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IMPACT OF FERMENTATION ON PROXIMATE COMPOSITION AND ISOLATION OF LACTIC ACID BACTERIA FROM KUNU BEVERAGE

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ABSTRACT
Fermentation is crucial in modifying nutritional content and enhancing microbial activities in food and beverages. This study investigated the proximate composition of fermented Kunu beverage and the isolated and identified lactic acid bacteria (LAB) through biochemical and molecular methods. A three-day fermented Kunu (sorghum and tiger nut) constituents, proximate analysis and LAB isolates were biochemically characterised using standard methods in aseptic conditions. Additionally, 16S rRNA gene sequencing was employed to identify the bacterial strains. The results revealed a significant decrease in moisture content from 63.59% to 13.82% while ash content increased from 1.34% to 1.74% by day 3. Fat content remarkably rose from 0.98% to 9.28%, and crude fibre content increased from 2.79% to 8.48%. Protein content also rose significantly from 7.29% to 11.96%, and carbohydrate content more than doubled, increasing from 24.01% to 54.72%. These changes are indicative of active microbial metabolism and nutrient modification during fermentation. The isolated LAB species, Lactobacillus fermentum and Lactobacillus delbrueckii, were identified with 97.83% and 99.26% sequence identity, respectively, and are known for their fermentative abilities and probiotic potential. This study demonstrates that fermentation enhances the nutritional composition of Kunu, with LAB playing a significant role in this process. The findings suggest that optimizing fermentation conditions and leveraging LAB strains could further improve the beverage's nutritional quality and functional properties. Future studies should explore co-fermentation strategies and investigate LAB application in other fermented food products.

Keywords*: Fermentation, Lactic Acid Bacteria, Nutritional composition, Non-Alcoholic beverage*

1.0 INTRODUCTION

Kunu, a traditional fermented non-alcoholic beverage widely consumed in northern Nigeria and has gained popularity among non-alcoholic drink enthusiasts in other parts of the country due to its refreshing properties (Amusa and Odunbaku, 2009). The variety of *Kunu* beverages is primarily determined by the grain substrate, supplements, and sensory characteristics used in its preparation. It can be customized to individual preferences as a porridge or a free-flowing gruel (Ezekiel *et al*., 2019). Cereal-based foods, including *Kunu*, are consumed by individuals of all ages and social classes across Africa, serving as breakfast for adults, weaning foods for infants, and beverages for rural communities (Izah *et al*., 2017). Grains such as acha (*Digitalis exilis*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), maize (*Zea mays*), millet (*Penisetum typhoides*), and wheat (*Triticum aestivum*) are commonly used in the production of *Kunu* and other beverages of African origin. These beverages are derived from various raw materials, including cereal grains, flowers, milk, plant juices, legumes, and fruits, and differ in composition and processing techniques depending on cultural and ethnic origins (Amadou *et al*., 2011). Supplementation with ingredients improves their protein, amino acid content, and antioxidant properties (Blandino *et al*., 2003).

Traditionally, the preparation of *Kunu* is artisanal, often carried out in homes (Nwaiwu *et al*., 2020). The specific raw materials and processing techniques used influence the type of *Kunu* produced, which, in turn, reflects the cultural and socio-economic characteristics of the consumers (Tafere, 2015). These beverages

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are often associated with lower-income groups, as they are locally produced and widely consumed by rural and middle-class populations. In rural Nigerian communities, beverages, both alcoholic and non-alcoholic, are integral to social gatherings and are used in various cultural rituals, such as hospitality or young girls' initiation ceremonies in countries like Namibia (Ezekiel *et al*., 2015). Besides their social significance, non-alcoholic beverages like *Kunu* offer nutritional and therapeutic benefits (Aka *et al*., 2014). The processing of *Kunu* typically involves steps such as malting, boiling, pasteurization, fermentation, and distillation (Fadahunsi *et al*., 2013). Several additives enhance the beverage's flavour, shelf-life, and nutritional profile. These techniques also help to digest complex compounds, eliminate toxins, and improve the overall quality of the beverage, as supported by previous research (Ezekiel *et al*., 2017). This study aims to analyze the proximate composition of *Kunu* made with sorghum and tigernut, isolated lactic acid bacteria and identify these bacteria isolates through biochemical and molecular methods.

2.0 MATERIALS AND METHODS

2.1. Sample Collection

Sorghum, tiger nut, coconut, ginger, and dates were purchased from a market (what is the name of the market) in Ado, Ekiti State, Nigeria, and analysis was done at the research laboratory at the Federal Polytechnic Ado, Ekiti State, for analysis.

2.2 Laboratory Preparation of Kunu Drink

Sorghum and tiger nuts to be used for the study will be sorted to remove dirts and spoilt ones from healthy ones. The sorted ones were weighed at the ratio of 500g and 250g respectively, washed thoroughly with distilled water, and soaked in water for 72 hours and the pH was noted at 12 hours intervals, drained and milled with the inclusion of other ingredients (dates, coconut and ginger) as spice which were added after the first 48hrs. The mixture was sieved and was further processed into Kunu (Adeyemi and Umar, 1994). The filtrate was then be stored in the refrigerator for further analysis.

2.3 Proximate analysis of the fermented Kunu drink

The fermented Kunu beverage was taken for proximate analysis. Moisture was determined by the gravimetric method of James, (1995), fat and crude protein compositions were determined by the soxhlet extraction method and the kjedahl method described by A.O.A.C (2009).

Moisture Content: 2.0g of the sample(s) was placed in an oven maintained at 100 - 103°C for 16 hours, with the weight of the wet sample and the weight after drying noted. The drying was repeated until a constant weight was obtained. The moisture content was expressed in terms of the loss in weight of the wet sample.

2.0g of each of the oven-dried samples in powder form was accurately weighed and placed in a crucible of known weight. These were ignited in a muffle furnace and ashed for 8 hours at 550°C. The crucible containing the ash was removed, cooled in a desiccator, and weighed, and the ash content was expressed in terms of the oven-dried weight of the sample.

The protein nitrogen in 1g of the dried samples were converted to ammonium sulphate by digestion with concentrated H2SO4 and in the presence of CuSO4 and Na2SO4. These were heated and the ammonia evolved was steam distilled into boric acid solution. The nitrogen from ammonia was deduced from the titration of the trapped ammonia with 0.1M HCl with Tashirus indicator (double indicator) until a purplish pink color is obtained. Crude protein was calculated by multiplying the value of the deduced nitrogen by the factor 6.25mg.

2.0g of each sample was weighed into separate beakers, the sample was extracted with petroleum ether by stirring, settling and decanting 3 times. The sample was air dried and transferred into a dried 100ml conical flask. 200cm3 of 0.127M sulphuric acid solution was added at room temperature to the samples. The first 40cm3 of the acid was used to disperse the sample and heated gently to boiling point and

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boiled for 30 minutes. The contents were filtered to remove insoluble materials, which was washed with distilled water, then with 1% HCI, next with twice ethanol and finally with diethyl ether. Finally, the oven-dried residue was ignited in a furnace at 550°C. The fibre content was measured by the weight left after ignition and expressed in term of the weight of the sample before ignition.

The lipid content was determined by extracting the fat from 10g of the samples using petroleum ether in a soxhlet apparatus. The weight of the lipid obtained after evaporating off the petroleum ether from the extract gave the weight of the crude fat in the sample.

The carbohydrate content of the samples will be determined as the difference obtained after subtracting the values of protein, lipid, ash and fibre from the total dry matter.

2.4 Isolation of Microorganisms from Test Samples

Preparation of media

The MRS agar used was prepared according to the manufacturer's instructions.

Serial dilution of test sample

Sterile test tubes were dispensed with 9ml of peptone water for each sample. One millimetre of the water sample was transferred into a test tube containing 9ml of peptone water dilution [10^{-1}]. Then using another micropipette, 1ml of the resulting dilution was transferred into a second test tube containing 9ml of distilled water [10^{-2}]. The procedure was repeated for further dilutions up to 10^{-6} dilutions and in the last dilution 1ml of the inoculum was discarded (Chessbrough, 2000).

Pour plates techniques for isolation of isolates

The method used for isolation was the pour plates technique. Sterile Petri dishes were arranged on a working bench for each sample collected and also for the type of organism to be cultivated. 1ml of dilution was poured into the Petri dishes that have already been arranged and properly labeled, and about 20ml of MRS agar was poured into each Petri dish. The plates were inverted and incubated in an anaerobic jar at 37^0C for 24-48 hours to allow bacterial growth. (Ishola and Adebayo-Tayo, 2012).

Identification of isolates

The organisms were subcultured and identified based on their cultural and morphological examination [Microscopic examination]. Colonial characteristics of all the various isolates were carried out by recording their characteristics and growth patterns on the plates and incubating them at 37°C for 24 hours.

Gram Staining of Isolates.

The working solution of reagents for the Gram staining technique was prepared according to the manufacturer's instructions. Staining was carried out by emulsifying approximately one isolated 18- 24-hour-old colony in a drop of water placed at the center of a clean, grease-free slide until a thin smear was made. The smear was heat-fixed by passing the slide through a Bunsen burner flame and then air-dried. The heat-fixed smear was flooded with a basic aniline dye [crystal violet] for 60 seconds. This was flooded with Lugo's iodine, allowed to remain for 60 seconds, and then rinsed off with running tap water. The smear was decolorized with 70% ethanol and immediately washed out to avoid total decolorization. The smear was counterstained with Safranin for 60 seconds, washed off with running tap water and blot-dried. The slide was be examined under an oil immersion objective microscope. Organisms that retain the purple colour of crystal violet-iodine complex [CV-1 complex] were recorded as Gram-positive, while those that appeared pink are Gram-negative (Chessbrough, 2000).

Biochemical Characteristics of the LAB Isolate

Biochemical tests were conducted on the Strains with Gram-positive reaction. The tests include catalase, motility for identification and oxidase test were be determined (Guiraud, 2008).

Sugar fermentation test

The fermentation of sugar test by the test organism was demonstrated by the production of acid and gas, phenol red [0.01g], sodium chloride [1.0g] and fermentable sugars [1.0g] were weighed into a conical flask containing 100ml of water. The mixture was swirled so that all components in it could dissolve. 9ml of preparation was dispensed into test tubes containing inverted durham tubes. The tubes were covered with cotton wool and aluminum foil and sterilized in the autoclave at a temperature of 121^{0}C for 15 minutes. Sugars to be used are glucose, fructose, lactose and sucrose. All test tubes were inoculated with respective test organisms aseptically and incubated at 37^{0}C for 3-5 days depending on how fast the organism can utilize the sugar (Harrigan and McCance, 2013).

2.5 Molecular Identification of the isolates.

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq@ Quick-Load@ 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented in Table 1. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit ™ , Catalogue No. D4050). The purified fragments were analysed on the ABI

Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, as listed in Section 1. BioEdit Sequence Alignment Editor version 7.2.5 was used to analyse the. abl files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

3.0 RESULTS AND DISCUSSION

Table 1 Proximate composition of fermented Kunu

Parameter (%)	Day 1	Day 2	Day 3
Moisture	63.59	54.23	13.82
Ash	1.34	1.51	1.74
Fat	0.98	2.21	9.28
Crude fibre	2.79	4.92	8.48
Protein	7.29	8.29	11.96
Cho	24.01	28.84	54.72

Table 2 Morphological characteristics of selected bacterial isolates

Bacterial isolates	Shape	Size	Texture	Elevation	Pigmentation	Opacity
S1	Irregular	Moderate	Rough	Raised	Creamy white	translucent
S2	Irregular	Moderate	Rough	Raised	Cream	translucent
S3	irregular	Small	Smooth	Raised	Cream	translucent
S4	irregular	Moderate	Smooth	Flat	Cream	translucent
S5	irregular	Small	Smooth	Raised	Cream	translucent

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Table 3 Biochemical characteristics of the isolates.

Bacterial isolates	Gram staining	Oxidase	Catalase	Motility	Sugar fermentation			
					Glucose	fructose	lactose	sucrose
S1	Gram Positive, rod	-	-	-	+	+	+	+
S2	Gram-Positive, Rods	-	-	-	+	+	+	+
S3	Gram-Positive, rod	-	-	-	+	+	+	+
S4	Gram-Postive, Rod-like	-	-	-	+	+	+	+
S5	Gram-Positive, diplococci	-	-	-	+	+	+	+

Figure 1: 16S report for the isolated organisms

RESULTS:

BLAST: The BLAST results correspond to the similarity between the sequence queried and the biological sequences within the NCBI database.

Name of sample	S3
Percentage ID	97.83%
Predicted Organism	*Lactobacillus fermentum*
GenBank Accession	MH880106.1

Name of sample	S5
Percentage ID	99.26%
Predicted Organism	*Lactobacillus delbrueckii*
GenBank Accession	CP045641.1

Table 4: BLAST results for the bacterial isolates based on their 16S rRNA gene sequence

Isolate Code	Accession Number	Percentage Identity (%)	E-value	Organism
S3	MH880106.1	97.83	0.0	*Lactobacillus fermentum*
S5	CP045641.1	99.26	0.0	*Lactobacillus delbrueckii*

Discussion

The data from this study indicates a substantial reduction in moisture content from 63.59% on Day 1 to 13.82% by Day 3 of the fermentation process. This significant decrease is typical in fermentation, as water is utilized by fermenting microorganisms and lost through evaporation, leading to the concentration of other constituents. Such a trend reflects active microbial metabolism, where water produced as a metabolic byproduct is less than the water consumed or evaporated. This observation aligns with previous fermentation studies examining moisture dynamics (Smith *et al*., 2022).

The increase in ash content from 1.34% to 1.74% over the fermentation period suggests a rise in mineral concentration. The relative proportion of inorganic components (ash) increases as moisture decreases. This pattern not only reflects the stability of mineral elements during fermentation but may also indicate enrichment through microbial metabolic byproducts (Lee and Park, 2019). The notable increase in fat content from 0.98% to 9.28% could be attributed to microbial lipolysis or fatty acid synthesis by fermentative microbes. While this is unusual in grain-based beverages, it may be explained by the activity of lipid-producing yeasts or bacteria capable of synthesizing or releasing fatty acids during fermentation (Johnson, 2021). There is a significant increase in crude fibre and protein content, with protein levels nearly doubling from 7.29% to 11.96% by Day 3. The rise in protein may result from microbial activity that releases amino acids or synthesizes proteinaceous substances during fermentation. The increase in crude fibre suggests that microbial enzymes might be modifying structural polysaccharides, making the fibre more detectable by standard analytical methods (Davies and Bianchi, 2020). The increase in carbohydrate content from 24.01% to 54.72% is likely due to microbial enzymatic hydrolysis of complex polysaccharides into simpler sugars. This process is commonly observed in cereal fermentation, where starches are broken down into simpler, more digestible sugars, contributing to the flavour and texture of the final product (Nguyen and Schwarz, 2018).

Studies on similar fermented beverages, such as the work by Okafor *et al*. (2021) on sorghum-based *Kunu*, have observed similar trends in moisture and ash content, supporting the typical dehydration and mineral concentration processes during cereal fermentation. Although the increase in fat content is less frequently reported, it aligns with findings by John *et al*. (2020), who noted lipid metabolism as a secondary function of lactic acid bacteria in various fermented substrates. The observed increases in protein and fibre are consistent with Singh and Raghuvanshi's (2019) findings, which emphasize microbial enzymatic activity as a crucial factor in enhancing the nutritional quality of fermented foods.

The morphological and biochemical analysis of the isolates identified a dominant presence of gram-positive bacteria, which are known for fermenting sugars—a characteristic of lactic acid bacteria (LAB). This was further confirmed by 16S rRNA gene sequencing, which identified species such as *Lactobacillus* *fermentum* and *Lactobacillus delbrueckii*, both recognized for their fermentative solid abilities and probiotic potential. These LAB species are widely used in food fermentation to improve the flavour, texture, and safety of fermented products (Martin and Patel, 2021).

The presence of these LAB strains is crucial, as they contribute to the production of organic acids, antimicrobial peptides, and aromatic compounds that shape the sensory profile of the beverage. Their high occurrence and significant E-values underscore their adaptability and stability in the fermentation environment of *Kunu*, echoing studies such as those by Zhao *et al*. (2019), which highlight the role of LAB in enhancing the safety and nutritional value of fermented beverages.

Conclusion and Recommendation

The proximate composition analysis reveals significant changes during fermentation, including a marked reduction in moisture content and notable increases in ash, fat, crude fibre, protein, and carbohydrates. These changes highlight the metabolic activities of microorganisms involved in the fermentation process. The unexpected rise in fat content may indicate microbial synthesis or lipolysis, while the increase in protein and fibre suggests enhanced nutritional quality due to microbial enzymatic activity. The identification of *Lactobacillus fermentum* and *Lactobacillus delbrueckii* confirms the critical role of lactic acid bacteria in improving the fermented product's safety, flavour, and nutritional value.

Further studies are recommended to explore the mechanisms behind microbial lipid synthesis during fermentation and optimize fermentation conditions to enhance nutrient yield. The application of *Lactobacillus* strains in other fermented products should also be investigated, along with the potential benefits of co-fermentation strategies to improve nutritional profiles and flavour characteristics further.

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