Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from Some Fermented Foods

Abdulsalam R.[1*] Bala J. D.[1] Abalaka M. E.[1] and Ocheme O. B.[2]

1. Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria

2. Department of Food Science Technology, Federal University of Technology, Minna, Niger State, Nigeria

* Corresponding author: a.ramatu@futminna.edu.ng

Abstract: Probiotics are live microorganisms which when consumed in adequate quantity; promote the health of the consumer. This study was carried out to evaluate probiotic properties of lactic acid bacteria (LAB) isolated from fermented foods (wara, ogi, orange juice, pickles, sauerkraut and grape juice). A total of eighty four isolates were identified as LAB species by morphological, Gram staining and short biochemical tests. All isolates were characterized for probiotic properties including NaCl tolerance, bile tolerance, sugar fermentation, proteolytic activity, acid tolerance, antibiotic susceptability assay and adherance to stainless plates. All isolates survived in 2%, 4%, 6.5% and 8% NaCl concentrations. Four isolates were tolerance to pH 1.5, 2.0, and 3.0 for 24 and 48 hours. The four acid tolerant isolates were found to tolerant 0.3% bile salt for 24 hours with 85 to 99% rate of survival. Results of fermentation test showed that most isolates fermented all sugars. All strains digested casein by producing protease enzyme in skim milk agar plate. All the four isolates were found inhibiting some pathogenic bacteria to varying degrees and also displayed varying susceptibility to different antibiotics. The *in vitro* adherence to stainless steel plates of the 4 screened isolates were ranged from 32.83 to 37.70% adhesion rate. The phylogenetic analysis and the 16S rDNA sequencing assigned all the four efficient LAB isolates with probiotic properties to genus *Lactobacillus, Leuconostoc* and *Weissella* and were identified as *Lactobacillus plantarum, Lactobacillus plantarum, Leuconostoc mesenteriodes* and *Weissella paramesenteroides.* The four LAB strains were found to be potentially useful to produce probiotic products. Further study is needed to find specific probiotics with specific benefit from fermented foods.

Key word: Probiotic, Pickles, Wara, Acid tolerance, *Lactobacillus plantarum.*

INTRODUCTION

Probiotics are live microorganisms that confer health benefit to human when administered in adequate quantity, generally by improving or restoring the gut microflora (Maftei *et al.,* 2024). Probiotics seem to function by influencing both intestinal epithelial and immune cells of the gut, but the details of these effects are still being unraveled (Sudan *et al.,* 2022). Therefore, probiotics, through their effects on the host immune system, might ameliorate diseases triggered by disordered immune responses. However, the beneficial effects of probiotics can vary between strains; therefore the selection of most suitable ones is crucial for their use in the prevention or treatment of specific diseases (Han, *et al.,* 2024). In order to exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form (Rabetafika *et al.,* 2023).

Probiotics effects can be strain specific, but may have more than one health benefits depending on their delivery method, host response or interaction with other microbes (Zommiti *et al.,* 2020). This study aims to evaluate the *in vitro* probiotic properties of lactic acid bacteria (LAB) isolated from fermented foods products wara, ogi, orange wine, pickles, sauerkraut and grape wine with respect to their potential probiotic properties against some pathogenic bacteria.

MATERIALS AND METHODS

Production of the fermented food Wara (Cheese): The sodom apple (*Calotropis procera*) stem were weighed and carefully washed with distilled water and sliced with a sterile knife. The sliced stem was manually mixed with disilled water in order for the extract to be collected. The mixture was further sieved with a sterile sieve cup to collect the extract. Fifty millilitres (50 ml) of the extract was added to the 1 L raw milk and mixed manually. The mixture was sieved to remove the sheaves and was transfer in a metallic pot place on hot plates,

heating was carried at a regulated temperature of 68 ^{o}C for 20 min. After the heating clotting occurs, the resulting mixture was cooled to renneting temperature of 31 ^{o}C. The curd was separated from the whey (noncoagulated part of the mixture) using a decontaminated sieve cup (Ogunlade, 2019).

Sauerkraut: The spotted and defective cabbage heads were trimed off. Cabbage was prepared by removing the outer leaves and core. The cabbage was shredded with a sterile knife into 2 mm x 10 mm. The shredded cabbages were weighed (1 kg) followed by the addition of 1.5 % non-iodized sodium chloride. The shredded cabbage salt was placed in alternating layers in a sterile glass jar. Pressure was applied to gently squeeze out the brine and 100 ml of water was added. The glass jar was covered with sterile lids and incubated at 27^{o}C for 7 days for the natural fermentation of the cabbage. During fermentation process the lactic acid bacteria were isolated; temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days (Bhosale and Sapre, 2021).

Orange juice: The fruits were washed, manually peeled, cut into halves with sterile knife using hand gloves and their seeds removed. The cut oranges (mesocarp) were pressed with a hand juicer squeezer to extract the juice. The juice and pulp obtained were homogenized (blended) in a sterile hand Monilex blender. The homogenate was clarified manually using a sterile muslin cloth to obtain a clear juice During fermentation process the lactic acid bacteria were isolated, temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days (Abdulla *et al.,* 2021).

Pickles: Cucumber was washed under running water.The cucumber was shredded with a sterile knife into 5 mm x 10 mm. The shredded cucumber were weighed (1 kg) followed by the addition of 1.5 % non-iodized sodium chloride. The shredded cucumber salt was placed in alternating layers in a sterile glass jar. Pressure was applied to gently squeeze out the brine and water was added (Zeng *et al.,* 2020). The glass jar was covered with sterile lids and incubated at room temperature for 7 days for the natural fermentation of the cucumber. During fermentation process the lactic acid bacteria were isolated; temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days.

Ogi: Ogi was produced from sorghum by adapting the method described by Eke-Ejiofor, (2018) and Banwo**,** *et al.* (2022). The sorghum grain was cleaned by hand to remove dirt like stones and chaff. Five hundred gram (500g) of cereal was steeped in 2 liters of distilled water in a sterile plastic buckets and allowed to ferment for 2 days at room temperature. The fermented grain was separated from the steeping water by decanting. Afterwards, the grain were wet milled using a blender and the resulting slurry passed through a sterile muslin cloth, and washed with excess water. The by-products in the muslin cloth were discarded, and the starch in the bucket were covered with muslin cloth and allowed to settle at 27^{o}C. The sieved starch was allowed to sediment and ferment naturally. During fermentation process the lactic acid bacteria were isolated; temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days.

Biochemical characterization of LAB: The isolated bacteria were characterized biochemically with Gram staining, catalase, indole, methyl- red, motility, citrate utilization test, following the procedures as explained by Erdoğmuş *et al.* (2021) and Vasiee *et al.* (2022).

Growth at different NaCl concentrations: The LAB isolates were tested for their tolerance different NaCl concentrations. For this purpose, 2%, 4%, 6.5% and 8% NaCl concentrations were used for testing. Similarly, test tubes with 5 ml of modified MRS broth containing bromecresol purple indicator were prepared according to the appropriate concentrations and were

inoculated separately with 50 µl of 1% of each overnight culture of LAB and incubated at 37°C for 48 h. The change of the color from purple to yellow was considered as proof of cell growth (Mulaw *et al.,* 2019).

Low pH tolerance test of LAB: The LAB were grown separately overnight in 5 ml MRS broth at 37°C under anaerobic conditions. A volume of 1 ml of 10^7 cfu/ml of each overnight-grown culture was inoculated into 10 ml of MRS broth to give an initial inoculum level of 10^6 cfu/ml. The culture was then centrifuged at 5000 rpm for 10 min at +4°C. The pellets were washed twice in phosphate buffer (pH 7.2). The pellets were resuspended in 5 ml MRS broth which was adjusted to pH values of 1.5, 2.0 and 3.0 using 1 N·HCl to simulate the gastric environment. The test tubes were incubated at 37°C for 24 and 48 hours. After an appropriate incubation period, 1 ml of the culture was diluted in sterile 9 ml phosphate buffer prepared according to the manufacturer's instruction (0.1 M, pH 6.2) in order to neutralize the medium acidity. Briefly, a 100 µl aliquot of the culture and its 10-fold serial dilutions were plated on the MRS agar medium. The inoculated plates were incubated at 37°C for 24 to 48 h under anaerobic condition using an anaerobic jar and the grown LAB colonies were expressed as colony-forming units per milliliter (cfu/ml). A positive control consisting of regular MRS broth inoculated with the culture was used (Gheziel *et al.* 2019). The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration:

$$\text{Survival rate (\%)} = \frac{\text{Log CFN}_1}{\text{Log CFN}_0} \text{ X } 100$$

Where N_1 is the viable count of isolates after incubation and N_0 is the initial viable count

Tolerance to bile salts: To estimate bile tolerance of acid-tolerant LAB, the isolates were separately grown overnight in MRS broth at 37°C under anaerobic conditions. Each culture with the initial concentration of 10^6cfu/ml was then centrifuged at 5000 rpm for 10 min at 4°C. The pellets were washed twice in the phosphate-saline buffer (PBS at pH 7.2). Cell pellets were resuspended in MRS broth supplemented with 0.3% (w/v) bile salt. Samples were taken at 24 h from the onset of incubation to determine the survivability of cells as (Mulaw *et al.,* 2019), positive control consisting of plain MRS broth without bile salts inoculated with each separate culture was simultaneously set up. After appropriate incubation, 1 ml of each separate culture was diluted separately in sterile 9 ml phosphate buffer (0.1 M, pH 6.2) in order to neutralize the medium. Concisely, a 100 µl aliquot of the culture and its 10-fold serial dilutions were plated on MRS agar medium. Plates were incubated at 37°C for 24 to 48 h under anaerobic condition using an anaerobic jar. The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration:

$$\text{Survival rate (\%)} = \frac{\text{Log CFN}_1}{\text{Log CFN}_0} \text{ X } 100$$

Where N_1 is the viable count of isolates after incubation and N_0 is the initial viable count

Sugar fermentation test: The ability of the isolates to ferment various sugars was determined using 1g sugar in 100ml MRS broth. (Glucose, Sucrose, Fructose, Lactose, Mannitol, xylose and sorbitol were used). Two drops of phenol red was used as indicator. Ten millilitre (10ml) of media was dispensed into a testtube and Durham's tube was inverted into each testtube. Isolates were inoculated and incubated at 37^{o}C for 24 h and 1 testtube of each fermentation broth was kept uninoculated as a negative control and setup was observed for colour change and gas formation (Vasiee *et al.,* 2022).

Casein digestion test: The protease activity was performed using MRS agar plate containing 1% skim milk solution. Bacterial cultures were inoculated and incubated for 48h at 37^{o}C. Clear zones around the cultures indicated protease activity (Gao *et al*, 2022). *Pseudomonas* spp. and *Klebsiella* spp. were

used as positive and negative control, respectively.

Antimicrobial activity against bacteria pathogens: Antibacterial activity of the acid-bile-tolerant LAB strains against some pathogens was determined using the agar-well diffusion method with some modifications of the protocol indicated by Zapa´snik *et al*. (2022). The selected acid-tolerant LAB isolates were inoculated from slants to fresh MRS broth containing 1% glucose and incubated overnight at 37°C. The overnight active culture broth of each isolate was centrifuged separately at 5000 rpm for 10 min at 4° C. The cell-free supernatant from each separate culture was collected as a crude extract for the antagonistic study against some food-borne pathogens. The pure cultures of pathogenic bacteria were inoculated from slants to brain heart infusion broth. After 24-hour incubation at 37°C, a volume of 100 µl of inoculum of each test bacteria was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. The plates were allowed to dry, and a sterile cork borer (diameter 5 mm) was used to cut uniform wells in the agar. Each well was filled with 100 µl culture-free filtrate obtained from each of the acid-bile-tolerant LAB isolates. After incubation at 37°C for 24 to 48 hours, the plates were observed for a zone of inhibition (ZOI) around the well. The diameter of the zone of inhibition was measured by calipers in millimeters, and a clear zone of 1 mm or more was considered positive inhibition (Benkova *et al.,* 2020).

Bacterial adhesion to stainless steel plates: The adherence assay of the lactic acid bacterial isolates was determined on stainless steel plates with some modifications by Mulaw et al. (2019). Lactic acid bacteria were cultured in sterile MRS broth. Thereafter, the overnight bacterial culture (500 µl) was deposited in a test tube, which was then filled with 450 µl of MRS broth, wherein the sterile stainless steel plate was deposited, and the test tubes were then incubated for 24 h at 37°C. The stainless steel plate was removed under aseptic conditions, washed with 10 ml of sterile 1% peptone water, and left for 5 min in a sterile 1% peptone water tube. The plate was then washed again in the same conditions and vortexed for 3 min in a sterile 1% peptone water tube (6 ml) consecutively to detach the bacterial cells adhering to the steel plate surface. The cell number was determined by counting on MRS agar after 24 h of incubation at 37°C. Simultaneously, the total initial cell numbers were estimated to calculate the percentage of adhered bacterial cells for each LAB.

Antibiotic susceptibility assay: Each of the acid -tolerant and antagonistic lactic acid bacteria isolates was assessed for its antibiotic resistance by the disc diffusion method against some antibiotics (ciprofloxacin, norfloxacin, chloramphenicol, erythromycin, gentamycin, ampiclox, amoxil, streptomycin, rifampicin, levofloxacin). In this case, a volume of 100 µl of actively growing cultures of each acid-tolerant and antagonistic lactic acid bacteria was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. After drying, the antibiotic discs were placed on the solidified agar surface, and the plates were left aside for 30 min at 4° C for the diffusion of antibiotics and then anaerobically incubated at 37°C for 24 to 48 h. Resistance was defined according to the disc diffusion method by using the above antibiotic discs, and the diameters of zones of inhibition were measured using calipers (Khan *et al.,* 2019), the zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21 mm); intermediate, I (16– 20 mm), and resistance, R (≤15 mm) (Gheziel *et al.* 2019).

Extraction of genomic DNA of the LAB: Single colonies grown on medium were transferred to 1.5 ml of liquid medium grown on a shaker for 48 h at 28 ºC and centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then

added (Adebowale *et al.,* 2021). The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. The DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours till ethanol drops disappeared completely. The dried sediment was dissolved in 30 µl TE buffer.

Polymerase chain reaction (PCR) of 16S rDNA: Polymerase chain reaction amplification of the extracted DNA was carried out with the 16S primer in a total volume of 25 µl containing 100mg of genomic DNA. Two point five microlitre (2.5 µl) of 10 x PCR buffer, 1 µl of 50mM $MgCl_2$, 2 µl of 2.5mM dNTPs (Thermo Scientific), 0.1 µl Taq polymerase (Thermo Scientific). One microlitre (1 µl) of DMSO, 1 µl each of forward and reverse primers and 11.3 µl H_2O. Touch-down PCR was used for amplification as follows: Initial denaturation step of 5 minutes at 94 ºC, follow by 9 cycles each consisting of a denaturation step of 20 seconds at 94 ºC, annealing step of 30 seconds at 65 ºC, and an extention step of 72 ºC for 45 seconds. This was followed by another 30 cycles each consisting of a denaturation step of 20 seconds at 94 ºC, annealing step of 30 seconds at 55ºC, and an extention step of 72ºC for 45 seconds. All amplification reactions were performed in a GeneAmp@ PCR System 9700, Applied Biosystems. Polymerase chain reaction amplicons were loaded on 1.5% agarose gel and run at 100volts for 2 hours (Lee, *et al.,* 2023).

DNA Sequencing: The amplicons were selected from the amplified products and purified using manufacturer's protocol, sequencing was performed using a big dye terminator cycle sequencing kit (Applied ioSystems), Unincorporated dye terminators were then purified and precipitated using ethanol EDTA solution. The pellets were then resolved in HiDi formamide buffer. Sequencing was performed using 3130 x 1 Genetic Analyser. The resulting pattern was then compared with the 16s rRNA nucleotide sequences present in BLAST tool of Genbank at NCBI (Sharma *et al.,* 2020).

RESULTS AND DISCUSSION

All the selected four potential probiotic bacteria were identified as LAB on the basis of their morphological, biochemical, and physiological characteristics, catalase negative and Gram positive rod. All tested isolates grows at 15°C, 37°C, 45°C and tolerate 2%, 4%, 6.5% and 8% NaCl concentration (Table 1). These findings were consistent with a previous study of Goa *et al*. (2022), testing the abilities of isolates growing at 15° C and 45°C.

Sugar fermentation and protease activity tests of the isolates

The isolates exhibied both heterofermentative and homofermentative types (Table 2) this finding is in accordance with Mulaw *et al.* (2019) who isolated Lactobacillus species from Ergo and found all isolates were grouped as homofermentative and heterofermentative types. The four selected isolates fermented and produce gas from glucose. Isolates CA4 fermented fructose, sucrose, manitol, sobitol and xylose except for lactose. Isolates NO5, W6 and W7 fermented all the sugars except for xylose. Isolates NO5, W6 and W7 would be useful for lactose intolerant people who cannot metabolize lactose due to lack of β-galactosidase enzyme. Lactose and dextrose utilization by LAB was confirmed by lactose and glucose utilization test. Lactic acid bacteia digests casein in order to grow in milk and subsequently utilize the degradation products (Aimee, 2022). In this present study, all the isolated strains were found to digest casein indicating LAB produced protease enzyme. Raveschot *et al.*

(2020) reported in their study that, Lactobacilli species utilized casein by proteolytic activity.

pH tolerance and bile salt tolerance tests of the isolated LAB

Out of 84 isolates, 6 isolates (7.14%), 12 isolates (14.28%), and 14 isolates (16.67%) tolerated pH values of 1.5, 2 and 3 for 24h, respectively. Upon further extension of the incubation period to 48 h, 5 and 8 isolates (tested at pH 1.5 and 2) survived, while 11 survived from 14 isolates with the extension of incubation period to 48 h at pH 3.0 (Table 3). Therefore, out of the total 84 LAB isolates, 4 (4.76%) isolates survived pH 1.5 upon exposure for 24 and 48 hours, and the mean value of the treatments was significantly different at $p < 0.05$ (Table 4). Among the fou selected isolates, 1 (25%) was isolated each from cabbage and ogi sample and 2 (50%) were isolated from wara. The survival rate of the isolates was ranged from 41.46 to 90.53% at different pH values for 24 and 48h incubation periods (Table 4). Isolates, like W6 and W7 were found highly tolerant and persisted above 50% for both 24 and 48h at pH 1.5. Isolate Ca4 and O5 upon exposure to pH 1.5 could not grow above 50%. However, incubation at low pH resulted in a significant decrease in the survival rate of all LAB isolates as reported in another study by Das *et al.* (2020) the authors observed that the viable counts of all lactic acid bacteria were significantly affected by low acidity, especially at pH 2.0.

The four acid tolerant LAB isolates showed high tolerance to bile salt conditions (85% to 95%) (Table 4). Similar to the present findings, the results in other studies have revealed that all the isolated strains displayed high tolerance to bile salt conditions and the survival rates of Lactobacillus strains ranged from 88% to 92% (Haghshenas *et al.*, 2023). In a related study, Akalu, *et al.* (2017) have also shown that out of the 30 tested LAB isolates, 17 Lactobacillus isolates obtained from Ethiopian traditionally fermented Shamita and Kocho showed remarkably high tolerance to an environment containing 0.3% bile salt. Inaddition, Biswal, *et al.* (2021) has reported that all of the LAB isolates demonstrated a high level of tolerance to bile salts by displaying surviving percentage above 50% on exposed to 0.3% bile salts after 24 h at 37°C.

Antimicrobial activities of the isolated LAB

The selected four potential probiotic lactic acid bacterial (W7, CA4, W6 and O5) exhibited varying degree of antagonism against *Staphylococcus aureus, Escherichia coli, Salmonella typimurium,* and *P. aeruginosa* (Table 5). All the selected potential probiotic LAB strains exhibited strong antimicrobial activity against the pathogens. Isolate O5 displayed the highest antagonistic activity against *E. coli*, *S. typhimurium* and *P. aeruginosa* with the zone of inhibition ranged from 20.08 to 20.85 mm in diameters, also isolates W7, CA4, W6 and O5 displayed highest antagonistic activity against *Staphylococcus aureus*, *E. coli, Salmonella typhi* and *P. aeruginosa* with the zone of inhibition ranged of 21.1mm, 21.00mm, 21.00 mm and 21.40 mm diameters respectively. In agreement to this study, Dejene *et al.* (2021) have verified that all the LAB isolates originated from Borde and finfish belonging to the genera *Lactobacillus*, found to inhibit the growth of the test strains such as *S. aureus, Salmonella spp.*, and *E. coli* with zone of inhibition that ranged from 15 to 17 mm in diameters. In addition, Gheziel *et al.* (2019) had demonstrated that 6 *Lactobacillus plantarum* strains isolated from faecal samples exposed high antibacterial activity against potential foodborne pathogens *E. coli* and *Staph. aureus*. Most LAB strains showed inhibitory activity against the growth pathogenic microorganisms either by competing with pathogenic bacteria for food or by producing antimicrobial compounds such as hydrogen peroxide, diacetyl, bacteriocins, naturally protective organic acids, and specific substances, such as antiviral peptides or low-molecular-weight peptides (Souza, 2021).

Among the main important characteristics of probiotic bacteria, adhesion to the intestinal mucosa is required. The screened probiotic LAB isolates possesses *in vitro* adherence property to stainless steel plates with the adhesion rate ranged from 32.83 to 37.70%. Isolate CA4 showed the highest (37.70%) adherence rates. However, isolate O5 showed the least 32.83% adherence rate (Table 6). In agreement with this study, Mulaw *et al.* (2019) have reported that the adhesion rate of lactic acid bacteria to stainless steel plates ranged from 32.75 to 36.30%, El-Jeni *et al.* (2020) have also revealed that the adhesion rate of lactic acid bacteria to stainless steel plates ranged from 32 to 35%.Generally, this suggests that the LAB isolates have a potential capacity to colonize the gastrointestinal (GI) tract mucosa.

Antibiotic susceptibility profile of the isolated LAB

All of the tested four Lactobacillus strains were found to be resistant to all the antibiotcs. However, CA4 and O5 are susceptible and intemediate to streptomycin and levofloxcin respectively but displayed resistance to other antibiotics (Table 6). These findings were in agreement with the report obtained by Yao *et al.* (2022), that isolates of LAB from curd were found to be resistant to erythromycin, streptomycin, gentamycin, ciprofloxacin and Norfloxacin. Similarly, Ferdouse *et al.* (2023) have reported that out of 120 isolates of LAB from four different Indonesian traditional fermented foods, 16 isolates were resistant to erythromycin. In line with this, Peng *et al*. (2023) observed that among the 12 Lactobacillus species obtained from Chinese fermented foods, 5 isolates were sensitive to kanamycin, 7 resistant to erythromycin, 9 resistant to ampicillin, and 8 isolates resistant to tetracyclin. On the contrary Tigu *et al.* (2016**)** reported that all of the LAB isolates obtained from traditional fermented condiments such as Datta and Awaze were susceptible to ampicillin, erythromycin, and tetracycline.

Identification of Probiotic LAB Isolates by 16S rRNA Gene Sequencing of the isolated LAB

The 16S rRNA gene sequences of the 4 LAB isolates with the best potential probiotic properties showed the highest homology to the known species of bacteria in the database (Figure 1). Accordingly, CA4 showed 99.80% similarity with *Leuconostoc mesenteriodes* NO5 showed 99.79% match with *Lactobacillus plantarum*, W6 showed 99.90% match with *Weissella paramesenteroides* and W7 showed 98.61% homology with *Lactobacillus plantarum*. Li, *et al.* (2023) have identified *Lactobacillus plantarum* with potential probiotic with anti-obesity and antioxidant anti-obesity and antioxidant properties using 16S rRNA genes analysis. Similarly, Zhao *et al.* (2022) have revealed the strain level identification of lactic acid bacteria with potent probiotic properties isolated from some Sichuan pickle using phylogenetic estimation of 16S rDNA genes.

Table 1: Morphological, biochemical and physiological characterization of the isolates

Isolates	Gram staining	Shape	Catalase	Citrate	Methyl red	Indole	Spore staining	Motility	Temperature (°C)			NaCl concentration (%)			
									15	37	45	2	4	6.5	8
CA4	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+
NO5	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+
W6	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+
W7	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+

Key:- = negative, + = positive

Table 2: Sugar fermentation and protease activity tests of the isolates

Isolate	Sugar							Protease activity
	Glucose	Fructose	Sucrose	Lactose	Manitol	Sorbitol	Xylose	
CA4	A^+G^+	A^+G^+	A^+G^-	A^-G^-	A^+G^-	A^+G^-	A^+G^-	+
NO5	A^+G^+	A^+G^+	A^+G^+	A^+G^-	A^+G^+	A^+G^+	A^-G^-	+
W6	A^+G^+	A^+G^+	A^+G^+	A^+G^-	A^+G^+	A^+G^+	A^-G^-	+
W7	A^+G^+	A^-G^-	A^+G^+	A^+G^+	A^+G^+	A^+G^-	A^-G^-	+

Key: - = negative, + = positive, A = assimilation, G = gas

Table 3: pH tolerance patterns of the isolates at different pH values after 24 and 48 hours exposure

Source	No. Isolate	No. of survived isolates (%)					
		24 hours			48 hours		
		1.5	2.0	3.0	1.5	2.0	3.0
Cabbage	14	1(7.14%)	2(14.28%)	2(14.28%)	1(7.14%)	1(7.14%)	2(14.28%)
Cucumber	14	-	1(7.14%)	2(14.28%)	-	1(7.14%)	2(14.28%)
Grape wine	14	-	-	1(7.14%)	-	-	1(7.14%)
Ogi	14	1(7.14%)	3(21.43%)	2(14.28%)	1(14.28%)	2(14.28%)	2(14.28%)
Orange wine	14	-	-	2(14.28%)	-	-	1(7.14%)
Wara	14	4(28.57%)	6(42.86%)	5(35.71%)	2(14.28%)	4(28.57%)	3(21.43%)
Total	84	6(7.14%)	12(14.28%)	14(16.67%)	4(4.76%)	8(9.52%)	11(13.09%)

Table 4: Percentage survival of probiotic LAB at different pH levels and 0.3% bile salt

Isolates	No. of survived isolates						Bile tolerance
	24 hours			48 hours			24 hours
	1.5	2.0	3.0	1.5	2.0	3.0	0.3%
Ca4	47.35 ± 0.00^b	70.51 ± 0.28^c	83.52 ± 0.70^c	43.68 ± 1.41^d	65.39 ± 0.01^b	78.64 ± 0.05^{ab}	95.00 ± 1.41^a
O5	43.38 ± 0.07^c	76.89 ± 0.70^b	74.00 ± 0.00^d	48.50 ± 0.14^c	66.50 ± 0.00^b	73.42 ± 0.0^{4b}	85.5 ± 0.70^c
W6	63.79 ± 0.70^a	87.19 ± 0.08^a	87.13 ± 0.02^b	55.34 ± 0.00^a	73.02 ± 0.07^a	86.60 ± 0.04^a	94.5 ± 2.12^{ab}
W7	61.55 ± 1.41^a	73.47 ± 0.58^b	90.53 ± 0.21^a	51.46 ± 0.07^b	70.11 ± 0.13^a	83.85 ± 0.07^a	91.5 ± 0.70^b

Key: Values are presented as mean ± standard deviation of three replicates. Values with different superscripts along column are significantly different at $p < 0.05$.

Table 5: Antimicrobial activities of the isolates against some pathogenic bacteria and adhesion of LAB to stainless steel plate

Diameter of inhibition zone (mm)					
Isolstes	*S. aureus*	*E. coli*	*S. typhimurium*	*P. aeruginosa*	Adherence (%)
Ca4	17.00 ± 0.00^b	19.15 ± 0.03^e	18.65 ± 0.01^a	20.00 ± 0.00^b	37.70
O5	17.05 ± 0.03^b	20.08 ± 0.04^d	20.85 ± 0.09^b	20.40 ± 0.06^c	32.83
W6	14.00 ± 0.00^a	16.06 ± 0.03^a	19.05 ± 0.03^{ab}	16.35 ± 0.20^a	35.84
W7	9.50 ± 0.06^c	17.00 ± 0.00^b	20.65 ± 0.03^b	20.05 ± 0.03^{bc}	37.33

Key: Values are presented as mean ± standard error of mean (SEM) of three replicates. Values with different superscripts along column are significantly different at $p < 0.05$.

Table 6: Antibiotic susceptibility profile of the isolates

Isolstes	CH	CPX	S	NB	APX	LEV	E	CN	AMX
Ca4	R	R	S	R	R	R	R	R	R
O5	R	R	R	R	R	I	R	R	R
W6	R	R	R	R	R	R	R	R	R
W7	R	R	R	R	R	R	R	R	R

CPX- Ciprofloxacin, NB- Norfloxacin, CH- Chloramphenicol, E- Erythromycin, CN- Gentamycin, APX- Ampiclox, AMX-Amoxil, S- Streptomycin, RD- Rifampicin, LEV- Levofloxacin. Zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21mm); intermediate, I (16–20mm); and resistance, R (≤15mm).

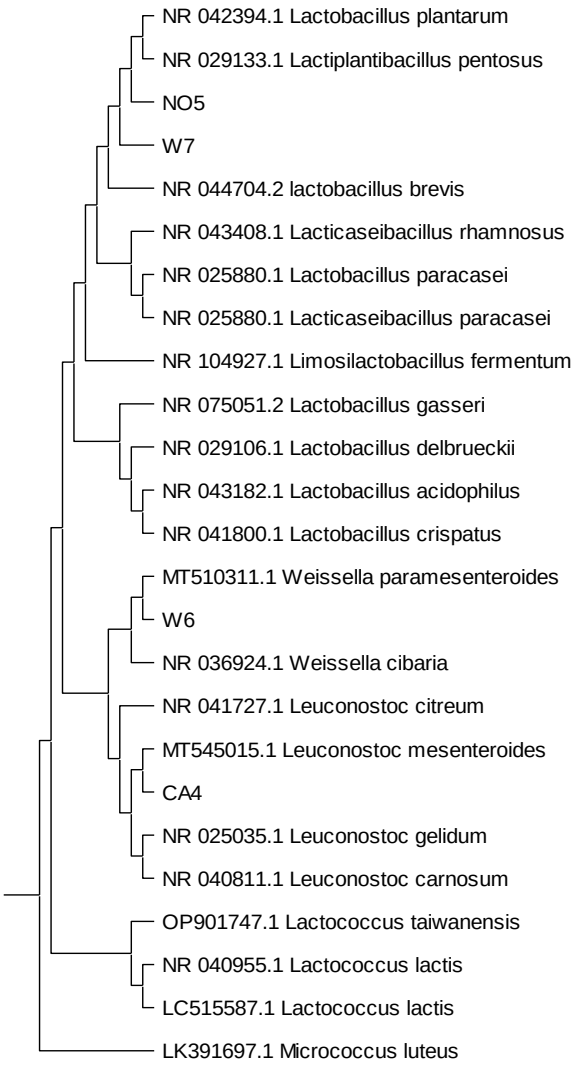

Figure 1: Neighbour-joining phylogenetic tree for LAB based on the 16S rRNA gene sequences. The relationship between isolated strains and related species were shown

CONCLUSION

The four LAB isolates from Wara, Cabbage and Ogi were the more desirable health-promoting bacteria with better acid and bile resistance, adherence property antimicrobial activity and antibiotics susceptibility test. It is suggested that these strains can be good candidates for food industries as the preservation of acidic foods at an industrial scale and can also serve as biopreservation of chilled food, which is mostly contaminated by spoilage pathogen. Since the increasing use of antibiotics leads to collateral damage to the host by disturbing the normal intestinal microbiota; therefore, consumption of fermented foods such as fermented milk and other enhances the proliferation of healthy GIT microbiota on the one hand and prevents the growth of undesirable microorganisms.This study reveals the functional properties of probiotics in fermented foods. Therefore, this fermented food product could be considered a valuable resource for probiotic strain screening and starter culture application.

REFERENCES

Abdulla, S.M., Mahmood, A.B., Mahmood, S.K. and Salh, S.K.H. (2021). A Comparative Study of the Physicochemical Properties and Sensory Evaluation of Commercial Orange Juice sold in the Sulaimani Market with Local Preparation of Orange Juice. *Tikrit journal for agricultural sciences*, *21*(4), 146-158.

Aimee, R. H., Renwick, C.J. D., Morris, V.K. and Gert-Jan, M. (2022). Fermentation of plant-based dairy alternatives by lactic acid bacteria. *Microbial Biotechnology* 15(5), 1404–1421

Akalu, N., Assefa, F. and Dessalegn, A. (2017)."In vitro evaluation of lactic acid bacteria isolated from traditional fermented Shamita and Kocho for their desirable characteristics as probiotics," *African Journal of Biotechnology*, 16 (12), 594–606.

Banwo, K., Oyeyipo, A., Mishra, L., Sarkar, D. and Shetty, K. (2022). Improving phenolic bioactive-linked functional qualities of traditional cereal-based fermented food (Ogi) of Nigeria using compatible food synergies with underutilized edible plants. *Nutrition and Food Science.* 17, 1 -12

Benkova, M., Soukup, O. and Marek, J., (2020). Antimicrobial susceptibility testing: currently used methods and devices and the near future in clinical practice. *Journal of applied microbiology*, *129*(4), 806-822.

Bhosale, S. and Sapre, V. (2021). GCMSD ALS analysis of bioactive compounds in fermented sauerkraut. *World Journal of Pharmaceutical and Life Sciences*, 7(2): 98 – 105

Biswal, P., Pal, A. and Das, A. P. (2021). Screening for Probiotic Potential of *Lactobacillus **r**hamnosus* strain CRD4. *Biointerface Research in Applied Chemistry* 11(3), 10174 – 10184.

Das, S., Mishra, B.K. and Hati, S. (2020). Techno-functional characterization of indigenous Lactobacillus isolates from the traditional fermented foods of Meghalaya, India. *Current Research in Food Science*, 3: .9-18.

Eke- Ejiofor, J. (2018). Evaluation of the Nutrient Content and Sensory Properties of Spiced Ogi (Gruel) Produced From Different Cereal Blends. *Journal of Nutrition Health Food Sciences* 6(6):1-8. DOI: 10.15226/jnhfs.2018.001148.

El-Jeni, R., El Bour, M. and Calo-Mata, P. (2015). "*In vitro* probiotic profiling of novel *Enterococcus faecium* and *Leuconostoc mesenteroides* from Tunisian freshwater fishes," *Canadian Journal of Microbiology*, 62(1): 60–71.

Erdoğmuş, S. F., Erişmiş, U. C.,and Uğuz, C. (2021). Isolation and identification of lactic acid bacteria from fermented meat products and evaluation of their antimicrobial effect. *Journal of Food Sciences*, 39(4):289-296.

Ferdouse, J., Paul, S., Chowdhury, T., Ali, F., Islam, S. and Hossain, T.J. (2023). Probiotic characteristics of *Pediococcus pentosaceus* and *Apilactobacillus kunkeei* strains: the lactic acid bacteria isolated from Bangladeshi natural honey. *Applied Food Biotechnology*, 10(1): 33-45.

Gao, S., Jiang, Y., Zhang, X., Cui, S., Liu, X., Zhao, J., Zhang, H. and Chen, W.(2022). Comparative peptidomics analysis of milk fermented by *Lactobacillu helveticus*. *Foods*, *11*: 3885-3896

Gheziel, C., Russo, P., Arena, M. P., Spano, G., Ouzari, H.-I., Kheroua, O., Saidi, D., Fiocco, D., Kaddouri, H., and Capozzi, V. (2019). Evaluating the probiotic potential of *Lactobacillus plantarum* strains from Algerian infant faeces: towards the design of probiotic starter cultures tailored for developing countries. *Probiotics and*

Antimicrobial Proteins, 11(1): 113-123.

Goa, T., Beyene, G., Mekonnen, M. and Gorems, K. (2022). Isolation and characterization of lactic acid bacteria from fermented milk produced in Jimma town, Southwest Ethiopia. *International Journal of Food Science*, 2022: 2076021-2076036.

Haghshenas, B., Kiani, A., Mansoori, S., Mohammadi-Noori, E. and Nami, Y. (2023). Probiotic properties and antimicrobial evaluation of silymarin-enriched Lactobacillus bacteria isolated from traditional curd. *Scientific Reports*, *13*(1): 10916-10931.

Han, C., Song, S., Cui, C., Cai, Y., Zhou, Y., Wang, J., Bei, W., Zhang, D., Guo, W. and Wang, S. (2024). Strain-Specific Benefits of Bacillus Probiotics in Hybrid Grouper: Growth Enhancement, Metabolic Health, Immune Modulation, and Vibrio harveyi Resistance. *Animals*, *14*(7):1062-1072.

Khan, Z.A., Siddiqui, M.F. and Park, S. (2019). Current and emerging methods of antibiotic susceptibility testing. *Diagnostics*, *9*(2): 49-50

Lee, S., McAnany, P.K. Chang, C.W., Norona, W., Short, M. L., Mulero, J. J. and Zhong, C. (2023).Developmental validation of Applied Biosystems YFiler Platinum Casework PCR Amplification Kit. *Scientific Reports,* 13: 14539-14544.

Li, K., Gu, Q., Yang, W. and Yu, X. (2023). *In vitro* screening and probiotic evaluation of anti-obesity and antioxidant lactic acid bacteria. *Food Bioscience*, *54*: 102844 - 102859.

Maftei, N.M., Raileanu, C.R., Balta, A.A., Ambrose, L., Boev, M., Marin, D.B. and Lisa, E.L.(2024). The potential impact of probiotics on human health. *Microorganisms*, *12*(2), .234 - 246.

Mulaw, G., Sisay Tessema, T., Muleta, D. and Tesfaye, A., (2019). In vitro evaluation of probiotic properties of lactic acid bacteria isolated from some traditionally fermented Ethiopian food products. *International journal of microbiology*, *19*: 1-18

Ogunlade, A.O., Oyetayo, V.O. and Ojokoh, A.O. (2019). Phytochemical screening and antioxidant properties of coagulants and soft cheese produced from goat milk using different biocoagulants of plant origin. *Asian Food Science Journal*, *7*(1): 1-8.

Peng, X., Ed-Dra, A. and Yue, M. (2023). Whole genome sequencing for the risk assessment of probiotic lactic acid bacteria. *Critical Reviews in Food Science and Nutrition*, *63*(32): 11244-11262.

Rabetafika, H.N., Razafindralambo, A., Ebenso, B. and Razafindralambo, H.L. (2023). Probiotics as antibiotic alternatives for human and animal applications. *Encyclopedia*, *3*(2): 561-581.

Raveschot, C., Cudennec, B., Deracinois, B., Frémont, M., Vaeremans, M., Dugersuren, J., Demberel, S., Drider, D., Dhulster, P., Coutte, F. and Flahaut, C. (2020). Proteolytic activity of *Lactobacillus* strains isolated from Mongolian traditional dairy products: A multiparametric analysis *Food Chemistry* 304: 125415 – 12531

Sharma, A., Lee, S. and Park, Y.S. (2020). Molecular typing tools for identifying and characterizing lactic acid bacteria: a review. *Food science and Biotechnology*, *29*: 1301-1318.

Souza, P. F. N. (2021). Synthetic peptides as promising antiviral molecules. *Future Virology*, 17(1): 9-11.

Sudan, S., Zhan, X. and Li, J. (2022). A novel probiotic *Bacillus subtilis* strain confers cytoprotection to host pig intestinal epithelial cells during enterotoxic *Escherichia coli* infection. *Microbiology Spectrum*, *10*(4): 01257-01278.

Tigu, F., Assefa, F., Mehari, T. and Ashenafi, M. (2016). "Probiotic property of lactic acid bacteria from traditional fermented condiments: Datta and Awaze," *International Food Research Journal*, 23(2): 770-779.

Vasiee, A., Falah, F. and Mortazavi, S. A. (2022).Evaluation of probiotic potential of autochthonous lactobacilli strains isolated from Zabuli yellow kashk, an Iranian dairy product, *Journal of Applied Microbiology*, 133(5): 3201–3214

Yao, J.,Gao, J., Guo, J., Wang, H., Zhang, E.N., Lin, Y., Chen, Z., Li, S. and Tao, S. (2022). Characterization of bacteria and antibiotic resistance in commercially produced cheeses sold in China. *Journal of Food Protection*, *85*(3):484-493.

Zapa´snik, A.; Sokołowska, B. and Bryła, M. (2022). Role of lactic acid acteria in food preservation and safety. *Foods*, 11: 1283-1300

Zeng, Y., Li, Y., Wu, Q.P., Zhang, J.M., Xie, X.Q., Ding, Y., Cai, S.Z., Ye, Q.H., Chen, M.T., Xue, L. and Wu, S. (2020). Evaluation of the antibacterial activity and probiotic potential of *Lactobacillus plantarum* isolated from Chinese homemade pickles. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 1-11.

Zhao, X., Hu, R., He, Y., Li, S., Yang, J., Zhang , J., Zhou, J., Xue, T. (2022). Screening of isolated potential probiotic lactic acid bacteria from Sichuan pickle for cholesterol lowering property and triglycerides lowering activity. *Food Sciences Technology*, 42: 09122-09128

Zommiti, M., Feuilloley, M. G. J. and Connil, N. (2020). Update of probiotics in human world: A nonstop source of benefactions till the end of time. M*icroorganisms 8*(12): 1907-1919.