Isolation and Characterization of Probiotic Lactic Acid Bacteria from Fermented Foods Using Conventional and Molecular Methods

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Abstract: The study aimed to isolate and characterize probiotic lactic acid bacteria from fermented foods using conventional and molecular methods. The isolation was done using De Man, Rogosa, and Sharpe (MRS) agar using standard procedure and standard biochemical tests for the identification of lactic acid bacteria. The isolates were confirmed using polymerase chain reaction (PCR), sequence analysis and comparison with Genbank resources were conducted to identify species levels. The isolates were evaluated for their probiotic potential using low pH tolerance, bile tolerance, and hemolytic tests, some antibiotic susceptibility pattern and antibacterial activity against enteric pathogens. The isolates identified were rod/bacilli and cocci, all Gram positive, but catalase, citrate, hydrogen sulfide (H_2S), indole, urease, Voges-Proskauer negative, and no NH_3 production from arginine. The isolates were positive for methyl-red and bile esculin salt. The sugar fermentation profile of eight different sugars and Molecular analysis identifies the isolates, which were found to be *Lactobacillus plantarum* in Kunun-Zaki, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* in Nono, *Streptococcus thermophilus* and *Lactobacillus bulgarus* in Yoghurt. Phylogenic tree revealed the isolates were related to the reference strain in the Genbank. The study showed that all the isolates were able to survive at low pH levels, tolerate bile concentrations, adhere to cover slips, were non-hemolytic and were susceptible to majority of the antibiotic tested; also the isolates had antagonistic activity against *Escherichia coli* and *Salmonella* Typhi, indicating their potential as probiotics.
Key word: Fermented foods, *Lactobacillus* species, probiotic, *Streptococcus thermophilus*

INTRODUCTION

Lactic acid bacteria (LAB) have been a crucial component in the fermentation process for centuries, contributing significantly to the development of flavor and chemical changes in various fermented foods. The term "Lactic Acid Bacteria" was first widely accepted in the early 20th century, replacing earlier terms like "milk souring" and "lactic acid producing" bacteria, which caused confusion among researchers (Coelho *et al*., 2022; Khalid, 2011). The work of Orla-Jensen in 1919 and Axelsson in 1989 played a significant role in the systematic classification of LAB (Khalid, 2011).

LAB are a major group of commensal bacteria, with certain strains exhibiting probiotic properties, particularly Lactobacilli, which are commonly found in fermented foods (Muhialdin *et al*., 2021). Many LAB strains isolated from fermented foods have been demonstrated to be beneficial to humans and have potential as probiotics (Vanniyasingam *et al*., 2019). The identification and classification of LAB populations have traditionally relied on phenotypic, biochemical, and physiological tests. However, these methods have limitations, such as the complexity of procedures, varying nutritional and growth needs of LAB, and lower discriminatory power (Bintsis, 2018). The emergence of molecular tools has addressed these limitations, enabling the accurate identification of LAB strains using PCR amplification, DNA sequencing, and other genotypic methods (Kuleshov *et al*., 2020).

There is an increased interest in probiotics, in face of their recorded safe use and recognized effects on human health (Khalid, 2011). Probiotics are live microorganisms when administered in large quantities; improve the health of the host (Coelho *et al*., 2023).

MATERIALS AND METHODS

Sample Collection: A total of fifteen (15) fermented food samples consisting of five samples each of Nono, Yoghurt, and Kunun-Zaki, were purchased and transported in ice pack immediately to the laboratory,

Department of Microbiology, Ahmadu Bello University, Zaria for analysis.

Isolation and Characterization: A stock sample was prepared by homogenizing 25ml of the sample with 225ml of sterile distilled water, serially diluted, and inoculated on MRS agar plates. The plates were incubated anaerobically at 37°C for 24 hours. Isolates with large whitish colonies after incubation at 37^0C for 18-24 hours were considered presumptive lactic acid bacteria (Neethu *et al.,* 2014). Isolates were identified based on Gram and Biochemical reactions (Catalase test, Citrate utilization test, H_2S production test, Urease test, Arginine hydrolysis test, Methyl Red - Voges-Proskauer test, Bile esculin salt test, Carbohydrate fermentation profile).

Confirmation of Isolate: The genomic DNA from a bacterial isolate was extracted using the Invisorb Spin DNA Extraction kit as describe by (Kuleshov *et al*., 2020). The 16S rRNA genes were amplified using Polymerase Chain Reaction (PCR) using bacterial universal primers (27F– AGAGTTTGATCCTGGCTCAG and 1492R– GGTTACCTTGTTACGACTT). The PCR reaction was conducted in a Techne PTC-100 Thermal Cycler, involving denaturation, 30 cycles of amplification, and extension. The PCR products were separated by electrophoresis on a 1% agarose TAE gel and visualized by UV transillumination. The amplicons were purified, sequenced for the 16S gene, and BLAST searched for similar sequences in the NCBI database. A phylogenetic tree was plotted to understand evolutionary relationships among organisms.

Evaluation of Probiotic Potential

Low pH tolerance: The bacterial cell culture was harvested and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the cell suspension was washed twice with pH7.22 phosphate buffer saline and re-suspend in pH6.9, pH4.5, and pH3.2 PBS. The aliquots were plated on sterile MRS agar and incubated at 37°C for 24 hours (Hussaini *et al.,* 2020).

Bile tolerance test: The bacterial cell culture was harvested, washed twice with pH 7.22 PBS, and re-suspended in PBS solution containing 0.3%, 0.5%, 1.0% and 1.5% bile salt. The resulting cell aliquots were plated on sterile MRS agar and incubated at 37°C for 24 hours (Hussaini *et al.,* 2020).

Adherence Test: Adherence test will be carried out by inoculating beakers containing 1X PBS with cover-slips in them. The cover-slips will then be stain with crystal violet and check for adherence under microscope after 5mins, 10mins and 60mins of incubation (Neethu *et al*., 2014).

Hemolytic Test: For testing the hemolytic activity, freshly prepared isolates was streaked on Blood agar plates 5% (w/v). The plates were incubated at 37°C for 48 hours. They were examined for any sign of hemolysis (Mathew *et al*., 2021).

Antibiotic Sensitivity Test: The LAB isolates were tested for antibiotic resistance using the disc diffusion method. The isolates were exposed to eight antibiotics: Cefotaxine, Vancomycin, Imipenem, Ofloxacin, Levofloxin, Ciprofloxine, erythromycin, and azithromycin. The disc was placed on Mueller-Hinton agar plates and incubated for 24 hours. The halo zone around the disc was measured, and isolates were classified as sensitive, intermediate, or resistance based on inhibition zone diameter (Neethu *et al*., 2014).

Antibacterial Activity of the Isolates against Escherichia coli and Salmonella Typhi: The agar well diffusion method was used to assess the antibacterial activity of *Escherichia coli* and *Salmonella* Typhi isolates. The isolates were obtained from the department of Microbiology and inoculated in MRS broth overnight. The cell-free supernatant was collected and spread onto Muller Hinton agar plates. Wells were made on each plate, filled with 100µL of the isolates cell-free supernatant. The plates were incubated for 24 hours at 37ºC and observed for zones of growth inhibition (Mathew *et al*., 2021).

RESULTS AND DISCUSSION

The Table 1 shows Gram-positive rod/bacilli and cocci from fermented food samples,

exhibiting negative results for catalase, citrate, H_2S, indole, urease, NH3 production from arginine, and Voges-Proskauer test, but positive results for methyl red and bile esculin salt tests. All isolates were able to ferment glucose to produce lactic acid, with *L. bulgaricus* and *S. thermophilus* fermenting galactose, lactose, and glucose. *L. plantarum* fermented all sugars except arabinose and mannitol, while *L. acidophilus* fermented all sugars but was weak with maltose and mannitol. These results are in line with previous studies, such as those by Thakur *et al*. (2013), Mathew *et al*. (2017), Agim-Ezenwaka *et al*. (2020), and Reuben *et al*. (2020). Plate I shows the gel electrophoresis picture of 16S rRNA amplification for identification of the selected isolates. Amplified gene shows distinct single DNA band with molecular weight of 1500bps which correspond to the expected amplicon size. BLAST analysis of the 16S rRNA sequences confirmed the identities of the isolates as *L. acidophilus, L. plantarum, L. fermentum, L. bulgaricus,* and *S. thermophilus*. The high percentage identity (92-100%) observed validated the accuracy of the biochemical tests used for identification. The LAB strains detected were related to reference strains from similar or similar origins, such as M26709 and M549 from the UK and India, M213 from Egypt and Azerbaijan, M8975 from China, M5645 from Nigeria and South Korea, and 627F from China. Table 2 shows all isolates tolerated low pH conditions (pH 6.9, 4.5, and 3.2).The significance of low pH tolerance in the selection of probiotic species is a crucial aspect highlighted by Vannisyasingan *et al*. (2019) and Hussain *et al*. (2020) in their respective studies. Both studies underscore the essential role of probiotic strains being able to survive acidic conditions, particularly in the stomach and small intestine, to exert their beneficial effects in the gut.

The human liver secretes bile into the small intestine daily, making probiotics a challenge due to exposure. Table 3 shows all isolates survived exposure to varying concentrations of bile, demonstrating their tolerance. Similar results were obtained by Hussain *et al*. (2020), and Shehata *et al*. (2020).

Table 1: Cultural, Microscopic and Biochemical Characteristics of LAB isolates from fermented foods

Isolate code	Cultural characteristics	GR	Cat	Cit	NH_3	H_2S	Ind	MR	VP	EBS	Glc	Fru	Suc	Lac	Mal	Man	Arab	Gal	Inference
K1	Milky white raisedmoist	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	+	*L. plantarum*
K2	White irregular, flat small	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	+	*L. plantarum*
K3	Milky white raisedmoist	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	+	*L. plantarum*
N1	Whitish, flat andmoist	+	-	-	-	-	-	+	-	+	+	+	+	+	+	-	-	+	*L. acidophilus*
N2	Milky white, Smooth raised	+	-	-	-	-	-	+	-	+	+	+	+	+	W	W	+	+	*L. fermentum*
N3	Milky white, Smooth raised	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	+	*L. plantarum*
Y1	Yellowish convex, round	+	-	-	-	-	-	+	-	+	+	-	+	+	+	-	+	-	*S. thermaph-ilus*
Y2	Creamy white, raised moist	+	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-	+	*L. bulgarus*
Y3	Creamy white, raised moist	+	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-	+	*L. bulgarus*

Key: GR= Gram reaction, Cat=catalase, cit=citrate, H_2S= hydrogen sulphide production test, Ind=Indole,MR= methyl red, VP= vogesproskauer, EBS=Esculin bile salt, Glu= glucose, Fru=fructose, Suc=sucrose, Lac=lactose, Mal=maltose, Man= mannitol, Arab=arabinose, Gal= galactose, +=positive, -=negative, W=weak reaction (after 24 hour)

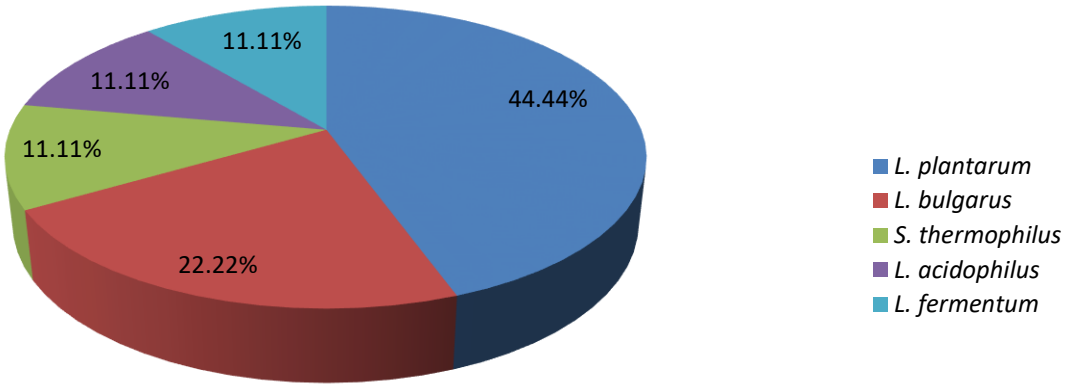

Figure1: Percentage distribution of Lactic Acid bacteria isolated from fermented products

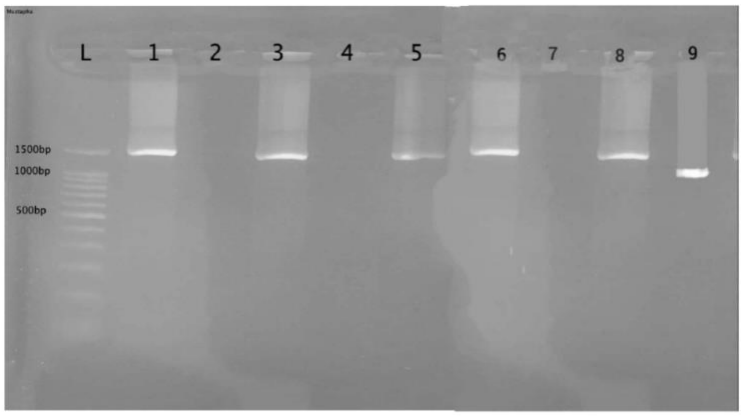

Plate1: Gel electrophorensis photo of amplified 16s rRNA gene of the LAB isolates Lane 1 – 9 samples; M = Molecular ladder (500 bp), Expected amplicon size 1500 bp

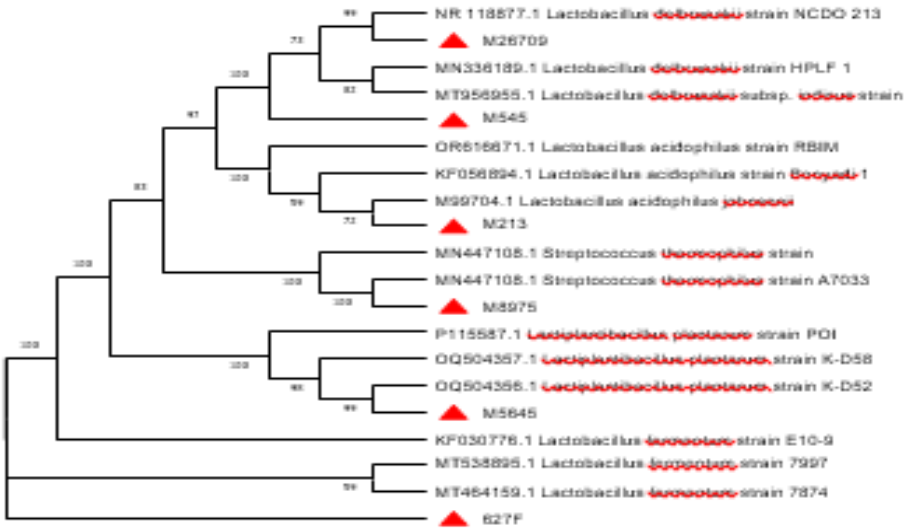

Figure 2: Neighbour joining tree showing the evolutionary relationship between the LAB genes sequence detected in the sample (1000 bootstrap) and the gene sequence detected in the Genbank

Table 2: Acid tolerance of the LAB isolates

Isolate code	pH		
	6.9	4.5	3.2
K1	+	+	+
K2	+	+	+
K3	+	+	+
N1	+	+	+
N2	+	+	+
N3	+	+	+
Y1	+	+	+
Y2	+	+	+
Y3	+	+	+

Key: + = growth; K= Kunun- Zaki; N= Nono; Y= Yoghurt

Table 3: Bile Torelance of the LAB isolates

Isolate code	Bile concentrations (%)			
	0.3	0.5	1.0	1.5
K1	+	+	+	+
K2	+	+	+	+
K3	+	+	+	+
NI	+	+	+	+
N2	+	+	+	+
N3	+	+	+	+
Y1	+	+	+	+
Y2	+	+	+	+
Y3	+	+	+	+

Key: +=growth; K= Kunun- Zaki; N= Nono; Y= Yoghurt

Table 4: Adherence properties of the LAB isolates

Isolate	Time Variation (mins)		
Code	5	10	60
K1	+	+	+
K2	+	+	+
K3	+	+	+
N1	+	+	+
N2	+	+	+
N3	+	+	+
Y1	+	+	+
Y2	+	+	+
Y3	+	+	+

Key: += isolates showed adherence properties; K= Kunun- Zaki; N= Nono; Y= Yoghurt

Table 5: Haemolytic activity of the LAB isolates

Isolate Code	Haemolytic Activity
K1	-
K2	-
K3	-
NI	-
N2	-
N3	-
Y1	-
Y2	-
Y3	-

Key: -= no haemolytic activity; K= Kunun- Zaki; N= Nono; Y= Yoghurt

Table 6: Antibiotic susceptibility profile of the LAB isolates

Isolate Code	CTX	VAN	IMP	OFX	LBC	CIP	ERY	AZN
K1	S	S	S	R	R	S	I	S
K2	S	S	S	R	R	S	S	S
K3	S	I	S	R	R	S	S	S
NI	S	I	S	S	R	R	R	I
N2	S	S	I	I	R	S	S	S
N3	S	S	I	I	I	I	R	I
Y1	S	S	R	I	S	R	I	S
Y2	S	I	S	S	I	S	R	S
Y3	S	I	S	S	I	S	R	S

Key: R=Resistant, I=Intermediate S=Susceptible, CTX=Cefotaxine-25µg VAN=Vancomycin-5µg IMP=Imipenem-10µg OFX=Ofloxacin-5µg, LBC=Levofloxin-5µg CIP=Ciprofloxine-5µg ERY=erythromycin 15µg AZN=azithromycin 15µg

Table 7: Antibacterial Activity of the LAB isolates against *E.coli* and *Salmonella* Typhi

Isolate Code	Zones of Inhibition (mm)	
	E. coli	*S.* Typhi
K1	12	7
K2	11	6
K3	11	7
NI	14	11
N2	7	0
N3	12	7
Y1	10	7
Y2	7	0
Y3	7	0

Key=K= Kunun- Zaki; N= Nono; Y= Yoghurt

Table 4 shows all isolates were able to adhere to coverslips at different time points (5 minutes, 10 minutes, and 60 minutes), suggesting their potential to colonize the gut. Similar report was obtained by Neethu *et al*. (2014) on Biopreservation of meat by probiotic bacteria isolated from dairy products in India. Table 5 shows none of the isolates exhibited any notable zones of hemolysis around the colonies, indicating their inability to produce hemolysins that could damage red blood cells. Similar results are reported by Neethu *et al*. (2014) and Hussain *et al.* (2020). Table 6 illustrated the susceptibility profile of all the LAB isolates to some commonly used antibiotics (n = 8). The isolates showed 100% susceptibility to cefotaxine, cetrotriaxone sulbactam, gentamycin, and azithromycin. They also demonstrated high susceptibility to amoxicillin and cefuroxime, but lower susceptibility to cefexime, ciprofloxine, ofloxine, levofloxin, and erythromycin. Similar result was reported by Shehata *et al.* (2020) on Antimicrobial activity and probiotic properties of Lactic Acid Bacteria isolated from traditional fermented dairy products in Egypt. Table 7 shows the antibacterial activity of the isolates against *Escherichia coli* and *Salmonella* Typhi, all isolates exhibited antagonistic activity against *E. coli*, with isolate N1 showing the highest zone of inhibition (14 mm). Isolates N2, Y2, and Y3 also had activity against *Salmonella* Typhi, with N1 having the highest zone of inhibition (11 mm), with N2, Y2 and Y3 showing no zone of inhibition against *Salmonella* Typhi.

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