Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Apr 15; 10(A):623-633.
https://doi.org/10.3889/oamjms.2022.8338
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Microbiology*

Genotyping of Probiotic Lactobacilli in Nigerian Fermented Condiments for Improved Food Safety

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Edited by: Slavica Hristomanova-Mitkovska
Citation: Obafemi Y, Oranusi S, Oluseyi AK, Akinduti P. Genotyping of Probiotic Lactobacilli in Nigerian Fermented Condiments for Improved Food Safety. Open-Access Maced J Med Sci. 2022 Apr 15; 10(A):623-633. https://doi.org/10.3889/oamjms.2022.8338
Keywords: Fermented foods; Condiments; Genomics; *Lactobacillus* species; Probiotics; Starter culture
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Received: 20-Dec-2021
Revised: 31-Mar-2022
Accepted: 05-Apr-2022

Funding: This study was supported by Nutricia Research Foundation
Competing Interest: The authors have declared that no competing interest exists

Abstract

BACKGROUND: Plant-based naturally fermented condiments usually result in poor quality products with various bacteria and fungi contaminants. Previous reports suggested the use of starter cultures from previously fermented condiments in fermentation processes to ensure health-promoting benefits, improved quality, shelf life, and organoleptic properties for the achievement of healthy nutrition, safe, and quality food.

AIM: This study aimed to genotype potential lactobacilli from locally fermented condiments for improved food safety.

METHODS: The lactobacilli colonies isolated from fermented condiments purchased from food markets in Southwest Nigeria were profiled for probiotic activities, hemolytic activities, antibiotics susceptibility, and inhibitory activities against food pathogens. Interesting probiotic lactobacilli were identified using 16S rRNA gene sequencing and evaluated for phylogenetic relatedness with other globally reported probiotic lactobacilli.

RESULTS: Lactobacillus species which expressed significant probiotics, γ-hemolysis, anti-spoilage, and anti-listerial activities ($P < 0.05$) with tolerable safety profiles were identified as Lactiplantibacillus plajomi YD001 (MW280136), Lactiplantibacillus plantarum YD002 (MW280139), L. plantarum YD003 (MW280137), and Lacticaseibacillus paracasei YD004 (MW280138) possessed 50.75, 50.61, 50.75, and 52.54 mol% DNA G+C contents, respectively. The species clustered into different phylogroups with high clonal relatedness with other potential lactobacilli metadata (≥96.80%) obtained from the public repository.

CONCLUSION: Obtained genotyped Lactobacillus species are potential starter cultures for improved fermentation processes, control of food pathogens, and spoilage organisms.

Significance of the Study

- Probiotic *Lactobacillus* species were isolated from Nigerian fermented condiments
- New *Lactobacillus* species were suggested as starter cultures for food fermentation industries
- Biotechnological approach toward real-life sustainable development applications.

Introduction

Fermented condiments are rich sources of legume-based proteins that are usually consumed in Nigeria to obtain affordable dietary protein needed to ease malnutrition [1]. Fermented condiments [locust beans "*Iru*" or "*Dawadawa*" (*Parkia biglobosa*); oil beans "*Ugba*" (*Pentaclethra macrophylla*); and castor seeds "*Ogiri*" (*Ricinus communis*)] are usually added to improve sensory properties of food and soups in many Nigerian homes [2]. Other *Leguminosae* used to produce fermented condiments included African Yam beans "*Owoh*" (*Sphenostylis stenocardia*), cotton plant seeds (*Gossypium hirsutum*), melon seeds "*Ogiri*" (*Citrullus vulgaris*), fluted pumpkin seeds "*Ogiri*" (*Telfairia occidentalis*), and *Prosopis Africana* seeds "*Okpiye*" (*P. africana*) [3], [4], [5]. These seeds are naturally fermented to detoxify, improve digestibility, and improve nutritional components before human and animal consumption. However, improved nutritional, safety, and shelf-life quality would be achieved with the application of starter cultures in the production process. The presence of potentially probiotic *Lactobacillus* species has been reported perspective as their application as starter cultures in fermentation enhanced nutritional benefits, digestibility, accessibility, safety, and quality of fermented foods [6], [7], [8], [9], [10]. At present, fermented condiments have only achieved local commercial acceptance to due poor hygiene during processing, transportation, and storage [11], [12], [13]. Prolonged fermentation of protein components in these fermented condiments by fermenting microorganisms during the spontaneous fermentation produces protease enzyme

which breaks down the proteins resulting in pungent odor, sticky texture, and characteristic taste due to release of λ-polyglutamic acid metabolite [14], [15]. This study aims to substantiate the benefits of probiotic lactobacilli in Nigerian fermented condiments as potential starter cultures that would ensure health-promoting benefits, organoleptic properties, and improve shelf-life qualities of these condiments.

Materials and Methods

Sampling and proximate analyses of fermented condiments

Freshly fermented condiments [(African locust beans "Iru" (*P. biglobosa*), castor oil seed "Ogiri" (*R. communis*), and African oil beans "Ugba" (*P. macrophylla)*] were randomly purchased in triplicates from major local food markets in Southwest Nigeria between August 2019 and January 2020. The coordinates of the points of purchase in the markets were recorded using a global positioning system device as described [16] to substantiate the geographical area where samples were obtained in the Nigerian most popular food markets. All the samples were transported in the cold chain to the Microbiology Laboratory, Department of Biological Sciences, Covenant University, Ota, Ogun State, Nigeria, for further analysis. The fermented samples were evaluated for proximate composition including the moisture content, ash content, crude fiber, fat, protein, and carbohydrate to quantify the nutritional components in samples as described [17].

Identification and probiotic activities of Lactobacillus species

One gram from each of the fermented condiment samples was serially diluted and inoculated into enriched de Mann Rogosa and Sharpe (MRS) agar (BD BBL, Franklin Lakes, NJ, USA) and incubated anaerobically at 37°C for 48 h. Growth colonies were identified for the sugar fermentation profile as described [3]. Pure cultures of selected *Lactobacillus* isolates were slanted on MRS agar with 20% (v/v) glycerol to await further analysis.

Acid tolerance assay

Each *Lactobacillus* culture (10^8 CFU/ml) was inoculated into sterile MRS broth 1% (v/v) at pH 2, 3, 4, and 5.5 and the control had inoculum at pH 7. Each culture was incubated at 37°C for 6 h and absorbance was analyzed using a microtiter plate reader (Labtech Auto Elisa P, Italy) at 630 nm wavelength. Then, 100 μL of 24 h cultures at different pH were plated by dropping in sterile MRS agar. The plates were incubated at 37°C for 48 h and the colonies were enumerated as described [18]. The survival rate was calculated using Equation 1 below.

Bile tolerance assay

The tolerance of *Lactobacillus* species to bile salt at different concentrations was evaluated using 96-well microtiter plates as described [19]. The MRS broth was supplemented with sodium thioglycolate (Bio-basic, Canada) at different concentrations of 0.1%, 0.3%, 0.7%, and 1.0%. The 96 wells were inoculated with *Lactobacillus* cultures and incubated anaerobically at 37°C for 48 h. After incubation, the absorbance was measured using a microtiter plate reader (Labtech Auto Elisa P, Italy) at 630 nm wavelength. The control setup was without bile salts. Then, 100 μL of 24-h MRS broth at different bile concentrations were plated by dropping in sterile MRS agar. The plates were incubated at 37°C for 48 h and the colonies were enumerated using Equation 1 below.

Phenol tolerance assay

Phenol tolerance was performed using overnight cultures of the bacterial isolates inoculated 1% (v/v) into MRS broth with 0.1, 0.3, 0.5, and 1.0% (v/v) phenol for without phenol. After 24 h of incubation at 37°C, viable counts on the MRS agar plates were observed and counted. The absorbance of the culture at 630 nm was determined using a spectrophotometer (Thermo Fisher Scientific 5225, USA) [20].

Cholesterol reduction assay

Cholesterol assimilation of *Lactobacillus* species was evaluated using the quantitative determination of total cholesterol reagent 200 g/ml (Biolabo SAS, France) using 96-well microtiter plates as described [21]. The medium was then inoculated with each tested *Lactobacillus* culture and incubated anaerobically at 37°C for 24 h. After incubation, absorbance measuring the optical density of cell culture using a microtiter plate reader (Labtech Auto Elisa P, Italy) at 630 nm wavelength was compared with control (without cholesterol reagent). Then, 100 μL of the 24 h culture was inoculated on MRS agar. The plates were incubated anaerobically at 37°C for 48 h and the colonies were enumerated using the Equation 1 below:

$$\text{Survival Rate } (\%) = \frac{\text{Number of viable cells survived } (\text{CFU/ml})}{\text{Number of Initially viable cells inoculated } (\text{CFU/ml})} \times 100 \quad (1)$$

Hemolytic and antibiotic susceptibility assay

The hemolytic activities of each of the selected Lactobacillus species was investigated. Fresh Lactobacillus cultures was inoculated on blood agar and incubated at 37°C for 48 hours. The agar plates were examined for hemolysis around the colonies as described by Clinical and Laboratory Standards Institute [22]. *Lactobacillus* isolates were profiled for antibiotics susceptibility using the Kirby–Bauer disk diffusion method. Briefly, each isolate culture of 0.5 McFarland turbidity was spread on Mueller-Hilton agar. The antibiotic disk of ceftazidime (30 μg), cefuroxime (30 μg), cefixime (30 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), gentamicin (10 μg), nitrofurantoin (30 μg), and augmentin (30 μg) was added and incubated at 37°C for 24 h. The zone of inhibition obtained was measured and evaluated [23].

Antibacterial activities of Lactobacillus isolates against food pathogens

The antibacterial activity of *Lactobacillus* isolates was determined by the spot overlay method [24], [25]. *Lactobacillus* isolates were inoculated in MRS broth anaerobically at 37°C for 24 h. The 24 h culture of each isolate was spotted sterile MRS agar and incubated anaerobically at 37°C for 24 h. Foodborne pathogens (indicator organisms) including *Listeria monocytogenes* DPC6579*, Pseudomonas aeruginosa* DPC6054, *Staphylococcus aureus* DPC7016*, Hafnia alvei* ATCC13337*,* and *Escherichia coli* ATCC20133 were cultured in Trypticase soy broth (10^5 CFU/ml) and incubated at 37°C for 24 h. About 100 μL of indicator cell culture were mixed with 10 ml of soft top agar (0.8% w/v sloppy agar) and used to overlay the spotted *Lactobacillus* isolates. The top agar was allowed to solidify and then incubated at 37°C for 24 h. The zones of inhibition around the spotted *Lactobacillus* isolates were recorded.

Genotyping and phylogenetic analysis of Lactobacillus species

Genomic DNA from *Lactobacillus* isolates was purified using a GenElute DNA extraction kit (Sigma-Aldrich, USA) following manufacturers' instructions. PCR amplification of 16S rRNA gene of selected *Lactobacillus* isolates was performed using the primers: 16SF: 5`-AGTTTGATCCTGGCTCAG-3` and 16SR: 5`-TACCTTGTTACGACTT-3` and carried out in a Hybrid PCR express unit (Hybrid Ltd., Middlesex, UK) at an initial denaturation temperature of 94°C for 5 min for one cycle which was followed by 35 cycles of denaturation at 95°C for 1 min. Annealing was at 55°C for 45 s and extension was at 72°C for 1 min. The final extension was carried out at 72°C for 7 min [26], [27]. DNA Amplicons were resolved on 1.0% agarose gel and electrophoresis was carried out at 120 V for 40 min. The DNA bands were visualized on agarose gel stained with SYBR Green with 1.0 kbp DNA weight marker (Solis BioDyne, USA) on photo-documented UV light. Amplicons were purified with a QIAquick PCR purification kit (Qiagen, USA) and sequenced using the ABI Prism Big Dye Terminator version 3.0 sequencer [28], [29]. Partial 16S rRNA gene sequences were blasted with Basic Local Alignment Search Tool concerning *Bacillus*, *Lactobacillus,* and *Lactococcus* genomes in NCBI GenBank, and DNA G+C contents were calculated [30], [31]. The 16S rRNA sequences obtained from probiotic lactobacilli were evaluated for phylogenetic relatedness with globally reported probiotics *Lactobacillus* species from fermented foods in the GenBank database. Closely related strains were decided using manual alignment with homologous sequences curated into the multiple sequence alignment in MEGA X software version 7 (http://www.megasoftware.net) to construct the phylogenetic trees using the neighbor-joining method considering the maximum parsimony and maximum-likelihood analysis with bootstrap consensus tree deduced from 1000 replicates [32], [33].

Statistical analysis

All experiments and measurements were done in triplicate. The results are expressed on a dry matter basis as mean ± standard deviation. The significance of the probiotic potential isolates was analyzed using SPSS Software version 20.0 using Chi-square at $P < 0.05$ to evaluate survival rates at different conditions while the antibiogram profile data were analyzed with descriptive statistical methods.

Results and Discussion

Geospatial distribution and nutritional composition of fermented condiments

This study aimed to genotype potential lactobacilli strains from the Nigerian fermented condiments and substantiates their potential use as starter cultures in the fermentation of Nigerian indigenous plant-based condiments. Fermented condiments (Figure 1) were purchased from eighteen major Nigerian food markets located on lat. 7.2571 ± 1.500° and long. 5.2058 ± 1.5500° (Figure 2). The markets served as a place where the farm produce and other locally produced food materials were displayed by consumers.

Proximate analysis of fermented condiments

The nutritional compositions in sampled fermented condiments are shown in Table 1. Percentage

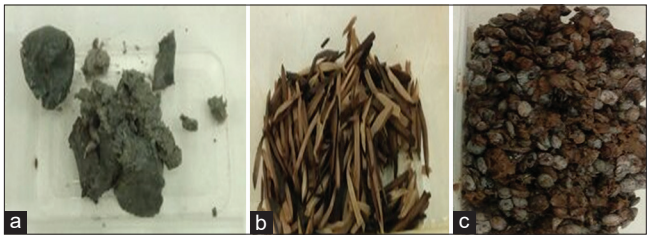

Figure 1: Retailed fermented condiments in Southwest Nigeria. (a = Castor oil seed "Ogiri" (Ricinus communis); B = African oil beans "Ugba" (Pentaclethra macrophylla); C = African locust beans "Iru" (Parkia biglobosa)

moisture content in the fermented condiments ranged 39.60 ± 0.42% – 52.82 ± 0.01% with fermented African locust beans (*Iru*) having significantly high moisture content (52.82 ± 0.01%). There was also moderate to low moisture content in fermented castor oil seed (*Ogiri*) and fermented oil beans (*Ugba*) as 39.60 ± 0.42% - 49.83 ± 0.42%, respectively. The percentage fat content in the samples ranged between 8.84 ± 0.02% and 16.51 ± 0.01%. The result revealed African locust beans (*Iru*) had high-fat content (16.51 ± 0.01%), fermented oil beans (*Ugba*) and castor oil seeds (*Ogiri*) had low-fat content 9.81 ± 0.01% and 8.84 ± 0.02%, respectively.

Table 1: Nutrient composition in selected fermented condiment samples retailed in Southwest Nigeria

Macromolecules	Percentage composition (%) Mean ± S. D			p-value
	Iru	Ogiri	Ugba	
Moisture content	52.82 ± 0.01^{c}	49.83 ± 0.21^{b}	39.60 ± 0.42^{a}	0.001
Fat content	16.51 ± 0.01^{c}	8.84 ± 0.02^{a}	9.81 ± 0.01^{b}	0.001
Crude fiber	4.12 ± 0.02^{c}	1.45 ± 0.04^{a}	2.21 ± 0.01^{b}	0.001
Crude protein	22.58 ± 0.01^{b}	22.02 ± 0.02^{a}	36.28 ± 0.02^{c}	0.001
Ash content	3.98 ± 0.01^{c}	1.65 ± 0.02^{a}	2.32 ± 0.02^{b}	0.001
Carbohydrate	16.05 ± 0.01^{b}	26.46 ± 0.02^{c}	15.55 ± 0.07^{a}	0.001

Iru: *Parkia biglobosa* (African locust beans), Ogiri: *Ricinus communis* (castor oil seed), Ugba: *Pentaclethra macrophylla* (African oil beans), S.D: Standard deviation. Values with superscripts ^{a,b,c}within the same row show significant differences.

The fermented condiment samples had very low crude fiber content with *Iru*, *Ogiri,* and *Ugba* containing 4.12 ± 0.02%, 1.45 ± 0.04%, and 2.21 ± 0.01%, respectively. Furthermore, fermented condiment the samples had significantly moderate crude protein content with *Iru*, *Ogiri,* and *Ugba* containing 22.58 ± 0.01%, 22.02 ± 0.02%, and 36.28 ± 0.02%, respectively. The fermented condiment samples used in this study had significantly low ash content with *Iru*, *Ogiri,* and *Ugba* containing 3.98 ± 0.01%, 1.65 ± 0.02%, and 2.32 ± 0.02%,

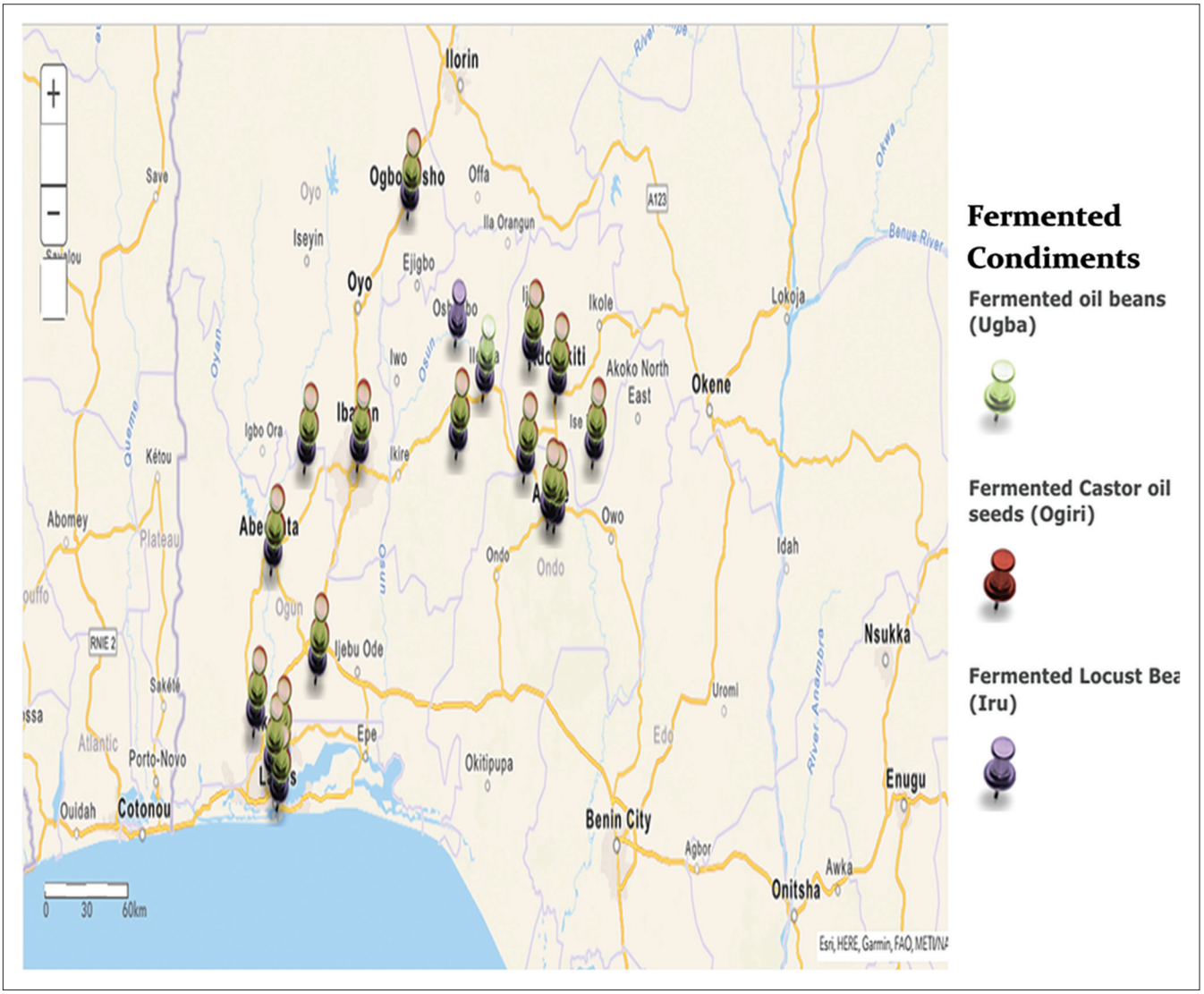

Figure 2: Geospatial mapping of sampled food markets in southwest, Nigeria (Map Attribution: www.esri.com)

respectively. There was significantly moderate carbohydrate content in the condiments with *Iru*, *Ogiri,* and *Ugba* containing 16.05 ± 0.01%, 26.46 ± 0.02%, and 15.55 ± 0.07%, respectively. Significantly high moisture content in fermented condiments in this study is in agreement with previously reported studies [34], [35] resulting from absorption of moisture during boiling, fermentation, and hydrolytic breakdown of the seed component. Observed crude protein content in this study was in agreement with the previous reports indicating proteolysis of the legume seeds amino by secretion of protease enzyme by fermenting microorganisms during the fermentation of these condiments [36]. Variations in carbohydrate contents in sampled fermented condiments resulted in conversion of complex oligosaccharides into simple utilizable sugars by biochemical activities of microbiota in the samples [37]. Similar carbohydrate contents in fermented locust beans, soybeans, and other fermented condiments containing carbohydrates classes (including stachyose, melibiose, raffinose, starch) was reported in Nigeria [38]. In addition, similar low ash content in fermented locust beans and soybean condiments was reported [37], [38]. Varied ash content is an indication of mineral abundance in the samples food was attributed to addition of local ash powder to raw seeds during processing of condiments in some local communities. Crude fiber content in analysed samples was lower than values previously reported for fermented locust bean, mung beans and soybean condiments [37], [38]. Observed low ash contents observed in the samples similarly reported in fermented locust beans, soybean, and “Soumbala” (commonly consumed fermented locust beans in Burkina Faso) due to the level of mineral abundance in the food products could be attributed to addition of local ash powder during processing of raw seeds [39]. High-fat content in Soumbala <40.47% was attributed to hydrolysis of fat contents in the seeds as reported to improve the organoleptic properties of food products [40]. Observed low crude fiber contents was, however, lower than previously reported values in fermented condiments as a result of processing methods of the legume-based seeds [41], [42].

Identification of Lactobacillus isolates

The isolated colonies appeared creamy in color with smooth edges on Difco™ Lactobacilli MRS agar (BD Biosciences, Ireland). Selected colonies were circular, with approximately 0.4–2.0 mm in diameter, 1.0–3.0 mm in length, and grew at pH 2.0–8.0 and optimum growth temperature of 30–37°C in anaerobic conditions for 24–48 h. Isolates were Gram-positive rod-shaped, catalase-negative, oxidase-negative, non-motile, facultative anaerobes, and non-spore-forming. Gas was not produced during the fermentation of glucose, lactose, and sucrose; however, gas production was observed with fermentation D-glucose, D-galactose, D-fructose, D-mannitol, and D-sorbitol (Table 2). Four *Lactobacillus* species found to be anaerobic, Gram positive, bacilli, catalase and oxidase negative, non-spore forming, and non-motile were isolated from Nigerian fermented condiments derived from leguminous seeds. The isolates exhibited morphologically, and biochemical resemblance with other previously reported probiotic strains of *Lactobacillus plajomi*, *Lactobacillus plantarum*, and *Lactobacillus paracasei* which were isolated from a wide variety of fermented foods [29], [43], [44].

Probiotic activities of Lactobacillus isolates

Selected *Lactobacillus* isolates from fermented condiments significantly exhibited above 89.0% survival rate in acidic media ranging from pH =2.0 and pH =5.5 for at least 6 h. The isolates survived the extremely acidic environment (pH =2.0) and had more survival rates in other acidic environments (Table 3). The isolates exhibited potential to survive in the GI tract at pH =3.0 with food passage for about 3 h. The result revealed these *Lactobacillus* isolates exhibited significantly above 98% survival rate in 0.3% bile and 100% survival rate in 1.0% bile after 6 h of incubation. Reduction of the fatty acids and lipid content in the cell membrane which eventually decreases the survival rate of bacteria in extreme bile environments (Bile =1.0%) was not observed in the studied isolates (Table 3). Selected *Lactobacillus* isolates exhibited ≥97.0% survival rate at $P < 0.05$ in 0.1% phenol concentration and ≥96% survival rate in 1.0% phenol concentration after 24 h. The presence of toxic metabolites in the cell membrane which may significantly decrease bacteria survival rate in extreme phenol environments (Phenol =1%) was not observed in the selected isolates (Table 3). The selected *Lactobacillus* isolates showed a good ability

Table 2: Phenotypic identification of *Lactobacillus* species in fermented condiments

Isolate code	Morphology	Gram’s reaction	Catalase	Oxidase	Coagulase	Citrate	Urease	MR-VP	Motility	Spore presence	Lactose	Maltose	Glucose	Sucrose	Mannitol	Identity of LAB isolates
LB004	Milk irregular, flat	Gram-positive rod diplobacilli	-	-	-	-	-	-	-	Ab	AG	AN	AN	AG	NR	*Lactobacillus* sp.
LB005	Milk irregular, flat	Gram-positive rod diplobacilli	-	-	-	-	-	-	-	Ab	AG	AN	AN	AG	NR	*Lactobacillus* sp.
LB006	Milk irregular, flat	Gram-positive rod diplobacilli	-	-	-	-	-	-	-	Ab	AG	AN	AN	AG	NR	*Lactobacillus* sp.
LB010	Milk irregular, flat	Gram-positive rod diplobacilli	-	-	-	-	-	-	-	Ab	AG	AN	AN	AG	NR	*Lactobacillus* sp.

+: Positive, -: Negative, Ab: Absent, Pr: Present, AN: Acid no gas, AG: Acid and gas production, NR: No reaction.

Table 3: Probiotic activities of *Lactobacillus* species

Probiotic properties	Parameter	Survival rate of *Lactobacillus* isolates (%) ± standard deviation (SD)						
		LB004	LB005	LB006	LB010	LB6680	95% CI	p-value
Acid tolerance	pH=2	103.98 ± 0.01^{b}	196.96 ± 0.07^{e}	102.72 ± 0.07^{a}	127.67 ± 0.02^{d}	126.78 ± 0.01^{c}	105.70-157.54	0.001
	pH=3	77.45 ± 0.02^{a}	140.85 ± 0.02^{e}	85.94 ± 0.07^{b}	109.81 ± 0.02^{c}	120.72 ± 0.08^{d}	89.56-124.34	0.001
	pH=4	77.45 ± 0.04^{a}	127.05 ± 0.08^{d}	90.96 ± 0.01^{b}	101.43 ± 0.01^{c}	116.74 ± 0.09^{d}	89.38-116.08	0.001
	pH=5.5	87.17 ± 0.03^{a}	112.21 ± 0.01^{d}	109.89 ± 0.01^{c}	103.77 ± 0.02^{b}	111.85 ± 0.03^{d}	97.88-112.08	0.001
Bile tolerance	Bile=0.1%	129.05 ± 0.07^{e}	102.88 ± 0.01^{b}	77.15 ± 0.02^{a}	119.40 ± 0.02^{c}	120.01 ± 0.01^{d}	95.87 – 123.52	0.002
	Bile=0.3%	135.64 ± 0.01^{e}	120.25 ± 0.01^{b}	118.27 ± 0.01^{a}	128.10 ± 0.07^{d}	125.05 ± 0.04^{c}	120.80 – 130.07	0.001
	Bile=0.7%	137.95 ± 0.01^{e}	101.30 ± 0.01^{b}	80.91 ± 0.01^{a}	120.14 ± 0.01^{c}	128.03 ± 0.04^{d}	98.82 – 128.98	0.004
	Bile=1.0%	137.96 ± 0.01^{e}	89.09 ± 0.01^{b}	88.97 ± 0.01^{a}	114.95 ± 0.02^{c}	126.82 ± 0.03^{d}	96.64 – 126.47	0.001
Phenol tolerance	Phenol=0.1%	122.81 ± 0.01^{e}	105.64 ± 0.01^{b}	110.81 ± 0.01^{c}	101.31 ± 0.01^{a}	116.14 ± 0.01^{d}	105.62-117.06	0.001
	Phenol=0.3%	112.26 ± 0.07^{e}	101.05 ± 0.07^{c}	93.85 ± 0.01^{a}	100.94 ± 0.01^{b}	105.47 ± 0.01^{d}	98.15-107.28	0.001
	Phenol=0.4%	118.48 ± 0.07^{c}	123.76 ± 0.01^{e}	102.79 ± 0.01^{a}	123.61 ± 0.01^{d}	110.35 ± 0.07^{b}	109.50-124.81	0.009
	Phenol=1.0%	104.10 ± 0.07^{e}	101.06 ± 0.07^{d}	84.91 ± 0.01^{a}	100.75 ± 0.01^{b}	100.91 ± 0.01^{c}	91.10-104.41	0.001
Cholesterol assimilation	200mg/dl	93.88 ± 0.03^{d}	90.56 ± 0.02^{b}	90.57 ± 0.00^{c}	90.29 ± 0.01^{a}	95.55 ± 0.07^{e}	90.55 – 93.79	0.001

S.D: Standard deviation. Values with superscripts ^{a,b,c}within the same row show significant difference at $P<0.05$.

to assimilate cholesterol after 24 h of incubation. The isolates significantly assimilated the cholesterol at $P < 0.05$ with a survival rate ≥90.0% (Table 3). The initial study for screening of *Lactobacillus* isolates for probiotics activities to mimic extreme environments found in the human gut system. These conditions may inhibit the growth of probiotic bacteria, including low pH, bile salts tolerance, phenol tolerance, and good cholesterol assimilation capabilities. *Lactobacillus* isolates were tolerant to pH ≥2.0, 1.0% bile salts, 1.0% phenol, and 200 mg/dl cholesterol with over 80% survival rate in agreement with studies previously reported [45], [46], [47], [48].

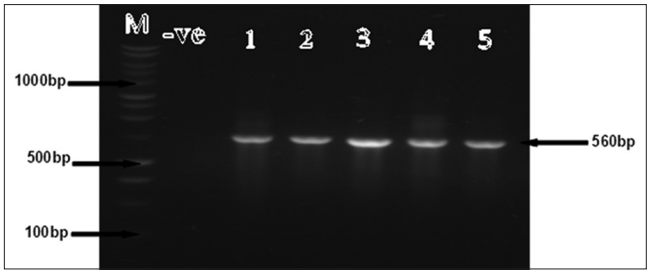

Figure 3: Gel electrophoresis of 16S rRNA genes obtained from polymerase chain reaction amplicons of Lactobacillus species.

Hemolytic and antibiotics susceptibility assay

The selected *Lactobacillus* isolates exhibited γ-hemolytic activities (no zones of inhibition) on blood agar. This signifies an absence of virulence activities in the isolates which may be a significant criterion for potential probiotic strains selection (plates not shown). The result also revealed generally regarded as safe status of isolated *Lactobacillus* isolates as implicated by γ-hemolysis (no hemolysis) on blood agar. The negative hemolytic result signifies an absence of virulence factors in the isolated *Lactobacillus* species which may be a crucial benchmark for potential probiotic strains selection. Our result supported other previous reports showing negative hemolysis in many *Lactobacillus* species [49], [50], [51]. *Lactobacillus* isolates were sensitive (with the zone of inhibition ≥20 mm in diameter) to erythromycin 5 μg, ceftriaxone 30 μg, cefuroxime 30 μg, gentamicin 10 μg, and ofloxacin 5 μg. Moderate susceptibility to potentially acquired resistance to other antibiotics such as ceftazidime 30 μg, cloxacillin 5 μg, and amoxycillin clavulanic acid 30 μg which protect activities of β-lactamase enzymes was also observed (Table 4). Susceptibility to inhibitors of cell wall synthesis and protein synthesis by these bacteria served as important selection criteria for the safety evaluation of potential probiotic bacteria for biotechnological applications. Our result was in agreement with the previous reports revealing the antibiotic sensitivity of selected *Lactobacillus* isolates to antibiotics such as erythromycin 5 μg, ceftriaxone 30 μg, cefuroxime 30 μg, gentamicin 10 μg, and ofloxacin 5 μg [52]. Moderate susceptibility observed to other antibiotics such as ceftazidime 30 μg, cloxacillin 5 μg, and amoxycillin clavulanic acid 30 μg which protected activities of β-lactamase enzymes observed among selected *Lactobacillus* species was also observed in the previous reports [53], [54].

Table 4: Antibiotic susceptibility profile of *Lactobacillus* isolates

Antibiotics	Concentration (μg/disk)	*Lactobacillus* isolates			
		LB004	LB005	LB006	LB010
Ceftazidime (CAZ)	30.00	R	R	R	R
Cefuroxime (CRX)	30.00	R	R	R	S
Ceftriaxone (CTR)	30.00	S	I	S	S
Ofloxacin (OFL)	5.00	R	R	R	S
Ciprofloxacin (CIP)	5.00	R	R	R	I
Cloxacillin (CXC)	5.00	R	R	R	R
Amoxycillin clavulanic acid (AUG)	30.00	R	R	R	I
Erythromycin (ERY)	5.00	S	S	S	S
Gentamicin (GEN)	10.00	R	R	R	S

The zone of inhibitions was interpreted as R (Resistance) = 0–14 mm; I (Intermediate) = 15–19 mm; S (Sensitive) = Above 20 mm

Antimicrobial activities of Lactobacillus species

Lactobacillus isolates were selected based on their ability to produce a maximum radial zone of inhibition (≥20 mm) when overlaid with sloppy agar seeded with indicator strain such as *L. monocytogenes* DPC6579, *P. aeruginosa DPC6054*, *S. aureus* DPC7016, *H. alvei* ATCC 13337, and *E. coli* ATCC2013 (Table 5). *Lactobacillus* isolates produced antimicrobial substances that inhibited the growth of food pathogens during the fermentation of food products. Our result revealed the inhibitory activities of the identified probiotic lactobacilli strains on the growth of common food pathogens including *L. monocytogenes DPC6579*,

Table 5: Antimicrobial activities of selected *Lactobacillus* isolates against food pathogens

Lactobacillus species	Mean zone of Inhibition (mm) ± SD					p-value
	Listeria monocytogenes DPC6579	*Pseudomonas aeruginosa* DPC6054	*Staphylococcus aureus* DPC7016	*Hafnia alvei* ATCC13337	*Escherichia coli* ATCC20133	
LB004	25.0 ± 0.5^{e}	21.0 ± 0.1^{c}	24.0 ± 0.6^{d}	13.0 ± 0.3^{a}	15.0 ± 0.5^{b}	0.001
LB005	24.0 ± 0.1^{d}	20.0 ± 0.1^{c}	25.0 ± 0.5^{e}	15.0 ± 0.1^{b}	12.0 ± 0.2^{a}	0.034
LB006	25.0 ± 0.2^{d}	26.0 ± 0.3^{e}	24.0 ± 0.3^{c}	19.0 ± 0.2^{b}	10.0 ± 0.7^{a}	0.002
LB010	23.0 ± 0.4^{c}	24.0 ± 0.4^{d}	25.0 ± 0.4^{e}	14.0 ± 0.1^{a}	18.0 ± 0.3^{b}	0.002

S.D: Standard deviation. Values with superscripts ^{a,b,c,d,e}within the same row show significant difference at $P<0.05$.

P. aeruginosa DPC6054, *S. aureus* DPC7016, *H. alvei* ATCC 13337, and *E. coli* ATCC20133 by the production of acidic organic metabolites into the surrounding medium; however, there was no inhibition against closely related *Lactobacillus* strains including *L. plantarum* DPC6730. Our result is related to some previous reports showing the antimicrobial activities of some *Lactobacillus* species such as *L. plantarum*

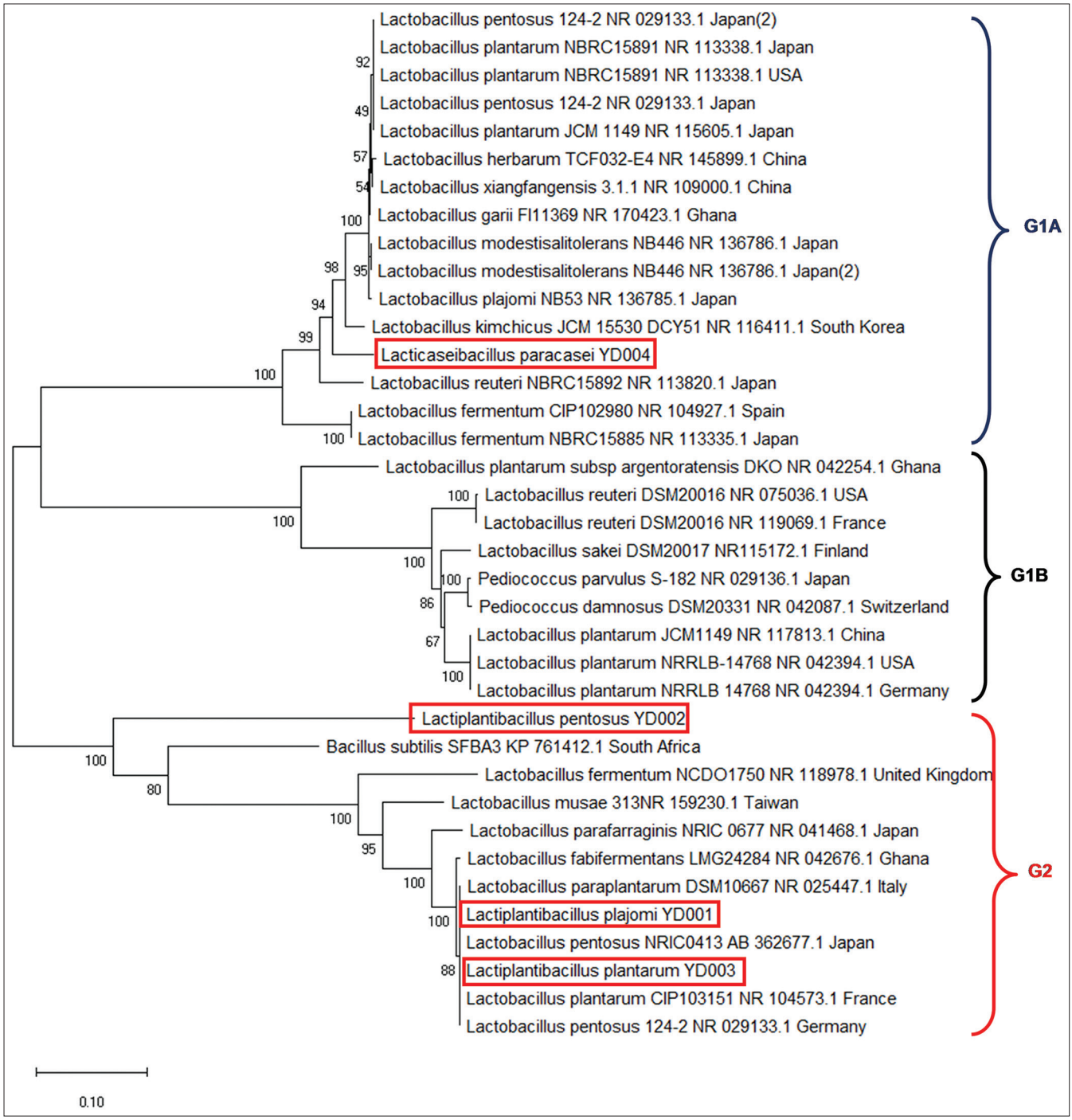

Figure 4: Phylogenetic tree constructed using neighbor-joining algorithm in MEGA X with 1000 bootstrap replicates to compare isolated with related Lactobacillus species from based on 16S rRNA gene sequences. G1–G2 represents the diversity of groups while taxa in red represent Lactobacillus species isolated from Nigerian fermented condiments

GCC_19M1 and *L. plantarum* isolated from fermented milk and vegetable, respectively [28], [55], [56], [57].

Genotyping of Lactobacillus species

Purified DNA concentrations of >50 ng/μL and A280 / 260 ratio >1.60 were used for the PCR amplification. High-molecular-weight bands observed on agarose gel. A negative control (molecular grade water) revealed no band (-ve) while bands in lane 1 are specific for the positive control (*L. plantarum* DPC 6682) and lanes 2–5 confirmed that the other four isolates belong to *Lactobacillus* genus (Figure 3). Analysis of partial 16S rRNA gene sequence identified isolates LB004, LB005, LB006, and LB010 as *Lactiplantibacillus plajomi* YD001 (MW280136), *Lactiplantibacillus plantarum* YD002 (MW280139), *L. plantarum* YD003 (MW280137), and *Lacticaseibacillus paracasei* YD004 (MW280138), respectively (Table 6).

Table 6: Genotypes of *Lactobacillus* species isolated from Nigerian fermented condiments

Isolate ID	Food Source	NCBI GenBank closest match	Sequence similarity index (%)	Identity	G+C (%)	Accession number
LB004	*Iru*	*Lactobacillus plajomi* NB53	99.80	*Lactiplantibacillus plajomi* YD001	50.75	MW280136
LB005	*Ugba*	*Lactobacillus plantarum* R12	96.68	*Lactiplantibacillus plantarum* YD002	50.61	MW280139
LB006	*Iru*	*Lactobacillus plantarum* JCM 1149	99.80	*Lactiplantibacillus plantarum* YD003	50.75	MW280137
LB010	*Ogiri*	*Lactobacillus paracasei* NBRC 15906	99.18	*Lacticaseibacillus paracasei* YD004	52.54	MW280138

Species identity based on new *Lactobacillus* species reclassification 2020.

Phylogenetic relatedness of Lactobacillus species with other probiotic isolates

Phylo-diversity of isolated *Lactobacillus* species was grouped into three groups (G1A, G1B, and G2) with 28 nucleotide sequences of probiotic lactobacilli isolated from different parts of the world (GenBank database) (Figure 4). There was a close genetic relationship between *L. paracasei* YD004 (MW280138) and other *Lactobacillus* species in Group 1A and Group 1B while *L. plajomi* YD001 (MW280136), *L. plantarum* YD002 (MW280139), and *L. plantarum* YD003 (MW280137) were closely related with reported *Lactobacillus* and *Bacillus* species in Group 2. These groups of probiotic lactobacilli were reported to have health-promoting benefits and were suggested as potential starter cultures for biotechnology processes. The 16S rRNA gene sequence from these isolates LB004, LB005, LB006, and LB010 identified as *L. plajomi* YD001 (MW280136), *L. plantarum* YD002 (MW280139), *L. plantarum* YD003 (MW280137), and *L. paracasei* YD004 (MW280138); exhibited high similarity with *L. plajomi* NB53 (99.80%), *L. plantarum 012 (*96.80%), *L. plantarum* JCM 1149 (99.80%), and *L. paracasei NBRC 15906 (*99.18%). The DNA G+C contents were 50.75, 50.61, 50.75, and 52.54 mol% for isolates LB004, LB005, LB006, and LB010, respectively. Phylogenetic analysis showed that selected *Lactobacillus* strains from Nigeria fermented condiments were highly related with other 28-nucleotide sequences of probiotic lactobacilli obtained from the public repository. It was also observed that *L. paracasei* YD004 (MW280138) was closely related with *Lactobacillus* species in Group 1A and Group 1B while *L. plajomi* YD001 (MW280136), *L. plantarum* YD002 (MW280139), and *L. plantarum* YD003 (MW280137) were also closely related with other previously identified *Lactobacillus* and *Bacillus* species in Group 2. All of these grouped probiotic lactobacilli were reported to possess health-promoting benefits and suggested as potential starter cultures for biotechnology processes [58], [59], [60], [61], [62], [63].

Conclusion

The results presented in this study revealed that genotyped *L. plajomi* YD001 (MW280136), *L. plantarum* YD002 (MW280139), *L. plantarum* YD003 (MW280137), and *L. paracasei* YD004 (MW280138) isolated from Nigerian fermented condiments could be probiotic safe species that can be used as potential starter cultures for fermentation processes and biological preservatives in foods. This study affirms our positive contribution toward the enhancement of real-life sustainable development in Nigeria through the provision of healthy nutrition and empowerment initiatives for the low-skilled women who are major producers of these condiments.

Authors' Contributions

Background concept: YDO and SUO; sample collection and experimental design: YDO and PAA, manuscript preparation and editing: YDO and PAA; manuscript reviewing: PAA, SUO, and KOA; and supervision: SUO and KOA. All authors read and approved the final manuscript.

Acknowledgments

The authors hereby appreciate the fellowship funding received from the Nutricia Research Foundation, Netherland (Award 2020-T1) to genotype the probiotic lactobacilli used for this study. We thank the Covenant University Centre for Research Innovation

and Development (CUCRID), Nigeria for provision of publication support for this manuscript.

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