

PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM SPONTANEOUSLY FERMENTED KUNUN-ZAKI

Abstract

This study was conducted to investigate the probiotic properties of twenty lactic acid bacteria (LAB) previously isolated and identified from naturally fermented kunun-zaki (a Nigerian fermented cereal beverage). The probiotic properties of the twenty lactic acid bacteria (LAB) isolates were assessed using different standard methods and agar well diffusion method. The results showed that all the isolates tested could tolerate acidic pH except one *Lactobacillus plantarum* and one *Lactobacillus brevis*; they survived at 30-40°C except one *Lactobacillus plantarum*. All the tested LAB isolates grew at 1% NaCl concentration and remarkably, six LAB isolates {*L. brevis* (3) and *L. plantarum* (3)} exhibited good growth at higher NaCl concentrations (10-15%). The highest percentage of cellular auto-aggregation was observed in *L. plantarum* (84.86%) and the lowest was obtained in *L. brevis* (27.44%). All the twenty LAB isolates tested in-vitro in this study grew in bile salt; fermented glucose; produced good aroma; produced no hemolysis revealing they were not pathogenic and produced antagonistic activity against indicated pathogens (*Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae* and *Escherichia coli*) which made them suitable and safe for human consumption as potential probiotics and for industrial purposes.

Comment [A1]: Correct and revise the sentence

Key words: Probiotic properties, Lactic acid bacteria, Kunun-zaki.

Introduction

Kunun-zaki is one of popular the indigenous fermented non-alcoholic beverages prepared from millet (*Pennisetum glaucum* L.), maize (*Zea mays*), or rice (*Oryza sativa*). Its wide acceptance has extended beyond the savannah region of Nigeria (Nwokolo, 2019) and also, is consumed at any time of the day by both adults and children as breakfast drink, food complement, refreshing drink for visitors, appetizers and are commonly served in social gatherings (Aleru *et al.*, 2017). The most promising microorganisms selected as starter culture are those that are isolated from the native microbiota of traditional products (Pereira, 2020) since they are well adapted to the

environmental conditions of food and are capable of controlling spoilage and pathogenic microbiota of food (Loranjo, 2019). To select a microorganism as a starter or starter culture, it is necessary to carry out a proper study regarding its metabolism and activities, since in some cases, its effects and/or properties may vary between laboratory conditions and food products (Diez and Saraiva, 2021). Also, starter culture must be recognized as safe, capable of being produced on a large scale and remain viable and stable during storage (Taskaila, 2017). The starter cultures that are mostly used to produce fermented foods and beverages, particularly acidic fermented products, belong to lactic acid bacteria (LAB). Such bacteria include members of *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Oenococcus*, and *Pediococcus*, and some of them may exert direct beneficial effects on health as live probiotic microbes (Moneim and Sulieman, 2017).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits on the host (Puebla-Barragan and Reid, 2021; Jeygowri *et al.*, 2015). Probiotic organisms used in foods must have the ability to resist gastric juices, exposure to bile, and be able to proliferate and colonize the digestive tract (Bari and Al- Mamun, 2021). The beneficial effects of probiotic foods on human health and nutrition are constantly increasing popularly being used as bio-ingredients in many functional fermented foods (Galdeano *et al.*, 2019; Tamang *et al.*, 2016). The most commonly used probiotic bacteria belong to the heterogeneous group of LAB (*Lactobacillus spp*, *Enterococcus spp*) and to the genus *Bifidobacterium spp*, however, yeasts and other microbes have also been developed as potential probiotics in recent years (Sen and Mansell, 2020). Products containing probiotic bacteria generally include foods and supplements (Soemarie *et al.*, 2021). Fermented milk products are the most traditional source of probiotic strains of lactobacilli (Khorshidian *et al.*, 2020);

however, commercial probiotic lactobacilli have also been added to meat products, snacks, fruit juice, etc. (Fenster *et al.*, 2019).

Comment [A2]: Incomplete sentence

The most commonly used *Lactobacillus* species in probiotic settings include *L. acidophilus*, *L. plantarum*, *L. casei*, *L. paracasei*, *L. johnsonii*, *L. reuteri*, and *L. rhamnosus* (Ahmed *et al.*, 2019). Lactic acid bacterial fermentation is used to improve sensory and nutritional properties of foods (Marthur *et al.*, 2020) and to produce products of high and consistent qualities, the fermentation process has to be controlled using tailor made starter culture; this would improve the products' shelf-life quality thereby reducing microbial risks associated with traditionally food process (Bintis,2018). Metabolic activities are associated with production of many beneficial compounds such as organic acids, polyols, exopolysaccharides and antimicrobial compounds (Bintis, 2018). Probiotic strains of *Lactobacilli* particularly adhere to the GI tract of the host cells, which is dependent on their aggregation ability (Nath *et al.*, 2020). They also act as a barrier to prevent the adherence of pathogenic bacteria, by interfering with colonization and proliferation of pathogens, thereby preventing the manifestation of infections (Patrignani *et al.*, 2019). *Lactobacilli* species also produces antimicrobial peptides (bacteriocins), organic acids (acetic acid and lactic acid), and other metabolites (hydrogen peroxide), which are reported to inhibit the growth of pathogenic bacteria (Li and Gu, 2018). Probiotic strains of *Lactobacillus plantarum* also participate in the absorption of water and sodium in the colon and decrease diarrhoea symptoms. They also exert holistic health benefits for medical conditions such as psoriasis, allergies, and nervous system diseases (Nath *et al.*, 2020). Among the potential benefits of probiotic lactic acid bacteria (LAB) is their practical use for bio-preservation of foods, fungal decontamination, and novel biotherapy (Egbere *et al.*, 2022). The aim of this study

was to evaluate the probiotic and safety properties of lactic acid bacteria isolated from spontaneously fermented kunun-zaki and to assess their potentials use in the food industry.

Comment [A3]: ?

Materials and methods.

Twenty LABs {*L. brevis* (7); *L. plantarum* (12) and *L. acidophilus* (1)} isolated from another study (Daniel *et al.*, 2023) from spontaneously fermented kunun-zaki and identified using RAPD-PCR analysis were used in this study. These twenty lactic acid bacteria were isolated from various kunun-zaki prepared from different cereal substrates (millet, sorghum, maize, wheat and paddy rice) (Daniel *et al.*, 2023).

Test for pH tolerance

The modified methods described by Ayo-Omogie and Okorie (2016) was used to determine the tolerance of the lactic acid bacteria (Table 1) obtained from another study to acidic pH when tested *in vitro*. 1 ml of each lactic acid bacterial culture at 1.5×10^8 Cfu/ml (fresh cultures of the isolates grown on MRS agar plates were harvested with sterile cotton swab and suspended separately in 1ml of sterile saline, 0.85% NaCl; to prepare a suspension equivalent to 0.5 McFarland turbidity standard) was inoculated into sterile de Man Rogosa and Sharpe (MRS) broth tubes of pH 2, 3 and 4. The pH was adjusted with HCl or NaOH using a pH meter (Hanna pocket-sized pH meter, model H1-98128) and incubated anaerobically at 37°C for 24h. After incubation, 1 ml inoculum from each tube was inoculated into MRS agar medium using pour plating technique and incubated anaerobically in an anaerobic jar at 37°C for 48 h. Presence or absence of growth of lactic acid bacteria on agar MRS was used to designate isolate as pH tolerant.

Sensitivity to temperature.

Test LAB culture (24h old) was inoculated into 10 ml sterile MRS broth for each of the isolate and incubated anaerobically at varying temperatures from 25 to 40°C for 48 to 72h. Thereafter, 1ml each of inoculum was pour plated on MRS agar and incubated at 37°C for 48h. The growth of LAB on MRS agar plates was used to designate isolates as temperature tolerant (Ayo-Omogie and Okorie, 2016).

NaCl tolerance test

MRS agar plates were prepared in eight different concentrations (%) of NaCl (0, 0.5, 1, 5, 7.5, 10, 12.5, and 15). Test LAB isolates grown on MRS broth for 24h were streaked on hardened MRS agar plates and these were incubated at 37°C for 24 h. The influence of NaCl concentrations on the degree of inhibition of bacterial growth was recorded (Nath *et al.*, 2020).

Cellular auto-aggregation assay

The methods described by Nath *et al.* (2020) was used; LAB grown on MRS broth overnight were centrifuged at 5000 rpm for 10 min to harvest the cell pellets. Pellets were repeatedly washed with phosphate-buffered saline solution (PBS) (pH 7.2), re-suspended in PBS , and the initial absorbance was noted at 600 nm, these bacterial suspensions was then incubated at 37°C for 2h, following which the final absorbance of the supernatant were measured at 600 nm. The percentage of cellular auto-aggregation was measured by the formula:-

Auto-aggregation rate (%) = $\frac{OD\ initial - OD\ final}{OD\ initial} \times 100$ (Nath *et al.*, 2020).

Bile tolerance test

One hundred microlitres (100µl) of overnight grown LAB cultures were inoculated in freshly prepared MRS broth containing 0.3% bile salt (Himedia Pvt. Ltd). Test isolates were also inoculated in MRS broth without bile (as control). Both test tubes (with and without bile salts) containing test isolates were incubated at 37°C for 4h, and growths at different time interval (0h,1h,2h,3h,4h) and percentage resistances recorded by measuring the absorbance of MRS broth at 600 nm. After the incubation period of 4 h, the viability of the LABs in 0.3% bile was also evaluated by spreading 100 µl of the bacterial samples onto the MRS agar plate (Nath *et al.*, 2020).

Glucose fermentation test

Eighteen-hour-old LAB cultures grown on MRS broth were centrifuged at 4000 rpm for 15 min, and the bacterial pellets were recovered. The pellets were washed twice by PBS buffer and re-suspended in the PBS buffer. Thereafter, 500 µl of PBS buffer containing the bacterial cells were inoculated into MRS broth supplemented by 1% glucose and 0.5% phenol red (dye) and incubated at 37°C for 24 h. Change in colour from purple to yellow indicates positive glucose fermentation, whereas no change in colour indicates negative glucose fermentation (Nath *et al.*, 2020).

Hemolytic activity

Hemolytic activity is a determining factor for probiotic bacteria, and the absence of hemolytic activity indicates that the particular bacteria were non-virulent. Sheep blood agar was prepared by weighing 2.8g of nutrient agar into 95mls of sterile distil water; sterilized at 121°C for 15

minutes in an autoclave and allow to cool to 45°C. 5% (vol/ vol) sterile defibrinated blood that has been warmed to room temperature was added and mix gently but well to avoid air and this was dispensed into sterile plates and allow to solidify. 24h LAB cultures were streaked on sheep blood agar and incubated at 37°C for 48 h. The zone formed around the colonies was observed, which is categorized as β -haemolysis (clear zone), α -haemolysis (green-hued zone), or γ -haemolysis (no zone) (Nath *et al.*, 2020).

Extraction of antibacterial agents and evaluation of their antagonistic activity

About 5 ml of 48h old LAB isolates were mixed with an equal volume of ethyl acetate and shaken at 20 rpm for 10 min in a rotary shaker (Table Top Model, PLT 207). The sample was centrifuged, and the supernatant was transferred into a fresh tube to evaporate the ethyl acetate. The remaining content was used to study the antagonistic effect on the selected test pathogen. The test pathogens were *Escherichia coli*, *Salmonella* species, *Shigella dysenteriae* and *Staphylococcus aureus* obtained from Afe Babalola University Ado-Ekiti (ABUAD) clinic. The pathogens were reactivated at 37°C for 24h on agar plates under aerobic condition as follows: Eosin methylene blue agar for *Escherichia coli*; Salmonella-Shigella agar for *Salmonella typhi* and *Shigella dysenteriae*; manitol salt agar for *Staphylococcus aureus*. The effectiveness of bacterial metabolites was studied by agar well diffusion method (Shakhatreh *et al.*, 2017). MHA plates of each test pathogen were prepared, and wells were made using a sterile cork borer (5 mm). The wells were loaded with of 60 μ l aliquots of the supernatant and incubated at 37°C for 24h. Dimethyl sulfoxide was used as a control. The diameters of the zones of growth inhibition were measured and classified as sensitive or +++ (<20 mm), moderate or ++ (10–20 mm), low or + (>10 mm) and resistant or – (0mm) (Prabhurajeshwar and Chandrakanth, 2017).

Potential use in fermentation

The acidification ability of isolated LAB cultures was evaluated on their potential use in food fermentation according to the methods described by Wang *et al.* (2018) in triplicates. LAB cultures were inoculated in reconstituted sterile skimmed milk powder (10% w/v) at 1%. A pH meter was used to determine the pH value changes in the milk at different time (h) intervals (3, 6 and 24) of incubation at 37°C. The acidification rate was calculated as:

$$\Delta\text{pH} = \text{pH}_0 - \Delta\text{pH}_t$$

Values of ΔpH after 3h (ΔpH_3), 6h (ΔpH_6) and 24h (ΔpH_{24}) were used to compare the strain-acidifying activity (Wang *et al.*, 2018).

Pure cultures of the twenty identified LABs were tested singly for their potential use as starter culture for kunun-zaki production. Fresh cultures of the isolates grown on MRS agar plates were harvested with sterile cotton swabs and suspended (separately) in 1ml of sterile saline (0.85% NaCl) to prepare a dense suspension (equivalent to a no. 2 McFarland turbidity standard; Agarry *et al.*, 2010); this was used to inoculate singly or in various combinations of two or three LAB isolates into 10ml of sterile Hydrolyzed cereal starch Broth (HCS-broth: 500g gelatinized cereal starch was hydrolyzed with 200g ground malted rice to which 2g of soy bean flour was added and sterilized at 121°C for 10 min) and incubated at ambient temperature (30±2°C) for 24h (Agarry *et al.*, 2010). After incubation, the samples were subjected to sensory evaluation by a 5 member trained expert profile panelist that can distinguish between intensity of aroma

differences. Production of good aroma (buttery) was used as quality indicator to establish their potential use in inoculum development (Agarry *et al.*, 2010).

Comment [A4]: ?

Comment [A5]: Please provide in detail organoleptic evaluation and discuss other parameters of evaluation.

Results and Discussion

pH tolerance of LAB tested.

Comment [A6]: Correct the heading

Tolerance to low pH is one of the most important criteria in the selection of probiotic species and of the twenty lactic acid bacteria (LAB) isolates used in this study. Eight (8) of the isolates comprising of *Lactobacillus brevis* (3); *Lactobacillus plantarum* (4) and *Lactobacillus acidophilus* (1) were grown at pH 2, this was in accordance with the reports of Ayo-Omogie *et al.* (2016) that *Lactobacillus acidophilus* and *Lactobacillus plantarum* tolerated pH 2.0. Fourteen of the isolates comprising of *Lactobacillus brevis* (5); *Lactobacillus plantarum* (8) and *Lactobacillus acidophilus* (1) grew at pH 3 (Table 1). Interestingly, most of the LAB isolates studied were observed to grow at pH 4, however, two LAB isolates comprising of *L. brevis* (1) and *L. plantarum* (1) did not grow at any of the pH (2, 3, 4) used in this study (Table 1).

Sensitivity to temperature result

Most of the lactic acid bacteria isolates studied survived the selected temperature range of 25-40°C. Nine isolates of *L. plantarum* and isolates of *L. brevis* grew at 25°C. All the twenty LAB isolates were able to grow and survive at temperatures of 30°C and 35°C (Table 1). This was in conformity with the result obtained by Ayo-Omogie and Okorie (2016) that most of the isolates grew at 25°C-40°C. These results may be an indication of their potential to survive temperature of the human gut and the selected temperature ranges was chosen to simulate the normal human body temperature.

UNDER PEER REVIEW

Comment [A7]: Correct the table caption

Table 1: LAB isolates growth characteristics at different pH, temperature and NaCl salt concentrations.																
S/N	LAB isolates used	Growth pH			Growth temperature (°C)				Growth in NaCl (% , 100g/100ml)							
		2	3	4	25	30	35	40	0	0.5	1	5	7.5	10	12.5	15
1	<i>L. plantarum</i>	-	-	-	+	+	+	-	+	+	+	-	-	-	-	-
2	<i>L. brevis</i>	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-
3	<i>L. brevis</i>	-	+	+	-	+	+	+	+	+	+	+	-	+	-	-
4	<i>L. brevis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
5	<i>L. brevis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
6	<i>L. plantarum</i>	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-
7	<i>L. brevis</i>	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
8	<i>L. plantarum</i>	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-
9	<i>L. plantarum</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	<i>L. brevis</i>	-	+	+	-	+	+	+	+	+	+	+	+	-	-	-
11	<i>L. plantarum</i>	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-
12	<i>L. plantarum</i>	-	-	+	-	+	+	+	+	+	+	+	-	-	-	-
13	<i>L. plantarum</i>	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-
14	<i>L. plantarum</i>	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-
15	<i>L. plantarum</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
16	<i>L. plantarum</i>	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-
17	<i>L. plantarum</i>	-	+	+	-	+	+	+	+	+	+	-	-	-	-	-
18	<i>L. brevis</i>	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
19	<i>L. acidophilus</i>	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-
20	<i>L. plantarum</i>	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-

These lactic acid bacteria were probably of different strains even though they were from the same species. +: Growth;- No growth

Sodium chloride tolerance test result

As shown in Table 1, nineteen of the LAB isolates studied were able to tolerate growth on 0, 0.5 and 1% NaCl concentrations, only one isolate of *L. plantarum* that did not grow in 1% NaCl concentration and likewise at 5% NaCl concentration. Fifteen out of the twenty lactic acid bacteria studied grew at 5% NaCl concentration while 8 isolates survived and grew in 7.5% NaCl concentration (Table 1). Furthermore, 6 of the LAB isolates comprising of *L. brevis* (3) and *L. plantarum* (3) tolerated 10% NaCl concentrations. Nath *et al.* (2020) reported no growth above 10% NaCl concentration. Remarkably, the isolates in this study tolerated higher salt concentrations than those reported by Ayo-Omogie and Okorie (2016) and Nath *et al.* (2020). Ayo-Omogie and Okorie (2016) reported that all their isolates grew at 4-6% NaCl concentrations and Nath *et al.* (2020) reported that no growth was observed above 10% NaCl concentration. However, at 12.5% NaCl concentration, two of the *L. plantarum* grew well and similarly at 15% NaCl concentration, one *L. brevis* and one *L. plantarum* exhibited good growth in this study (Table 1).

Comment [A8]: Some more explanation will make your argument more stronger.

Glucose fermentation test result

Also shown in Table 2, all the twenty LAB isolates fermented glucose. The colour of the medium changed from red to yellow which indicated that this sugar was fermented to produce organic acids thereby reducing the pH of the medium. This was in conformity with the report of Nath *et al.* (2020) that the *Lactobacillus plantarum* isolated was capable of fermenting glucose.

Comment [A9]: You can use the word "selected" instead of "indicated"

Table 2: Hemolytic activity, glucose fermentation, degree of aroma and antagonistic activity of lactic acid bacteria species against indicated pathogens

S/N	LAB used	Hemolytic activity	Glucose fermentation	Degree of aroma	LAB isolates inhibition characteristics			
					<i>E coli</i>	<i>S aureus</i>	<i>S typhi</i>	<i>S dysenteriae</i>
1	<i>L plantarum</i>	-	+	+	+++	+++	+	-
2	<i>L. brevis</i>	-	+	+	+	++	-	+
3	<i>L. brevis</i>	-	+	+	+	+++	+	++
4	<i>L. brevis</i>	-	+	+++	+	+++	+	++
5	<i>L. brevis</i>	-	+	+	-	++	+	-
6	<i>L plantarum</i>	-	+	+++	++	++	++	+
7	<i>L. brevis</i>	-	+	+++	-	++	+	++
8	<i>L plantarum</i>	-	+	+++	++	+++	++	+++
9	<i>L plantarum</i>	-	+	+++	++	+++	++	++
10	<i>L. brevis</i>	-	+	+	++	+++	++	++
11	<i>L plantarum</i>	-	+	+++	+	+++	++	++
12	<i>L plantarum</i>	-	+	+++	++	+++	+	+++
13	<i>L plantarum</i>	-	+	+++	++	+	-	++
14	<i>L plantarum</i>	-	+	+	+	+++	+	+++
15	<i>L plantarum</i>	-	+	+++	+	++	++	+
16	<i>L plantarum</i>	-	+	+++	-	++	++	++
17	<i>L plantarum</i>	-	+	+++	-	+++	+	+++
18	<i>L. brevis</i>	-	+	+++	+++	+++	++	++
19	<i>L.acidophilus</i>	-	+	+++	+	+++	++	++
20	<i>L plantarum</i>	-	+	+++	++	++	+	

These lactic acid bacteria were probably of different strains even though they were from the same species but from different cereal substrates.=Hemolytic activity: -no hemolysis; Glucose fermentation: + ability of isolates to ferment glucose; Degree of aroma: +++-strong buttery aroma present,+ -weak aroma; LAB isolates inhibition characteristics:- +++ =20-30 mm zone of inhibition, ++ =10-20mm zone of inhibition, + =1-10mm zone of inhibition, - = no zone of inhibition.

Hemolytic activity

The hemolytic activity of the twenty selected Lactic acid bacteria isolates were evaluated on sheep blood agar plates and no hemolytic effects were observed (γ -hemolysis). This result implies that all the LAB isolates were non-virulent (Table 2). This was in conformity with the report of Nath *et al.* (2020) that the in-vitro screening of probiotic properties of *Lactobacillus plantarum* from fermented milk product did not show any hemolysis when tested. Similarly, Wang *et al.* (2018) reported that all the ten selected strains of lactic acid bacteria screened showed no hemolytic activity.

Comment [A10]: ?

Extraction of antibacterial agents and evaluation of their antagonistic activity

The antagonistic activity of the twenty selected Lactic acid bacteria used in this study as against four pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Shigella dysenteriae*) is represented in Table 2. Twelve lactic acid bacteria isolates comprising of *L. brevis* (3), *L. plantarum* (8) and *L. acidophilus* (1) showed a strong antagonistic activity of 20-30 mm against *Staphylococcus aureus*. However, *L. plantarum* showed the highest zone of inhibition (30mm) against *S. aureus* and another *L. plantarum* showed the least (<10 mm) against the same organism (Table 2). A high antagonistic activity of 20mm was also observed in *L. plantarum* against *Shigella dysenteriae* whereas the same isolate did not show any antagonistic activity against *Escherichia coli* (0 mm). Furthermore, *L. brevis* showed the highest antagonistic activity of 30mm against *Escherichia coli* (Table 2). Ayo-Omogie and Okorie (2016) reported that the highest inhibition of 39mm was displayed by *Lactobacillus acidophilus* against *Salmonella enterica subsp. typhi* while the most significant inhibition against *Escherichia coli* was shown by *Lactobacillus plantarum* (35mm).

Comment [A11]: Correct the sentence

Potential use in fermentation

The salt tolerance, acidification ability as well as the LAB's ability to produce good aroma when inoculated into sterile HCS broth were examined on selected probiotic LAB strains to evaluate their potential use in food fermentation (Table 2). For acidification ability (Table 4), *Lactobacillus plantarum* (1) produced the highest value of ΔpH after 3h incubation ($\Delta\text{pH}_3= 1$) followed by three other *Lactobacillus plantarum* isolates ($\Delta\text{pH}_3= 0.7$ for the three isolates). However, after 6h of incubation, the highest acidification rate ΔpH_6 of (1.3) was observed in *L. plantarum* while after 24h of incubation, the highest acidification rate (ΔpH_{24}) of 3 was still observed in *L. plantarum*. It was also observed that as the hour of fermentation is increasing, acidification rate increased (Table 4). All these isolates were probably different strains of *Lactobacillus plantarum*. The degree of aroma produced by the LAB isolates differed (Table 2). Fourteen LAB isolates comprising of *Lactobacillus brevis* (3), *Lactobacillus plantarum* (10), *Lactobacillus acidophilus* (1) were able to produce strong (good) buttery aroma while six isolates produced weak aroma (Table 2). Onyimba *et al.*(2017) reported that *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus fermentum* produced the best aroma while fermenting hydrolysed sorghum starch (HSS). Production of good aroma (buttery) was used as quality indicator to establish their potential use in inoculum development.

Bile tolerance test

Bile tolerance is one of the most crucial properties for probiotic bacteria, as it determines its ability to survive in the small intestine, and consequently its capacity to play its functional role as a probiotic (Nath *et al.*, 2020). The results obtained in this study (Table 4) show that the twenty

lactic acid bacteria (LAB) were able to grow in the medium containing 0.3% bile salt. Nath *et al.* (2020) reported a survival rate of 83.70% after 3 hours of incubation.

Cellular auto-aggregation assay result

The ability to aggregate is a desirable property for probiotics in health promoting foods and in this study, highest percentage of cellular auto aggregation was observed in a strain of *Lactobacillus plantarum*, LAB-46MB-1, which was 84.86% followed by another strain of *Lactobacillus plantarum*, LAB-49MB-1, which was 79.23% and the lowest cellular auto-aggregation of 27.44% was recorded in one strain of *Lactobacillus brevis*, LAB-10SB-6 (Table 4).

S/N	LAB isolates	Absorbance of isolates grown with bile salts					Absorbance of isolates grown without bile salts					Growth on MRS agar	
		0	1	2	3	4	0	1	2	3	4	With bile salt	Without bile salt
1	<i>L. plantarum</i>	0.7	0.8	0.9	1.0	1.2	0.7	0.6	0.8	0.9	1.4	+	+
2	<i>L. brevis</i>	0.8	0.7	0.9	1.9	1.5	0.7	0.6	1.0	1.1	1.7	+	+
3	<i>L. brevis</i>	0.7	0.8	1.0	1.2	1.5	0.8	0.6	0.2	1.7	2.2	+	+
4	<i>L. brevis</i>	0.7	0.8	0.9	1.1	1.2	0.8	0.7	0.9	0.9	1.1	+	+
5	<i>L. brevis</i>	1.0	1.1	0.9	1.0	1.4	1.1	0.8	0.9	1.3	2.0	+	+
6	<i>L. plantarum</i>	0.5	0.6	1.0	0.1	1.9	0.5	0.4	1.2	1.4	2.0	+	+
7	<i>L. brevis</i>	1.6	1.7	1.0	1.2	1.7	1.1	1.2	1.3	1.8	2.1	+	+
8	<i>L. plantarum</i>	0.7	0.8	1.0	1.3	1.5	0.5	0.5	0.9	1.2	1.8	+	+
9	<i>L. plantarum</i>	0.8	0.8	1.6	1.5	1.9	0.6	0.6	1.1	1.8	2.0	+	+
10	<i>L. brevis</i>	0.7	0.7	1.0	1.2	1.6	0.7	0.6	1.0	1.3	2.0	+	+
11	<i>L. plantarum</i>	0.7	0.8	0.8	1.0	1.2	0.6	0.6	0.9	1.0	1.1	+	+
12	<i>L. plantarum</i>	0.7	0.8	0.9	0.9	1.6	0.6	0.6	0.9	1.0	1.6	+	+
13	<i>L. plantarum</i>	1.2	1.2	0.9	1.1	1.3	0.8	0.6	0.9	1.0	1.4	+	+
14	<i>L. plantarum</i>	1.3	1.3	0.9	1.6	1.9	1.4	1.0	0.9	1.3	1.2	+	+
15	<i>L. plantarum</i>	1.5	1.5	1.4	1.4	2.0	1.0	0.9	0.9	1.2	2.0	+	+
16	<i>L. plantarum</i>	1.7	0.7	1.5	1.4	2.0	0.9	0.7	0.9	1.4	2.2	+	+
17	<i>L. plantarum</i>	0.8	0.8	1.0	1.3	1.7	0.7	0.6	0.9	1.1	2.2	+	+
18	<i>L. brevis</i>	0.6	0.6	1.0	1.3	1.7	0.6	0.6	0.9	1.3	2.2	+	+
19	<i>L. acidophilus</i>	0.8	1.0	0.8	1.3	1.9	0.7	0.6	1.0	1.5	2.2	+	+
20	<i>L. plantarum</i>	1.3	1.1	1.0	1.3	1.7	1.2	1.0	0.9	1.0	1.8	+	+

These lactic acid bacteria were probably of different strains even though they were from the same species but from different cereal substrates.

S/N	Lab Code	Cellular Auto-Aggregation Assay			LAB Growth (h) in 10% Skimmed Milk						
		Initial OD	Final OD	%	0	3	ΔpH_3 (3)	6	ΔpH_6 (6)	24	ΔpH_{24} (24)
1	<i>L. plantarum</i>	0.5	0.3	41.4	7.2	6.9	0.3	6.9	0.3	6.8	0.4
2	<i>L. brevis</i>	0.4	0.3	27.4	7.2	6.8	0.4	6.6	0.6	6.6	0.6
3	<i>L. brevis</i>	0.5	0.3	38.2	7.2	6.9	0.3	6.8	0.4	6	1.2
4	<i>L. brevis</i>	0.4	0.2	46.1	7.2	6.8	0.4	6.7	0.5	6.6	0.6
5	<i>L. brevis</i>	0.9	0.3	66.5	7.2	6.6	0.6	6.5	0.7	6.2	1
6	<i>L. plantarum</i>	0.4	0.2	41.3	7.2	6.8	0.4	6.7	0.5	6.3	0.9
7	<i>L. brevis</i>	0.5	0.3	44.8	7.2	6.9	0.3	6.8	0.4	5.8	1.4
8	<i>L. plantarum</i>	0.4	0.3	30.0	7.2	6.2	1	5.9	1.3	5.5	1.7
9	<i>L. plantarum</i>	0.4	0.2	49.7	7.2	6.5	0.7	6.1	1.1	5.2	2
10	<i>L. brevis</i>	0.5	0.3	45.6	7.2	6.7	0.5	6.7	0.5	6.3	0.9
11	<i>L. plantarum</i>	0.5	0.3	43.3	7.2	6.5	0.7	6	1.2	4.2	3
12	<i>L. plantarum</i>	0.4	0.2	51.0	7.2	6.6	0.6	6.2	1	4.4	2.8
13	<i>L. plantarum</i>	0.7	0.4	42.8	7.2	6.8	0.4	6.6	0.6	5.4	1.8
14	<i>L. plantarum</i>	0.7	0.4	45.5	7.2	6.8	0.4	6.6	0.6	6.1	1.1
15	<i>L. plantarum</i>	1.5	0.2	84.9	7.2	6.8	0.4	6.1	1.1	5.1	2.1
16	<i>L. plantarum</i>	1.5	0.3	79.2	7.2	6.7	0.5	6.7	0.5	5.4	1.8
17	<i>L. plantarum</i>	0.5	0.3	40.8	7.2	6.5	0.7	6.1	1.1	5.3	1.9
18	<i>L. brevis</i>	0.5	0.3	48.2	7.2	6.6	0.6	6.3	0.9	4.9	2.3
19	<i>L. acidophilus</i>	0.5	0.2	58.3	7.2	6.6	0.6	6.5	0.7	5	2.2
20	<i>L. plantarum</i>	0.9	0.5	50.3	7.2	6.7	0.5	6.6	0.6	5.7	1.5

These lactic acid bacteria were probably of different strains even though they were from the same species but from different cereal substrates; OD=optical density; ΔpH =acidification rate; +=presence; -=absence

Conclusion

In the present study, the lactic acid bacteria (*Lactobacillus brevis*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*) tested in-vitro possessed good probiotic characteristics. They were able to survive at low pH, they survived the selected temperature range (25-40°C), they survived at high NaCl concentrations; they exhibited good antimicrobial activities and produced no hemolysis. These organisms can be good candidates for food industries as prospective probiotic cultures.

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