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Assay of probiotic qualities and safety of lactic acid bacteria starter cultures isolated from fermented Fonio millet-based Ogi—a breakfast gruel

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Abstract

Aim: Lactic acid bacteria (LAB) starter cultures developed during the production of Fonio millet gruel, Ogi, were screened for their probiotic potential.

Methods: The starter cultures were assayed in vitro on the basis of their resistance to pH 2.0 and 0.3% bile salts, surface properties, antimicrobial, antibiotic, antioxidant activities and safety by evaluating their gelatinase, hemolytic, and deoxyribonuclease (DNase) activities.

Results: The starter cultures exhibited high survival rates (> 50%) at pH 2.0 and bile salts (0.3%) for 4 h. The zone of inhibition of the LAB starter cultures (LSC) ranged from 6 mm to 25 mm against the 6 tested enteric pathogens. The cell surface properties indicated hydrophobicity with xylene as high as 68.29%, microbial adhesion 79.55%, and high level of auto-aggregation (> 40%) at 20 h. Strains of *Lactiplantibacillus plantarum* CAM 7 and *Lactiplantibacillus plantarum* 2.7.17 exhibited desirable antioxidant properties (> 60%) for DPPH scavenging activity, probably owing to their unique metabolites. The safety of the starter cultures was confirmed by negative responses to the safety matrices.

Conclusions: The outcome of this study showed the probiotic potential of Fonio gruel LSC, which could find application as functional bio-control agents in food fermentation with promising health benefits.

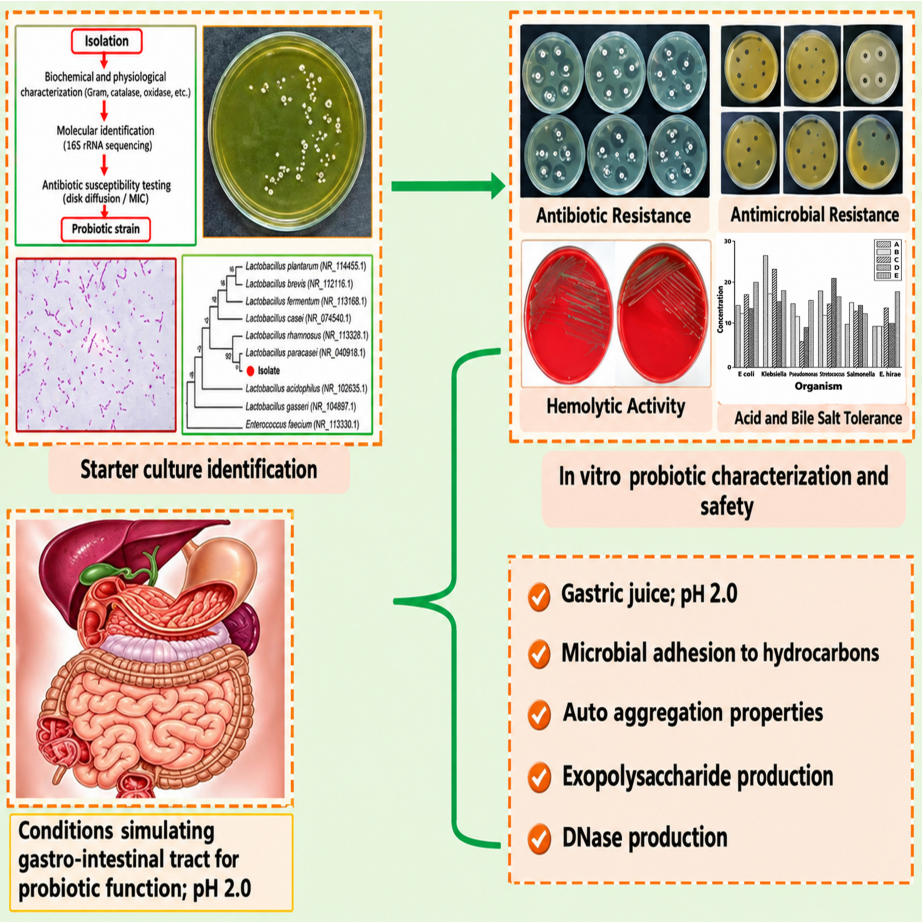
Keywords

Fonio millet, fermentation, starter culture, lactic acid bacteria, probiotic potential

Introduction

Fermented foods are recognized as sources of multifunctional salient and health promoting microbiomes produced by spontaneous processes that contain desirable nutrients, bioactive components, antimicrobial

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Graphical abstract. Graphical illustration of probiotic qualities of Ogi LAB.

compounds, and beneficial microorganisms such as lactic acid bacteria (LAB) [1, 2]. This group of microorganisms is Gram-positive, cocci or rod shaped, non-sporulating, acid-tolerant, and mainly lactic acid producers, with the most important genus *Lactobacillus*, known to have received "Generally Recognized As Safe" (GRAS) status [3, 4].

Presumptive LAB (n = 28) identified phenotypically to belong to the *Lactobacillus* and *Pediococcus* species isolated from fermented foods are widely employed as starter cultures in food fermentation and scientific research in order to accelerate the process, facilitate reproducibility, improve palatability and overall quality of the fermented food, as well as control opportunistic spoilage and pathogenic microbiota for safety and biopreservation [5–7]. The process of LAB fermentation involves the conversion of sugars, particularly glucose, either via a homo-lactic or hetero-lactic fermentation with the production of lactic acid through a rapid acidification process at values below pH 4.6. The acidification inhibits the growth of undesirable spoilage and pathogenic microorganisms that can serve as threat to public health [8]. Among LAB that play a pivotal role in gastrointestinal balance are health promoting microorganisms called probiotics [9], which have already received much publicity for their beneficial health effects on humans, animals, plants and foods. Probiotics have been associated with LAB fermentation as beneficial live microbiomes which, when consumed in adequate amounts [ca. 10^6–10^7 colony forming unit (CFU)/mL] survive and confer health benefits to the host gastrointestinal tract (GIT) [10, 11]. The gut modulation activity of probiotics is greatly influenced by prebiotics and postbiotics. Prebiotics are non-digestible substances, particularly dietary fibers, that promote the growth and activity of beneficial gut bacteria, while postbiotics exemplify the beneficial compounds produced by probiotics in the gut [9].

LAB strains, predominantly *Lactobacillus* species isolated from the fermentation of several African traditional foods, especially cereals ("Ogi", "kunun-zaki", "burukutu", and "pito") and tuber-based food products (e.g., "gari", "fufu", and "lafun"), have demonstrated desirable characteristics that make them safe

for use as probiotic microorganisms and even as starter cultures for controlled fermentation of African indigenous fermented foods [12–14]. However, despite their excellent roles as starters, data regarding their probiotic health benefits are still scanty.

The use of well-defined functional starter cultures with added nutritional benefits, particularly during traditional microbial fermentation, is documented to enhance the production of fermented foods with improved quality [15]. One such commonly consumed health sustaining readily available African fermented food is 'Ogi' [16], which is a cereal-based fermented gruel with a normally smooth, free-flowing consistency. It is obtained from wet-milled, fermented maize, sorghum, or millet grain. It offers several functional applications in the diet as a major weaning food for infants, a breakfast meal for both children and adults, and sometimes as food for the convalescents [17]. Recently, consumers have become more health- and nutrition-conscious; and consequently, different strains with various therapeutic properties are constantly being identified from traditional fermented foods from many countries [18]. At the same time, there is a paucity of probiotics with starter culture functionality for daily maintenance of the gastro-intestinal tract [19], which has necessitated insights into this study of probiotic LAB starter cultures (LSC).

Fonio millets are nutritionally excellent cereal grains, particularly native to Western African landrace systems, and are rapidly gaining research attention from different parts of the world due to their excellent nutritional properties. The two species common to sub-Saharan Africa are white Fonio millet (*Digitaria exilis*) (commonly referred to as acha) and black Fonio millet (*Digitaria iburua*) (commonly referred to as iburu). The grains have been researched and found to be uniquely rich in sulphur containing amino acids (methionine and cysteine) [20], suitable for celiac patients as a gluten free cereal [21] and used as nutraceuticals for the control of blood glucose. When cooked as a whole grain, it is light, easily digestible and can be included in many different cereal based recipes, making it an attractive ingredient for healthy food products, especially for those with gluten intolerance, poor health, or for complementary weaning food and other potential food applications [21].

Previous studies have focused intensely on improving the nutritional and sensorial properties of Ogi by adding legume based products [22–25]. However, only a few studies have focused on examining the health benefits of Ogi and improving its quality by optimizing some processes such as fermentation. The lactic acid and other desirable metabolites produced by probiotic LAB can contribute to the sour taste of Fonio Ogi, improving safety and consumer acceptability, as functional characterization of probiotics is not only strain specific but largely depends on origin [26]. Considering the ability of probiotics, prebiotics, and postbiotics to successfully modulate the gut microbiota, maintain better health, and prevent/treat disease, preparation of a diet that can promote this tripartite functionality is highly essential. Therefore, the aims of this study were to evaluate in vitro the probiotic properties and antioxidant activities of carefully selected starter cultures of *Lactiplantibacillus plantarum* CAM 7, *Pediococcus pentosaceus* NRIC 0123, *L. plantarum* 2.7.17, *P. pentosaceus* CP16 and *Levilactobacillus brevis* 15F1 strains isolated from traditionally fermented Fonio millet Ogi. The antioxidant activity, gelatinase production, DNase production and haemolytic activity matrices of the probiotic strains were also examined to ascertain their safety.

Materials and methods

Isolation and identification of probiotic strains

The LAB strains employed in this study were isolated from traditional fermented Fonio Ogi. One gram of the food sample was serially diluted and spread on de Man, Rogosa and Sharpe (MRS; Oxoid, Hampshire, England) agar at 37°C for 24 h. Individual colonies were then inoculated and incubated in MRS broth at 37°C for 24 h. The LSC were identified in a previous study reported by Adisa et al. [27] as *L. plantarum* CAM 7, *P. pentosaceus* NRIC 0123, *L. plantarum* 2.7.17, *P. pentosaceus* CP16 and *L. brevis* 15F1 by sequencing the 16S rRNA of the bacterial isolates. The nucleotide sequences indicated 93–100% similarities and were deposited in the Genbank of the National Centre for Biotechnology Information (NCBI).

Determination of potential probiotic properties of LSC

Survival under acidic conditions

Survival of the LSC cultures under acidic conditions was determined using the method described by Huligere et al. [28] and Mulaw et al. [29] with slight modifications. Precisely, l mL of each overnight grown culture was inoculated into 10 mL of sterile MRS broth and centrifuged at 3,500 × *g* for 10 min. The pellets were re-suspended in 5 mL sterile MRS broth and 5 mL of cellular suspension of 8 Log CFU/mL was infused into a 45 mL sterile normal saline solution (0.9% w/v) with the pH adjusted to 2.0 and using 5 N HCl to simulate the gastric environment. The test tubes were incubated under anaerobic conditions for 0 h, 2 h and 4 h at 37°C. Precisely, 100 µL aliquot of the samples were then taken every hour for 4 h and, using a 10-fold serial dilution prepared in 0.1% peptone water, the viable numbers of the probiotic cultures were enumerated by pour plate method on MRS agar medium and incubated at 37°C for 48 h, under anaerobic conditions. The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration:

$$\%\ \ log\ \ survival\ \ rate = \frac{Log\ \ X_1}{Log\ \ X_O} \times \ \ 100$$

where X_1 is the viable count (CFU/mL) of isolates after incubation, and X_0 is the initial viable count before incubation under harsh conditions.

Tolerance to bile salts

Bile tolerance of the LSC was measured by employing bile salts, according to the method described by Huligere et al. [28] and Mulaw et al. [29]. Individual isolates were grown overnight in MRS broth incubated at 37°C under anaerobic conditions. An initial concentration of 7 Log CFU/mL was taken and centrifuged at 3,500 × *g* for 10 min. The pellets were washed twice in phosphate saline buffer (PBS) pH 7.2, re-suspended in sterile MRS broth supplemented with (0.3%, w/v) Ox-bile (Oxoid, Basingstoke, Hampshire, England) and incubated anaerobically at 37°C for 4 h. Concisely, an aliquot (100 µL) of the samples was then taken at time intervals of 0 h, 2 h and 4 h, respectively, by using a 10-fold serial dilution prepared in 0.1% peptone water. The viable numbers of the starter cultures were enumerated by pour plate technique on MRS agar and incubated at 37°C for 48 h. Sterile MRS broth without bile salts was set up as the control. The counted LAB colonies were expressed as CFU/mL. The survival rate was calculated as LAB colonies (expressed as a percentage) grown on MRS agar compared to the initial bacterial concentration:

$$\%\ \ Survival\ \ rate\ \ = \ \ \frac{Log\ \ X}{Log\ \ X_0} \times \ \ 100$$

where X is the viable count (CFU/mL) of isolates after incubation, and X_0 is the initial viable count before incubation under harsh conditions.

Determination of antimicrobial activity of isolates

Antimicrobial activity was determined using the agar-well diffusion technique with slight modifications to the protocol described by Olatunde et al. [30]. The test organisms (*S. enteritica* Serova Typhimurium SKN 533, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC 700603, *Streptococcus pyogenes* ATCC 12344 and *Enterococcus hirae* ATCC 9790) were obtained from the Department of Microbiology, Ladoke Akintola University, Ogbomoso, Nigeria. The selected LSC were inoculated from slants into fresh MRS broth and incubated anaerobically at 37°C for 48 h, centrifuged (5,000 × *g*, 4°C for 10 min) and the cell-free supernatant (CFS) was obtained. Pure cultures of the test organisms were suspended in sterile normal saline (NaCl, 0.9%) to obtain a final concentration (ca. 10^6 CFU/mL). After 24-hour incubation at 37°C, a 100 µL aliquot of each indicator bacterium was mixed with MH (Mueller Hinton) soft agar (Oxoid, Hampshire, England) and swabbed evenly over the surface of the agar plates. A sterile cork borer (diameter 5 mm) was used to cut uniform wells in the agar, wherein each well was filled with 100 µL CFS obtained from each of the LSC isolates. After incubation at 37°C for 48 h, the diameter (mm) of the inhibition zone around the well was measured and a clear zone of ≥ 1 mm was considered positive inhibition.

Antibiotic resistance activity of the isolates

Antibiotic resistance was assayed by the disc diffusion method on MH agar plates as described by Ajayeoba and Ijabadeniyi [31] against some commercial antibiotic discs. Thus, 100 µL of fresh culture of each LSC was evenly swabbed over the surface of MH agar plates using a sterile cotton swab. After drying, the antibiotic discs were placed on the solidified agar surface and the plates were allowed to stand for 30 min at 4°C for the diffusion of antibiotics and then incubated anaerobically at 37°C for 48 h. The diameters (mm) of the zone of inhibition (ZOI) for each of the antibiotics were measured and expressed as susceptible, *S* (≥ 21 mm); intermediate, *I* (16–20 mm), and resistance, *R* (≤ 15 mm).

Adhesion ability to intestinal mucosa using hydrocarbons

The adhesion ability was assessed for the LSC by measuring cell surface hydrophobicity (CSH), microbial adhesion to hydrocarbons (MATH), and auto-aggregation indexes using the protocol described by Ajayeoba and Ijabadeniyi [31], with some modifications. The cells were grown in MRS broth at a temperature of 37°C for 24 h and centrifuged at 5,000 × *g* for 15 min. The pellets were then washed twice with PBS pH 7.0 and finally re-suspended in 3 mL of 0.1 M KNO_3 containing about 8 Log CFU/mL of bacteria. The absorbance was measured at 600 nm, after which 1 mL of the microbial suspension was added to 1 mL of different hydrocarbons (chloroform and xylene). The mixture was then vortexed for 30 sec and left to rest for 30 min for phase separation. The optical density (OD) of the aqueous separation was measured again at 600 nm and compared with the initial value. The percentage CSH was calculated using the equation:

$$\%\ \ hydrophobicity\ \ = \ \ \frac{A_i\ \ - A_f}{A_f} \times \ \ 100$$

where A_i is the cell pellet OD_{600}; A_f is the aqueous phase OD_{600} at the end of the experiment.

Autoaggregation assay

The LSC isolates were evaluated for their capacity to form self-aggregates via spectrophotometric assay by adopting the protocol described by Ajayeoba and Ijabadeniyi [31]. The LSC isolates were grown at 37°C for 48 h in MRS broth. The cells were subsequently harvested by centrifugation at 7,000 × *g* for 10 min, washed twice with phosphate buffered saline (pH 7.0), and re-suspended in PBS pH 7.0 at a concentration of approximately 8 Log CFU/mL corresponding to an OD of 0.25 measured at 600 nm. The bacterial suspension (4 mL) was mixed by vortexing for 10 sec and incubated at 37°C without shaking the cell suspension. Autoaggregation was observed by measuring the absorbance ($OD_{600\ nm}$) of the top clear suspension after 3 h and 20 h of incubation. The auto-aggregation percentage was expressed as:

$$Autoaggregtion\ (\%)\ \ \ = \ \ \left(1\ - \frac{A_T}{A_0}\right) \times \ \ 100$$

where A_T represents the absorbance at time t = 3, or 20 h and A_0 the absorbance at t = 0.

Exopolysaccharides production

Exopolysaccharides (EPS) released by LSC isolates were evaluated following the method of Abushelaibi et al. [32]. Overnight cultures of the selected LAB starter isolates were streaked on the surface of plates containing ruthenium red milk (g/l: skim milk powder 100.0, sucrose 10.0, ruthenium red 0.08, agar 20.0). After incubation at 37°C for 24 h, the appearance of white colonies was taken as capable EPS producing ropy isolates and red colonies as non-ropy isolates owing to the staining of microbial cell wall.

In vitro safety assessment of LSC

The safety assessments of the LSC isolates were carried out in vitro by determining antioxidant activity, gelatinase production, DNase production, and haemolytic activity.

Antioxidant properties

The antioxidant properties of LSC were determined in the CFS of strains in freshly prepared MRS broth after 24 h by 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) scavenging capacity as elaborated by Rai et al.

[33] and hydroxyl scavenging activity as outlined by Łepecka et al. [34]. The cultures in MRS broth were centrifuged at 8,000 × *g* for 10 min at 4°C and the MRS broth was washed with 0.1% PBS and centrifuged again at 8,000 × *g* for 10 min at 4°C. For DPPH scavenging property, the experiment was initiated by mixing 800 µL of freshly prepared 0.20 mmol/L of DPPH (Sigma-Aldrich, St. Louis, MO, USA) solution in 80% methanol with 400 µL of culture supernatant and vortexed for 30 sec. An equal volume of methanol was applied to replace the DPPH solution as the blank. The mixture was incubated at 37°C in the dark for 30 min. Scavenging ability was determined by the decrease in absorbance at 517 nm. Un-inoculated MRS broth was used as a control sample. The DPPH radical (%) scavenging activity was calculated using the following equation:

$$1 - \left[\frac{Asample - Ablank}{Acontrol}\right] \times \quad 100$$

For hydroxyl radical scavenging activity using the Fenton reaction, the reaction mixture comprised 1.0 mL of brilliant green (0.435 mmol), 2.0 mL of FeSO4 (0.5 mmol), and 1.5 mL of H_2O_2 (3.0%, w/v). Culture samples, 1 mL culture at various concentrations (0.5 to 4 mg/mL) were added and incubated at tropical ambient temperature (28 ± 2°C) for 20 min. The absorbance was measured at 624 nm. The hydroxyl radical scavenging activity (%) was calculated using the equation:

$$\frac{A_S - A_0}{A - A_0} \times 100$$

A_S = absorbance of the sample;

A_0 = absorbance of the control;

A = absorbance of the deionized water without the sample and Fenton reaction.

Gelatinase production

Gelatinase production was determined using the method of Gupta and Malik [35]. This was carried out by inoculating overnight cultures of the LSC on nutrient agar plates, supplemented with 0.4% gelatin and then incubated at 37°C for 48 h. The incubated plates were flooded with saturated ammonium sulphate solution. Subsequently, the development of clear zones around the spots against the opaque background indicated a positive reaction, while the absence of clear zones indicated a negative result.

DNase production

The protocol described by Gupta and Malik [35] was adopted. DNase agar was used to check the production of DNase enzyme. A loopful of the LSC was streaked on the surface of the agar and incubated at a temperature of 30°C for 48 h. After incubation, a clear pinkish zone around the colonies against dark-blue background was taken as positive for DNase production.

Haemolytic activity

This was determined as described by Hawaz [36]. Fresh overnight bacterial cultures were streaked in triplicates on Columbia agar (Merck, Darmstadt, Germany) plates, containing 5% sheep blood and incubated at 30°C for 48 h. The haemolytic activities of the bacterial cultures, were examined for signs of β-haemolysis (conspicuous clear zones around colonies), α-haemolysis (visible green zones around colonies) or γ-haemolysis (no zone around colonies) on sheep blood agar plates.

Statistical analysis

The experimental procedures were carried out in triplicate determinations, and data obtained were subjected to one-way analysis of variance (ANOVA) to determine the significant differences at 5% level ($p < 0.05$). The values were expressed as mean ± SD and separated using New Duncan's Multiple Range Test.

Results

Acid tolerance activity of LSC strains

Impacts of the harsh conditions of low pH on the viability of the starter cultures are presented in Table 1. All the LSC exhibited excellent survival rates at pH 2.0 during 2 h and 4 h incubation periods at 37°C. The initial counts of the LSC showed very high cell counts and differed significantly ($p < 0.05$), with values ranging from 8.11 to 8.34 Log CFU/mL. The cell viability was observed to decrease after 2 h and 4 h exposure under the stated conditions. The three strains that showed the highest survival rates after 2 h digestion period were *L. plantarum* 2.7.17 (78.85%), *P. pentosaceus* CP I6 (76.0%) and *L. plantarum* CAM 7 (75.02%) while at the termination of the 4 h exposure time, the highest survival rate was observed for *L. plantarum* strains 2.7.17 and CAM 7 which showed survival rates of (73.12% and 73.95%) respectively.

Table 1. Acid tolerance (Log CFU/mL) of LAB microbial consortium at pH 2.0 for 2 h and 4 h.

Isolates	T_0	2 h	% Survival rate at 2 h	4 h	% Survival rate at 4 h
A	8.34 ± 0.06^{a}	6.26 ± 0.03^{a}	75.02 ± 0.91^{a}	6.17 ± 0.17^{a}	73.95 ± 2.60^{a}
B	8.11 ± 0.01^{c}	5.03 ± 0.02^{c}	62.05 ± 0.21^{b}	4.54 ± 0.77^{d}	56.01 ± 1.00^{d}
C	8.30 ± 0.02^{ab}	6.38 ± 0.05^{a}	78.85 ± 0.80^{a}	6.09 ± 0.57^{a}	73.12 ± 1.30^{ab}
D	8.17 ± 0.02^{c}	6.21 ± 0.11^{a}	76.0 ± 1.50^{a}	5.34 ± 0.24^{c}	65.59 ± 3.04^{c}
E	8.26 ± 0.01^{b}	5.71 ± 0.11^{b}	69.12 ± 1.54^{bc}	5.48 ± 0.35^{b}	66.34 ± 4.19^{b}
C_T	8.24 ± 0.02^{b}	6.22 ± 0.01^{a}	75.44 ± 0.03^{a}	6.17 ± 0.01^{a}	74.84 ± 0.21^{a}

Mean ± standard deviation. Values with different superscript letters in the same column are significantly different at ($p < 0.05$) using Duncan's multiple range test. A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; E: *L. brevis* 15F1; and C_T: Control.

Bile salts tolerance of selected starter culture isolates

As shown in Table 2, starter cultures of *L. plantarum* had the highest survival rates at 2 h and 4 h, respectively, with strains of *L. plantarum* 2.7.17 having values of (74.16% and 72.59%), and *L. plantarum* CAM 7 (72.49% and 71.71%), whilst *P. pentosaceus* NRIC 0123 showed survival rate of 71.28% and 70.71%, and the least value (51.30% and 50.03%) was obtained for *L. brevis* 15F1.

Table 2. Tolerance Activity (Log CFU/mL) of LAB microbial consortium in 0.3% bile salt for 2 h and 4 h.

Isolates	T_0	2 h	% Survival rate at 2 h	4 h	% Survival rate at 4 h
A	8.34 ± 0.28^{a}	6.04 ± 0.05^{b}	72.49 ± 0.84^{bc}	5.97 ± 0.03^{b}	71.71 ± 0.99^{b}
B	8.20 ± 0.04^{a}	5.84 ± 0.01^{c}	71.28 ± 0.28^{cd}	5.87 ± 0.04^{b}	70.71 ± 1.20^{bc}
C	8.30 ± 0.14^{a}	6.16 ± 0.11^{ab}	74.16 ± 1.15^{ab}	6.03 ± 0.04^{ab}	72.59 ± 0.55^{ab}
D	8.15 ± 0.13^{a}	5.65 ± 0.04^{d}	69.41 ± 1.65^{d}	5.55 ± 0.78^{c}	68.22 ± 1.94^{c}
E	8.14 ± 0.15^{a}	4.12 ± 0.00^{e}	51.30 ± 0.00^{e}	4.07 ± 0.99^{d}	50.03 ± 0.30^{d}
C_T	8.27 ± 0.04^{a}	6.27 ± 0.01^{a}	75.86 ± 0.16^{a}	6.17 ± 0.09^{a}	74.59 ± 0.79^{a}

Mean ± standard deviation. Values with different superscript letters in the same column are significantly different at ($p < 0.05$) using Duncan's multiple range test. A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; E: *L. brevis* 15F1; and C_T: Control.

Antimicrobial property of selected LSCs

The antimicrobial spectrum of the starter cultures against selected indicator food borne pathogens is illustrated in Figure 1. The high inhibitory activity observed in the results of this study showed that all the LSC strains favorably suppressed the six bacterial pathogens. Zones of inhibition ranged from 11.5 mm to 20.0 mm against *E. coli*, which is a Gram-negative organism, with the highest ZOI displayed by *L. brevis* 15F1, closely followed by *L. plantarum* 2.7.17 with a ZOI of 15.0 mm. Strains of *L. plantarum* CAM 7 and *L. plantarum* 2.7.17 demonstrated the highest ZOI of 25.0 mm and 22.5 mm, respectively, against *Klebsiella pneumoniae*. Against Gram-positive *Streptococcus pyogenes*, the strain of *P. pentosaceus* CP16 displayed the highest ZOI of 21.0 mm. Strains of *P. pentosaceus* NRIC 0123 and CP16 both displayed the highest ZOI of

14.0 mm for *Salmonella typhimurium*, and *L. brevis* showed the highest ZOI of 22.5 mm against *E. hirae*, which is also a Gram-positive organism.

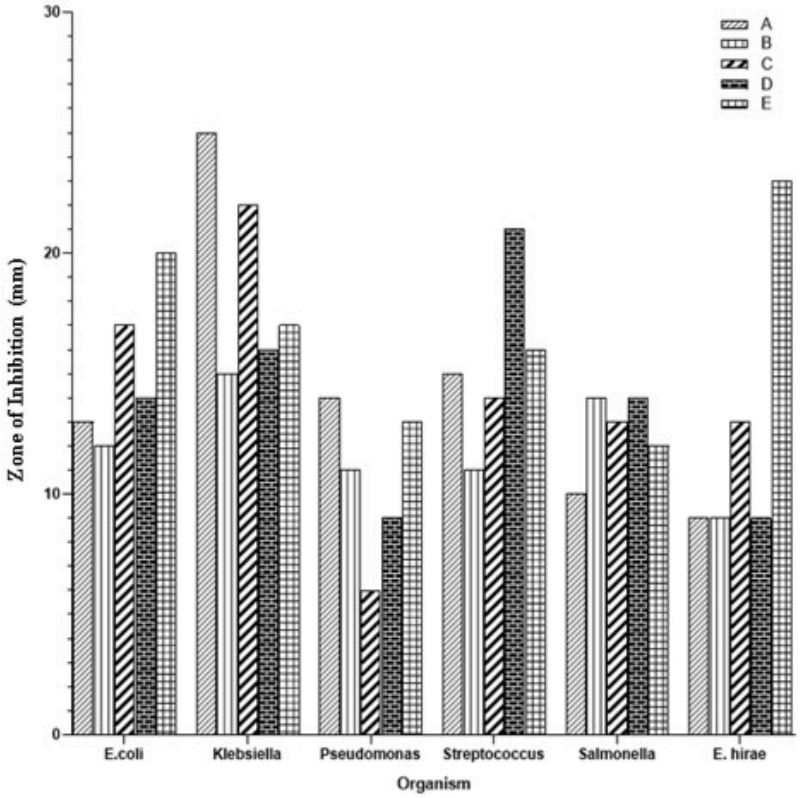


Figure 1. Antimicrobial activity of isolates against selected pathogens. A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; E: *L. brevis* 15F1. Values are mean ± SD of triplicate measurement.

Antibiotic susceptibility pattern of LSC

The LSC screened for probiotic activity (Table 3) were observed to show variable antibiotic sensitivity to all antibiotics tested, with values ranging from 10.5 mm to 32.0 mm. A general susceptibility of all the LSC was observed towards β-lactamas group (AMX and APX) and the aminoglycosides (S and NB). The two strains of *L. plantarum* showed strong and broad resistance to ciprofloxacin and norfloxacin, which belong to the functional group of quinolones, while they were particularly sensitive to erythromycin, which belongs to the class of macrolides. All of the starter cultures showed sensitivity towards chloramphenicol and rifampicin except *L. plantarum* CAM 7 which displayed tolerance with diameter of the ZOI of 13.5 and 11 mm respectively.

Table 3. Antibiotic resistance (mm) pattern of selected starter culture isolates.

Strains	CPX	NB	S	AMX	R	APX	E	CN	CH	LEV
	10 µg	10 µg	30 µg	20 µg	20 µg	20 µg	30 µg	10 µg	30 µg	10 µg
A	12(R)	13(R)	14.5(I)	23(S)	11(R)	29.5(S)	25(S)	27(S)	13.5(R)	18(I)
B	11(R)	10.5(R)	15(I)	16(I)	19.5(S)	25(S)	10(R)	32(S)	20(S)	12.5(R)
C	13.5(R)	11.5(R)	13.5(R)	26(S)	21(S)	16(I)	26(S)	16(I)	17.5(I)	13.5(R)
D	20(S)	20(S)	25(S)	27(S)	14.5(I)	15.5(I)	23(S)	20.5(S)	27.5(S)	20.5(S)
E	22(S)	23(S)	14.5(I)	24.5(S)	27(S)	26.5(S)	21(S)	27(S)	28.5(S)	24(S)

A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; E: *L. brevis* 15F1. CPX: ciprofloxacin; B: gentamycin; S: streptomycin; AMX: amoxillin; R: rifampicin; APX: ampiclox; E: erythromycin; CN: norfloxacin; CH: chloramphenicol; LEV: levofloxacin.

CSH of LSC isolates

The data presented in Table 4 revealed significant differences ($p < 0.05$) in the cell surface hydrophobic capacity of the selected starter cultures. The highest values of percentage hydrophobicity (66.21% and 68.29%) were observed for *L. plantarum* 2.7.17 in both hydrocarbons of n-hexane and xylene, respectively. The lowest percentage hydrophobicity was observed for *L. plantarum* CAM 7 (13.39% and 12.26%) and *P. pentosaceus* CP16 (14.54% and 11.31%) in n-hexane and xylene, respectively.

Table 4. Cell surface hydrophobicity of starter culture isolates.

Isolates	% Cell surface hydrophobicity	
	n-Hexane	Xylene
A	13.39 ± 0.16^{e}	12.26 ± 0.19^{d}
B	14.33 ± 0.03^{d}	22.43 ± 0.03^{c}
C	66.21 ± 0.11^{a}	68.29 ± 0.09^{a}
D	14.54 ± 0.04^{c}	11.31 ± 0.02^{e}
E	40.71 ± 0.09^{b}	25.22 ± 0.02^{b}

Mean ± standard deviation. Values with different superscript letters in the same column are significantly different ($p < 0.05$) using Duncan's multiple range test. A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; and E: *L. brevis* 15F1.

Adherence ability of starter culture isolates to hydrocarbon

Microbial adhesion to hydrocarbons (MATH) is usually regarded as a measure of an organism's cell surface hydrophobic property. The result obtained in this study showed that all the LSC isolates displayed good adherence capacity to both hydrocarbons, chloroform, and xylene, with values ranging from 13.45% to 79.55% (Table 5). The best MATH was observed for strains of *L. plantarum* 2.7.17, *L. plantarum* CAM 7, and *L. brevis* 15F1, which also showed a moderate hydrophobicity of 39.20%,79.55%, and 53.35% for the tested hydrocarbons.

Table 5. Adherence capability of starter culture isolates to hydrocarbons.

Isolates	% Adherence to hydrocarbon	
	Chloroform	Xylene
A	52.60 ± 0.40^{d}	79.55 ± 0.15^{a}
B	69.50 ± 0.20^{c}	30.40 ± 0.10^{d}
C	73.60 ± 0.95^{b}	39.20 ± 0.10^{c}
D	22.45 ± 0.25^{e}	13.45 ± 0.15^{e}
E	79.55 ± 0.15^{a}	53.35 ± 0.05^{b}

Mean ± standard deviation. Values with different superscript letters in the same column are significantly different ($p < 0.05$) using Duncan's multiple range test. A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; and E: *L. brevis* 15F1.

Autoaggregation ability of starter culture isolates

All the selected strains exhibited an excellent high percentage of auto-aggregation between 3 h and 20 h, with values ranging between 17% to 41% and 44% to 94%, respectively as shown in Figure 2 and Table 6; and it shows that the ability to auto-aggregate for all the examined isolates increased over time. The percentage of auto-aggregation for all the selected starter culture strains after 3 h was greater than 40%, which is an indication of good adhesion ability.

Table 6. Percent co-aggregation property after 4 h.

Isolates	*E. coli*	*Salmonella typhimurium*
A	37.83 ± 0.20^{a}	34.62 ± 0.21^{a}
B	24.36 ± 0.08^{b}	23.92 ± 0.66^{b}

Table 6. Percent co-aggregation property after 4 h. (*continued*)

Isolates	*E. coli*	*Salmonella typhimurium*
C	22.59 ± 0.41[b]	21.88 ± 0.05[b]
D	21.64 ± 0.04[b]	23.01 ± 0.11[b]
E	17.13 ± 0.34[c]	16.76 ± 0.11[c]

Mean ± standard deviation. Values with different superscript letters in the same column are significantly different ($p < 0.05$) using Duncan's multiple range test. A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; and E: *L. brevis* 15F1.

In vitro safety properties of potential probiotic LSC

The abilities of the selected LSC strains to produce EPS, DNase, gelatinase and hemolytic activity are shown in Table 7. All the strains studied exhibited EPS production ability, which was detected by the presence of ropy white mucus on skimmed milk plates containing the ruthenium red pigment. This is attributed to the presence of growth factors such as large quantities of amino acids and short peptides in the cultivation medium.

Table 7. In vitro safety properties of LAB starter culture isolates.

Strain	EPS production	DNase production	Gelatinase activity	Hemolysis
A	+	–	–	–
B	+	–	–	–
C	+	–	–	–
D	+	–	–	–
E	+	–	–	–

+: present; –: absent; EPS: exopolysaccharide; A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; E: *L. brevis* 15F1.

Antioxidant activity of potential LSC isolates

Antioxidant properties of the identified LAB strains are illustrated in Figures 3 and 4. The percent DPPH radical scavenging activity was observed to be in the following listed order: *L. plantarum* 2.7.17 (77%); *L. plantarum* CAM 7 (70%); *P. pentosaceus* CP16 (50%); *P. pentosaceus* NRIC 0123 (40%); and *L. brevis* 15F1 (35.5%). The result of the –OH radical scavenging activity, as illustrated in Figure 4, show that the lowest antioxidant effect was demonstrated by *L. brevis* 15F1 (30%), while *L. plantarum* 2.7.17 (60%) presented the most superior antioxidant activity.

Discussion

The ability of isolated LAB strains to withstand harsh conditions of low pH and high concentrations of bile salts in the gastrointestinal tract is an important characteristic for the selection of probiotic cultures [37, 38]. In most in vitro assays, a pH 3.0 limit has been set as the preferred standard for acid tolerance for probiotic culture [36, 39, 40] owing to the fact that a meaningful decrease in the viability of strains is usually observed at pH 2.0 and below [41]. However, findings from this study did not support this hypothesis and showed viability counts at pH 2.0, which is well supported by the findings of [4, 32, 42, 43]. The high survival rates observed in this study at pH 2.0 may be attributed to the known fact that the mechanism of acid tolerance is both species-dependent and strain-specific. This is attributed to generations of proton motive forces that expel protons from most cells, thus maintaining a normal intracellular pH [44, 45]. Observations of Olajugbagbe et al. [37] also supported the findings of this present study where they indicated that *P. acidilactici* strains showed high acid tolerance ability by surviving exposures to pH 2.0. African fermented cereal foods have been found to be a suitable substrate for the delivery of much-needed probiotics to the human GIT owing to the fact that LAB isolates particularly from lactic acid fermentations, such as Ogi, fufu, nunu, kunu have been previously known to possess the ability to grow and survive low pH environment [30, 46, 47]. Since they are inherently able to withstand the harsh fermentation conditions, they are also likely to be able to effectively survive passage through the GIT [48] and thus help in improving

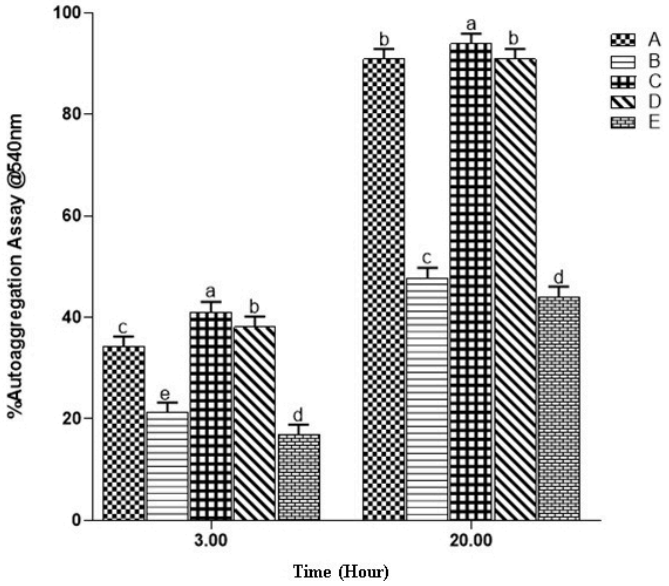


Figure 2. Auto-aggregation assay of selected starter cultures. Bars with different superscript letters within the same time frame are significantly different at $p < 0.05$. A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; and E: *L. brevis* 15F1.

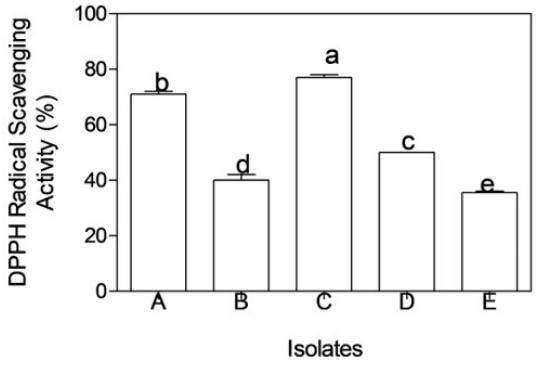


Figure 3. DPPH radical scavenging activity of isolates. Values are the mean ± SD of triplicate measurements. Bars with different superscript letters are significantly different ($p < 0.05$). A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; E: *L. brevis* 15F1.

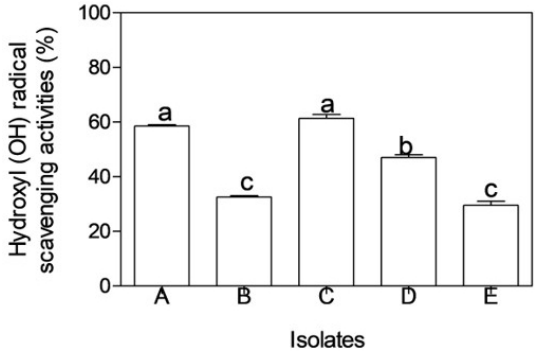


Figure 4. OH radical scavenging activity of starter culture isolates. Values are mean ± SD of triplicate measurement. Bars with different superscript letters are significantly different ($p < 0.05$). A: *L. plantarum* CAM 7; B: *P. pentosaceus* NRIC 0123; C: *L. plantarum* 2.7.17; D: *P. pentosaceus* CP16; E: *L. brevis* 15F1.

the gut microbiota and maintain overall health of the host [10, 20, 49]. The importance of acid tolerance of LAB is not only to support gastric stress, but also function as a prerequisite for its application as a dietary adjunct as well as show the ability of the strains to last longer in foods with high acidic environments [43].

All the selected starter culture isolates were viable at 0.30% concentration of bile salts being the average bile concentration in the human GIT, and set as standard by other researchers [37, 50]. This is an indication that strains of *L. plantarum* had considerable resistance to bile salts. The ability of strains of *L.*

plantarum to survive gastric transit while colonizing the intestinal tract of humans and other mammals had previously been proven [50–52], and several strains have been employed for the development of functional and therapeutic foods [53]. Generally, the results of this work confirm previous observations by other researchers that bile salt tolerance is not just related to the species of LAB, but may also be strain-specific [48]. High viability observed for the starter cultures after exposure to these harsh conditions is attributed to the ability of some LAB to produce bile salt hydrolase (BSH), which is known to hydrolyze conjugated bile salts, thus lowering their toxicity [54].

With respect to bile salts, tolerance is considered to be a prerequisite for colonization and unhindered metabolic activity of bacteria in the small intestine of the host, thus contributing to the balance of the intestinal microbiota [55]. In evaluating the potential of LAB as effective probiotics, it is always necessary to evaluate their ability to resist the lethal effects of bile acids. The concentration of bile salts used in this study represents the extreme concentration normally encountered in the human intestine during the first hour of digestion [56]. Thus, the selected LSC demonstrated significant resistance against 0.30% bile salts, which suggests that these LAB isolates could be able to potentially colonize the GIT when employed as probiotics.

The inhibitory activities of the isolates observed against known pathogens can be attributed to the low pH, production of antimicrobial substances such as hydrogen peroxide, organic acids (such as lactic, acetic, butyric and propionic acids), bacteriocins, and their co-aggregation properties, which represent a defensive tool for the colonization of pathogens [30, 50, 57]. Antimicrobial activity is generally considered an important and desirable property for probiotic characterization, as a reduction in the growth and multiplication of pathogenic microorganisms within the large intestine could be a positive force in decreasing the incidence of gastroenteritis and incidence of food poisoning [58, 59]. Past studies have shown that LAB species isolated from African traditionally fermented foods displayed antibacterial activity against Gram-positive and Gram-negative bacteria [20, 58–60]. The antagonistic activity showed that all the selected starter cultures significantly ($p < 0.05$) inhibited the growth of *E. coli*, *S. typhimurium*, *S. pyogenes*, *K. pneumoniae* and *E. hirae* tested in this study. The high ZOI exhibited against the Gram-negative bacteria can be aligned to the capacity of the LSC supernatant to affect the cells of Gram-negative bacteria more quickly than the Gram-positive bacteria owing to the thin peptidoglycan layer of the former [30, 61]. The result obtained in the present study is in agreement with the findings of Adeniran et al. [62] who examined the inhibitory activity of some LAB species on Gram-positive and Gram-negative bacteria such as *E. coli* and *Staphylococcus aureus*, and reported ZOI in the range of 3.0 mm to 9.0 mm against *S. aureus* and 1.0 mm to 5.0 mm against *E. coli*. Olatunde et al. [30] reported ZOI of LAB isolates, having probiotic potentials, to range from 7.0 mm to 24.7 mm against strains of *E. coli*, *S. aureus* and *S. typhimurium.*

In line with the recommendation of FAO/WHO [63] that as a safety measure, antibiotic resistance profile of a prospective probiotic organism should be evaluated, the susceptibility pattern recorded in this work is an indication that susceptibility of LAB to antibiotics may be different and species dependent [64]. However, Abriouel et al. [65] and Jaber et al. [66] reported that the resistance displayed by LAB was not transferable as the antibiotic resistance genes are located in the chromosome.

The high resistance exhibited by strains of *L. plantarum* CAM 7, and *L. plantarum* 2.7.17 in this study can be substantiated with the reports of Nawaz et al. [67] and Duche et al. [68] which supported the presence of intrinsic resistance mechanism of LAB action towards the antibiotic group of quinolones. Intrinsic resistance is usually non-transferable and does not pose any risk in LAB [69, 70], thus alleviating the concern of transferability of resistant gene to pathogens. Tolerance towards quinolones can be attributed to mutation in topoisomerase IV and enzymatic inactivation, which is the basic target for ciprofloxacin and blocks the binding of the antibiotics with their specific target [71]. However, strains of *L. brevis* 15F1 and *P. pentosaceus* CP16 identified in this study presented good sensitivity to the quinolones. This result is in agreement with the works of Duche et al. [68] and Sharma et al. [72], who also reported quinolone resistance in maximum numbers of LAB isolates.

This study revealed that the LSC strains were resistant to a few of the generally prescribed antibiotics (norfloxacin, chloramphenicol, ciprofloxacin, rifampicin, levofloxacin) in Nigeria, which presupposes that their administration in combination with the LAB cultures will not negatively affect their survival in the GIT [73]. Susceptibility of all the isolates towards β-lactamas (AMX and APX) group obtained in this study is in agreement with the research documented by Duche et al. [68] who reported susceptibility of LAB species towards ampicillin and penicillin which are antibiotics belonging to the β-lactamas group in which their mechanism of action specializes in inhibiting synthesis of the cell wall.

Hydrophobicity is considered one of the important suggestive parameters of probiotic strain properties initiating the first contact between bacteria and host cells [74]. Hydrophobicity is regarded as the ability of bacteria to promote inter-linkages with mucosal and epithelial cells of the host [75]. Hydrophobicity of above 40% is generally considered desirable for most probiotic strains [76] and only *L. plantarum* 2.7.17 in this regard qualified as a potential probiotic candidate. CSH is often measured by evaluating the affinity of probiotic bacteria to a hydrocarbon solvent, and bacteria with higher hydrophobicity have been documented to possess a better binding capacity to epithelial cells and thus influence the adhesion capacity to some extent [28, 77]. Differences in the CSHs are attributed to variation in the structurally and chemically heterogeneous bacterial surface, the level of expression of cell surface proteins and polysaccharides among strains of the same species, and environmental conditions affecting the expression of the surface proteins [75, 78]. The CSH result in this study is in good consonance with some documented work showing that hydrophobicity is strain specific [77, 79].

To be able to colonize the GIT, probiotics should be able to adhere strictly to the intestinal mucosal epithelial cells and resist being removed from the colon during peristalsis [80]. In the present study, all the selected strains exhibited favorable adherence capacity to chloroform and xylene with strains of *L. plantarum* CAM 7 and *L. brevis* 15F1 demonstrating the best adherence capacity of 79.55% to chloroform and xylene. Previous studies have documented that hydrophobicity correlates with the adhesion ability of probiotics; and bacteria with higher percent hydrophobicity results have a higher ability to attach to epithelial cells thereby colonizing the GIT and promoting health of the host [45, 77, 81, 82]. The trend obtained in this study is an indication that hydrophobicity and adhesion capacity may be strain specific. For example, strains of *L. plantarum* CAM 7, *L. brevis* 15F1 and *P. pentosaceus* NRIC 0123 also revealed high adherence capacity to the tested hydrocarbons. *L. plantarum* CAM 7 which is the best adhering strain in this study, showed high adherence of 52.60% and 79.6% to chloroform and xylene, but revealed hydrophobicity values of 13.39% and 12.26% to n-hexane and xylene, respectively. A similar trend was revealed for *P. pentosaceus* NRIC 0123, which showed good adhesion rates of 69.5% and 30.40% to the tested hydrocarbons but presented low hydrophobicity rates of 14.3% and 22.43%. The result of this study agrees with the reports of [48, 74] who also documented a contradiction to the hypothesis that higher hydrophobicity of cells results in higher adhesion capacity. In general, strains of *L. plantarum* have been documented as well defined probiotic species [10, 12, 50]. Although *L. brevis* is not a commonly applied probiotics, it has received the GRAS status [83], plus other researchers have included *L. brevis* in a list of possible probiotic strains [48].

In the literature, there is no standard percentage of auto-aggregation that a probiotic organism is expected to possess. However, according to Wang et al. [79] auto-aggregation of 40% and above is considered good while values of 10% and below are taken as weak. The auto-aggregation ability of microorganisms is a property which is related to the adherence capacity of the strains to the intestinal epithelium of the host cells [74]. To achieve the intended benefit of probiotic bacteria, the organisms are required to form a reasonably large biomass through aggregation, and when the cells aggregate, it increases adhesion to the intestines and thus improves the colonization observed in the gastrointestinal tract [84]. It is therefore an important requirement for strains to be integrated into the intestinal microbiota and consequently able to protect the GIT of the host cell by successfully outcompeting pathogens through distinct blockage of cell receptors and immunomodulatory action [31, 77, 85]. The result is in conformity with the contributions of previous authors who have earlier reported strains of homofermentative *L.*

plantarum to exhibit higher values of auto-aggregation [3, 28, 75, 86, 87]. Ajayeoba and Ijabadeniyi [31] and Krausova et al. [74] also documented strains of *P. pentosaceus* and *L. brevis* to possess a high auto-aggregation capacity. The findings of this study revealed that all the selected starter cultures presented a good cell surface property which is one of the preliminary screening tests for their potential as probiotics.

The result obtained for safety assessment agrees with the observation of Adesulu-Dahunsi et al. [4], who reported on EPS produced by strains of *L. plantarum* Y0175 and 0F101 isolated from traditionally fermented cereal beverages. In contributing to safety, EPS play a major part in cell recognition, adhesion to intestinal surfaces, and formation of extracellular biofilm matrix, which facilitate colonization of various ecosystems to protect microorganisms from adverse factors such as temperature, pH, host immune defenses, desiccation, phagocytosis, phage attack, osmotic stress, antibiotics, and other toxic compounds [45, 88], thus conferring health benefits such as anti-tumor, anti-ulcer, immune modulating, antioxidant, antimicrobial, cholesterol lowering effects and serving as prebiotic agents [89].

As part of safety concerns, the starter cultures demonstrated a negative response to hemolysis, gelatinase activity, and DNase production, thus proving the non-pathogenic status of the LSC for their probiotic potentiality, which was confirmed by the 'no zone' in the test plates inoculated with all the starter cultures studied. Hemolysis activity would normally break down the epithelial layer, while, on the other hand, gelatinase activity would derange the mucoid lining. These actions would generally interfere with the normal functioning of the mucosal linings and the many physiological substances exchanged, leading to or creating a pathway for infections. The absence of DNase enzyme, which may cause hydrolysis of the DNA molecules in the strains, supports their safe use in food fermentations [28]. An unbalanced gut environment and mucosal lining is commonly recognized as a source of allergic autoimmune disease and acute chronic infections [90]. It is therefore important for a probiotic strain to be free of hemolytic and gelatinase activity.

DPPH and hydroxyl radical scavenging activity of LAB isolates has been observed to be due to the colonization of the viable cells, their ability to proliferate in the GIT, and the presence of non-enzymatic substances and intracellular antioxidant activities [91, 92]. Antioxidant enzymes such as NADH oxidase, glutathione reductase, glutathione S-transferase, catalase, glutathione peroxidase, and feruloyl esterase have been reported to be synthesized by LAB and counteract oxidative stress [93, 94]. LAB possess the ability to scavenge free radicals, thereby contributing to the prevention and treatment of several diseases associated with oxidative stress, such as high blood pressure, diabetes, stroke, Alzheimer's disease, and gastrointestinal disorders, and also contribute to improved immune response related to the cell surface properties and composition of bacteria such as proteins, polysaccharides, and lipoteichoic acid [31, 92].

In conclusion, this study investigated the probiotic potential of five LSC, belonging to strains of *L. plantarum* 2.7.17, *L. plantarum* CAM 7, *P. pentosaceus* CP16, *P. pentosaceus* NRIC 0123, and *L. brevis* 15F1 developed from Fonio Ogi. The probiotic starter cultures all exhibited beneficial and promising in vitro probiotic properties, with respect to acid and bile salt tolerance, excellent levels of antimicrobial and antioxidant activities, and strong adhesion ability to intestinal mucosa. At the same time, some of the safety prerequisites desirable for the potential use of LAB as probiotic bacteria support their functionality as probiotic starter cultures and are thus judged to be safe for use in a wide range of food applications. Strains of *P. pentosaceus* CP16, *L. plantarum* 2.7.17 and *L. plantarum* CAM 7 emerged as the most promising probiotic strains. Therefore, supporting these findings with in vivo experiments to assess their potential health benefits will be the subject of our future work.

Abbreviations

CFS: cell-free supernatant

CFU: colony forming unit

CSH: cell surface hydrophobicity

EPS: exopolysaccharides

LAB: lactic acid bacteria

LSC: lactic acid bacteria starter cultures

MH: Mueller Hinton

NCBI: National Centre for Biotechnology Information

OD: optical density

PBS: phosphate buffered saline

ZOI: zone of inhibition

Declarations

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Author contributions

AMA: Methodology, Investigation, Writing—original draft. PJA: Formal analysis, Software, Writing—review & editing. BOTI: Resources, Methodology, Supervision. AAB: Supervision, Validation, Data curation, Writing—review & editing. VNE: Conceptualization, Supervision, Writing—review & editing, Project administration, Visualization. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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