



## Effect of Enhanced Fermentation on the Antioxidant, Proximate and Shelf Life Properties of Kunu

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### Abstract

The significance of antioxidants in human health cannot be overemphasized, owing to the brilliant advances in understanding the mechanisms of their reaction with free radicals to reduce the likelihood of disease manifestation. The effect of enhanced fermentation with species of *Lactobacillus* (*L. reuteri*, *L. plantarum* and *L. acidophilus*) on the antioxidant potentials, shelf life and nutrient composition of Kunu, a widely accepted and enjoyed local beverage in Nigeria made from millet, was investigated in this study. Proximate analysis (using the AOAC assay methods), spoilage bacterial density (aerobic colony forming unit assay) and the antioxidant potential ( $H_2O_2$  scavenging assay) of Kunu produced through enhanced fermentation with *Lactobacillus* species were determined. At the 5<sup>th</sup> day of storage, the microbial abundance of Kunu made through naturally and enhanced fermentation were  $570 \times 10^5$  and  $410 \times 10^5$  CFU/mL respectively. Proximate compositions (fat, ash, total solid, acidity, crude protein) were higher in Kunu fermented with *Lactobacillus* species, with crude protein having the highest recorded difference (0.77 %) when compared to samples produced through natural fermentation. It appeared Kunu samples (made from millet) generally possess better antioxidant activities when compared to ascorbic acid standard. At 10% stock dilution, linear regression plot revealed  $H_2O_2$  inhibition by ascorbic acid, Kunu with natural and enhanced fermentation as 58, 73 and 80% respectively. It is therefore suggestive (from the results) that the improvement of nutrient qualities, shelf life properties and antioxidant potentials of Kunu could be achieved through enhanced fermentation of processed millet with species of *Lactobacillus*.

### 1 Introduction

Cereals are the most consumed food crop with a 2015 estimated global production of 2500 million tonnes. These crops are important sources of basic nutrients like vitamins, minerals, dietary fiber, protein, bioactive compounds and even calorie. However, the optimal exploitation of nutrients locked in these cereals is a challenge because of the natural taste, unappealing nature of the grains, presence of anti-nutritional factors, poor digestibility, as well as their deficiency in certain nutrients<sup>1,2</sup>. Consequently, these grains are processed into more acceptable value-added forms. Kunu, a traditional, non-alcoholic beverage usually made from millet (*Pennisetum glaucum*, a nutrient-rich cereal known to

possess some level of antioxidant properties), is one of the most widely consumed beverages in Nigeria; fermentation of processed millet into Kunu offers a simple and economical means to improve the nutritional value and sensory attributes of this cereal grains<sup>3</sup>. This beverage became more widely accepted all over the country, owing to its refreshing qualities and nutritional benefits mostly derived from the source cereal grain and processing method<sup>4</sup>. Millet is more than an interesting alternative to other grains in the production of Kunu; it is a good source of some very important nutrients including copper, manganese, phosphorus and magnesium. The health benefits of millet include its heart protective properties, development and repair of body tissues,

prevention of gallstones, protection against breast cancer and other antioxidant protective properties<sup>5</sup>.

The bio-enrichment, reduction of anti-nutritional compounds, elongation of shelf-life, improvement of organoleptic and probiotic properties of Kunu can be achieved by systematically selecting microbial strains as starter cultures or to augment natural fermentation of this beverage<sup>3</sup>, these microorganisms could be massively produced from growth media composed from agricultural waste. In addition to their ability to release protective metabolites and unlock millet-based nutrients, some lactic acid bacteria (LAB) strains (especially, species of *Lactobacillus*) have the potentials to overproduce and release important nutrients during fermentation. The production of B-group vitamins such as riboflavin, folate and cobalamin by lactic acid bacteria has been extensively reviewed by LeBlanc *et al.*<sup>6</sup>. LAB species selected for millet bioprocessing are Generally Recognized as Safe (GRAS), they are probiotics whose inclusion in cereal processing are considered harmless<sup>7</sup> and they may, to a large extent, improve the antioxidant (free radical scavenging) potentials of the final Kunu product.

Several biochemical reactions and pathways in the body lead to the production of free radicals which have been implicated in oxidative stress and mediators of many diseases, including arthritis, vasculitis, glomerulonephritis, lupus erythematosus, adult respiratory diseases syndrome, ischemic diseases, hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension, preeclampsia, neurological disorder and many others<sup>8</sup>. Although, these free radicals are regularly scavenged by a number of antioxidant compounds produced *in vivo*, the endogenous antioxidants are insufficient to adequately remove the radicals and maintain a healthy balance. Consequently, dietary antioxidants are constantly required to counteract excess free radicals. Important free radicals recurrently generated in human body include the super oxide radicals, hydroxyl radicals, peroxy radicals, and single oxygen<sup>9</sup>. This study was aimed at establishing the nutrient qualities, shelf life properties and antioxidant potentials of Kunu produced through enhanced fermentation of processed millet with species of *Lactobacillus*.

## 2 Materials and Methods

### 2.1 Production of Kunu

Millet (1000g) was weighed and properly washed with boiled cooled (BC) water; the cereal was thereafter steeped in 2000mL BC water for 24 hours at ambient temperature. The grain was then rewashed, drained and again steeped in BC water containing ginger (6g), black pepper (2g) and clove (2g) for 5 minutes; after which, the preparation was drained and ground to paste. The paste was divided into unequal portions (1:3 v/v); while the larger portion was gelatinized with boiling water (1:1 v/v), the smaller portion was prepared for enhanced or natural fermentation. For

enhanced fermentation, 20mL of  $2 \times 10^8$  cfu/mL each of *L. reuteri*, *L. plantarum* and *L. acidophilus* was added to the ungelatinized portion at 10% (v/v) before thoroughly mixing with the gelatinized portion and the mixture was allowed to ferment for 6 hours at ambient temperature, following which, it was sieved to remove the pomace present in the preparation<sup>10</sup>. Natural fermentation was achieved with the addition of sterile distilled water (10% v/v) to the ungelatinized portion in place of *Lactobacillus* species. The recovered Kunu samples in both cases (after fermentation) were subsequently regarded as the stuck samples from which further dilutions were made for subsequent analysis.

### 2.2 Estimation of microbial density and proximate analysis

The aerobic spoilage bacterial enumeration was carried out using nutrient agar (NA; oxoid grade) with the pour-plate isolation method. The samples were serially diluted, after which, 1ml of appropriate dilutions ( $10^{-5}$  and  $10^{-6}$ ) were inoculated each on petri plates in triplicates to determine the average colony forming units (CFU). The culture plates were then incubated at 37°C for 24 h. After incubation, the colonies in each plate were counted using the Gallenkamp colony counter<sup>5</sup>. The pH and acidity of the beverage samples were determined, as well as other proximate parameters including the ash content, moisture content, crude fiber, crude protein and total solid; all carried out using the AOAC<sup>11</sup> standard methods.

### 2.3 Hydrogen peroxide scavenging activity

The probiotic potentials of fermented Kunu and ascorbic acid (standard antioxidant) were determined using the peroxide inhibition assay. Scavenging of hydrogen peroxide by samples made through natural and enhanced fermentation processes were determined by the method of Ruch *et al.*<sup>12</sup>. Samples (4 ml each) prepared in distilled water at various concentrations were mixed with 0.6 ml of 4 mM H<sub>2</sub>O<sub>2</sub> solution prepared in phosphate buffer (0.1M, pH 7.4) and incubated for 10 min. The absorbance of each reaction mixture was taken at the Soil Science Laboratory, Federal College of Agriculture, Ibadan with a spectrophotometer (CECIL 2041, 2000 series) at 230 nm against a blank solution containing the beverage (or standard) without hydrogen peroxide. However, this assay was modified by the preparation of dilutions from a 1000 µg/mL stock of standard ascorbic acid sample.

$$\% \text{ Inhibition} = \frac{\text{CAbs} - \text{SAbs}}{\text{CAbs}} \times 100$$

Where CAbs = Absorbance of control and SAbs = Absorbance of sample.

## 3 Results and Discussions

### 3.1 Estimation of microbial abundance

Antibacterial fermentative LAB and secondary metabolites have been extensively reported as potential substitutes for synthetic compounds to guarantee the stability and safety of cereal

fermented products<sup>13</sup>. After 24 hours of storage, the microbial abundance of naturally and enhanced-fermented Kunu were  $233 \times 10^5$  and  $153 \times 10^5$  CFU/mL respectively, while at the fifth day of storage, the aerobic bacterial abundance were  $570 \times 10^5$  and  $410 \times 10^5$  CFU/mL respectively (Table 1). The reduction in the aerobic colony forming units observed for Kunu samples produced through enhanced LAB fermentation might have been as a result of the antagonistic properties (through the possible production of bioprotective metabolites) of these LAB, which could in turn extend the shelf life of Kunu through the suppression of spoilage microorganisms.

**Table 1: Bacterial abundance in Kunu samples**

Storage days	Abundance (CFU/mL)	
	Natural fermentation	Enhanced fermentation
1	$233 \times 10^5$	$153 \times 10^5$
2	$390 \times 10^5$	$300 \times 10^5$
3	$450 \times 10^5$	$390 \times 10^5$
4	$470 \times 10^5$	$370 \times 10^5$
5	$570 \times 10^5$	$410 \times 10^5$

### 3.2 Proximate analysis

The proximate analysis report (Table 2) revealed that crude protein, fat, ash, total solid and acidity contents at 5.18, 1.22, 1.31, 5.49 and 0.07% respectively were higher for samples produced through enhanced fermentation.

**Table 2: Proximate analysis of Kunu samples**

Proximate parameter	Natural fermentation	Enhanced fermentation
Crude protein (%)	$4.41 \pm 0.005$	$5.18 \pm 0.006$
Crude fiber (%)	$0.11 \pm 0.007$	$0.10 \pm 0.000$
Fat (%)	$1.14 \pm 0.005$	$1.22 \pm 0.005$
Ash (%)	$1.59 \pm 0.015$	$1.31 \pm 0.005$
Moisture content (%)	$89.81 \pm 0.005$	$89.51 \pm 0.010$
Total solid (%)	$5.23 \pm 0.035$	$5.49 \pm 0.011$
Acidity (%)	$0.05 \pm 0.001$	$0.07 \pm 0.001$
pH	$4.53 \pm 0.025$	$4.05 \pm 0.051$

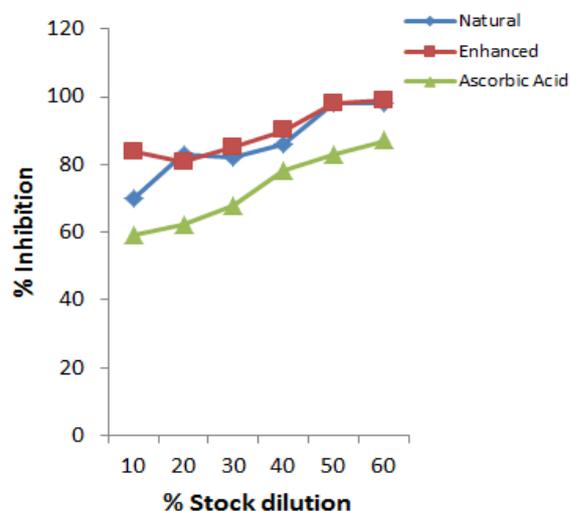
Values are expressed as mean  $\pm$ SE for each parameter

Moisture content and pH were however higher in Kunu samples subjected to natural fermentation, this could have been due to the metabolic activities (including the production of lactic acid and

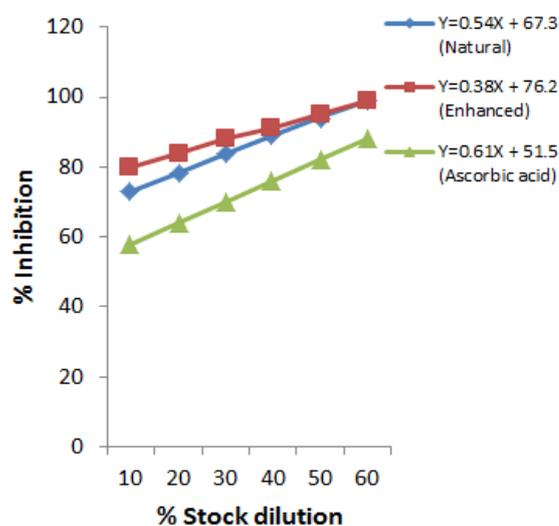
other extracellular metabolites) of the *Lactobacillus* species added to samples produced through enhanced fermentation. LeBlanc *et al.*<sup>6</sup>, Onipede *et al.*<sup>14</sup> and Ogunremi *et al.*<sup>15</sup> all reported astonishing improvement in the nutritional qualities of cereals fermented with selected LAB strains.

### 3.3 Hydrogen peroxide scavenging activity

From the results obtained, it appeared Kunu samples (made from millet) generally possess better antioxidant activities when compared to ascorbic acid standard. Samples produced through enhanced fermentation however, appeared to possess better antioxidant potentials than those fermented naturally. At the dilution of 10% stock, sample produced through enhanced fermentation appeared to scavenge the test antioxidant ( $H_2O_2$ ) better with 14% inhibition difference (Fig. 1). Like the linear regression plot of the standard ascorbic acid antioxidant assay (Fig. 2), percentage inhibition of hydrogen peroxide by Kunu samples decreased with increment in dilution.



**Fig 1: H<sub>2</sub>O<sub>2</sub> scavenging by Kunu and standard samples**



**Fig 2: Linear regression plot of H<sub>2</sub>O<sub>2</sub> inhibition**

Inhibition of samples fermented naturally decreased from 99% to 72% stock with a stock dilution concentration of 60% to 10%, while Kunu produced through enhanced fermentation with *Lactobacillus* species inhibited H<sub>2</sub>O<sub>2</sub> at a range of 99% to 80% with sample dilutions of 60% to 10% stock. Ogunremi *et al.*<sup>15</sup> also reported the possibility of better probiotic activities of cereal-based food produced with carefully selected combinations of started cultures or through an enhanced fermentation process.

There were significant correlations between the increments in inhibition of free radical by the ascorbic acid standard compared to the scavenging activities of Kunu samples produced through natural as well as through enhanced fermentation processes with coefficient of determinations of 0.98 and 0.96 respectively. The IC<sub>50</sub>, defined as the linear regression plot concentration or proportion of antioxidant required to inhibit 50% of the radicals<sup>16</sup>, for ascorbic acid standard, samples produced through natural and enhanced fermentation were all found to be at stock dilutions below 10%.

#### 4 Conclusion

Enhanced fermentation is an easily applicable, economical, sustainable and consumer friendly way to improve the nutritional value of millet. In addition to the improvement in antioxidant activity revealed through H<sub>2</sub>O<sub>2</sub> scavenging, enhanced fermentation with species of *Lacto bacillus* appeared to also improve the nutrient composition as well as reduce the microbial load of Kunu product, thereby contributing to improving the value and prolonging the shelf-life of this beverage.

#### 5 Conflict of interest

None

#### 6 Author's contributions

AOM carried out the Laboratory assay of beverage samples and manuscript writing, FOA was involved in the assay of samples fermented with *Lactobacillus* species, OSO provided relevant instructions and aided in the publication process, OAO provided relevant instructions and IKO provided ideas and involved in the publication process.

Authors declare no conflict of interest.

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