica***	***Pseudomonas aeruginosa***
J1	0.00 ± 0.00	10.93 ± 0.06	13.03 ± 0.15	12.80 ± 0.15	0.00 ± 0.00	12.03 ± 0.15
J2	11.52 ± 0.08	0.00 ± 0.00	13.77 ± 0.15	11.70 ± 0.20	14.23 ± 0.06	11.13 ± 0.06
J3	9.33 ± 0.15	12.13 ± 0.06	9.78 ± 0.08	13.13 ± 0.06	14.50 ± 0.20	12.70 ± 0.10
J4	13.23 ± 0.25	10.60 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	13.30 ± 0.10	13.50 ± 0.20
J5	8.93 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	12.93 ± 0.06	14.83 ± 0.06	0.00 ± 0.00

DISCUSSION

Discussion

This study successfully isolated, characterized and identified lactic acid bacteria (LABs) from traditionally fermented *ogi* (a cereal-based porridge) produced from maize, millet, and guinea corn. The research further evaluated the probiotic potential of these isolates, specifically their tolerance to physiological stresses (acid, bile, salt, and phenol), and assessed the antimicrobial efficacy of their crude bacteriocins against multidrug-resistant (MDR) pathogenic bacteria. The findings present significant insights into the microbial ecology of this indigenous fermented food and highlight the potential of its native LABs as source of functional probiotics and natural antimicrobial agents in an era of rising antibiotic resistance.

The total bacterial counts in all the *ogi* samples ranged from $1.12 \pm 2.1\times10^4$ to $9.00 \pm 0.2 \times 10^4$ CFU/ml, confirming active fermentation and a substantial microbial load, consistent with other spontaneously fermented cereal products as discovered by Oguntoyinbo *et al*. (2016). Notably, only 7 out of the 15 samples yielded cultivable LAB, with counts between $1.80 \pm 2.1\times10^4$ and $8.90 \pm 0.2\times 10^4$ CFU/ml. The absence of detectable

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LAB in 8 samples is intriguing and could be attributed to several factors: the stage of fermentation at the time of sampling (late stages may see higher viable LAB growths), the competitive presence of other microorganisms like yeasts and acetic acid bacteria, or the use of cultivation-dependent methods that may fail to recover stressed or viable-but-non-culturable (VBNC) cells (Giraffa, 2014). This underscores the limitation of culture-based techniques and suggests a more complex microbial consortium than detected.

The study identified five LAB isolates through phenotypic methods as *Pediococcus acidilactici*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Leuconostoc mesenteroides*. However, molecular identification (16S rRNA sequencing) of the three Lactobacilli confirmed them to be *Lactiplantibacillus plantarum* (formerly *Lb. plantarum*) and *Lacticaseibacillus rhamnosus* with 100% matches. This discrepancy between phenotypic and genotypic identification is common and highlights the necessity of molecular techniques for accurate species-level taxonomy, as phenotypic traits can be variable and misleading (Salvetti *et al*., 2018). The predominance of *Lactiplantibacillus plantarum* is a common finding in fermented cereals worldwide, attributed to its remarkable metabolic versatility and resilience to various environmental stresses (Siezen and van Hylckama Vlieg, 2011). The presence of *Lacticaseibacillus rhamnosus*, a well-documented probiotic species, is of particular interest due to its known health benefits as confirmed by Segers and Lebeer (2014).

Studies on Nigerian ogi consistently report high microbial loads and the dominance of *Lactobacillus* species, particularly L. plantarum. For instance, research by Olanbiwoninu and Odunfa (2015) on improving ogi production also identified *L. plantarum* as a key fermenter. The discrepancy between phenotypic and genotypic identification observed in the thesis is a common challenge in Nigerian labs where biochemical tests are often used first due to cost, a point noted by Adegboye *et al*. (2021) in their review of African fermented foods. The finding of *L. rhamnosus* is less common in traditional ogi might indicate cross-contamination or a unique microbial selection in the specific samples studied. The variability in LAB detection aligns with findings from other African studies, which attribute it to fermentation stage, substrate, and geographical location, as discussed by Mokoena (2017).

The six clinical isolates after reconstitution and culturing confirmed them to be the organisms procured. Three were confirmed to be gram positive and three Gram negative. They are mostly among the organisms that depict Multidrug resistance.

For a bacterium to function as a probiotic, it must survive passage through the harsh conditions of the gastrointestinal tract (GIT), including gastric acid and bile salts (FAO/WHO, 2002). The isolates exhibited varying degrees of tolerance to low pH and bile salts. Isolate J3 (*Lactiplantibacillus plantarum*) demonstrated superior bile tolerance, maintaining viability (3.1×10^2 CFU/ml) even after 6 hours of exposure. This aligns with the known robustness of this species, which often possesses bile salt hydrolase (BSH) enzymes that confer a survival advantage in the gut (Begley *et al*., 2006). The rapid decline or complete loss of viability in other isolates (J1, J4 and J5) after 3-4 hours suggests they may not survive prolonged intestinal transit. This variability is expected, as stress tolerance is a strain-specific property (Ruiz *et al*., 2013).

Salt tolerance is crucial for LAB survival in high-osmolarity environments like certain foods and the gut. All isolates grew best at 2% and 4% NaCl, with growth declining significantly at 6% and 8%. This pattern is typical for many LABs, which are generally non-halophilic (Leroy and De Vuyst, 2004). The phenol tolerance assay, often used as a proxy for tolerance to toxic microbial metabolites in the gut, showed that all isolates could withstand up to 0.3% phenol, with significant growth at 0.4%. This indicates a capacity to endure the stressful environment of the distal intestine, a positive trait for potential probiotics (Zommiti *et al*., 2020). Several Nigerian studies have screened LAB from traditional foods for probiotic properties. Olonisakin *et al*. (2017) found LAB from kunun-zaki that could tolerate 0.3% bile salts, which is lower than the 6-hour tolerance demonstrated by J3 in this research. The salt tolerance (up to 4%) is consistent with findings by Adeyemo and Onilude (2018) for LAB from Nigerian fermented cereals, confirming their non-halophilic but osmotolerant nature. Research on probiotic LABs from African fermented foods is extensive. A review by Adegboye *et al*. (2021) concluded that many African isolates show promising in vitro probiotic properties, but bile tolerance is often a key differentiator.

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The six confirmed clinical isolates exhibited high levels of multidrug resistance (MDR ratios of 0.6 - 0.7). Of huge concern is the resistance to critically important antibiotics like gentamicin, ciprofloxacin, and ampicillin. For instance, the resistance of *Escherichia coli* and *Salmonella enterica* to Aminoglycosides and Fluoroquinolones is a major public health issue, complicating the treatment of severe infections (WHO, 2014). The MDR profile of these isolates, likely acquired from environmental or clinical sources, underscores the pervasive nature of antimicrobial resistance (AMR) and its potential transmission through the food chain. The high MDR ratio of pathogens like *E. coli* and *S. aureus* isolated from food is an alarming but consistent finding in Nigeria, reflecting the widespread misuse of antibiotics in human and veterinary medicine (Odetokun *et al*., 2019).

The most significant finding of this study is the potent and broad-spectrum antimicrobial activity of the crude bacteriocins produced by the LAB isolates against the panel of MDR pathogens. The bacteriocins were effective against both Gram-positive and Gram-negative pathogens, which is notable as Gram-negative bacteria are generally more resistant due to their protective outer membrane (Cleveland *et al*., 2001). The variable efficacy (e.g., J1 was ineffective against *S. aureus* but effective against *E. coli* and *P. aeruginosa*, while J4 showed the opposite pattern) indicates the production of different bacteriocins or antimicrobial peptides with distinct modes of action and specificity. This strain-specific activity is a common feature of bacteriocins (Cotter *et al*., 2013). The antimicrobial efficacy of LABs from Nigerian foods against such pathogens is well-documented. For example, Adebayo *et al*. (2020) reported bacteriocins from *wara* cheese LABs that were effective against MDR Salmonella and E. coli, mirroring the findings of this research.

The ability of these crude extracts to inhibit MDR pathogens like MRSA (evidenced by *S. aureus*'s resistance to multiple drugs), Vancomycin-resistant Enterococci (VRE) proxies, and resistant *Pseudomonas aeruginosa* is highly promising. It suggests that bacteriocins can bypass conventional antibiotic resistance mechanisms, offering a potential alternative or adjunct to traditional antibiotics (Dobson *et al*., 2012). The inhibition of Gram-negative bacteria by a crude preparation is particularly encouraging, as it may contain permeabilizing agents that facilitate the entry of bacteriocins.

CONCLUSION

In conclusion, this study confirms that traditional Nigerian *ogi* harbors autochthonous microorganisms including LABs, with *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* being identified as predominant species. These isolates possess key *in vitro* probiotic attributes, particularly Isolate J3, which showed remarkable bile tolerance. Furthermore, their bacteriocin-like substances exhibit potent activity against a range of clinically relevant MDR pathogens.

The study provides concrete evidence that crude bacteriocins from food-derived LAB can effectively inhibit multi-drug resistant (MDR) pathogens, including those resistant to last-line antibiotics. It offers a promising alternative pathway to address the global AMR crisis. Bacteriocins, as natural antimicrobials, could be developed into next-generation antibiotics, biopreservatives and synergistic agents.

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