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Research Article

Investigation of the Probiotic Potentials of Lactic Acid Bacteria Isolated from Palm Wine, Cabbage, Ogi, and Tiger Nuts

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ABSTRACT

Probiotics are live microorganisms which when consumed provide health benefits to the consumer. The aim of this study was to isolate and characterize lactic acid bacteria with probiotic potentials from fresh palm wine, fermented cabbage, ogi and tiger nuts collected from different locations in Port Harcourt. The samples were screened for the presence of lactic acid bacteria using standard microbiological techniques including genotypic techniques. A total of five presumptive lactic acid bacteria were identified. Two lactic acid bacterial isolates were obtained from palm wine, one each from cabbage, ogi and tiger nuts respectively. These isolates were further subjected to test for their probiotic potentials including tolerance to pH 2.0 and pH3.0, bile salts (0.3%, 1.0% and 2.0%), and antimicrobial activity against *Escherichia coli*, *Bacillus subtilis*, *Shigella flexneri*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Of the five isolates, 100%, 40% and 20% had over 80% survival rate when exposed to 0.3%, 1.0% and 2.0% bile salt respectively. All the isolates had over 80% survival rate in acidic conditions. The lactic acid bacterial isolates variedly inhibited 1 – 4 bacterial isolates. However, *Staphylococcus aureus* was inhibited by all the lactic acid bacterial isolates. The result of this study demonstrates the presence of probiotic lactic acid bacteria in palm wine, fermented cabbage, ogi and tiger nuts, which can be harnessed for the health benefits they provide.

Keywords: Lactic acid bacteria, *Lactobacillus*, probiotics, bile salt, antimicrobial activity, *S. aureus*

Introduction

The term "probiotic" was used for the first time by Lilly and Stillwell in 1965 to describe the secreted substances by a microorganism which triggers the growth of other microorganisms (Gupta and Garg, 2009). Probiotics are living microorganisms which provide health benefits to the consumer when consumed. This is because they promote the growth of microorganisms in the gut (Kechagia *et al*., 2013). Detrimental effects on the gastrointestinal tract flora have occurred as a result of the use of antibiotics, irradiation, as well as immunosuppressive therapy. The use of probiotics has been found to re-establish the equilibrium of the microbial flora in the gastrointestinal tract, thereby preventing disease. The microorganisms, *Lactobacillus* and *Bifidobacterium* are mainly used as probiotics (Vlasova *et al*., 2016).

Palm wine is a sweet, clear drink which contains lots of vitamins and minerals and serves as a medium for microorganisms such as lactic acid and acetic acid bacteria (Opara *et al*., 2013).

Ogi (or Akamu, called pap in English) is a fermented cereal food with custard-like consistency typically made from maize, sorghum, guinea corn or millet using yeast and lactic acid bacteria (Obire and Amadi, 2015). These microorganisms play a role in enhancing the flavour and aroma. Ogi is rich in minerals and carbohydrates and is eaten in Nigeria and across West Africa. (Omemu and Faniran, 2011; Obire and Amadi, 2015). Tiger nuts are consumed in Nigeria because of their high fiber, minerals and protein content. Fermented cabbage, otherwise known as sauerkraut contains a large amount of lactic acid bacteria which aid in the fermentation process.

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Sauerkraut is a great source of vitamins, fibre and minerals. This study aims to assess the probiotic potentials of the lactic acid bacteria isolated from these four sources (palm wine, ogi, tiger nuts and fermented cabbage).

Materials and Methods

Collection of Samples and Identification of *Lactobacillus* species

Fifteen samples of palm wine, five samples of cabbage, ten samples of Ogi and ten samples of tiger nuts were purchased and collected in sterile containers from different locations in Port Harcourt, Nigeria. The cabbage samples were fermented as described by Siddeeg *et al*. (2022) in order to prevent contaminants. The samples were transported to the laboratory, weighed to 1g and added into 9ml of 0.85% (w/v) normal saline in order to homogenize them. Serial dilution was carried out, followed by plating in De Man, Rogosa and Sharpe (MRS) agar medium. The plates were incubated anaerobically using an anaerobic jar and gas pak, at 30°C for 72 hours. Colonies formed were purified by sub-culturing them and identification was done by biochemical tests such as Gram stain, sugar fermentation, catalase and oxidase (Karami *et al*., 2017). The *Lactobacillus* cells were retrieved and resuspended as described by Lee *et al*., 2021. For molecular identification of the presumptive *Lactobacillus* species, genomic DNA was extracted by using ZR Fungal/Bacterial DNA Miniprep (manufactured by Zymo Research). Bacterial 16S rRNA gene sequences from each sample was amplified by PCR using the 27F-1525R primers. The sequencing kit used was BigDye terminator v3.1 cycle sequencing kit, while MEGA 6 and Bio-Edit software were used to carry out genetic analysis.

Evaluation of *Lactobacillus* Probiotic Potentials

In order to confirm the probiotic potentials of the *Lactobacillus* species, pH tolerance test, bile salt tolerance test, antibiotic susceptibility test and antimicrobial activity test were carried out.

The Resistance of *Lactobacillus* species to Different pH levels was determined by the inoculation of the *Lactobacillus* isolateinto MRS broth adjusted to pH 2.0, 3.0 and 6.4 using Hydrochloric (HCl) acid and incubated aerobically for 24 hours at 37°C. The MRS broth adjusted to pH 6.4 was used as the control. The optical density (OD) values at 600nm were measured at 0 hour and 4 hours using the VIS 721 spectrophotometer. The survival percentage of the *Lactobacillus* species to the different pH levels were calculated using the following formula:

$$\text{Survival rate (\%)} = \frac{\text{OD(After treatment)}}{\text{OD(Before treatment)}} \text{ x } 100$$

Isolates with over 80% survival rate were selected for further probiotic potential analysis (Mazlumi *et al*., 2022).

Resistance of *Lactobacillus* species to Different Bile Salt Concentrations

Bovine bile salts at 0.3, 1.0, and 2.0% w/v were added to MRS broth. The *Lactobacillus* species were cultured in the broth and incubated under aerobic conditions at 37°C for 24 hours. The MRS broth without bile salts was used as control. The optical density (OD) values at 600 nm were measured at 0 hour and 4 hours using the VIS 721 spectrophotometer. The survival percentage of the *Lactobacillus* species to the different bile salt concentrations were calculated using the following formula:

$$\text{Survival rate (\%)} = \frac{\text{OD(After treatment)}}{\text{OD(Before treatment)}} \text{ x } 100$$

Isolates with over 80% survival rate were selected for further probiotic potential analysis (Mazlumi *et al*., 2022).

Resistance of *Lactobacillus* species to Different Antibiotics

The antibiotic disc diffusion method was utilized for the determination of the antibiotic susceptibility pattern of the *Lactobacillus* species. The *Lactobacillus* species were inoculated by the spread plate method on the solidified surface of MRS agar medium.

The antibiotic discs were then placed in the plates and incubated for 48 hours at 37°C. Augmentin (30µg), vancomycin (30µg), ampicillin (10µg), tetracycline (10µg), erythromycin (5µg), gentamycin (10µg), ciprofloxacin (5µg), cefoperazone (µg), cephalexin (1.5µg), meropenem (10µg), cotrimoxazole (25µg), and cefuroxime (10µg) were the antibiotics used to assess the antibiotic susceptibility pattern of the *Lactobacillus* isolates.

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The inhibition zone diameter was measured and the results were presented as susceptible, intermediate, and resistant. The multiple antibiotic resistance index (MARi) of the isolates was also calculated using the formular:

$$\text{MAR index} = \frac{x}{y}$$

where "x" is the number of antibiotics that the isolate is resistant to, and "y" is the total number of antibiotics tested.

Assessment of Antimicrobial Activity

To assess antimicrobial activity, the *Lactobacillus* isolates were grown in MRS broth at 37 °C for 24 h, after which the fully grown cultures were centrifuged (3000 g, 4 °C for 45 min). The supernatant was separated and sterilized by passage through a 0.2-µm membrane filter (Whatman, Sigma-Aldrich, St. Louis, MO).

The sterilized supernatant was then tested against the pathogenic microorganisms isolated from the mice (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Shigella flexneri*). The antimicrobial activity of the *Lactobacillus* isolates with potential probiotic properties against these pathogens was tested using the agar well diffusion method, as per the method of Korhonen *et al*., 2010.

Statistical Analysis

All data in this study were expressed as mean ± standard deviation. Statistical analysis was carried out by two-way analysis of variance (ANOVA) to compute statistically significant differences at $p < 0.05$. Turkey's pairwise comparison was used to separate the means. All graphics were constructed using SPSS 16.0.

Results

The population and prevalence of the *Lactobacillus* species isolated from palm wine, fermented cabbage, tiger nuts and ogi were expressed as mean and standard deviation, and are presented in Table 1.

Among the four samples analysed, fermented cabbage had the highest count of *Lactobacillus* (7.71×10^5 CFU/g), while ogi had the least (2.21×10^5 CFU/g). Among the four *Lactobacillus* species, *L. fermentum* occurred most at 40.0%. On the other hand, *L. casei*, *L. delbrueckii* and *L. plantarum* occurred the least at 20.0% each.

The GenBank accession number and percentage of the identified *Lactobacillus* species is presented in Table 2.

Table 1: Population and prevalence of *Lactobacillus* species in cabbage, ogi, palm wine and tiger nuts samples

Samples	Population (CFU/g)	*L. casei*	*L. delbrueckii*	*L. fermentum*	*L. plantarum*
Cabbage	$7.71 \pm 1.8 \times 10^5$	1 (20.0%)	-	1 (20.0%)	-
Ogi	$2.21 \pm 1.8 \times 10^5$	-	-	1 (20.0%)	-
Palm Wine	$5.32 \pm 1.1 \times 10^5$	-	1 (20.0%)	-	-
Tiger Nuts	$1.75 \pm 0.5 \times 10^5$	-	-	-	1 (20.0%)
Total		**1 (20.0%)**	**1 (20.0%)**	**2 (40.0%)**	**1 (20.0%)**

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Table 2: Identified *Lactobacillus* species 16S rRNA Sequences Relatedness and their Assigned GenBank Accession Numbers

Isolate code	Tentative Identity	GenBank closest Relative	Relatedness (%)	GenBank Accession Number
Pa7	*Lactobacillus delbrueckii*	*Lactobacillus delbrueckii*	100	NR029106.1
Pa11	*Lactobacillus fermentum*	*Lactobacillus fermentum*	100	KI546172.1
Cab3	*Lactobacillus casei*	*Lactobacillus casei*	99.9	CP006690.1
Cab4	*Lactobacillus fermentum*	*Lactobacillus fermentum*	99.9	CP082359.1
Og1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	100	NR117813.1

The survival percentage of the *Lactobacillus* species at different bile salt concentrations is presented in Fig. 1. All the isolates had more than 80% survival rate when exposed to 0.3% bile salt concentration. The highest survival rate was recorded in Og1 (*L. plantarum*) at 94.53±1.36%, while the lowest was recorded in Pa11 (*L. fermentum*) at 82.17±0.83%. When exposed to 1.0% bile salt concentration, only Cab3 (*L. casei*) and Og1 (*L. plantarum*) had over 80% survival rate, at 83.53±1.08% and 81.30±0.90% respectively. Thus, Cab3 (*L. casei*) had the highest survival rate when exposed to 1.0% bile salt concentration, while Pa11 (*L. fermentum*) had the lowest survival rate at 65.53±2.08%. When exposed to 2.0% bile salt concentration, only Cab3 (*L. casei*) had over 80% survival rate, with the highest rate of 82.57±0.55%. The lowest survival rate at 2.0% bile salt concentration was recorded in Pa11 (*L. fermentum*) at 46.73±0.47%.

The survival percentage of the *Lactobacillus* species at different pH levels is presented in Fig. 2. When the isolates were exposed to pH 2.0, 3.0 and 6.4, they all had over 80% survival rate. When exposed to pH 2.0, 3.0, and 6.4, the isolate, Cab3 identified as *L. casei* had the highest survival rate at 85.97±2.15%, 94.53±1.23%, and 94.70±1.61% respectively. On the other hand, the isolate, Pa11 identified as *L. fermentum* had the lowest survival rate when exposed to pH 2.0, 3.0, and 6.4 at 81.63±1.40%, 84.67±2.63, and 89.17±2.11 respectively.

From the results of the antibiotic sensitivity presented in Table 3, all the *Lactobacillus* isolates (*L. casei, L. delbrueckii, L. fermentum* and *L. plantarum*) were susceptible to Tetracycline. All the *Lactobacillus* isolates were resistant to Vancomycin, Cephalexin, Meropenem and Ampicillin. The species, *L. delbrueckii* and *L. fermentum* were resistant to seven out of the twelve antibiotics tested for. On the other hand, the species, *L. casei* and *L. plantarum* were resistant to six out of the twelve antibiotics tested for. Thus, they all had MAR indices > 0.2.

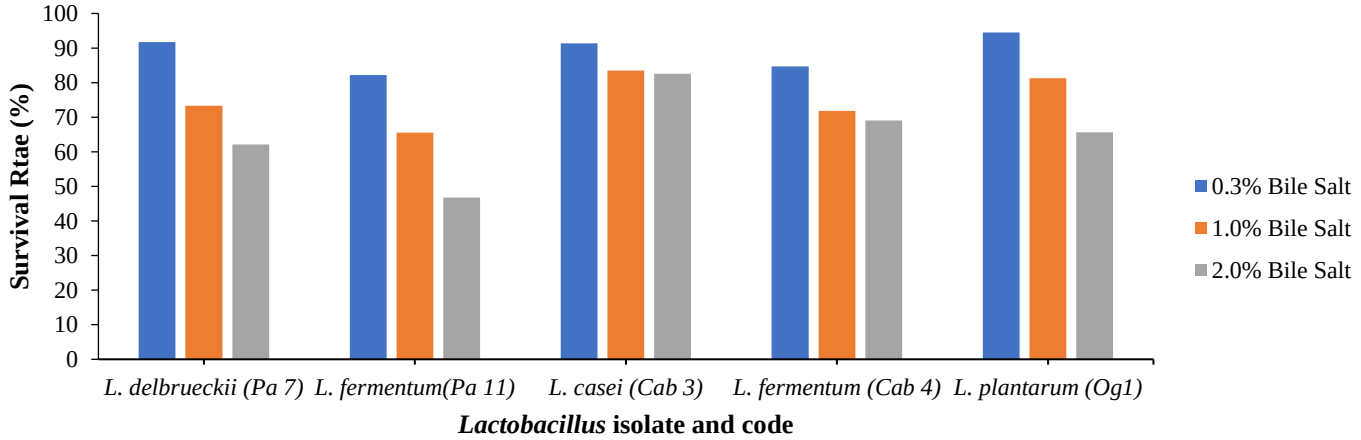

Fig. 1: Effect of different bile salt concentrations on the survival rate (%) of the *Lactobacillus* Isolates

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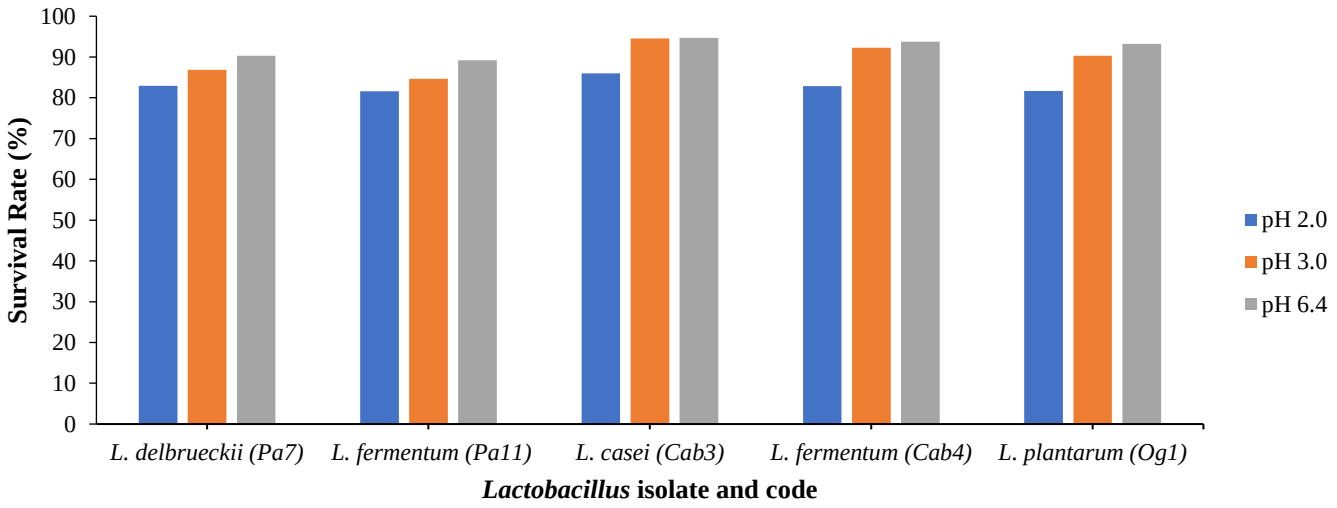

Fig. 2: Effect of different pH levels on the survival rate (%) of the *Lactobacillus* isolates

Table 3: Percentage Antibiotic Susceptibility Pattern of the *Lactobacillus* species

Antibiotics (conc.)	***L. casei*** **n = 1**	***L. delbrueckii*** **n = 1**	***L. fermentum*** **n = 2**	***L. plantarum*** **n = 1**
AUG (30µg)	S	R	I	I
VAN (30µg)	R	R	R	R
CPZ (10µg)	R	R	R	I
CP (1.5µg)	R	R	R	R
MEM (10µg)	R	R	R	R
ERY (5 µg)	S	I	S	S
TET (10µg)	S	S	S	S
COT (25µg)	S	I	S	I
CRX (10µg)	R	I	I	R
GEN (10µg)	I	R	R	R
CIP (5µg)	S	S	R	I
AMP (10µg)	R	R	R	R
MAR index	**0.50**	**0.58**	**0.58**	**0.50**

Key: AUG: Augmentin, VAN: Vancomycin, CPZ: Cefoperazone CP: Cephalexin, MEM: Meropenem, ERY: Erythromycin, TET: Tetracycline, COT: Co-trimoxazole, CRX: Cefuroxime, GEN: Gentamycin, CIP: Ciprofloxacin, AMP: Ampicillin, R: Resistant, I: Intermediate, S: Susceptible, MAR: Multiple antibiotic resistance

The antimicrobial activity of the *Lactobacillus* species is presented in Table 4. All the *Lactobacillus* species inhibited the growth of *Staphylococcus aureus*. The isolate, Cab4 identified as *L. fermentum* inhibited all the organisms expect *Pseudomonas aeruginosa*. Thus, *L. fermentum* Cab4 inhibited the highest number of organisms. On the other hand, the isolate, Cab3 identified as *L. casei* inhibited the least number of

organisms, as it only inhibited *Staphylococcus aureus*. The *L. fermentum* Pa11 and *L. plantarum* Og1 isolates both inhibited *Escherichia coli* and *Staphylococcus aureus*. However, *L. fermentum* Pa11 also inhibited *Pseudomonas aeruginosa*. The organisms, *S. aureus, Bacillus subtilis* and *Shigella flexneri* were inhibited by *L. delbrueckii* Pa7.

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Table 4: Assessment of Antibiosis as shown by their Zones of Inhibition in Diameter (mm)

***Lactobacillus* Species**	**Zone of Inhibition of *Lactobacillus* in Diameter (mm) against bacteria**				
	E. coli	***S. aureus***	***B. subtilis***	***P. aeruginosa***	***S. flexneri***
L. delbrueckii (Pa7)	0	13	10	0	10
L. fermentum (Pa11)	10	11	0	11	0
L. casei (Cab3)	0	14	0	0	0
L. fermentum (Cab4)	13	11	10	0	11
L. plantarum (Og1)	11	11	0	0	0
Ciprofloxacin (Control)	27	28	30	27	27

Discussion

The presence of gastric juice in the stomach and bile salts in the duodenum inhibit the action of bacteria (Singhal *et al*., 2010). The bile salts in the body cause damage to the fatty acids and lipids in the cell membrane, which reduces the survival rate of organisms. Thus, it is imperative that probiotics have the ability to survive in the presence of bile salts, as they pass through the duodenum where bile salt levels get as high as 0.7%. Probiotics should also be able to survive upon getting into the ileum where they are required to increase in population and carry out their functions (Byakika *et al*., 2020). These probiotics are able to survive in the presence of bile salts because they produce an enzyme called, bile salt hydrolase (BSH) which causes the conjugated bile salts to undergo hydrolysis. This decreases the toxicity of these bile salts towards the probiotics (Byakika *et al*., 2020). According to Byakika *et al.*, (2020), the pH level of the stomach could get as low as 3.0. Thus, it is essential for probiotics to survive at this low pH for 2-4 hours, which is the time it takes for food to digest and move from the stomach to the small intestine. The findings of this study are in agreement with a study by Nath *et al*., (2020), which reported optimal growth patterns of *L. plantarum* at pH 3.0 after 3 hours of incubation. The results of this study demonstrate that *L. plantarum* and *L. casei* have tolerance to bile salts and acidic pH. These results are in agreement with a study by Singhal, *et al*., (2010) that analysed the ability of *L. casei* and *L. plantarum* to tolerate bile salts and acidic pH.

All the *Lactobacillus* species showed resistance to three or more antibiotics, which indicates multi-drug resistance. The knowledge that antibiotic resistance genes could be transferred from probiotic bacteria to pathogenic bacteria makes it a worthwhile evaluation when choosing a probiotic (Khanal *et al*., 2019; Temmerman *et al*., 2003). Tetracycline inhibited all the *Lactobacillus* species, thus, giving a 100% inhibition rate. This is attributed to the fact that *Lactobacillus* species are susceptible to antibiotics that are involved in protein synthesis inhibition, such as tetracycline, chloramphenicol and lincomycin (Sharma *et al*., 2021). This result agrees with the study by Sharma *et al*., (2016), which also reported a low resistance to tetracycline.

Among the several mechanisms with which *Lactobacillus* species inhibit pathogens which include antimicrobial compounds production and substrate competition (Belicova *et al*., 2013, Byakika *et al*., 2020), the major mechanism that confers the antimicrobial ability to *Lactobacillus* species is the production of organic acids or antimicrobial compounds (Zago *et al*., 2011; Zhang *et al*., 2011). Lactic acid, hydrogen peroxide, acetic acid and bacteriocins are the antimicrobial compounds produced by *Lactobacillus* species that are inhibitory or toxic to pathogens. The amount of organic acid produced determines the degree of pathogen inhibition, and varies from one *Lactobacillus* strain to the other (Tejero-Sarinena *et al*., 2012).

In conclusion, the results of this study show that probiotics are present in sauerkraut (fermented cabbage), tiger nuts, palm wine and ogi. The phenotypically and genotypically identified *Lactobacillus* species isolated from the samples proved to have the required probiotic characteristics such as tolerance to bile salts, acidic conditions, antimicrobial activity and antibiotic susceptibility. Thus, the *Lactobacillus* species, *L. delbrueckii* NR029106.1, *L. fermentum* KI546172.1, *L. fermentum* CP082359.1, *L. casei* CP006690.1 and *L. plantarum* NR117813.1 all have good probiotic potential.

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