



# ***In vitro* Probiotic Potential of Autochthonous Lactic Acid Bacteria and Microbiology of Kunu Made from Mixed Grains**

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## **Authors' contributions**

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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## **ABSTRACT**

**Aims:** This study investigated the microbiological quality of commercially-prepared kunu in comparison with those prepared under laboratory conditions using two cereals, millet and sorghum, and the *in vitro* probiotic potential of the autochthonous lactic acid bacteria.

**Study Design:** Randomized complete block design.

**Place and Duration of Study:** Food Processing Laboratory, Food Science and Technology Department, Federal University of Technology, Akure, and Akure metropolis of Ondo State, Nigeria in June 2015.

**Methodology:** Eight Kunu samples were used; 5 obtained from different commercial processors and 3 prepared in the laboratory from a combination of two cereal grains. The samples were subjected to physicochemical and microbiological analyses. Microorganisms isolated were characterized using conventional identification tests and the lactic acid bacteria were screened *in vitro* for their probiotic potential.

**Results:** Crude protein (%dry weight) of samples ranged from 33.85-58.68%; with the sample prepared from a combination of sorghum and millet having the highest content. Total ash (3.84-

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6.26%) and solids (7.00-9.11%) varied significantly ( $P=0.05$ ) between samples. Values obtained for pH and acidity of the samples ranged from 4.14-5.01 and 1.22-3.45%. Out of 13 microbes isolated from the Kunu samples, 6 were lactic acid bacteria, 2 *Bacillus* spp., 3 other bacteria, 1 mould and 1 yeast. Lactic acid bacteria identified include *L. acidophilus*, *L. plantarum*, *Lactobacillus jensenii*, *Lactobacillus cellibiosus*, *Leuconostoc mesenteroides*, *Leuconostoc cremoris*. *Lactobacillus acidophilus* was predominant and showed the most significant antimicrobial inhibition against all three pathogenic strains tested (*Escherichia coli*, *Salmonella enterica* subsp. *typhi* and *Shigella dysenteriae*), followed by *Leuconostoc mesenteroides*; *L. plantarum* and *L. jensenii* varied in their activity, while *L. cellibiosus* showed the least activity. The isolates showed high acid tolerance, out of which *L. plantarum* and *L. acidophilus* showed the highest tolerance.

**Conclusion:** The selected lactic acid bacteria exhibited excellent probiotic characteristics and thus can serve as potential probiotics, hence indicating that spontaneously-fermented kunu can serve as a probiotic drink.

**Keywords:** Antimicrobial inhibition; autochthonous; fermented foods; in vitro; Kunu; lactic acid bacteria; probiotics; spontaneous fermentations.

## 1. INTRODUCTION

Probiotics have been defined as live microorganisms, which when consumed in adequate amounts as part of food; confer a health benefit on the host [1]. Probiotics are present in food, or can be incorporated into foods, and yield health benefits related to their interactions with the GIT. Consumption of probiotics can help balance the flora, by increasing the number of helpful flora, and reducing or inhibiting the growth of harmful bacteria in the intestine. They can modify the gut immune response, improve its barrier function, and modulate or adjust the activity of the immune system, thus helping to control or reduce the development of certain allergies. Probiotic foods contain large numbers of naturally occurring live bacteria, such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Lactococcus* spp. [2]. The types of bacteria studied for their probiotic potential include *Lactobacillus* sp. (*L. acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *L. johnsonii*, *L. plantarum*, *Lactobacillus rhamnosus*), *Bifidobacterium* sp. (*Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium animalis/lactis*, *Bifidobacterium longum*, *Bifidobacterium breve*), *Enterococcus faecalis*, *E. coli*, and *Bacillus cereus*. However, the most widely recognized living probiotic bacteria in use today are lactobacilli (e.g., *L. rhamnosus*, *L. paracasei*, *L. acidophilus*) and bifidobacteria (mainly *B. animalis* subsp. *lactis*) [3].

Fermented milks have been reported to be the most common food carriers for probiotics which are known to contain large numbers of naturally occurring live bacteria, such as *Lactobacillus* sp, *Bifidobacterium* spp. and *Lactococcus* spp. More

recently, probiotics have been incorporated into other foods apart from dairy products such as fruit, berry juices and drinks, recovery drinks, cereal-based drinks, and snacks [4]; however, there is need to investigate other fermented drinks from other sources for possible presence of probiotics. This is very important particularly from the economic standpoint of view and affordability of an average African family since fermented milk products are often relatively unaffordable for an average Africa family. It is therefore important to shift focus to affordable, available food products. African spontaneously fermented products meet the needs of being easily accessible, accepted by the population and are low in cost. In addition, the fermented products contain large numbers of LAB.

Fermented cereal foods and drinks have been used from ancient times in Africa as weaning foods for infants and refreshing drinks for adults. Apart from being enjoyed for their refreshing and taste-quenching properties, these fermented drinks have been reportedly used for medicinal purposes because of the presence of some health-promoting bacteria which have been reported to contribute to health and wellbeing of the consumers. In developing countries like Nigeria, alcoholic and non-alcoholic fermented beverages play a very important role in the dietary pattern of people serving as after-meal or refreshing drinks. Most of these beverages are often made from submerged fermented cereals, mixed with sugar, flavouring agents and sometimes preservatives. Some of these drinks include burukutu, pito, kunu, among others. The fermentation of these drinks has been reported to involve probiotics which confer health benefits to their consumers [5-7].

Kunu is a nutritious, non-alcoholic fermented beverage previously common consumed in northern Nigeria, but is presently being widely consumed in southern Nigeria. It is a complex mixture which contains protein, carbohydrates and lipids; and is taken after meal as a supplement or as a refreshing drink to quench thirst [8,9]. Although several reports have isolated, characterized and reported the presence of lactic acid bacteria in kunu fermentation [9-13], it is important to screen these LAB for their probiotic potential. This will encourage enhanced utilization of kunu as a probiotic drink, especially among the urban dwellers since kunu is presently being consumed more by rural dwellers. Also, most of the studies reported the microbiology of kunu prepared under strict hygienic laboratory conditions, it is important to also investigate kunu prepared by commercial processors (who often have little or no knowledge of hygiene), to not only determine the probiotic potential of the LAB present in the commercial samples but also to determine the bacteriological safety of the kunu since most consumers of kunu often patronize these commercial processors. This study has therefore investigated the microbiological quality of commercially-prepared kunu in comparison with those prepared in the laboratory using a combination of two cereals, millet and sorghum. The isolated lactic acid bacteria have also been screened for their *in vitro* probiotic potential using antagonistic and simulated gastric tests.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Design

The experimental design used for this study is the randomized complete block design.

### 2.2 Sample Acquisition

Two cereal grains, sorghum and millet, and spices (ginger, dried red pepper, cloves) were purchased from the major market in Akure, Ondo State, Nigeria. Five (5) samples of kunu packaged in polyethylene (PET) bottles were also randomly purchased from different producers 6 h after preparation in Akure-south metropolis, Ondo State.

### 2.3 Laboratory Preparation of Kunu

Three (3) samples of kunu were prepared in the laboratory using sorghum, millet and a

combination of sorghum and millet (ratio 1:1). The kunu was prepared using the methods of Adeyemi and Umar [10] and Gaffa [14] with slight modification. The grains were sorted removing stones and all solid impurities, washed in tap water and about 700 g were steeped separately in clean tap water for 12 h. The steeped grains were drained, mixed with the spices (30 g each) and peeled sweet potatoes (300 g each) and wet milled using an attrition mill. The resulting paste was divided into two parts; one part of the slurry (3/4 volume) was gelatinized with boiled water while the remaining ungelatinized part was mixed with the gelatinized part when the temperature was about 60–70°C. The mixture was left overnight at room temperature for chance fermentation, filtered using a clean muslin cloth the next morning and bottled. Sugar was added as a sweetener according to preference.

## 2.4 Analyses

### 2.4.1 Physicochemical analyses

Proximate composition of the samples was determined using official AOAC methods [15] for moisture (14.004), crude fat (14.081), crude fiber (7.0006), ash (14.006) and crude protein (47.021). A nitrogen-protein conversion factor of 6.25 was used. Carbohydrate was calculated by difference. Triplicate determination of pH was done by the potentiometric method using Jenway pH meter (Model 3505, serial number 03132, Barloworld Scientific Ltd, Dunmow Essex UK) as described by Pearson [16]. The meter was first calibrated with buffer solutions of pH 4 and 7. Titratable acidity was also determined as described by Pearson [16] by titrating 10 ml of the samples against 0.1N NaOH using phenolphthalein as indicator.

### 2.4.2 Microbiological analyses

The freshly-prepared and purchased kunu samples (1 ml each) were serially diluted using 9ml sterile physiological saline (0.85% NaCl solution) and an aliquot of 1 ml plated in duplicate on sterile standard plate count agar (APHA), MacConkey, de Man Rogosa and Sharpe (MRSA) (Oxoid, Hampshire, UK), deoxycholate citrate, Eosin Methylene blue (Levine), and acidified potato dextrose agars using the pour plate method of Harrigan and McCance [17] for viable mesophilic bacterial, coliform, lactic acid bacterial, Salmonella/Shigella, *E. coli*, and mould/yeast counts, respectively. All plates were thereafter incubated

at 35°C±2 for 24 h, except plates of MRSA which were incubated anaerobically at 37°C for 72 h and PDA plates incubated at ambient temperature for 48-72 h. Discrete, visible colonies were counted at the end of the incubation period using an electronic Quebec counter. Colonies with different colonial characteristics were randomly picked from the standard plate count agar and MRSA plates and streaked severally on fresh sterile nutrient agar and MRSA plates until pure colonies were obtained. The pure isolates were then preserved on nutrient agar slants and MRS broth in screw-capped McCartney bottles and maintained at 4°C. The isolates were identified using conventional taxonomic tools described by Holding and Collee [18] and Buchanan and Gibbons [19].

#### **2.4.3 In vitro probiotic potential of the lactic acid bacteria**

The probiotic potential of the lactic acid bacteria isolated from the kunu samples was studied *in vitro* using growth inhibition of three pathogenic bacteria, resistance to low pH, growth at different NaCl concentrations and temperature tolerance [20-23].

##### ***2.4.3.1 Antimicrobial activities against pathogenic strains***

The pathogenic strains used were *Salmonella enterica* subsp. *typhi*, *Escherichia coli* and *Shigella dysenteriae* obtained from the Medical Microbiology Culture Collection of the Department of Microbiology, Federal University of Technology, Akure, Nigeria. The pathogens were reactivated at 37°C for 24 h on agar plates as follows: Eosin Methylene blue agar under aerobic conditions for *Escherichia coli*, and deoxycholate citrate agar under aerobic conditions for *Salmonella enterica* subsp. *typhi* and *Shigella dysenteriae*. Lactic acid bacteria screened include *L. acidophilus*, *L. cellibiosus*, *L. plantarum*, *L. jensenii*, and *Leuconostoc mesenteroides*. Stock cultures of the test lactic acid bacteria were transferred from de Man Rogosa and Sharpe broth medium (Oxoid, Hampshire, UK) into 10 ml of fresh and sterile MRS broth and incubated anaerobically at 37°C for 48 h. After incubation, cells were centrifuged (Labofuge 200, Kendro Laboratory Products, Germany) at 6,000×g for 10 min to obtain a cell suspension. The suspended cells were cultured by the pour plate technique to determine cultures with 1×10<sup>8</sup> cfu/ml (colony forming units per milliliter).

The antimicrobial activity of the potential probiotics against test pathogenic strains was evaluated using the agar spot test [20,21]. 8 µL of each probiotic suspension at 1×10<sup>8</sup> cfu/ml was spotted onto the centre of the surface of MRS agar plates and incubated anaerobically at 37°C for 48 h. The test pathogenic strains were then inoculated in 5 mL of soft agar (containing 0.7% agar) in the appropriate medium as described above, at a final concentration of 1×10<sup>7</sup> cfu/ml, and poured onto MRS agar with probiotic spots. The plates were incubated at 37°C for 24 h, after which the diameter of the zone of inhibition around the LAB spot was measured. The diameter of the clear zone (mm) was determined by measuring the diameter between LAB colonies and four different points of the clear zone surrounding the colonies and reporting the average.

##### ***2.4.3.2 Tolerance of isolated LAB to acidic pH***

The tolerance of the probiotic bacteria to acidic pH was tested *in vitro* as described by Pelinescu et al. [22]. 1 ml of each LAB culture at 1×10<sup>8</sup> cfu/ml was inoculated into sterile MRS broth and incubated anaerobically at 37°C overnight, then sub-cultured into fresh MRS broth tubes of pH 2-4 (broth was adjusted by a pH meter using HCl and NaOH) and incubated anaerobically at 37°C for 24 h. After incubation, 1 ml inoculums from each tube was inoculated into MRS agar medium using pour plate technique and incubated anaerobically at 37°C for 48 h. The growth (indicated by presence or absence of growth) of LAB on MRS agar was used to designate isolates as pH tolerant.

##### ***2.4.3.3 NaCl tolerance***

Tested LAB cultures were inoculated into 10 ml sterile MRS broth with NaCl concentration between 4-6% and incubated at 37°C for 48 h. Growth was monitored by visual inspection of the test tubes and NaCl tolerance was evaluated after 1 ml was plated using sterile MRS agar, allowed to set and incubated at 37°C for a period of 48 h [23]. Positive control experiments were made of tubes containing LAB cultures without additional NaCl, while negative control experiments were tubes with added NaCl but without LAB cultures.

##### ***2.4.3.4 Sensitivity to temperature***

The selected LAB cultures were inoculated into 10 ml sterile MRS broth and incubated

anaerobically at varying temperatures, from 25-40°C for 48-72 h. Thereafter, 1 ml inoculums was transferred to MRS agar plates by pour plate method and incubated at 37°C for 48 h. The growth of LAB on MRS agar plates was used to designate isolates as temperature tolerant [23].

#### **2.4.4 Statistical analysis of data**

Triplicate data obtained for the physicochemical composition of the kunu samples and diameters of inhibition of growth of the organisms were subjected to analysis of variance (ANOVA) using SPSS 16.0 for windows computer software package. Values were expressed as means  $\pm$  standard deviation and the difference in means was compared using the Duncan's new Multiple Range test and significant level was established at  $P=0.05$ .

### **3. RESULTS**

#### **3.1 Physicochemical Composition of Kunu**

Presented in Table 1 is the physicochemical composition of the commercial kunu samples and those prepared in the laboratory using different cereals. Crude protein content (% dry weight) of the samples varied considerably, ranging from 33.85-58.68%; with the laboratory sample H prepared from a combination of sorghum and millet grains having the highest content. Total ash content varied from 3.84-6.26%; the commercial samples seemed to have higher ash content than the laboratory samples, except for sample E. Total solids contents varied significantly ( $P=0.05$ ) from 7.00-9.11%; while commercial sample D had the highest value, laboratory-prepared kunu (sorghum + millet) had the lowest. Although pH and total titratable acidity (TTA) of the samples differed significantly ( $P=0.05$ ), there was no significant correlation between the values. Values obtained ranged from 4.29-4.96 and 4.14-5.01; 1.41-3.45% and 1.22-2.37% for pH and TTA of commercial and laboratory-prepared samples, respectively.

#### **3.2 Microbial Quality of the Kunu**

Results presented in Table 2 showed that higher viable mesophilic counts were obtained for the commercial kunu samples as compared to the laboratory-prepared samples. The highest mesophilic counts was obtained in sample B ( $5.70 \times 10^4$  cfu/ml), followed by  $4.93 \times 10^4$  cfu/ml,

$4.20 \times 10^4$  cfu/ml,  $4.10 \times 10^4$  cfu/ml and  $1.97 \times 10^4$  cfu/ml for samples A, D, E, and C respectively. However, significantly lower counts ranging from  $2.38 \times 10^3$ - $3.27 \times 10^3$  cfu/ml were obtained for the laboratory-prepared samples. A similar trend was observed for yeast count. There was no presence of *E. coli*, coliforms (except in commercial samples B and C), *Salmonella* spp. (except in commercial samples C and D), *Shigella* spp. and mould (except in commercial samples D) in the samples. The results also showed that counts obtained for mesophilic bacteria and yeasts were higher than for others.

Thirteen (13) microbes were isolated from the Kunu samples (Table 3); 46% (6) of them were lactic acid bacteria (LAB) and 15% (2) *Bacillus* spp. Other bacteria made up 23% (3), while 1 mould and yeast each were present. The organisms include *L. plantarum*, *L. acidophilus*, *L. jensenii*, *L. cellibiosus*, *Leuconostoc cremoris*, *Leuconostoc mesenteroides*, *Bacillus subtilis*, *Bacillus brevis*, *Kurthia* spp., *Corynebacterium ovis*, *Micrococcus* spp., *Aspergillus niger* and *Saccharomyces cerevisiae*. In both commercial and laboratory samples, *L. acidophilus* had the highest rate of occurrence, followed by *L. plantarum*, *Leuconostoc mesenteroides*, *L. cellibiosus*, *Saccharomyces cerevisiae*, *Bacillus subtilis* and *L. jensenii*. *Leuconostoc cremoris* (sample E), *Kurthia* spp., *Aspergillus niger*, *Bacillus brevis* (sample D) were only detected in one of the commercial samples, while *Corynebacterium ovis* and *Micrococcus varians* each only occurred in two of the commercial samples (Table 3).

#### **3.3 Probiotic Potential and Antimicrobial Activity of Lactic Acid Bacteria**

Growth resistance of the test probiotics against simulated gastric conditions (evaluated by growth tolerance in low pH, NaCl and temperature) as presented in Table 4 showed that the organisms tolerated and were able to grow at temperatures ranging from 25-40°C, however growth of *Leuconostoc cremoris* was inhibited at 40°C. Similarly, this organism could not withstand acidic pH (2.0 and 4.0), although only *Lactobacillus acidophilus* and *Lactobacillus plantarum* tolerated pH 2.0. With respect to salt tolerance, all the organisms tolerated and grew at NaCl concentration of 4-6%.

The antagonistic activity of the probiotic strains against three pathogens is presented in Table 5. The probiotics displayed varying levels of growth

inhibition against the pathogens, *Salmonella enteric* subsp. *typhi*, *Escherichia coli* and *Shigella dysenteriae*. The highest inhibition was displayed by *Lactobacillus acidophilus* (39 mm) against *Salmonella enteric* subsp. *typhi*, while the most significant inhibition against *Escherichia coli* was shown by *Lactobacillus plantarum* (35 mm), and *Lactobacillus cellibiosus* displayed the least inhibition (10 mm). Both *Lactobacillus acidophilus* and *Leuconostoc mesenteroides*

**Table 1. Physicochemical composition of Kunu**

Samples	Moisture content (%)	Crude protein (% dw)	Total Ash (% dw)	Total solid (%)	pH	TTA (%)
A	91.25 <sup>bc</sup> ±0.21	46.40 <sup>d</sup> ±0.01	5.70 <sup>b</sup> ±0.02	8.74 <sup>b</sup> ±0.21	4.31 <sup>c</sup> ±0.07	2.35 <sup>c</sup> ±0.05
B	90.85 <sup>c</sup> ±0.20	45.91 <sup>f</sup> ±0.02	5.23 <sup>d</sup> ±0.01	9.02 <sup>a</sup> ±0.20	4.29 <sup>c</sup> ±0.05	3.45 <sup>a</sup> ±0.10
C	92.32 <sup>a</sup> ±0.08	56.51 <sup>b</sup> ±0.01	6.26 <sup>a</sup> ±0.02	7.68 <sup>d</sup> ±0.08	4.96 <sup>a</sup> ±0.06	3.42 <sup>a</sup> ±0.09
D	90.89 <sup>c</sup> ±0.02	46.78 <sup>c</sup> ±0.02	5.36 <sup>c</sup> ±0.00	9.11 <sup>a</sup> ±0.02	4.71 <sup>b</sup> ±0.05	1.41 <sup>d</sup> ±0.05
E	87.03 <sup>d</sup> ±0.32	33.85 <sup>h</sup> ±0.20	3.84 <sup>g</sup> ±0.00	7.81 <sup>d</sup> ±0.08	4.88 <sup>ab</sup> ±0.11	3.00 <sup>b</sup> ±0.05
F	91.00 <sup>bc</sup> ±0.22	46.33 <sup>e</sup> ±0.01	4.88 <sup>e</sup> ±0.01	8.15 <sup>c</sup> ±0.25	5.01 <sup>a</sup> ±0.00	1.22 <sup>e</sup> ±0.05
G	91.39 <sup>b</sup> ±0.52	39.25 <sup>g</sup> ±0.01	4.61 <sup>f</sup> ±0.05	7.61 <sup>d</sup> ±0.05	4.14 <sup>c</sup> ±0.02	2.37 <sup>c</sup> ±0.13
H	92.12 <sup>a</sup> ±0.19	58.68 <sup>a</sup> ±0.03	5.20 <sup>d</sup> ±0.03	7.00 <sup>e</sup> ±0.01	4.70 <sup>b</sup> ±0.28	2.37 <sup>c</sup> ±0.13

Key: A-E (commercial samples), F-sorghum, G-millet, H-sorghum and millet, TTA-total titratable acid, (% dw) -% dry weight. Values represent the mean of triplicate determinations±SD. Values having the same superscript within the same column do not differ significantly (P=0.05)

**Table 2. Microbial count (cfu/ml) of the Kunu samples**

Samples	TVC	LAB	<i>E. coli</i>	Coliforms	<i>Salmonella/Shigella</i>	Yeast	Mould
A	4.93×10 <sup>4</sup>	9.75X10	0	0	0	8.36×10 <sup>4</sup>	0
B	5.70×10 <sup>4</sup>	9.50X10	0	1.25x10 <sup>2</sup>	0	1.08×10 <sup>4</sup>	0
C	1.97×10 <sup>4</sup>	1.13X10 <sup>2</sup>	0	8.00x10	1.75x10 <sup>2</sup>	9.00×10 <sup>3</sup>	0
D	4.20×10 <sup>4</sup>	1.50X10 <sup>2</sup>	0	0	5.50x10	8.20×10 <sup>3</sup>	3.83×10 <sup>3</sup>
E	4.10×10 <sup>4</sup>	2.81X10 <sup>2</sup>	0	0	0	4.70×10 <sup>3</sup>	0
F	2.38x10 <sup>3</sup>	3.00X10 <sup>2</sup>	0	0	0	3.30×10	0
G	2.60x10 <sup>3</sup>	3.50X10 <sup>2</sup>	0	0	0	1.00×10 <sup>2</sup>	0
H	3.27x10 <sup>3</sup>	2.00X10 <sup>2</sup>	0	0	0	4.00×10 <sup>2</sup>	0

Key: A-E (commercial samples), F-sorghum, G-millet, H-sorghum and millet, TVC- total viable count of mesophilic bacteria, LAB- lactic acid bacteria

**Table 3. Frequency occurrence of isolated microorganisms in the Kunu samples**

Isolates	Samples							
	A	B	C	D	E	F	G	H
<i>Leuconostoc cremoris</i>	-	-	-	-	+	-	-	-
<i>Leuconostoc mesenteroides</i>	-	-	+	+	-	+	-	+
<i>Lactobacillus plantarum</i>	+	+	-	-	+	-	+	+
<i>Lactobacillus acidophilus</i>	+	+	+	+	-	+	+	+
<i>Lactobacillus jensenii</i>	-	-	+	-	-	+	-	+
<i>Lactobacillus cellibiosus</i>	+	-	+	-	-	-	+	+
<i>Bacillus subtilis</i>	+	+	-	-	-	-	+	+
<i>Bacillus brevis</i>	-	-	-	+	-	-	-	-
<i>Kurthia</i> spp.	-	-	-	+	-	-	-	-
<i>Corynebacterium ovis</i>	-	-	-	+	+	-	-	-
<i>Micrococcus varians</i>	+	+	-	-	-	-	-	-
<i>Aspergillus niger</i>	-	-	-	+	-	-	-	-
<i>Saccharomyces cerevisiae</i>	-	+	+	+	-	+	+	+

+ = Organism present in sample; - = Organism absent in sample; A-E (commercial samples), F-sorghum, G-millet, H-sorghum and millet

**Table 4. pH, salt and temperature tolerance of the lactic acid bacteria**

Isolate	Growth at				Growth in		pH tolerance	
	25°C	30°C	35°C	40°C	4% NaCl	6% NaCl	2.0	4.0
<i>Leuconostoc cremoris</i>	+	+	+	-	+	+	-	-
<i>L. acidophilus</i>	+	+	+	+	+	+	+	+
<i>L. plantarum</i>	+	+	+	+	+	+	+	+
<i>L. cellibiosus</i>	+	+	+	+	+	+	-	+
<i>L. jensenii</i>	+	+	+	+	+	+	-	+
<i>Leuconostoc mesenteroides</i>	+	+	+	+	+	+	-	+

+ = Growth Present; - = Growth Absent

**Table 5. Growth inhibition of the pathogenic strains by the test probiotics expressed in diameter of inhibition (mm)**

Isolate	Pathogenic strains		
	<i>E. coli</i>	<i>Shigella dysenteriae</i>	<i>Salmonella enteric subsp typhi</i>
<i>Lactobacillus acidophilus</i>	25±0.7 <sup>d</sup>	34±0.4 <sup>a</sup>	39±0.5 <sup>a</sup>
<i>Lactobacillus cellibiosus</i>	10±1.2 <sup>e</sup>	20±1.9 <sup>d</sup>	11±0.5 <sup>e</sup>
<i>Lactobacillus plantarum</i>	35±0.5 <sup>a</sup>	31±0.3 <sup>c</sup>	22±0.3 <sup>d</sup>
<i>Lactobacillus jensenii</i>	31±0.09 <sup>b</sup>	32±0.5 <sup>b</sup>	25±1.2 <sup>c</sup>
<i>Leuconostoc mesenteroides</i>	29±0.3 <sup>c</sup>	34±0.6 <sup>a</sup>	34±2.0 <sup>b</sup>

Values represent the mean of triplicate determinations ± SD. Values having the same superscript within the same column do not differ significantly (P=0.05)

displayed the same and highest resistance against *Shigella dysenteriae* (34 mm), followed by *Lactobacillus jensenii* (32 mm), *Lactobacillus plantarum* (31 mm), and the least inhibition by *Lactobacillus cellibiosus* (20 mm). Overall, *Lactobacillus acidophilus* displayed the most significant inhibition against two of the pathogens (*Salmonella enteric subsp. typhi* and *Shigella dysenteriae*), while *Lactobacillus cellibiosus* showed the least significant inhibition against all three pathogens.

#### 4. DISCUSSION

This study investigated the physicochemical composition and microbiological quality of commercially prepared kunu in comparison with kunu prepared under laboratory conditions with the aim of determining microbiological status and the *in vitro* probiotic potential of the isolated lactic acid bacteria. The variation in the crude protein content of the kunu samples may be as a result of different additives used by individual processors during preparation. This is corroborated by the results obtained from the laboratory samples prepared from different cereals which showed that samples prepared with millet and sorghum alone had lower protein content as compared to that prepared with a combination of the cereals, which had significantly (P=0.05) higher content. Hence, cereal-based drinks would have higher protein

content when combinations of cereals are used. Also, the amount of protein in this drink makes it more nutritious as compared to the commercial carbonated drinks which do not contain protein. The fairly high ash content of the samples is an indication of the amount of mineral elements present in the sample. It has also been reported that the value of ash is a useful quality grading assessment criterion for certain edible materials [24].

The low pH obtained in this study is in conformity with several reports on kunu as an acid-fermented beverage resulting from production of organic acids during the fermentation of sugars by the fermenting microorganisms, mainly lactic acid bacteria and yeasts [10]. The variation in the pH may be attributed to the fact that the commercial samples were obtained from different processors who would have brought in a few variations in their processing, while the different cereals used in the laboratory-prepared samples may account for variation in their pH values, hence indicating varying amounts of organic acid was produced from fermentation of each cereal.

The higher mesophilic counts of the commercial samples may be attributed to unsanitary practices during processing observed by the producers who are most times unlearned local women who have little or no knowledge of hygiene. This is further corroborated by the

presence of coliforms in commercial samples B and C and Salmonella in samples C and D. These microorganisms may have been introduced into the samples from the use of unclean water, unsterilized packaging materials, spices and other ingredients. However, the absence of *E. coli*, coliforms, *Salmonella* spp. and *Shigella* spp. in most of the samples indicates that most of the samples are safe for consumption and potentially free of pathogens. The comparably high counts obtained for yeast is an indication of the importance of yeasts alongside the lactic acid bacteria in kunu fermentation as have been previously reported [10,9]. Some yeasts have been reported to be probiotic, hence their presence in kunu will further enhance the status of kunu as a probiotic drink.

The presence of 6 lactic acid bacteria out of the 13 organisms identified in this study is an indication of the predominance of lactic acid bacteria in kunu fermentation as have been reported by several authors. Adeyemi and Umar [10] had earlier reported the presence of *Bacillus subtilis*, *Bacillus pumilus*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Micrococcus* spp., *Staphylococcus aureus*, *Streptococcus* sp., *Mucor* spp., *Rhizopus* spp and *Saccharomyces cerevisiae* in kunu prepared with a combination of sorghum and millet; while Osuntogun and Aboaba [11] isolated *Lactobacillus*, *Streptococcus*, *Aspergillus* and *Penicillium*. However, Olasupo et al. [25] reported the presence of only lactic acid bacteria in kunu, including *Lactobacillus salivarius*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus jensenii*, *Lactobacillus cellobiosus* and *Lactobacillus plantarum*. Adebayo et al. [12], on the other hand, did not isolate lactic acid bacteria and yeast from kunu but other organisms including *Bacillus subtilis*, *Micrococcus species*, *Escherichia coli*, *Staphylococcus aureus*, *streptococcus* sp., *Mucor*, *Rhizopus stolonifer*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus nidulans*. The heavy presence and activities of the lactic acid bacteria is usually responsible for the sour taste resulting from lactic acid production from fermentation of sugars.

The occurrence of some of the isolates in only 1 or 2 of the commercial samples shows that they are not important in the fermentation of kunu and are probably contaminants. The presence of *Bacillus subtilis* in 50% of the samples may be indicative of its importance in the fermentation of

kunu corroborating the reports of several authors who have reported the isolation of *Bacillus* spp. from fermented cereal products. Although *Bacillus subtilis* has been widely reported to be involved in the fermentation of protein-rich oil seeds being a proteolytic organism, the diversity of the *Bacillus* genus may explain its presence in these samples. The *Bacillus* genus is made up of different species with various physiological and biochemical characteristics; collective features including degradation of almost all substrates derived from plant and animal sources including cellulose, starch, pectin, proteins and hydrocarbons due to their ability to synthesize various enzymes, particularly the extracellular protease enzyme which they are able to secrete in large amounts [26,27]. Hence, the presence of *Bacillus subtilis* in kunu may involve elaboration of protease enzymes for hydrolysis of proteins. However, Adegoke et al. [28] have attributed the ropiness associated with fermented drinks to be due to the presence of both *Pseudomonas* spp. and *Bacillus subtilis*. *Lactobacillus acidophilus* may be said to be the most important and predominant organism in the fermentation of kunu since it was present in 7 of the 8 samples and it is closely followed by *Saccharomyces cerevisiae* which occurred in 6 samples. This further explains the importance and predominance of these two organisms in kunu fermentation as have been previously reported by several researchers that the fermentation of kunu involves mainly lactic acid bacteria and yeast.

The ability of the lactic acid bacteria to survive at the selected temperature range (25-40°C) (except *L. cremoris* whose growth was inhibited at 40°C) may be an indication of their potential to survive temperature of the human gut since temperature is an important requirement for bacterial growth, and the selected temperature range was chosen to simulate the normal human body temperature. This factor is very important in determining the effectiveness of probiotics since growth/viability during storage and use is one of the important determining factors for functionality of probiotics [29]. Moreso, the tolerance of all the isolates to high NaCl concentration (4-6%) further indicates their potential to survive the harsh conditions and bile salt of the intestine. The observed variation in the inhibition of the test pathogens by the lactic acid bacteria is an indication that the organisms possess varying abilities to exert antimicrobial effects on pathogens and this corroborates the report of Grimoud et al. [21] that antimicrobial effects



exerted by lactic acid bacteria are strain-specific. This observation is a very important factor for determining potential probiotics in kunu because pathogen inhibition is also a major probiotic selection criterion involved in the restoration of gut microbiota balance [30]. This has been reported to have significant positive effects in various physiological functions and in the reduction of pathologies such as inflammatory bowel disease or colorectal cancer [31,32]. Although all the lactic acid bacteria exerted significant antimicrobial effects against the test pathogens (except *L. cellibiosus* which displayed very weak antimicrobial effect against the 3 tested pathogens, *Lactobacillus acidophilus* followed by *Lactobacillus plantarum* exerted the most significant pathogen inhibition. Overall, *Lactobacillus acidophilus* may be the most effective probiotics in kunu.

## 5. CONCLUSION

This study has shown that combining cereals for kunu production results in kunu of higher protein content as compared to kunu prepared from a single cereal. Secondly, the study has shown that lactic acid bacteria especially *Lactobacillus acidophilus* and *L. plantarum* and the yeast *Saccharomyces cerevisiae* may be the most important organisms involved in kunu fermentation. Furthermore, *Lactobacillus acidophilus* has been shown to be the most effective probiotic which exerted the highest antimicrobial effect against the test pathogens. Also, the viability of the lactic acid bacteria was shown to have been retained in the final product, a factor important in determining the functionality of probiotics. Since good viability is generally considered a prerequisite for optimal probiotic functionality in that probiotic products should contain high enough levels of the specific probiotic strain(s) throughout storage and during consumption; the short storage life and duration of consumption of kunu which is usually within 24 hours of preparation may also guarantee the viability of the probiotics, and hence the potential of kunu to serve as a probiotic drink.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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