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Characterization and antibacterial activity of *lactobacillus* species isolated from a traditionally fermented cereal beverage (*kunun-zaki*) in Nigeria

Ola Ajibola Joshua*[1], Adamu Aliero Almustapha[1], Aminu Fardami Yusuf[2], Amina Muhammad[2], Abbas Yusuf Bazata[3] Mubarak Bodinga Musa [4], Marwana Magaji [4]

[1]*Department of Microbiology, Kebbi State University of Science and Technology, Aliero, Nigeria.*
[2]*Department of Microbiology, Usmanu Danfodiyo University, Sokoto, Nigeria.*
[3]*Department of Microbiology, Federal University, Birnin-Kebbi, Kebbi, Nigeria.*
[4]*Department of Medical Laboratory, Sultan Abdulrahman College of Health Technology, Gwadabawa, Nigeria.*

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ABSTRACT

Kunun-zaki is an indigenous cereal beverage in Nigeria that undergoes spontaneous fermentation by mixed cultures of autochthonous bacterial species. Given the antimicrobial compounds synthesized by lactic acid bacteria (LAB), this study aimed to evaluate the antibacterial activities of *Lactobacillus* species isolated from Kunun-zaki. The traditionally fermented cereal beverage (Kunun-zaki) was analyzed for pH and LAB counts. *Lactobacillus* isolates were identified using both phenotypic tests and molecular method (16S rRNA amplification). Then the representative isolates were subjected to 16S rRNA amplification analysis using universal primers (27F/1492R). Cell-free supernatant (CFS) extracts of the isolates were tested for antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., and *Klebsiella* spp., by using the agar well diffusion method. The pH values of traditionally fermented Kunun-zaki ranges from 4.50 ± 0.10 to 6.83 ± 0.05, while LAB count range from $2.45 \times 10^3 \pm 1.80 \times 10^2$ cfu/mL to $2.87 \times 10^3 \pm 4.36 \times 10^1$ cfu/mL within 0-48 h after preparation. Eight isolates were obtained and identified as *Lactiplantibacillus plantarum* (35%), *Limosilactobacillus fermentum* (25%), *Lacticaseibacillus acidophilus* (25%), and *Ligilactobacillus salivarius* (12.5%) The CFS extracts of *Lactobacillus* isolates exhibitedsignificant inhibitory activity against *Staphylococcus aureus*, *E. coli*, *Salmonella* spp., and *Klebsiella* spp., with zones of inhibition ranging from 8.16 ± 0.28 mm to 20.30±0.60 mm. Notably, only *Salmonella* spp. was resistance to some *Lactobacillus* isolates (LBK0-23, LBK4-24, LBK4-26), showing a pattern of selective resistance. Tukey's HSD post-hoc analysis confirmed significant differences ($p<0.05$) among the inhibition profiles of individual isolates. These results indicate the potential of *Lactobacillus* spp. in traditional production of Kunun-zaki beverages and their role as a promising antibacterial agent.

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1. Introduction

Traditionally fermented foods have been consumed for hundreds of years and have had a significant cultural impact on millions of people worldwide (1)

Kunun-zaki is a cereal-based beverage prepared from millets or sorghum, commonly obtained from public places such as markets, schools, motor parks and also served as refreshments during social events such as

*Corresponding author. Tel.: +2348100189084
E-mail address: olamyjoshua@gmail.com

marriage ceremonies, festivities and naming ceremonies (2).

The traditional method of preparing Kunun-zaki involves grain steeping, grinding and sieving. The resulting liquid is subjected to spontaneous fermentation, which utilizes mixed cultures of autochthonous bacteria species and sweetened or flavoured before consumption (3,4).

Lactic Acid Bacteria (LAB) species such as *Lactobacillus* spp., *Lactococcus* spp., and *Leuconostoc* spp. are the predominant bacteria in the fermentation of *Kunun-zaki*. Yeasts such as *Saccharomyces cerevisiae and Candida milleri* are also present, along with molds such as *Aspergillus* spp. and *Penicillium* spp. (5,6). Okechukwu et al. (6) further demonstrated that *Lactobacillus* spp. are the most effective fermenters in Kunun-zaki.

Lactobacillus spp. represents a diverse group of Gram-positive microaerophilic bacteria that appear as short to long rods under a microscope. The majority of the species in this genus are catalase-negative and either homo- or hetero-fermentative in terms of hexose metabolism (7). Based on studies concerning the antimicrobial agents synthesized by LAB, several *Lactobacillus* species, particularly *L. acidophilus*, *L. plantarum, L. brevis, and L. salivarius*, have been shown to produce organic acids (lactic or acetic acid), hydrogen peroxide, diacetyl, antifungal substances like fatty acids or phenyllactic acid, and/or bacteriocins which protect food against spoilage and pathogenic microorganisms (8,9,10).

Similarly, Kaewchomphunuch et al. (11) reported that cell-free culture supernatants (CFCS) produced from Lactic Acid Bacteria (LAB) contain active metabolites, including bacteriocins, that can inhibit the growth of *E. coli* O157:H7, *Gardnerella vaginalis*, *Listeria monocytogenes*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella flexneri, Shigella sonnei*, *Staphylococcus aureus*, and *Streptococcus suis.*

In recent years, the antimicrobial drug resistance of pathogens has become a serious threat to public health, as it reduces the effectiveness of antibiotics in treating infections, and makes bacterial diseases more difficult to manage (12). Therefore, there is a need for natural ways to control bacterial pathogens. The objective of this study is to isolate *Lactobacillus* spp. with antibacterial properties from a traditionally fermented cereal beverage (Kunun-zaki).

2. Material and Methods

2.1. Sample collection

Sorghum grains, ginger, red pepper, and clove were obtained from Aliero market, Aliero Local Government Area of Kebbi State, Nigeria. The samples were collected in sterile polyethylene bags and immediately transported to the laboratory of the Department of Microbiology, Federal University Birnin-Kebbi, for further analysis.

2.2. Preparation of traditionally fermented Kunun-zaki

Traditionally fermented *kunun-zaki* was prepared following the indigenous processing method as illustrated in Fig 1. The production process began with the preparation of the cereal substrate. Five hundred grams (500 g) of sorghum grains were thoroughly washed with clean tap water to remove dirt and extraneous materials. The cleaned grains were then drained and soaked in 1,000 mL of tap water in a clean plastic bucket and left overnight for approximately 12 h. This soaking step was necessary to soften the grains and enhance subsequent grinding and fermentation.

After soaking, the sorghum grains were washed again and blended with pre-measured spices to improve flavor and aroma. The spices consisted of ginger (10 g), clove (5 g), and red pepper (5 g). The mixture of soaked grains and spices was then ground into a smooth paste using a clean and sterilized blender to minimize contamination and ensure uniformity of the slurry.

The resulting paste was divided into two portions. Two-thirds (2/3) of the paste was transferred into a clean container and thickened by the gradual addition of boiling water, followed by vigorous stirring for about 2–3 min. This step partially gelatinized the starch, contributing to the characteristic consistency of kunun-zaki. Subsequently, the remaining one-third (1/3) of the uncooked paste was added to the thickened portion and stirred thoroughly to obtain a homogenous mixture.

The slurry was then filtered using a clean muslin cloth to separate the liquid extract from the chaff and coarse particles. After sieving, sweetening was achieved by adding 50 g of sugar to the filtrate and stirring until completely dissolved. The sweetened filtrate was left to undergo spontaneous fermentation at ambient temperature for 6 h, during which natural microflora facilitated the fermentation process.

At the end of the fermentation period, the final product, kunun-zaki, was obtained. This product served as the stock sample for subsequent microbiological and physicochemical analyses.

2.3. Isolation of *Lactobacillus* spp.

2.3.1. Preparation

One milliliter (1 mL) of the Kunun-zaki sample was aseptically taken from the fermenting vessel and dispensed into test tube containing 9 mL of distilled water. Then the first dilution (10^{-1}) was shaken to homogenize the mixture. Subsequently, one milliliter (1 mL) of this dilution (10^{-1}) was aseptically dispensed into another sterile test tube containing 9 mL of sterile distilled water to make a mixture (10^{-2}). The serial dilution continued until a 10^{-6} was attained. This process was carried out at 0 h, 24 h, and 48 h after the preparation of Kunun-zaki (15).

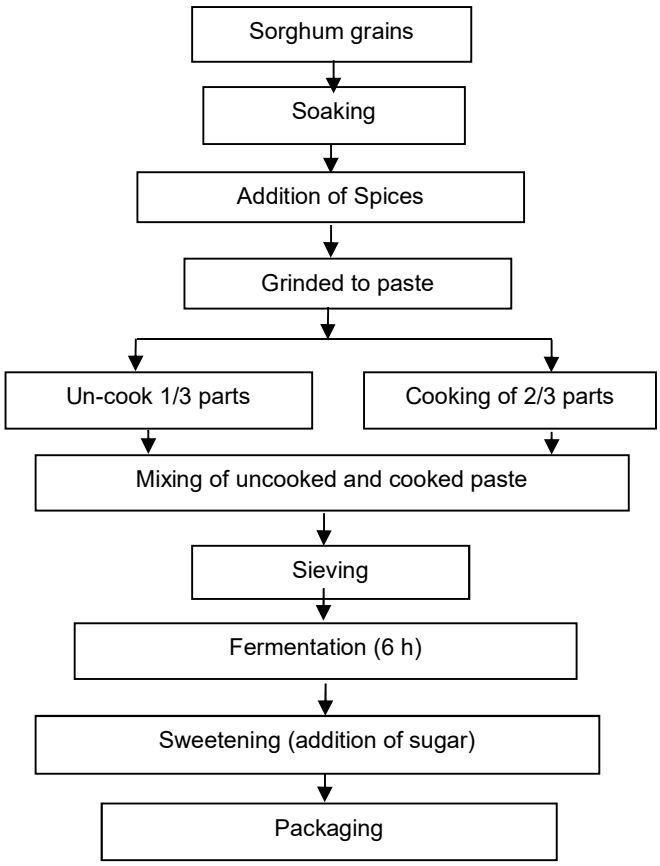

Figure 1. Flow diagram for the processing of traditionally fermented *Kunun-zaki* (13,14).

2.3.2. Determination of pH

Ten milliliters (10 mL) of traditionally fermented Kunun-zaki samples were aseptically removed at 0 h, 24 h and 48 h after preparation. The pH was determined by using a pH meter (Corning 475343 Benchtop) which was initially standardized by buffer solution of pH 4.0. Readings were taken in triplicate (16).

2.3.3. Plating (pour plating) and enumeration of lactic acid bacteria

Once the dilutions were made, one milliliter (1 mL) from dilution tube 10^{-2} and tube 10^{-4} was carefully dispensed into separate Petri dishes. After that, melted MRS agars were poured onto the dishes, mixed well and then allowed to cool and solidify. The inoculated plates were then incubated anaerobically at 37 ^{o}C for 24 – 48 h using an anaerobic jar. The colonies observed were counted on a colony counter and expressed in colony forming units per milliliter (cfu/mL). All plates were prepared in triplicate (16).

2.4. Morphological and biochemical characterization *Lactobacillus* spp.

Identification of isolates was based on their cultural, morphological, and biochemical characteristics following Bergey's Manual of systematic bacteriology (17). The isolates obtained were subjected to growth at different temperatures (15 ^{o}C and 45 ^{o}C), gram staining and microscopy, catalase test, endospore, oxidase test, motility, indole and urea (MIU) tests and sugar fermentations.

2.4.1. Growth at different temperatures (15 ^{o}C and 45 ^{o}C)

Colonies of the isolates were picked and carefully inoculated into MRS broth in sterile test tubes using a wire loop. The test tubes were gently swirled and incubated anaerobically at 15 ˚C and 45 ˚C respectively for 24 – 48 h. After 24 h of incubation, the test tubes were examined for medium cloudiness, which indicated bacterial growth (positive result), whereas a clear medium indicated no growth (negative result) (18).

2.4.2. Gram staining and microscopy

Using an overnight pure culture, smear was prepared by spreading a loopful of the isolate on a glass slide and passing it over a low flame three times to fix it. The smear was covered with crystal violet for 1 min and rinsed with water. Lugol's iodine solution was then applied for 1 min and rinsed off, after which 95% ethanol was used for decolarization for about 10 s, followed by rinsing with water. The smear was then counterstained with safranin for 30 s and washed with distilled water. After staining, the slides were air-dried and examined under a light microscope by using × 100 oil immersion objectives. Gram-positive rod-shaped bacteria cells appeared purple against background (14).

2.4.3. Catalase test

A loopful of culture from each isolate was placed on a clean, grease-free glass slide. A few drops of 3% hydrogen peroxide were added to the culture. The slides were immediately observed for the formation of bubbles; where active effervescence indicated positive catalase reactions (19).

2.4.4. Endospore test

Smear from each of the isolate were prepared on clean, grease-free slides and air-dried. The slides were then placed over a beaker of boiling water, resting on the rim with the bacterial film upward. After 40 s, the smears were flooded with 5% aqueous solution of malachite green and steamed for 2 min while the water continues

to boil. The slides were then removed steam source, rinsed with distilled water, and counterstained with safranin for 30 s. After staining, the slides were rinsed again with distilled water, air-dried and examined under light microscope using × 100 oil immersion objectives. Endospores appeared green, while vegetative cells were stained red (19).

2.4.5. Oxidase test

An aliquot of each colony was picked from the isolates and rubbed onto a strip of a filter paper impregnated with oxidase reagent (tetramethyl-p-phenalenediamine dihydrochloride). The development of dark purple colour within 10 s indicated a positive oxidase reaction, whereas the absence of colour change indicated a negative result (20).

2.4.6. Motility, indole and urea (MIU) test

Isolates were picked using a sterile wire loop and inoculated into sterile slant test tubes containing motility indole urea (MIU) Medium to a depth of approximately two-thirds of the medium. The tubes were then incubated at 37 °C for 24 h. Motility was indicated by diffuse growth away from the line of inoculation, whereas growth restricted to the stab line indicated non-motile organisms (18). A change in the colour of the MIU medium to pink or red indicated a positive urease reaction, while no colour change indicated a negative result. After the addition of Kovac's reagent, the formation of pink or red ring at the surface of the medium indicated a positive indole reaction, whereas the absence of a colour change indicated a negative result (3).

2.5. Sugar fermentation profile of *Lactobacillus* isolates

Isolates confirmed to be rod-shaped, gram-positive, catalase-negative, and non-spore-forming were subjected to sugar fermentation tests. Prior to the testing, each isolate was streaked onto solidified MRS agar within petri dishes and incubated anaerobically at 35 °C for 48 h.

The sugar fermentation profiles of *Lactobacillus* isolates were determined by using fourteen different sugars: xylose, galactose, glucose, gluconate, rhamnose, mannitol, sorbitol, salicin, cellobiose, lactose, melibiose, trehalose, raffinose and sucrose. A total of 1.5 g of phenol red broth base medium was suspended into 100 mL of distilled water and gently heated to dissolve. Subsequently, 0.1 g (0.1 % w/v) of each sugar substrate was added to 100 mL of the broth medium. Approximately, 5 mL of the mixture was dispensed into 13 × 100 mm test tubes, and inverted Durham tube was inserted into each. All the tubes were sterilized at 121 °C for 15 min.

After sterilization, each tube was inoculated with a single colony of the *Lactobacillus* isolates using a sterile wire loop and incubated at 37 °C for 48 h. A positive fermentation reaction was indicated by a change in the colour of the medium from red to yellow and presence of air bubbles in the Durham tubes, while no colour change indicated a negative reaction (21).

2.6. Molecular characterization of LAB isolates

2.6.1. DNA extraction

Genomic DNA was extracted from six LAB isolates using the QIAamp DNA Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions. Isolates were subcultured on MRS agar and incubated at 30°C for 48 to 72 h. Single colonies were suspended in nuclease-free microtubes, lysed with buffer AVL, and mixed with absolute ethanol to facilitate DNA binding. The lysates were transferred onto QIAamp

Mini spin columns and subjected to sequential washing with buffers AW1 and AW2. DNA was eluted in 50 µL of Buffer AE and stored at −20°C until PCR analysis.

2.6.2. PCR amplification of the 16S rRNA gene

The 16S rRNA gene was amplified using universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), which yield an expected fragment of approximately 1,500 bp (17). Each 25 µL PCR reaction contained 12.5 µL of Qiagen Master Mix, 1 µL of each primer (10 µM), 3 µL of nuclease-free water, and 5 µL of DNA template. Amplification was performed in an Applied Biosystems GeneAmp PCR System 9700 using the following conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 60°C for 2 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were stored at 4°C prior to electrophoresis.

2.6.3. Agarose gel electrophoresis

PCR products were analyzed by agarose gel electrophoresis using a 1.5 percent gel prepared in 1X Tris-Acetate EDTA (TAE) buffer. Ethidium bromide was incorporated to allow visualization. A 100-bp molecular weight ladder (Biolabs, UK) was used as a size marker. For each sample, 8 µL of amplicon was mixed with loading dye and transferred into the wells. Electrophoresis was carried out at 70 V for 1 h. A known LAB strain served as the positive control and nuclease-free water as the negative control. Gels were visualized under UV illumination using a Bio-Rad documentation system**.** Then clear PCR bands of the expected size were sequenced commercially. Resulting sequences were edited and compared with reference sequences in the NCBI GenBank database using BLAST.

2.7. Determination of antibacterial activity of *Lactobacillus* spp.

2.7.1. Collection and preparation of test organisms

The test organisms; *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., and *Klebsiella* spp were obtained from Microbiology Laboratory in the Department of Microbiology, Federal University Birnin-Kebbi, Kebbi State Nigeria. The organisms were subjected to confirmatory tests such as gram staining, catalase test, coagulase test and oxidase test. Thereafter, they were sub-cultured into their respective selective media. *Staphylococcus aureus* was sub-cultured onto Mannitol Salt Agar Medium, while *Escherichia coli, Salmonella* spp., and *Klebsiella* spp were sub-cultured onto MacConkey Agar Medium prior to use.

Inoculum suspensions of the test organisms were prepared by transferring colonies of 24 h cultures into 5 mL of sterile normal saline using a sterile wire loop. The turbidity of each suspension was adjusted to match the 0.5 McFarland standards (equivalent to 1.5×10^8 cfu/mL). Suspension with higher turbidity were diluted with sterile normal saline, while those with lower turbidity were adjusted by adding additional colonies to achieve the requires density (22).

2.7.2. Preparation of cell-free supernatant (CFS) extracts of *Lactobacillus* isolates

Cell-free supernatants (CFS) of *Lactobacillus* isolates were prepared following the method described by Hudzicki et al., (22). Eight isolates of *Lactobacillus* spp. were separately grown in 10 mL MRS broth for 24 h at 37 ^{o}C under anaerobic conditions. The cultures were centrifuged at 10,000 × g for 15 min. The resulting

supernatants were carefully collected using sterile pipettes, and the pH was adjusted to 6.5 with 1 N NaOH to eliminate the effect of organic acids. The adjusted supernatants were then filtered through 0.22 µm membrane filters to obtain sterile cell-free supernatants.

2.7.3. Antibacterial activity of *Lactobacillus* spp.

Antibacterial assay was carried out using the agar well diffusion method as described by Adeyemo et al. (23). Sterile Mueller-Hinton Agar (MHA) was poured into petri dishes and allowed to solidify. From the standard inoculums (0.5 McFarland Standard) of each test organism, 0.1 mL was pipetted and evenly spread over the surface of the MHA using a sterile glass rod. The inoculated plates were allowed to dry for 5 min. Wells of approximately 6 mm in diameter were aseptically made in the agar by using a sterile cork-borer, and each well was filled with 0.1 mL CFS of *Lactobacillus* isolates. The plates were incubated aerobically at 37 ^{o}C for 24 h. Streptomycin (25 mg/mL) and sterile distilled water served as the positive and negative control, respectively. After incubation, the diameters of the zones of inhibition around the wells were measured in millimetres. All assays were performed in triplicate.

2.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 20. Data were expressed as mean ± standard deviation (mean ± SD) from three independent replicates. Differences among treatment means were evaluated using one-way analysis of variance (ANOVA). When significant differences were detected ($p<0.05$), Tukey's Honestly Significant Difference (HSD) test was applied to determine pairwise differences among inhibition zone means.

3. Results

3.1. Determination of pH of traditionally fermented Kunun-zaki

The pH values obtained from traditionally fermented Kunun-zaki at different time intervals after preparation are presented in Table 1. At 0 h, the initial pH mean value was 6.83 ± 0.05. After 24 h, the mean pH value decreased to 5.63 ± 0.05 and at 48 h, it further declined to 4.5 ± 0.10.

3.2. The enumeration of lactic acid bacteria (LAB) from traditionally fermented Kunun-zaki

The enumeration of Lactic Acid Bacteria (LAB) at different dilution factors of the traditionally fermented Kunun-zaki samples on MRS agar medium were presented in Table 1.

For the 10^{-2} dilution, the mean colony count at 0 h was $2.45 \times 10^{3} \pm 1.80 \times 10^{2}$ cfu/mL, while at 24 h, the mean count increased to $2.75 \times 10^{3} \pm 7.57 \times 10^{2}$ cfu/mL. At 48 h, the colonies were too numerous to count (TNTC).

For the 10^{-4} dilution, the mean colony count at 0 h was $1.14 \times 10^{3} \pm 5.14 \times 10^{1}$ cfu/mL, at 24 h it was $1.78 \times 10^{3} \pm 3.51 \times 10^{1}$ cfu/mL, and at 48 h, the mean colony count was $2.87 \times 10^{3} \pm 4.36 \times 10^{1}$ cfu/mL.

Table 1. pH Values/enumeration of LAB in traditionally fermented Kunun-zaki

Storage time	pH value	Dilution factors (cfu/mL)	
		10^{-2}	10^{-4}
0 h	6.83 ± 0.05	$2.45\times10^{3} \pm 1.80\times10^{2}$	$1.14\times10^{3} \pm 5.14\times10^{1}$
24 h	5.63 ± 0.05	$2.75\times10^{3} \pm 7.57\times10^{2}$	$1.78\times10^{3} \pm 3.51\times10^{1}$
48 h	4.50 ± 0.10	TNTC	$2.87\times10^{3} \pm 4.36\times10^{1}$

KEY: TNTC= too numerous to count, TFTC= too few to count, cfu/mL = colony forming units per milliliter, h = hours

Table 2. Morphological and biochemical characterization of *Lactobacillus* isolates

Isolate Codes	Colonies appearance	Growth Temperature		G/S	Sha	Cat	Oxi	Spo	MIU test			Probable Bacteria
		15 °C	45 °C						Motility	Indole	Urea	
LBK0-23	Creamy	+	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.
LBK2-23	White	-	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.
LBK2-22	White	-	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.
LBK4-12	White	+	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.
LBK4-23	Creamy	-	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.
LBK4-24	White	+	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.
LBK4-15	White	-	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.
LBK4-26	Creamy	-	+	+	Rod	-	-	-	-	-	-	*Lactobacillus* spp.

KEYS: G/S=Gram Staining, Sha = Shape, Cat=Catalase, Oxi=Oxidase, Spo=Spore, MIU=Motility Indole and Urea test
– = negative reaction, + = positive reaction

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3.3. Morphological and biochemical characterization of *Lactobacillus* isolates

The morphological and biochemical characteristics of the identified *Lactobacillus* isolates (LBK0-23, LBK2-23, LBK2-22, LBK4-12, LBK4-23, LBK4-24, LBK4-15 and LBK4-26) obtained from samples of traditionally fermented Kunun-zaki are presented in Table 2. All the presumptive isolates appeared whitish or creamy on MRS agar medium. The isolates were rod-shaped, gram-positive organisms and exhibited negative reactions to catalase, oxidase, spore formation, motility, indole and urease tests. Only *Lactobacillus* isolates of LBK0-23, LBK4-12, and LBK4-24 showed growth when incubated at 15 °C for 24 – 48 h, whereas all the *Lactobacillus* isolates exhibited turbidity when incubated at 45 °C for 24 – 48 h.

3.4. Sugar fermentation profile of *Lactobacillus* isolates

The results of the sugar fermentation profiles of *Lactobacillus* isolates (Table 3) from traditionally fermented Kunun-zaki showed that all the isolates exhibited positive reactions to the fermentation of galactose, glucose, sucrose, and lactose, while none of the isolates exhibiteda positive reaction to rhamnose. There were varying reaction to xylose, mannitol, sorbitol, salicin, cellobiose, melibiose, trehalose, raffinose and gluconate among the different *Lactobacillus* isolates. These results indicate the presence of eight distinct *Lactobacillus* isolates identified as *Lactobacillus plantarum* (LBK0-23, LBK4-12, LBK4-24), *Lactobacillus fermentum* (LBK4-23, LBK4-26), *Lactobacillus acidophilus* (LBK2-23, LBK2-22), and *Lactobacillus salivarius* (LBK4-15) from the traditionally fermented Kunun-zaki beverage.

3.5. Molecular identification

PCR amplification of the 16S rRNA gene from representative isolates produced single amplicons of approximately 1,500 bp (Fig. 2). Analysis of the 16S rRNA gene sequences using the NCBI BLAST database revealed that the representative isolates shared 98-100% sequence similarity with reference strain. Based on these similarities, the isolates were identified as *Lactiplantibacillus plantarum* (37.5%), *Lacticaseibacillus acidophilus* (25%), *Limosilactobacillus fermentum* (25%) *and Ligilactobacillus salivarius* (12.5). Hence, the obtained sequences were used solely for species confirmation and were not deposited in GenBank.

3.6. Distribution of *Lactobacillus* species isolated from traditionally fermented Kunun-zaki

The distribution of *Lactobacillus* species isolated from traditionally fermented Kunun-zaki revealed *Lactobacillus plantarum* as the most frequently occurring species (37.5%), representing three isolates (LBK0-23, LBK4-12, LBK4-24). This was followed by *Lactobacillus acidophilus* (25%) with two isolates (LBK2-23, LBK2-22), *Lactobacillus fermentum* (25%) with two isolates (LBK4-23, LBK4-26), and *Lactobacillus salivarius* (12.5%) with one isolate (LBK4-15) as presented in Table 4.

Table 3. Sugar Fermentation Profile of *Lactobacillus* Isolates

Sugar fermentation	*Lactobacillus* Isolates							
	LBK0-23	LBK2-23	LBK2-22	LBK4-12	LBK4-23	LBK4-24	LBK4-15	LBK4-26
xylose	+	-	-	+	-	+	-	-
Galactose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-
Mannitol	+	-	-	+	-	+	+	-
Sorbitol	+	-	-	+	-	+	+	-
Salicin	-	+	+	-	-	-	-	-
Cellobiose	+	+	+	+	-	+	-	-
Lactose	+	+	+	+	+	+	+	+
Melibiose	+	-	-	+	+	+	+	+
Trehalose	+	-	-	+	-	+	+	-
Raffinose	-	+	+	-	+	-	-	+
Gluconate	+	-	-	+	-	+	-	-

Keys: + = Positive reaction, - = Negative reaction,

Table 4. Distribution of *Lactobacillus* species isolated from traditionally fermented

Identified *Lactobacillus* species	Isolate codes	Frequency	Percentages
Lactobacillus plantarum	LBK0-23, LBK4-12, LBK4-24	3	37.5 %
Lactobacillus acidophilus	LBK2-23, LBK2-22	2	25 %
Lactobacillus fermentum	LBK4-23, LBK4-26	2	25 %
Lactobacillus salivarius	LBK4-15	1	12.5 %
Total		**8**	**100 %**

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Table 5. Antibacterial activity (mm) of cell free supernatant extract of *Lactobacillus* isolates on test organisms

***Lactobacillus* isolates**	**Test organisms/zone of inhibition (mm)**			
	S. aureus	***E. coli***	***Salmonella* spp.**	***Klebsiella* spp.**
LBK0-23	14.83 ± 0.28[a]	11.86 ± 0.15[a]	00.00[a]	12.50 ± 0.45[a]
LBK2-23	13.76 ± 0.25[b]	14.20 ± 0.30[b]	8.90 ± .0.20[b]	14.43 ± 0.56[b]
LBK2-22	14.00 ± 0.36[a]	13.90 ± 0.10[b]	14.23 ± 0.49[c]	13.46 ± 0.41[c]
LBK4-12	14.13 ± 0.41[a]	18.60 ± 0.52[c]	13.90 ± 0.01[c]	9.36 ± 0.55[d]
LBK4-23	19.57 ± 0.58[c]	20.30 ± 0.60[d]	9.50 ± 0.50[d]	13.13 ± 0.32[c]
LBK4-24	13.47 ± 0.50[b]	18.46 ± 0.45[c]	00.00[a]	15.36 ± 0.56[e]
LBK4-15	14.30 ± 0.50[a]	12.26 ± 0.55[a]	8.16 ± 0.28[b]	17.73 ± 0.36[f]
LBK4-26	18.26 ± 0.46[c]	10.26 ± 0.64[e]	00.00[a]	19.23 ± 0.25[g]
Streptomycin (25 mg/mL)	22.50 ± 0.50[d]	18.80 ± 0.39[c]	20.50 ± 0.19[e]	21.50 ± 1.00[h]
Negative control	00.00[e]	00.00[f]	00.00[a]	00.00[i]

Note: Values are in mean ± SD of three replicates experiments. Mean values with different superscript letter within the same column differ significantly ($p<0.05$), according to Tukey's honestly significant difference (HSD) post hoc test.
Keys: Positive control = Streptomycin (25 mg/mL), Negative control = distilled water

Table 6. ANOVA Table for antibacterial activity (mm) of cell free supernatant extract of *Lactobacillus* isolates on test organisms

CFS of *Lactobacillus* isolates	**Sum of squares**	**df**	**Mean square**	**F**	**Sig.**
Between groups	991.759	9	110.195	4.289	.001
Within groups	770.791	30	25.693		
Total	1762.550	39			

3.7. Antibacterial activity of *Lactobacillus* spp.

The results of the antibacterial activity of cell-free supernatant (CFS) extracts from various *Lactobacillus* isolates (LBK2-23, LBK2-22, LBK4-12, LBK4-23, LBK4-15, LBK0-23, LBK4-24 and LBK4-26) against *Staphylococcus aureus, Escherichia coli, Salmonella* spp., and *Klebsiella* spp. are presented in Table 5. The CFS extracts of isolates LBK2-23, LBK2-22, LBK4-12, LBK4-23 and LBK4-15 exhibited inhibitory effects against all the test organisms. However, isolates LBK0-23, LBK4-24 and LBK4-26 showed no activity against *Salmonella* spp.

For *S. aureus*, isolates LBK4-23 and LBK4-26 produced the largest inhibition zones (19.57 ± 0.58 mm and 18.26 ± 0.46 mm, respectively), significantly greater than those of the other isolates ($p < 0.05$, Tukey's test), although smaller than that of streptomycin (22.50 ± 0.50 mm).

Against *E. coli*, LBK4-23 showed the strongest activity, with an inhibition zone of 20.30 ± 0.60 mm, significantly larger than those of all other isolates ($p<0.05$). Isolates LBK4-12 and LBK4-24 formed a second group with inhibition zones around 18.5 mm, similar to that of streptomycin (18.80 ± 0.39 mm). LBK0-23, LBK2-23, LBK2-22 and LBK4-15 had moderate activity, while LBK4-26 showed the lowest inhibition against *E. coli* (10.26 ± 0.64 mm).

Salmonella spp. was the least susceptible test organism. CFS from LBK0-23, LBK4-24 and LBK4-26 produced no inhibition zones, similar to the negative control. Among the active isolates, LBK2-22 and LBK4-12 had the largest zones (14.23 ± 0.49 mm and 13.90 ± 0.01 mm, respectively), significantly higher than those of LBK2-23, LBK4-23 and LBK4-15 (8.16–9.50 mm), but all were markedly lower than that of streptomycin (20.50 ± 0.19 mm).

Against *Klebsiella* spp., all isolates exhibited inhibitory activity. LBK4-26 and LBK4-15 showed the highest inhibition (19.23 ± 0.25 mm and 17.73 ± 0.36 mm, respectively), followed by LBK4-24 and LBK2-23. LBK4-12 had the smallest inhibition zone (9.36 ± 0.55 mm). Streptomycin again showed the largest inhibition zone (21.50 ± 1.00 mm), and the negative control produced no inhibition.

ANOVA indicated significant differences ($p<0.05$) among mean inhibition zones for each test organism, and Tukey's HSD test distinguished multiple groups, as indicated by the superscript letters in Table 5. The summarised ANOVA is shown in Table 6.

4. Discussion

The pH of traditionally fermented Kunun-zaki decreased progressively over time, which contributes to its characteristic sour taste. The pH values of the sample ranged from 6.83 ± 0.05 at 0 h after preparation to 4.5 ± 0.10 at 48 h (Table 1). The result consistent with the findings of Akoma et al. (24), who reported a pH of 4.20 for laboratory prepared Kunun-zaki. Similarly, Agarry et al. (4) observed pH variations ranging from 3.94 ± 0.01 to 5.44 ± 0.04 within 6 h of fermentation.

This observed pH decline reflects the metabolic activities of fermenting microorganisms, particularly lactic acid bacteria, which convert fermentable sugars into organic acids. This acidification plays a crucial role in the fermentation process by influencing flavor development and enhancing the microbial stability of the beverage. The acidic environment created during fermentation inhibits the growth of spoilage organisms and potential pathogens, thereby contributing to the safety and shelf life of Kunun-zaki. This observation

agrees with the report of by Wakil and Osamwonyi (25), which highlighted the antimicrobial effect of acidification in traditionally fermented beverages.

The enumeration of LAB at different dilution factors (10^{-2} and 10^{-4}) of the traditionally fermented Kunun-zaki revealed a progressive increase of microbial population over time (Table 1). At 0 h after preparation, the dilution factor of 10^{-2} exhibited a LAB mean count of $2.45 \times 10^3 \pm 1.80 \times 10^2$ cfu/mL, indicating the presence of an initial microbial population at the onset of fermentation. After 24 h, the LAB counts increased, reflecting active microbial proliferation during the early stage of fermentation. By 48 h, the dilution factor of 10^{-2} showed colonies too numerous to count (TNTC), signifying a substantial increase in LAB population as fermentation progressed.

At 10^{-4} dilution, the LAB mean count was $1.14 \times 10^3 \pm 5.14 \times 10^1$ cfu/mL at 0 h, with relatively lower counts recorded at 24 and 48 h compared to the 10^{-2} dilution. This difference demonstrates the influence of dilution factors on observed colony counts and underscores the dense microbial growth occurring at lower dilutions during fermentation.

LAB counts increased over time, showing active proliferation of acidogenic bacteria. This trend agrees with previous studies on fermented cereal beverages, in which LAB populations increased during fermentation and were associated with pH reduction and flavour development (24). The proliferation of LAB is essential for maintaining product safety and quality.

Eight LAB isolates from Kunun-zaki showed phenotypic properties consistent with members of the former genus *Lactobacillus.* All were gram-positive, non-spore-forming rods, catalase- and oxidase-negative and mostly unable to grow at 15°C but tolerant of 45°C, which is typical of many *Lactobacilli*. Their sugar fermentation profiles allowed initial assignment to *L. plantarum*, *L. fermentum*, *L. acidophilus* and *L. salivarius*. Molecular analysis by 16S rRNA gene sequencing confirmed these identifications and enabled updating of taxonomy according to Wakil and Osamwonyi (25) resulting in their reassignment to *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum* and *Ligilactobacillus salivarius*, while *L. acidophilus* remained within the genus *Lactobacillus*. *Lactiplantibacillus plantarum* was the most prevalent species, which agrees with reports that this species commonly dominates the LAB community in Kunun-zaki and other African cereal fermentations (26).

All the *Lactobacillus* isolates exhibited varying antibacterial activity against selected food-contaminating bacteria using the agar well diffusion method (Table 5). Cell-free supernatants (CFS) of isolates LBK2-23, LBK2-22, LBK4-12, LBK4-23, LBK4-15, LBK0-23, LBK4-24, and LBK4-26 were tested against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., and *Klebsiella* spp. Antibacterial activity was determined by measuring the zones of inhibition (in millimeters) around each well where bacterial growth was inhibited. The inhibition zones ranged from 8.16 ± 0.28 mm to 20.30 ± 0.60 mm, with the highest activity recorded for isolate LBK4-23 against *E. coli* and the lowest activity observed for isolate LBK4-15 against *Salmonella* spp. Statistical analysis revealed a significant difference ($p<0.05$) in the antibacterial activities of the CFS from different isolates against the test organisms.

The observed antibacterial effects can be attributed to the production of various antimicrobial compounds by *Lactobacillus* species, including organic acids, hydrogen peroxide, and bacteriocins, which inhibit the growth of a wide range of bacteria (27). These findings are consistent with the report of Aka-Gbezo et al. (28), who demonstrated that *Lactobacillus* spp. inhibited the growth of indicator organisms such as *B. subtilis*, *E. coli*, *S. aureus*, *E. faecalis*, *S. typhi*, and *P. aeruginosa*, with inhibition zones ranging from 7 mm to 21 mm. Similarly, Victor-Aduloju et al. (16) highlighted that the antimicrobial effects of lactic acid bacteria are strain-dependent, which aligns with the varying antibacterial activities observed among the isolates in this study.

Salmonella spp. was the only test organism that was completely resistant to the cell-free supernatant of some isolates (LBK0-23, LBK4-24 and LBK4-26), and even when inhibition occurred, the zones were smaller than those observed for *Staphylococcus aureus* or *Klebsiella* spp. This pattern is consistent with the known lower susceptibility of many gram-negative bacteria to metabolites produced by lactic acid bacteria. The outer membrane of *Salmonella* acts as a permeability barrier that limits the entry of many bacteriocins and other hydrophobic antimicrobial molecules, whereas gram-positive bacteria lack this additional protective layer. In addition, our CFS were adjusted to pH 6.5 before testing, which reduces the direct effect of lactic and acetic acids and makes inhibition more dependent on non-acidic metabolites such as bacteriocins. Many bacteriocins produced by *Lactobacillus* and related genera have a narrow activity spectrum that targets Gram-positive bacteria through specific receptors that are absent in *Salmonella*. These structural and functional features can therefore explain why some of the isolates that were active against *S. aureus*, *E. coli* and *Klebsiella* spp. showed little or no effect on *Salmonella* spp.

Another factor that may contribute to the reduced susceptibility of *Salmonella* spp. is its well-documented acid tolerance response. Exposure to mildly acidic environments can induce stress response systems in *Salmonella* that increase its ability to survive at lower pH and in the presence of organic acids. Such adaptive mechanisms, together with efflux pumps and modifications of the lipopolysaccharide layer, have been associated with increased resistance to various antimicrobial agents. In the present work, the *Salmonella* strain was not serotyped, so it is not possible to relate its behaviour to a particular serovar or to specific resistance determinants.

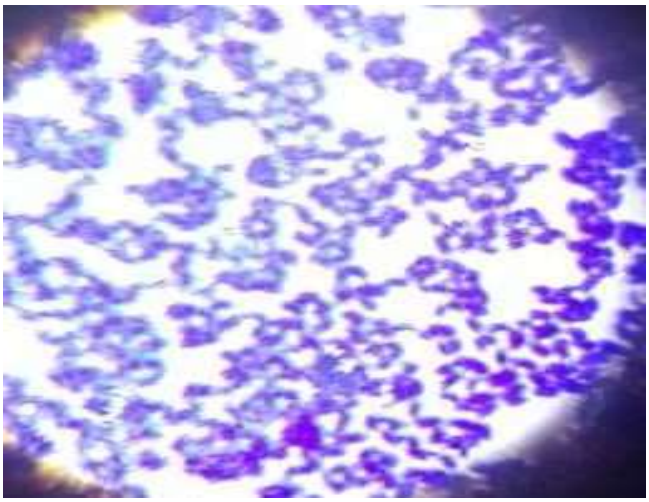

Figure 2. Microscopic view of gram-positive *Lactobacillus* isolate

Fig. 2 shows the microscopic appearance of a *Lactobacillus* isolate following gram staining. The bacterial cells appear purple, indicating a gram-positive reaction, which is characteristic of organisms possessing a thick peptidoglycan layer in their cell wall. The cells are observed as slender, elongated rods,

occurring singly and in short chains, a typical morphological feature of *Lactobacillus* species.

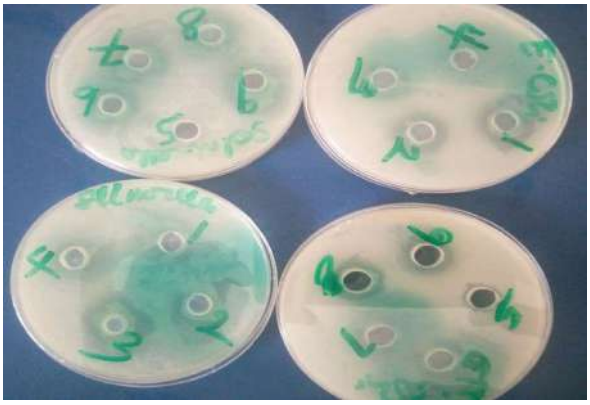

Figure 3. Agar medium showing zones of inhibition

Figure 3 shows the antibacterial activity of the test isolate as demonstrated by the presence of clear zones of inhibition on the agar medium. The transparent regions surrounding the wells indicate areas where the growth of the test organisms was suppressed. These zones are a direct measure of the inhibitory effect exerted by antimicrobial substances produced by the isolate.

5. Conclusion

In this study, eight species of *Lactobacillus* were isolated from traditionally fermented Kunun-zaki. These species were identified through their morphological and biochemical characteristics and include *Lactobacillus plantarum* (LBK0-23, LBK4-12, LBK4-24), *Lactobacillus fermentum* (LBK4-23, LBK4-26), *Lactobacillus acidophilus* (LBK2-23, LBK2-22), and *Lactobacillus salivarius* (LBK4-15).

The cell-free supernatants (CFS) from these isolates showed different levels of antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., and *Klebsiella* spp. Among them, the CFS from isolate LBK4-23 recorded the highest inhibition against *E. coli*. These results show that *Lactobacillus* species found in *Kunun-zaki* play an active role during fermentation and also possess antibacterial properties. This supports their potential use both in traditional beverage production and as natural antimicrobial agents.

Further studies are therefore recommended to optimize the fermentation process for *kunun-zaki* production; to evaluate the antibacterial activity of *Lactobacillus* spp. against a wider range of foodborne pathogens for food safety applications; and to investigate the bioactive compounds produced by these isolates in order to determine their chemical nature, health-promoting properties, and safety for use in food systems.

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Authorship contribution

Ola Ajibola Joshua conceptualized the study, carried out the methodology and investigation, curated and analyzed the data, prepared the visualizations, and wrote the original draft of the manuscript as part of his M.Sc. thesis. Abbas Yusuf Bazata provided major supervision, contributed to the study conceptualization and methodology, validated the results, and supplied essential resources. Adamu Aliero Almustapha contributed to the methodology, coordinated the project, supported supervision, and assisted in shaping the thesis. Aminu Fardami Yusuf assisted with validation of results and manuscript review. Marwana Magaji contributed to manuscript writing through review and editing. Mubarak Bodinga Musa and

Amina Muhammad supported the study through manuscript review and editorial input. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there are no competing interests of any kind.

Data availability

The data generated and analyzed during this study are available from the corresponding author upon reasonable request.

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