

# Microbial Load and Keeping Quality of Kunu under Various Preservative Regimes

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## Abstract

Kunu beverage was prepared from two different cereals (Millet and Guinea corn), by standard native procedures the process of cleaning, steeping, wet milling; wet sieving, settling, decantation and slurry recovery were applied in its preparation. The following preservatives: lime, lemon, *Phyllanthus*, Sodium benzoate and a combination of 'Lime and Lemon' as mixed preservatives were added under cold and room temperatures and shelf-life monitoring was carried out. The sensory qualities (colour, taste, texture, flavour and odour) were also assessed on a 9-point Hedonic scale showed that the sample with the mixed preservatives, (lime and lemon) had the best sensory qualities, with that of *Phyllanthus*, being the poorest. Lime and lemon combination led to a reduction in microbial load unlike the individual preservatives which represents high cfu/ml in their samples. Statistical analysis showed that the bacterial load of Kunu with Lime and lemon was significantly ( $P < 0.05$ ) lower than that of other samples. Molecular characterisation confirmed fungal contamination was mainly due to *Aspergillus niger* IMI 500340 and *Neurospora sitophila* IMI 500339 A combination of lime and lemon (1:1 v/v) was able to keep the beverage for up to 3days.

**Keywords:** Kunu; Microbial quality; Keeping quality; Preservatives

## Introduction

Kunu zaki, commonly referred to as Kunu, is a product of fermentation of malted grains of Sorghum and millet, used singly or in composite form and is one of the most highly consumed cereal-based non-alcoholic, non-carbonated beverages in Nigeria [1,2]. Processing is with local household traditional utensils [3]. Spicy ingredients like ginger, clove, pepper together with saccharifying agents such as paste of sweet potato tubers, malted rice, malted sorghum, crude extract from dried *Cadaba farinose* (Dangarafa or Legel) stem are also added [4]. The final product is a thin free-flowing gruel. Its acceptability is fast spreading in Nigeria just as consumption cuts across all social classes and age groups. It is almost replacing the conventional carbonated drinks in part because the raw materials are cheap and readily available. Although it has low nutrient content [5], the water content is high with an attraction that derives from a soothing feeling on consumption. It is on record that cereal-based food products contain some carbohydrates but are generally low in protein and several of the essential amino acids [6]. These major raw materials belong to the family *Poaceae* with slightly varying composition in protein, ash, fat, starch and vitamin. Both millet and guinea corn have at least 82% starch each [7].

The aim of this study was to investigate possible changes in quality parameters of this beverage when subjected to some preservation regimes. The arrest of oxidative susceptibility of Kunu under low and room temperatures in the presence of citrus juice with a view for substitution of synthetic preservatives was also studied.

## Materials and Methods

### Preparation of Kunu

The raw materials for the preparation of the Kunu were purchased from a major market in Ilishan- Remo, Ogun State, South Western Nigeria. These materials were maize, millet and guinea corn, alligator pepper, black pepper, red pepper, kakandoro, granulated sugar and Ginger. Sands and other solid impurities were removed through physical sorting, from the millet and guinea corm. Each was then soaked separately for about 24 h after which the ingredients like ginger,

alligator pepper, red pepper, black pepper and kakandoro were added. It was ground very well in a hygienic way and sieved with a very clean and white cloth. The filtrate was fermented for 24 h, during which the slurry was allowed to settle and sediment. The supernatant liquid was decanted and the residue was mixed with water and divided into two. Half of the residue was boiled and the second half was poured into it to produce Kunu. After this, water was added to meet the generally acceptable consistency and texture i.e., not too watery and not too thick. Sugar was added to taste. The final product subsequently served as the stock for further experiments.

The following preservatives were used, Sodium benzoate, Lime, lemon, *Phyllanthus* and Lime and Lemon together as mixed preservatives. The samples were prepared, one set for storage at room temperature and the other, at low temperature, in the refrigerator. Each experiment and the control, which had no preservative was in duplicate.

### Preparation of preservatives

**Sodium benzoate:** Two grams of Sodium benzoate was weighed and dissolved in 2 ml of distilled water. The Sodium benzoate solution was added to a 100 ml bottle of Kunu sample and was mixed thoroughly.

**Lime:** A piece of Lime fruit was sliced into two equal halves and squeezed out into a sterile beaker. With a sterile syringe, 5 ml was removed and added into a 100 ml bottle of Kunu sample and shaken thoroughly.

**Lemon:** A piece of Lemon fruit was sliced into two equal halves

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and squeezed out into a sterile beaker. With a sterile syringe, 5 ml was removed and added into a 100 ml bottle of Kunu sample and shaken thoroughly.

**Phyllanthus spp.:** *Phyllanthus* leaves were washed with sterile water. With no water or solvent added, 100 grams of the leaves were ground with mortar and pestle. With a sterile syringe, 10 ml was removed and added into a 50 ml bottle of Kunu sample.

**Lime and lemon (mixed preservatives):** Using gloves, a piece each of lime and lemon were sliced into two equal halves and squeezed out into two different sterile beakers. With a sterile syringe, 2.5 ml were taken from both beakers and added into a 100 ml bottle of Kunu sample.

### Sensory evaluation

Five panellists (mainly microbiology students) who are familiar with Kunu zaki were invited to evaluate the Kunu zaki samples with the different preservatives for taste, colour, texture, odour and flavour. The samples were served in clean cups for each panellist. Sample presentation to the panellists was at random and one at a time. A 9-point Hedonic scale modified by Williams (1982) was adopted in the scoring with 1 being 'very unacceptable or unwholesome' and 9 being 'very acceptable or wholesome'. The mean scores were subjected to analysis of variance at 5% and the Least Significant Difference (LSD) test used to determine the degree of difference between the samples.

### Microbial analysis

The pour plate method using Nutrient Agar and Potato Dextrose Agar for total aerobic bacteria and total yeast count respectively were used on all samples. One ml of 10<sup>-3</sup> dilution was inoculated each into sterile Petri dishes. Prepared media were poured into each Petri dish containing the samples and allowed to gel. Petri dishes containing Nutrient agar were incubated at 37°C for 18-24 hours for bacterial growth while fungal growth was examined on Potato Dextrose agar incubated at 28°C for 3-5 days. Streptomycin sulphate at 200ml/L was added to prevent bacterial contaminants. Observations were taken each day for 72 hrs for room temperature and those at low temperature were allowed to turn cold for 24 hrs before microbial analysis was done. The total bacterial count for all samples was recorded for each day. Identification of fungi was based on morphology, microscopy and molecular sequencing as carried out by the Commonwealth Agricultural Bureau Institute (CABI), UK.

### Storage

The Kunu samples containing the preservatives were made in duplicates. The first set were stored in low temperature, (4°C) and the second batch stored in room temperature to check for the effectiveness of the various preservatives in retaining the qualities with time. For the two variables, preservatives and temperature – a control setup was made.

### Amino acid profile

**Solvents:** Butanol : acetic acid : water (4:1:5, v/v) served as the mobile phase.

**Detection spray:** Ninhydrin (0.2 g% in absolute ethanol) : pyridine (1.5 ml)

**Principle:** Amino acids are partitioned in solvents based on their partition coefficient determined by their degree of hydrophobicity.

**Procedure:** Tank was equilibrated for at least 30 minutes with

chromatographic solvent. Kunu samples and standard (Sigma Aldrich) were spotted on chromatographic paper at about 1 cm to the bottom of the paper. The mobile phase was. The solvent front was allowed to reach the topmost portion of the paper. Paper was dried thoroughly in air followed by spraying with ninhydrin reagent. Paper was now placed in an oven at 80 -100°C to enable spots (pink) to become visible.

## Results

### Sensory evaluation

For the samples at room temperature, Kunu with lime and lemon still retained its sensory characteristics to a point of satisfaction according to the five panellists followed by the sample with Sodium benzoate, which is a synthetic preservative, then the sample with lemon and then lime, with the sample containing *Phyllanthus* as the poorest sample to the panellists because of its characteristic bitter taste. Low temperature is the characteristic 'comfort zone' for Kunu, at low temperatures; it can stay close to 7 days. Storage at low temperature in this study is to act as a control or check against storage at room temperature. The sample with lemon and lemon was also rated as the best according to the panellists with the sample with *Phyllanthus* being the poorest (Table 1-5). The samples that were stored at low temperature however became darker in appearance and more viscous. This could be as a result of chill injury [8].

	D1RT	D2RT	D3RT	D1LT	D2LT
Control	5.00±0.71	4.20±1.64	3.00±1.87	5.80±0.45	6.40±0.55
<i>Phyllanthus</i>	2.40±0.89	2.00±1.00	2.40±2.07	6.20±0.89	5.80±0.55
Lime	5.40±0.89	6.00±1.58	4.80±0.64	3.60±1.10	3.40±0.71
Lemon	5.20±0.84	5.40±0.89	4.60±1.52	6.20±0.45	6.00±0.84
Na benzoate	5.20±0.45	5.40±1.95	5.20±1.10	4.80±0.45	4.60±0.89
Li & Lemon	8.80±0.45	8.60±0.55	7.80±0.45	9.00±0.00	8.60±0.55

D1RT: Day 1, Room Temperature; D2RT: Day 2, Room Temperature; D3RT: Day 3, Room Temperature; D1LT: Day 1, Low Temperature; D2LT: Day 2, Low Temperature

**Table 1:** Mean and Standard deviation values of Taste of Kunu stored for 3 days using various preservatives under room and 4°C.

	D1RT	D2RT	D3RT	D1LT	D2LT
Control	8.60±0.55	8.00±0.00	7.00±1.22	7.60±0.89	6.80±0.84
<i>Phyllanthus</i>	4.00±1.58	4.40±1.14	4.40±1.67	5.00±1.58	5.00±0.71
Lime	9.00±0.00	8.00±0.71	6.60±0.89	7.20±1.30	5.60±0.55
lemon	8.60±0.55	7.80±0.45	6.00±0.71	7.40±1.34	6.80±1.10
Na benzoate	9.00±0.00	8.00±0.71	7.00±0.71	6.80±0.84	6.40±0.55
Lime & Lemon	9.00±0.00	8.20±0.84	8.60±0.55	8.00±1.00	8.40±0.55

D1RT: Day 1, Room Temperature; D2RT: Day 2, Room Temperature; D3RT: Day 3, Room Temperature; D1LT: Day 1, Low Temperature; D2LT: Day 2, Low Temperature

**Table 2:** Mean and Standard deviation values of Colour of Kunu stored for 3 days using various preservatives under room and 4°C.

	D1RT	D2RT	D3RT	D1LT	D2LT
Control	8.60±0.55	8.00±0.00	7.00±1.00	7.60±0.89	6.60±1.14
<i>Phyllanthus</i>	6.60±2.88	5.60±2.19	4.60±2.07	5.60±1.14	5.20±1.10
Lime	8.80±0.45	8.00±1.22	5.60±1.67	7.20±1.30	6.00±1.00
lemon	8.80±0.45	7.60±0.89	5.60±1.34	7.40±1.34	6.20±1.10
Na benzoate	8.80±0.45	7.60±1.52	5.60±2.19	5.60±1.34	5.80±1.48
Lime & Lemon	8.80±0.45	8.40±0.89	8.40±0.55	8.40±0.55	8.00±0.71

D1RT: Day 1, Room Temperature; D2RT: Day 2, Room Temperature; D3RT: Day 3, Room Temperature; D1LT: Day 1, Low Temperature; D2LT: Day 2, Low Temperature

**Table 3:** Mean and Standard deviation values of Texture of Kunu stored for 3 days using various preservatives under room and 4°C.

	D1RT	D2RT	D3RT	D1LT	D2LT
Control	8.60±0.55	7.40±0.89	4.60±1.67	5.80±1.10	5.40±1.52
Phyllanthus	5.60±2.07	4.40±1.82	3.00±1.22	5.40±1.52	4.40±1.34
Lime	6.40±2.07	5.80±1.48	5.20±1.48	6.80±0.84	5.80±1.10
Lemon	7.80±1.10	7.20±0.84	4.60±2.30	6.60±0.89	6.20±1.64
Na benzoate	6.80± 2.05	6.20±1.10	6.40±1.52	6.00±1.00	4.80±0.84
Lime & Lemon	8.60±0.55	8.00±0.71	7.80±1.10	8.40±0.55	8.20±0.84

D1RT: Day 1, Room Temperature; D2RT: Day 2, Room Temperature; D3RT: Day 3, Room Temperature; D1LT: Day 1, Low Temperature; D2LT: Day 2, Low Temperature

**Table 4:** Mean and Standard deviation values of Odour of Kunu stored for 3 days using various preservatives under room and 4°C.

	D1RT	D2RT	D3RT	D1LT	D2LT
Control	5.40±2.30	4.40±1.67	3.40±1.14	6.20±1.30	6.00±1.58
Phyllanthus	1.80±0.45	2.20±1.84	2.60±0.52	4.40±1.67	3.60± 1.14
Lime	5.80±2.28	4.80±1.30	5.20±2.05	6.60±1.14	5.20±1.84
Lemon	5.80±0.45	6.20±1.10	5.60±1.34	6.80±1.10	5.80±1.10
Na benzoate	5.80±0.45	5.40±1.52	4.80±1.64	6.00±1.41	5.60±0.89
Lime & Lemon	8.40±0.55	8.40±0.55	8.20±0.45	8.80±0.45	8.25±0.71

D1RT: Day 1, Room Temperature; D2RT: Day 2, Room Temperature; D3RT: Day 3, Room Temperature; D1LT: Day 1, Low Temperature; D2LT: Day 2, Low Temperature

**Table 5:** Mean and Standard deviation values of Flavour of Kunu stored for 3 days using various preservatives under room and 4°C.

	D1RT	D2RT	D3RT	D1LT	D2LT
Control	1.78 x 10 <sup>5b</sup>	1.75 x 10 <sup>4a</sup>	4.6 x 10 <sup>4cd</sup>	2.85 x 10 <sup>5a</sup>	6.9 x 10 <sup>4a</sup>
Phyllanthus	1.64 x 10 <sup>5d</sup>	5.5 x 10 <sup>3a</sup>	6.9 x 10 <sup>4b</sup>	1.3 x 10 <sup>4c</sup>	1.4 x 10 <sup>4d</sup>
Lime	1.75 x 10 <sup>5bc</sup>	4.0 x 10 <sup>4a</sup>	7.1 x 10 <sup>4ab</sup>	2.35 x 10 <sup>5b</sup>	1.4 x 10 <sup>4d</sup>
Lemon	1.63 x 10 <sup>5d</sup>	5.5 x 10 <sup>4a</sup>	5.9 x 10 <sup>4bc</sup>	1.6 x 10 <sup>4c</sup>	5.5 x 10 <sup>4b</sup>
Sodium Benzoate	1.70 x 10 <sup>5c</sup>	2.3 x 10 <sup>4a</sup>	8.6 x 10 <sup>4a</sup>	1.8 x 10 <sup>4c</sup>	3.4 x 10 <sup>4c</sup>
Lime & Lemon	1.89 x 10 <sup>5a</sup>	1.8 x 10 <sup>4a</sup>	3.0 x 10 <sup>4d</sup>	4.5 x 10 <sup>3c</sup>	2.1 x 10 <sup>4d</sup>

Means with different alphabets in a column are significantly different at P<0.05. D1RT: Day 1, Room Temperature; D2RT: Day 2, Room Temperature; D3RT: Day 3, Room Temperature; D1LT: Day 1, Low Temperature; D2LT: Day 2, Low Temperature

**Table 6:** Mean Bacterial Load in Kunu Samples at Room temperature and 4°C

### Microbial analysis

**Bacterial load:** The bacterial loads for all samples were taken after incubation for 18 hours. The bacteria loads were recorded for samples in both room temperature and low temperature for the period of 3 days (72 hours). The bacterial loads of samples are shown in the Table 6. The bacterial load of the Kunu sample containing lime and lemon was significantly (P < 0.05) higher than the bacterial load of all other samples on the first day at room temperature. There was no significant (P > 0.05) difference on the second day at room temperature among all the samples. On the third day at room temperature, the bacterial load of the sample with lime and lemon was again significantly (P < 0.05) lower than the microbial load of the control setup and other samples. The sample containing lime and lemon also had a significantly (P < 0.05) lower microbial difference on the first and second day at low temperature.

**Mycological analysis:** The fungal load was examined for each sample with different preservatives at the two different temperatures. Using the ITS DNA sequencing, the fungal species were identified *Neurospora sitophila* IMI 500339 and *Aspergillus niger* IMI 500340 as shown in Figure 1 and 2. The sequences of the fungal isolates were deposited with CABI.

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TACAGAGTTGCAAACTCCACAAACCATCGCGAATCTTACCCGTACGGTTCCTCGGGCTGGCGGT
CCGAAAGGCCCTCGGGTCTCCGGATCTCCGGTCTCCCGCTCGGGGAGGCTGCCCGCGGAGTG
CCGAAACTAACTCTTGATATTTATGCTCTCTGAGTAACTTTAAATAAGTAAAACCTTCAACAA
CGGATCTCTTGGTCTGGCATCGATGAAGAACGACGCGAAATGCGATAAGTAATGTGAATTGCAGAA
TCAGTGAATCATCGAATCTTTGAACGCACATTCGGCTCGCCAGTATTTCTGGCGAGCATGCCTGTTCGAG
CGTCAATTC AACCATCAAGCTCTGCTTGGGTTGGGGATCCCGCGGCTCCCGCGGTCCCTCAAAATCAGT
GGCGGGCTCGTAGTACACCGAGCGTAGTAACCTACATC
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**Figure 1:** Sequence of Internal Transcribed Spacer (ITS) region of *Neurospora sitophila* IMI 500339 isolated from Kunu.

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CCGAGTGGGGTCTTTGGGCCAACCTCCATCCGTGCTATTGTACCTGTTGCTTCGGCGGGCCCG
CCGCTTGTTCGGCCCGGGGGGGCGCTCTGCCCGCGGGCCGTGCCCGCGGAGACCCCAACACGA
ACACTGTCTGAAGCGTGCAGTCTGAGTTGATTAATGCAATCAGTTAAACTTTCAACAATGGATCT
CTTGGTCCGGATCGATGAAGAACGACGCGAAATGCGATAACTAATGTGAATTGCAAAATTCAGTGA
ATCATCGAGTCTTTGAACGCACATTGCGCCCTGGTATTCGGGGGGCATGCCTCGCAGGCGTCATT
GCTGCCCTCAAGCCCGGCTTGTGTGGTTCGGCTCCCTCTCCGGGGGACGGGCCGAAAGGC
AGCGGGCACCGCTCCGATCTCGAGCGTATGGGGCTTTGTACATGCTCTGAGGATTTGGCGCGC
GCCTCGCGACGTTTTCAACCATCTTCCAGGTTGACCTCGGATCAGGATCAGGATACCCCTGCACT
```

**Figure 2:** Sequence of Internal Transcribed Spacer (ITS) region of *Aspergillus niger* IMI 500340 isolated from Kunu.

**Amino acid profile:** The total amino acid profile was done for the control against the sample with the best preservative (Kunu with lime & lemon). The following amino acids were detected through paper chromatography using their various standards: Glycine, Phenylalanine, Methionine, Cysteine & Lysine. The total amino acids were calculated in mg/100g. The detection using RF values that corresponded to the standards confirmed the presence or absence of the amino acids. The standard amino acids were got from Sigma-Aldrich. Standard deviation was determined as the square root of variance, by using standard statistical procedures.

### Discussion

The attention on Kunu zaki is significant because many traditional fermented drinks possess therapeutic properties due to the existence of probiotic bacteria which are known to lower cholesterol levels through their activity. Also probiotic-rich fermented products can slow down aging process, ease digestive distress, and boost energy and immunity. Cereal based products are reported to harbour probiotic bacterial communities including *Bifidobacterium spp*, *Lactobacillus brevis*, *Weissella confusa*, *Streptococcus lutetiensis*, *Streptococcus gallolyticus* [9,10], since these cereals and their components may be exploited as a fermentation substrate capable of imparting even pre-biotic effects [11]. Although *Neurospora sitophila* was found in the edible preparation, this fungus, usually encountered in its anamorphic form, is generally regarded as 'safe' because it has not been implicated in mycotoxin production. *Neurospora* is regarded as benign and in fact been put to use in food and beverages industries [12-15]. *Aspergillus niger* is capable of producing mycotoxins like malformins which are toxic, killing chicks and mice after being fed moldy soybeans and wheat. Specifically, and of recent, this fungus has been implicated in the production of fumonisin in grapes and derived products [16]. Although some advanced countries grouped it under harmless category, good manufacturing and cultural practices that guarantee excellent hygiene will solve the problem of *Aspergillus* contamination in Kunu preparation. The keeping quality improved when a combination of lime and lemon, in equal volumes, was added without significant change in organoleptic attributes although amino acid level reduced (Table 7). Lime has been reported as antimicrobial and therefore a convenient preservative in some instances [17]. Focus on the shelf stability and optimization of storage conditions of Kunu zaki should be enhanced now that there is an emerging and increasing interest in probiotic products of non-dairy

	Control (mg/100g)	With lime & Lemon (mg/100g)
1st reading	327	115
2nd reading	309	103
Mean	318	109
Standard Deviation	12.72792	8.485281

Table 7: Total Amino Acid Composition.

sources. Although some previous works [18,19] touched on storage stability; they did not address the present storage applications.

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