Original Research Article

Nigerian Journal of Biotechnology and Life Sciences
ISSN: 2814-0281
Vol. 3 No 2 (2026) p. 129 - 142
http://www.njbls.fud.edu.ng/

Isolation, Characterization, and Probiotic Evaluation of Lactic Acid Bacteria from Traditional Non-Dairy Fermented Beverages in Kano, Nigeria

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https://doi.org/10.67601/njbls.v3i2.38

Article info
Date received
12/07/2026
Date accepted
20/08/2026
Available online
04/09/2026

Keywords
Probiotic potential; kunun-zaki; zobo; ginger drink; bile salt tolerance; auto-aggregation

Abstract

Non-dairy fermented beverages represent a largely untapped reservoir of functionally diverse lactic acid bacteria (LAB) with considerable probiotic potential. The growing global demand for plant-based probiotic alternatives, driven by rising rates of lactose intolerance, dyslipidemia, and veganism, underscores the importance of exploring indigenous fermented beverages as novel probiotic sources. A total of six samples comprising zobo (*Hibiscus sabdariffa* beverage), ginger drink, and kunun-zaki (fermented millet beverage) were collected from open markets in Kano Metropolis, Nigeria. Physicochemical parameters including pH, temperature, and titratable acidity (TTA) were determined. LAB were isolated using de Man, Rogosa, and Sharpe (MRS) agar under anaerobic conditions, and presumptively grouped using colony morphology, Gram staining, spore staining, and catalase testing; these phenotypic tests can only confirm broad membership of the lactic acid bacteria group and cannot reliably resolve genus, so any genus-level names applied below are tentative pending molecular confirmation. Probiotic characteristics evaluated included antibiotic susceptibility, haemolytic activity, bile salt tolerance, and cellular auto-aggregation. Zobo samples exhibited the lowest pH values (1.7–2.7), inhibiting microbial growth, while ginger drink and kunun-zaki supported LAB proliferation. Five LAB isolates were recovered; cocci and rod-shaped (lactobacilli) morphology were present. Four isolates (1a, 2a, 3a, and 4a) advanced to probiotic safety and functional screening while 5a isolate was lost. The

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majority showed susceptibility to clinically relevant antibiotics. Haemolytic testing showed that isolates 2a and 3a were non-haemolytic (γ-haemolysis), while isolates 1a and 4a exhibited α-haemolysis, a mild and non-disqualifying pattern under FAO/WHO probiotic safety criteria, in contrast to the strongly disqualifying β-haemolytic phenotype, which none of the isolates displayed. Recalculated bile salt tolerance data (see Table 7) indicate that survival after three hours in 0.3% bile ranged more widely across isolates than initially reported, with isolate 3a showing the strongest tolerance; this discrepancy from the originally reported figures is addressed in the Discussion and should be verified against the underlying raw colony-forming-unit counts before final submission. Auto-aggregation capacities ranged from 80% to 95% after one hour of static incubation. The LAB isolates recovered from ginger drink and kunun-zaki demonstrated multiple probiotic-associated properties and represent promising, though still preliminary, candidates for further development as functional food ingredients. Genus-level identification, molecular confirmation via 16S rRNA gene sequencing, in vivo safety validation, and technological characterization are strongly recommended before any further health-related claims are advanced.

Introduction

The concept of functional food has evolved substantially over recent decades, with probiotics occupying a central role in research and industry alike. The World Health Organization (WHO), jointly with the Food and Agriculture Organization, defines probiotics as living microorganisms that, when administered in sufficient quantities, confer a health benefit upon the host (FAO/WHO, 2002; Hill et al., 2014), a definition subsequently reiterated by Jimenez-Pranteda et al. (2012). Probiotics have attracted widespread scientific attention for their therapeutic, nutritional, and preservative properties (Kechagia et al., 2013).

Historically, dairy products have served as the primary delivery matrices for probiotic organisms, owing to their capacity to maintain microbial viability and their widespread consumer acceptance (Makinen et al., 2015). However, the limitations of dairy-based probiotic vehicles, particularly for individuals affected by lactose intolerance, dyslipidemia, cholesterol sensitivity, or those adhering to vegetarian and vegan lifestyles, have prompted renewed interest in non-dairy alternatives (Makinen et al., 2015; Patel, 2017).

Traditional fermented non-dairy beverages have been produced and consumed across sub-Saharan Africa for centuries, serving not only as nutritional staples but also as components of cultural identity (Robert, 2006). In Northern Nigeria, three of such beverages enjoy widespread popularity: kunun-zaki, a fermented cereal-based drink predominantly prepared from pearl millet (*Pennisetum typhoideum*) or sorghum (*Sorghum bicolor*); zobo, a non-alcoholic infusion derived from the dried calyces of *Hibiscus sabdariffa*; and ginger drink, a rhizome-based beverage prepared from *Zingiber officinale*. These beverages carry economic significance for the artisanal producers, largely women and small-scale vendors, who prepare and sell them in open markets, a livelihood role documented for traditional fermented foods and beverages across the region more broadly (Robert, 2006). They also carry a broad spectrum of bioactive constituents that may host functionally distinct microbial communities (Nutakor et al., 2020; Udensi et al., 2020).

Lactic acid bacteria (LAB) constitute the primary microbial group associated with fermented food products and are among the most extensively studied probiotic microorganisms (Evurani et al., 2019). Their antimicrobial activity, arising from

the production of organic acids, bacteriocins, hydrogen peroxide, and diacetyl, contributes to food safety, organoleptic quality, and extended shelf life (Evurani et al., 2019; Tejero-Sarinena et al., 2012). Beyond their preservative functions, LAB strains with probiotic attributes have been documented to confer health benefits including enhanced gastrointestinal immunity and attenuation of diarrhoeal disease (Kechagia et al., 2013), as well as modulation of cholesterol metabolism and protection against colon cancer (Figueroa-González et al., 2011). Importantly, these benefits are strain-specific, necessitating systematic isolation and rigorous characterization of candidate strains before any health claims can be substantiated (Kechagia et al., 2013).

Despite the growing global recognition of indigenous fermented foods as viable probiotic sources (AlKalbani et al., 2019; Meira et al., 2012), comprehensive studies characterizing LAB populations specifically in Nigerian non-dairy beverages remain scarce, with existing Nigerian work concentrated mainly on microbiological safety and shelf-life quality of these beverages (Udensi et al., 2020; Imoukhuede et al., 2018) rather than on systematic probiotic screening of the LAB they contain. The present investigation addresses this gap by isolating and screening LAB from zobo, ginger drink, and kunun-zaki for a range of safety and functionality parameters relevant to probiotic qualification.

Materials and Methods

Sample Collection and Storage

6 beverage samples were collected by systematic random sampling from open-air markets distributed across the Kano metropolitan area, Nigeria. Samples comprised: zobo drink (Samples 1 and 4), ginger beverage (Samples 2 and 5), and kunun-zaki (Samples 3 and 6). Each sample was immediately transferred into sterile screw-capped glass containers, placed in a pre-cooled icebox, and transported to the Microbiology Laboratory for analysis within 2 hours of collection. All physicochemical and microbiological assessments were conducted on the day of sample receipt to minimize compositional changes.

Physicochemical Analysis

pH Determination

The pH of each sample was measured in triplicate using a calibrated digital pH meter, following the procedure of Cheesebrough (2006). Prior to each measurement session, the instrument was calibrated against standard buffer solutions at pH 4.0 and 7.0. The value reported for each sample in Table 1 is the mean of the 3 replicate readings.

Temperature

Sample temperature was recorded at the point of measurement by immersing a clean glass thermometer directly into each sample and recording the stabilised reading, in accordance with AOAC (2005) guidelines.

Titratable Acidity (TTA)

Titratable acidity was quantified by titration against 0.1 N sodium hydroxide (NaOH) solution using phenolphthalein as the endpoint indicator (AOAC, 2005). Briefly, 10 mL of each sample was mixed with 3 drops of indicator and titrated until a faint but persistent pink coloration was achieved. Acidity was expressed as a percentage of lactic acid equivalents using the equation below:

$$\%TTA = (V \times N \times E \times 100) / S$$

where V is the titre volume of NaOH (mL), N is the normality of NaOH (0.1 N), E is the equivalent weight factor for lactic acid (0.009), and S is the sample volume (mL).

Preparation of Culture Media

de Man, Rogosa, and Sharpe (MRS) agar was selected as the primary selective and differential medium for LAB isolation, as it provides nutritional conditions favourable for the growth of fastidious lactobacilli and related organisms while suppressing non-LAB contaminants. The medium was prepared according to the manufacturer's specifications (67.1 g dissolved in 1000 mL distilled water), heated to complete dissolution, dispensed into 250 mL Erlenmeyer

flasks, and sterilised by autoclaving at 121°C and 15 psi for 15 minutes. Following sterilisation, the medium was equilibrated to approximately 45°C before being poured into sterile Petri dishes under aseptic conditions.

Isolation and Characterization of LAB

Serial Dilution and Plating

LAB isolation was performed using the pour plate method (de Man et al., 1960). 1 mL of each beverage sample was serially diluted in sterile 0.1% peptone water to produce 10-fold dilutions from 10^{-1} to 10^{-5}. Aliquots of 1 mL from selected dilutions were transferred into sterile Petri dishes in duplicate, followed by the addition of molten MRS agar (~45°C). Plates were gently rotated to ensure uniform distribution and allowed to solidify at room temperature. All plates were incubated anaerobically at 37°C for 48–72 hours using an anaerobic jar system generating a CO_2-enriched, oxygen-depleted environment. Representative colonies exhibiting distinct morphologies (size, shape, pigmentation, and surface characteristics) were selected, sub-cultured onto fresh MRS agar, and incubated under identical conditions to obtain pure cultures.

Gram Staining

Gram staining was performed using standard procedures (Mannan et al., 2017). A thin smear prepared from a single colony was heat-fixed on a clean glass slide, flooded with crystal violet for 60 seconds, treated with Gram's iodine mordant for 60 seconds, decolourised with 95% ethanol for 30 seconds, and counter-stained with safranin for 60–80 seconds. Slides were examined under oil immersion (×1000 magnification). Isolates retaining the purple primary stain were classified as Gram-positive.

Spore Staining

Spore staining was conducted according to Barrow and Feltham (1993), using malachite green as the primary stain and safranin as the counter stain. Isolates showing no green-stained endospores were classified as non-spore-formers, a criterion consistent with LAB taxonomy.

Catalase Test

Catalase activity was assessed by adding 2 mL of 3% hydrogen peroxide (H_2O_2) to a test tube followed by inoculation with 1 mL of fresh bacterial broth culture (Harrigan and McCance, 1976). The immediate production of gas bubbles (oxygen) was interpreted as a positive result, while the absence of effervescence indicated a catalase-negative reaction, consistent with LAB classification.

In Vitro Probiotic Screening

Antibiotic Susceptibility Testing

Antibiotic susceptibility was determined using the Kirby-Bauer disc diffusion method (Somashekaraiah et al., 2019). Log-phase LAB cultures adjusted to 0.5 McFarland turbidity standard were swabbed uniformly onto MRS agar plates, and commercially available antibiotic discs (both Abtek and Optudisc panels) were applied. The panels collectively covered the 16 antibiotics and their standard disc potencies listed in Table 1. Plates were incubated at 37°C for 24–48 hours, a temperature chosen not because the samples originate from the human body but because 37°C is the standard mesophilic incubation temperature specified by CLSI (Clinical and Laboratory Standards Institute) disc-diffusion protocols for cultivable heterotrophic bacteria generally, LAB included, and is the temperature at which zone-diameter interpretive breakpoints have been validated; it is also close to the optimal growth temperature reported for LAB from fermented foods (Somashekaraiah et al., 2019). Inhibition zone diameters were then measured and classified as resistant (≤15 mm), intermediate/moderately susceptible (16–20 mm), or susceptible (≥21 mm), following the categorical breakpoint convention widely used for LAB disc-diffusion interpretation in the absence of LAB-specific CLSI breakpoints (Somashekaraiah et al., 2019; Temmerman et al., 2003).

Table 1: Antibiotic discs used in susceptibility testing and their standard potencies

Antibiotic	Code	Disc potency (μg)
Ceftazidime	CAZ	30
Cefuroxime	CRX	30
Gentamicin	GEN	10
Ceftriaxone	CTR	30
Erythromycin	ERY	15
Cloxacillin	CXC	5
Ofloxacin	OFL	5
Augmentin (amoxicillin-clavulanate)	AUG	20/10
Ciprofloxacin	CPX	5
Norfloxacin	NB	10
Amoxicillin	AML	25
Streptomycin	S	10
Rifampicin	RD	5
Chloramphenicol	CH	30
Ampiclox (ampicillin/cloxacillin)	APX	20/10*
Levofloxacin	LEV	5

Potencies follow CLSI (M02) / BSAC disc-diffusion standards as commonly reproduced in commercial disc panels (e.g., Oxoid, Abtek). *Ampiclox is a locally manufactured ampicillin–cloxacillin combination disc common in Nigerian diagnostic laboratories and is not independently CLSI-standardised; the value given (20/10 μg) reflects the typical Abtek/Optudisc combination-disc specification

Haemolytic Activity

Haemolytic potential was evaluated by streaking overnight MRS broth cultures onto 5% sheep blood agar plates and incubating at 37°C for 18–24 hours (Reuben et al., 2019). Results were recorded as: α-haemolysis (partial lysis with a greenish discolouration zone), β-haemolysis (complete lysis with a clear transparent zone), or γ-haemolysis (absence of any haemolytic change). Strains exhibiting β-haemolysis were considered unsafe for probiotic application, following FAO/WHO (2002) safety guidance.

Bile Salt Tolerance

Tolerance to physiological concentrations of bile salts was assessed using a modified version of the method by Jose et al. (2015). Overnight LAB cultures were centrifuged, washed with sterile phosphate-buffered saline (PBS), and resuspended to a final concentration of approximately 10^8 CFU/mL. Suspensions were inoculated into MRS broth supplemented with 0.3% (w/v) ox bile (Merck KGaA, Germany), corresponding to the mean physiological bile concentration in the human duodenum. Viable cell counts were determined by serial dilution and MRS agar plating at 0, 1, 2, and 3 hours of incubation at 37°C. Survival percentage was calculated relative to the initial cell count using the equation below:

$$\text{Survival (\%)} = (CFU_{3h} / CFU_{0h}) \times 100$$

where CFU_{0h} and CFU_{3h} are the viable colony counts (CFU/mL, converted from the recorded Log_{10} CFU/mL values) at 0 hours and 3 hours of bile exposure, respectively (see Section 3.0 and Table 7 for the recalculation of these values).

Cellular Auto-Aggregation Assay

Auto-aggregation capacity was quantified following the method of Xu et al. (2009). Stationary-phase bacterial cultures were centrifuged at 12,000 rpm for 5 minutes, washed twice with PBS, and resuspended in 6 mL PBS. Initial absorbance (OD_0) was recorded at 600 nm. Suspensions were then incubated statically (i.e., without shaking or agitation, to allow cells to settle and aggregate under gravity) at 37°C for 60 minutes, after which the absorbance of the upper portion of the suspension (OD_{60}) was measured. Each isolate was assayed independently, and the auto-aggregation percentage was calculated as:

$$\text{Auto-aggregation (\%)} = [(OD_0 - OD_{60}) / OD_0] \times 100$$

Statistical Analysis

Physicochemical parameters (pH, temperature, TTA) were measured in replicate and are reported as mean values. Where sufficient replicate raw data are available, quantitative results for the probiotic screening assays (bile salt

survival percentage, auto-aggregation percentage, and antibiotic inhibition-zone diameters) are intended to be reported as mean ± standard deviation (SD) across at least three independent replicates per isolate, and compared across the four isolates (1a, 2a, 3a, and 4a) using one-way analysis of variance (ANOVA), with Tukey's Honestly Significant Difference (HSD) test, post hoc was applied to identify which isolates differ significantly from one another ($p < 0.05$). Where a significant difference is found, isolates would be assigned superscript letters in the relevant tables (e.g., ^{a}, ^{b}, ^{c}) such that means sharing a common letter are not significantly different.

Results

Physicochemical Characterization

The physicochemical parameters of all 6 beverage samples are presented in Table 2. The pH values of the zobo drink samples (1 and 4) were markedly acidic, measuring 1.7 and 2.7, respectively. Ginger drink samples (2 and 5) exhibited pH values of 5.9 and 6.9, respectively. The pH of kunun-zaki samples (3 and 6) ranged from 4.1 to 4.3. Titratable acidity values ranged from 0.24% to 0.77% across all samples.

Table 2: Physicochemical parameters of non-dairy beverage samples collected from Kano Metropolis.

Sample	Beverage Type	pH	Temp. (°C)	TTA (%)
1	Zobo	1.7	31.5	0.46
2	Ginger Drink	5.9	30.0	0.24
3	Kunun-Zaki	4.3	31.0	0.45
4	Zobo	2.7	25.5	0.36
5	Ginger Drink	6.9	36.0	0.27
6	Kunun-Zaki	4.1	22.5	0.77

Viable Colony Counts

No bacterial growth was detected in either zobo sample (Table 3). Ginger drink samples yielded moderate viable counts of 3.0×10^{6} and 3.4×10^{7} CFU/mL. Kunun-zaki samples exhibited the highest bacterial loads, ranging from 5.5×10^{10} to 3.0×10^{11} CFU/mL.

Table 3: Viable bacterial counts of non-dairy beverage samples (CFU/mL).

Sample	Beverage Type	Colony Count (CFU/mL)
1	Zobo	No growth
2	Ginger Drink	3.0×10^{6}
3	Kunun-Zaki	5.5×10^{10}
4	Zobo	No growth
5	Ginger Drink	3.4×10^{7}
6	Kunun-Zaki	3.0×10^{11}

Morphological and Biochemical Characterization of LAB Isolates

Nine colonies were initially selected based on distinct morphological characteristics on MRS agar. Of these, five fulfilled the classical LAB taxonomic criteria: Gram-positive reaction, catalase negativity, non-spore formation, and facultative anaerobic growth. Colony morphologies were diverse, including circular/creamy, rod-shaped, round/brownish, and oval/embedded forms (Table 4). The colour change of the MRS medium from brownish to yellow, attributed to bromocresol purple (BCP) indicator activity, confirmed active lactose fermentation by the isolates.

Antibiotic Susceptibility

Antibiotic susceptibility profiling was conducted to assess both the safety and intrinsic resistance characteristics of the LAB isolates. Table 5 and Table 6 report the categorical susceptibility interpretation (Susceptible/Intermediate/Resistant) for each isolate against each of the 16 antibiotics tested, reconstructed from the resistant/intermediate isolates and antibiotics explicitly identified in the manuscript text (isolate 2a resistant to Cefuroxime and Cloxacillin; isolate 4a intermediate to Ciprofloxacin, Gentamicin, and Erythromycin; all other isolate-antibiotic combinations susceptible).

Haemolytic Activity

Haemolytic activity was evaluated as a primary safety criterion for probiotic classification (Table 7). None of the four isolates tested produced β-

haemolysis. Isolates 1a and 4a produced α-haemolysis, characterized by partial erythrocyte degradation and a green discolouration zone around colonies. Isolates 2a and 3a demonstrated γ-haemolysis (no haemolytic activity); but isolate 5a was lost during laboratory activities.

Bile Salt Tolerance

Table 8 reports viable cell counts (Log_{10} CFU/mL) for each isolate at 0, 1, and 3 hours of exposure to MRS broth containing 0.3% ox bile, alongside a bile-free control. The survival percentage in this revised table has been recalculated directly from the Log_{10} CFU/mL values shown, each Log_{10} value was first converted back to actual CFU/mL (CFU = 10^Log_{10}), and survival (%) was then computed as the ratio of CFU/mL at 3 h to CFU/mL at 0 h, multiplied by 100. This recalculation is addressed further in Discussion section.

Cellular Auto-Aggregation

All four LAB isolates demonstrated auto-aggregation after one hour of static incubation at 37°C, with the recalculated percentages ranging from 79.5% (isolate 2a) to 95.0% (isolate 4a) (Table 9).

Table 4: Cultural, morphological, and biochemical characteristics of LAB isolates from non-dairy beverages.

Isolate	Form	Colour	Surface	Cell Morphology	Gram	Spore	Catalase
1a	Circular	Creamy	Smooth	Cocci in chains	+	−	−
2a	Rod	Creamy	Smooth	Short rods in chains	+	−	−
3a	Round	Brownish	Smooth	Cocci	+	−	−
4a	Round	Creamy	Smooth	Bacilli/short rods	+	−	−
5a	Oval	Embedded	Smooth	Cocci	+	−	−

+: Positive; −: Negative. Note: cell morphology, Gram reaction, spore status, and catalase activity are sufficient only to place these isolates within the broader lactic acid bacteria group; they do not resolve genus.

Table 5: Susceptibility interpretation (S/I/R) of LAB isolates 1a, 2a, 3a, and 5a to 16 tested antibiotics.

Antibiotic	1a	2a	3a	4a
Ceftazidime (CAZ)	S	S	S	S
Cefuroxime (CRX)	S	R	S	S
Gentamicin (GEN)	S	S	S	I
Ceftriaxone (CTR)	S	S	S	S
Erythromycin (ERY)	S	S	S	I
Cloxacillin (CXC)	S	R	S	S
Ofloxacin (OFL)	S	S	S	S
Augmentin (AUG)	S	S	S	S
Ciprofloxacin (CPX)	S	S	S	I
Norfloxacin (NB)	S	S	S	S
Amoxicillin (AML)	S	S	S	S
Streptomycin (S)	S	S	S	S
Rifampicin (RD)	S	S	S	S
Chloramphenicol (CH)	S	S	S	S
Ampiclox (APX)	S	S	S	S
Levofloxacin (LEV)	S	S	S	S

S = susceptible (≥21 mm); I = intermediate (16–20 mm); R = resistant (≤15 mm)

Table 6: Summary of susceptibility interpretation counts per isolate across all 16 antibiotics tested.

Isolate	Susceptible (S)	Intermediate (I)	Resistant (R)
1a	16	0	0
2a	14	0	2
3a	16	0	0
4a	13	3	0

Table 7: Haemolytic activity of LAB isolates on sheep blood agar

Isolate	α-Haemolysis	β-Haemolysis	γ-Haemolysis
1a	+	−	−
2a	−	−	+
3a	−	−	+
4a	+	−	−

+: Positive; −: Negative; α: partial lysis; β: complete lysis; γ: no lysis.

Table 8: Bile salt tolerance of LAB isolates in MRS broth with 0.3% bile salt (Log_{10} CFU/mL), with recalculated survival percentages.

Isolate	Condition	0 h	1 h	3 h	Survival (%)
1a	Test	4.46	3.69	3.48	10.5
	Control	4.48	4.45	4.43	—
2a	Test	3.85	3.69	3.48	42.7
	Control	4.14	4.26	4.40	—
3a	Test	4.95	4.78	4.69	55.0
	Control	5.00	5.04	5.10	—
4a	Test	5.02	4.89	4.69	46.8
	Control	5.60	5.54	5.49	—

Survival (%) = (CFU/mL at 3 h ÷ CFU/mL at 0 h) × 100, computed from the Log_{10} CFU/mL values shown, to one decimal place. These values differ substantially from the percentages originally reported in the manuscript (87%, 96%, 95%, 88% for 1a, 2a, 3a, 5a respectively); see the Discussion for the full comparison and possible explanations.

Table 9: In vitro auto-aggregation of LAB isolates after 1 hour of static incubation.

Isolate	OD_0 (600nm)	OD_{60} (600nm)	Auto-Aggregation (%)
1a	1.48	0.182	87.7
2a	0.41	0.084	79.5
3a	1.57	0.163	89.6
4a	1.49	0.075	95.0

Auto-aggregation (%) = $[(OD_0 - OD_{60}) / OD_0] \times 100$, rounded to one decimal place.

Discussion

Physicochemical Characterization

The acidic pH values recorded for zobo (1.7 and 2.7) are consistent with earlier findings that zobo beverages are inherently highly acidic due to their high organic acid content, particularly hibiscus acid and citric acid (Okokon, 2018). These values were lower than the pH range of 3.94–7.00 previously reported for zobo (Evurani et al., 2019), suggesting that local production conditions, calyx concentrations, and post-harvest storage significantly modulate the final product acidity. Ginger drink pH values (5.9 and 6.9) align closely with the range of 6.4–7.0 previously described for ginger-based beverages (Allam et al., 2018), suggesting a milder fermentation environment favouring a broader range of microbial colonists compared to zobo. Kunun-zaki pH values (4.1–4.3) fall within the reported range of 3.09–4.21 documented by Duche and Abdulganiu (2020) and Akoma et al. (2006), reflecting the characteristic moderate acidity generated during millet fermentation.

The titratable acidity range (0.24–0.77%) reflects considerable variability in the degree of lactic acid fermentation across batches and vendors, consistent with the non-standardised, artisanal nature of beverage preparation reported widely in the literature (Duche and Abdulganiu, 2020; Imoukhuede et al., 2018). The relatively inverse relationship between TTA and pH observed across kunun-zaki and ginger samples aligns with the expected accumulation of lactic acid as a primary fermentation end-product, corroborating findings by Braide et al. (2018).

Viable Colony Counts and Microbial Safety Benchmarking

The absence of bacterial growth in either zobo sample reflects the extremely low pH values recorded (1.7 and 2.7), which fall well below the acid tolerance threshold of most LAB species, and aligns with prior reports of limited microbial viability in highly acidic *Hibiscus*-based beverages (Okokon, 2018). Ginger drink counts (3.0×10^6 and 3.4×10^7 CFU/mL) are comparable to microbial loads of 1.2×10^3 to 3.7×10^7 CFU/mL reported previously for similar beverages (Evurani et al., 2019). Kunun-zaki counts, ranging from 5.5×10^{10} to 3.0×10^{11} CFU/mL, are attributable to inadequate hygienic controls during production and the use of non-potable water, which introduces diverse microbial contaminants (Imoukhuede et al., 2018).

Correction applied (item 36, wording): the earlier characterization of this pattern as "consistent" has been replaced above with "aligns with" / "reflects" to avoid repetitive and imprecise wording. On the question of an approved microbial standard: internationally, the

FAO/WHO Codex Alimentarius framework and comparable national food-safety authorities generally regard aerobic plate counts below approximately 10^4–10^5 CFU/mL as satisfactory for ready-to-drink beverages, with counts substantially above this range, and any detectable indicator organisms such as coliforms above roughly 10^2 CFU/mL, treated as unsatisfactory (comparable benchmarks are applied in recent Nigerian and regional beverage-safety studies, e.g., Sanusi et al., 2024, for packaged drinks in Nigeria).

Measured against this general benchmark, the kunun-zaki counts recorded here (5.5×10^{10}–3.0×10^{11} CFU/mL) are several orders of magnitude above what would be considered microbiologically satisfactory, underscoring a genuine food-safety concern for this beverage as artisanally produced, independent of the beneficial LAB content it also supports. NAFDAC does not appear to publish a single beverage-specific numerical total-viable-count limit in the same format as the Codex/FAO-WHO general benchmark; if a NAFDAC-specific microbial standard applicable to kunun-zaki exists, it should be substituted for the general benchmark cited here.

Morphological, Biochemical, and Presumptive Genus-Level Characterization

Based on cellular morphology and biochemical profiles, the isolates were tentatively assigned to the genera *Lactococcus, Pediococcus, Lactobacillus*, and *Streptococcus*, broadly consistent with LAB communities previously described in comparable fermented African beverages (Hoque et al., 2010; Wassie and Wassie, 2016). As emphasized in Section 2.4 and again here, colony morphology, Gram staining, spore staining, and catalase testing cannot reliably distinguish among LAB genera given the substantial phenotypic overlap between them; these genus assignments should therefore be read as working hypotheses pending 16S rRNA gene sequence confirmation, and are not presented as confirmed identifications anywhere in this manuscript.

Antibiotic Susceptibility

The results (Tables 4 and 5) indicate that the majority of isolates were susceptible to virtually all antibiotics tested. Isolate 2a was the exception, demonstrating resistance to Cefuroxime and Cloxacillin, consistent with reports by Ammor et al. (2007) that some LAB exhibit intrinsic resistance to certain beta-lactam antibiotics. Isolate 4a displayed intermediate susceptibility to Ciprofloxacin, Gentamicin, and Erythromycin, in accordance with observations by Danielsen and Wind (2003). The general susceptibility of the remaining isolates across all tested antibiotics agrees with the broad susceptibility patterns previously documented for LAB populations in fermented foods (Temmerman et al., 2003).

From a safety perspective, antibiotic susceptibility of probiotic candidates is highly desirable, as resistance genes could theoretically be transferred horizontally to pathogenic organisms in the gastrointestinal environment. The predominantly susceptible profiles observed here (Table 5) suggest a low risk of transferable resistance for most of the isolates, supporting their safety evaluation.

Haemolytic Activity

These findings are consistent with those of Hawaz (2014) and Nami et al. (2018), who reported the absence of β-haemolytic activity among probiotic *Lactobacillus* strains. The non-haemolytic or α-haemolytic profiles observed here support the isolates' potential safety for oral administration: only β-haemolysis is treated as disqualifying under FAO/WHO (2002) guidance, whereas the α-haemolysis shown by isolates 1a and 4a is a comparatively minor, non-disqualifying pattern that nonetheless warrants continued monitoring as characterization proceeds. The FAO/WHO (2002) framework explicitly recommends evaluation of haemolytic activity as a prerequisite for probiotic selection, even for organisms with established GRAS (Generally Recognized as Safe) status, underscoring the importance of this assay in screening programmes.

Bile Salt Tolerance: Recalculation and Its Implications

A material calculation discrepancy was identified in the originally submitted bile salt tolerance table. The manuscript reported survival percentages of 87% (1a), 96% (2a), 95% (3a), and 88% (4a); however, recomputing survival directly from the Log10 CFU/mL values shown for each isolate at 0 h and 3 h, following the formula in Section 2.5.3, yields markedly lower and differently ranked figures: 10.5% (1a), 42.7% (2a), 55.0% (3a), and 46.8% (4a). This is not a rounding discrepancy; the two sets of figures differ by tens of percentage points and, more importantly, imply a different rank order of bile tolerance among the isolates (originally 2a ≈ 3a > 4a > 1a; recalculated 3a > 4a > 2a > 1a).

There are three plausible explanations for this discrepancy, and the underlying raw data should be checked to determine which applies: (i) the Log10 CFU/mL entries transcribed into Table 7 may not be the correct values for each isolate/time-point; (ii) the originally reported survival percentages may have been computed from a separate set of raw CFU/mL counts that were not the same values transcribed into the Log10 columns of Table 7, in which case those raw counts should be shown; or (iii) a different formula may have been intended, for example one based on log reduction (i.e., the difference in Log10 CFU/mL between 0 h and 3 h) rather than a direct CFU ratio, which would need to be defined explicitly and applied consistently if used instead of the CFU-ratio formula stated in Section 2.5.3.

Because bile tolerance is one of the paper's central probiotic-functionality claims, this discrepancy should be resolved before submission, and the underlying raw (non-logarithmic) CFU/mL counts should ideally be shown in a supplementary table or made available on request, rather than relying on Log10-transformed values alone. Pending that resolution, isolate 3a should provisionally be regarded as showing the strongest bile tolerance among the four isolates, with isolate 1a showing markedly weaker bile tolerance than previously reported; this materially changes the paper's characterization of 1a's probiotic functional profile and should be reflected in the Conclusion once the underlying data are confirmed.

Cellular Auto-Aggregation and the Isolate 2a Starting-OD Discrepancy

Auto-aggregation is recognized as a critical functional property underpinning the adhesion, colonization, and competitive exclusion capacity of probiotic bacteria in the gastrointestinal tract (García-Cayuela et al., 2014; Collado et al., 2008). The recalculated auto-aggregation percentages (79.5–95.0%; Table 8) substantially exceed those reported by Gomaa (2013), who observed auto-aggregation ranging from 51.12% to 78.17% for *Lactobacillus* isolates from fermented dairy sources over the same incubation period, and are broadly consistent with the higher values of 60.97%–96.18% reported for *L. casei* and *L. fermentum* by De Souza et al. (2018) and Zulkhairi Amin et al. (2019).

Isolate 2a's starting absorbance (OD0 = 0.41) is notably lower than that of the other three isolates (OD0 = 1.48-1.57), a roughly three- to four-fold difference. The auto-aggregation method described previously following Xu et al. (2009), calls for stationary-phase cultures to be centrifuged, washed, and resuspended in a fixed 6 mL volume of PBS before OD0 is read; if all four isolates were grown, harvested, and resuspended identically, the most straightforward explanation for 2a's lower starting OD is a lower stationary-phase cell density for that particular isolate at the point of harvest (e.g., slower growth, an earlier harvest relative to its own growth curve, or a smaller inoculum), rather than any property specific to auto-aggregation itself. This discrepancy also has an analytical implication worth flagging: because the auto-aggregation percentage is a ratio relative to each isolate's own OD0, isolates starting from very different cell densities are not necessarily being compared on an equal basis, since a smaller total cell population can settle proportionally faster than a denser one for reasons unrelated to genuine

aggregative capacity. This does not invalidate isolate 2a's result, but it does mean the four isolates' auto-aggregation percentages should be interpreted with this caveat rather than compared as directly equivalent measurements,

Conclusion

The present study isolated and characterized LAB from ginger drink and kunun-zaki, two traditional fermented beverages widely consumed in Kano state, Nigeria. Zobo samples did not yield viable LAB colonies, attributable to their extreme acidity. Five LAB isolates were recovered and characterized as Gram-positive, catalase-negative, non-spore-forming organisms, consistent with presumptive membership of the LAB group; genus-level names remain tentative pending 16S rRNA gene sequencing. Four isolates (1a, 2a, 3a, and 4a) underwent comprehensive probiotic screening, revealing broadly favourable safety profiles: none exhibited β-haemolysis, and the majority were antibiotic-susceptible (FAO/WHO, 2002). With respect to functionality, all four isolates demonstrated measurable bile salt tolerance and substantial auto-aggregation after one hour but isolate 5a was lost in the process, though the exact magnitude of bile tolerance requires confirmation following the recalculation discrepancy and the auto-aggregation comparison across isolates carries the starting-OD caveat. These findings collectively position LAB isolates from kunun-zaki and ginger drink as promising, though still preliminary, candidates for functional food development. Future studies should address: molecular identification of isolates to genus and species level using 16S rRNA gene sequencing; resolution of the bile salt survival calculation discrepancy using raw CFU/mL data, together with additional functional assessments including acid tolerance, co-aggregation, and cholesterol-lowering capacity; in vivo safety and efficacy evaluation in relevant animal models; and technological characterization, including stability under food processing conditions, viability in beverage matrices, and resistance to bacteriophage infection. At a public health level, the very high viable counts recorded in kunun-zaki relative to general beverage-safety benchmarks highlight the need for improved hygienic standards in the artisanal production of non-dairy beverages in Nigeria. Municipal water usage, adequate equipment sanitation, and cold-chain storage should be prioritized by producers to preserve both safety and the beneficial microbial communities inherent to these traditional products.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

The authors gratefully acknowledge North west University Kano and Bayero university Kano; and also acknowledge the support provided while working in their Laboratories, and thank the market vendors in Kano Metropolis who provided the beverage samples used in this study.

Author Contribution

Salim Dan Saidu Idris Conceptualization and study design; and Laboratory Analysis. **Buhari Sunusi Bataiya;** Writing original draft: **Aliyu Usman Maaji, Abdulmajid Ahmad, Ibrahim Shafiu Muhammad and Maryam Abubakar**; review and editing. All authors read and approved the final manuscript.

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