

Microbial Assessment of Kunu Sold In Awka Metropolis

Abstract

Kunu is a non-alcoholic Nigerian beverage produced locally from cereals and has been widely accepted as a nutritional drink due to its cost hence, the need for evaluation of its nutritional and safety status. This research was carried out to determine the microbiological quality of “kunu-zaki” beverages sold in Awka metropolis. Three samples of “kunu-zaki” beverages were obtained from three different locations in Awka, Anambra state namely; Amansea, Eke-Akwa and Amawbia, labelled P, Q R respectively. These samples were tested for both bacterial and fungal contamination. The results show that for the Colony counts from the Kunu samples ranged from 0.5×10^5 to 3.2×10^5 for total coliform bacteria, 2.5×10^5 to 4.2×10^5 for total heterotrophic fungi for the three samples analyzed. Bacterial isolates identification revealed the presence of *Bacillus* sp, *Pseudomonas aeruginosa*, *Escherichia coli* and *Streptococcus* sp, while the fungal isolates include; *Aspergillus flavus*, *Penicillium* sp, *Fusarium* sp and *Saccharomyces cerevisiae*. The data revealed that all the Kunu drinks sold within the study area were contaminated and also contained different pathogenic microorganisms which can serve as sources of infections to human. Therefore, proper hygienic and sanitary measures need to be enforced during production, processing and packaging of this local beverage drink.

Introduction

Kunu, a non-alcoholic beverage used to be consumed mainly in the Northern parts of Nigeria is now widely acceptable in almost all parts of Nigeria, owing to its refreshing and nutritive qualities as well as it being cheaper as compared to carbonated drinks (Gaffa *et al.*, 2002; Adejuyitan *et al.*, 2008). Kunu is a popular cereal based non-alcoholic beverage that is prepared from either guinea corn (*Sorghum bicolor*), millet (*Penisetum typhoides*), maize (*Zea mays*), rice (*Oryza sativa*) or wheat (*Triticum aestivum*). Although there are various types of kunu which are processed and consumed in Nigeria. They include: kunuzaki, kunugyada, kunuakamu, kunutsamiya, kunubaule, kunujiko, kunuamshau, kunugayamba. However, kunuzaki is mostly consumed in every part of Nigeria.

Kunu-zaki is prepared traditionally using millet, maize, wheat or sorghum. It contains 11.6% of protein, 3.3% of fats, 1.9% of ash and 76.8% of carbohydrates and arrays of amino acid (Gaffa *et al.*, 2002). Apart from these cereals, kunu has been shown to be produced from tigernuts (Belewu and Abodunrin, 2006), guinea corn or rice (Umaru *et al.*, 2014). The appearance of kunu is milky cream and is usually consumed within few hours after its production (Adeleke and Abiodun, 2010). Because kunu is prepared in the traditional method, the ingredient concentrations are neither quantified nor standardized (Aboh and Oladosu, 2014). The production procedure varies depending on household, taste and cultural habits of the consumers. This leads to variation in the taste, quality and specifications of the product. According to Umaru *et al.*, 2014, the processes involved in the preparation of kunu-zaki include: steeping the whole grains in water for 6-24 hours, followed by wet milling usually with spices and sweet potato, gelatinization of a portion of the mixture in hot water and then pitching with about one quarter fresh (ungelled part of the mixture. Spices such as

ginger (*Zingiber officinales*), aligator pepper (*Aframomum melegueta*), red pepper (*Capsicum* species), black pepper (*Piper guinense*) and Kakandoru or Eruare usually utilized (Adelekan *et al.*, 2013). Apart from the nutritional benefits of kunu consumption, the drink has been shown to have other benefits including reduction in blood cholesterol, lowering the risks of diabetes, and prevention of blood clot formation (Ofudje *et al.*, 2016).

In spite of the benefits derived from this drink (kunu), there have been reports of microbial contamination which may cause spoilage. Some of the microorganisms implicated in the spoilage of kunu include lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Streptococcus* spp. and *Leuconostoc*spp as well as other food-borne microbial pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp. Among others (Bibek, 2001; Amusa *et al.*, 2005). Also, activities of the natural food enzymes could also contribute in the spoilage. Other factors include insects, rodents or pests present in the environment during the preparation, temperature and light. All these and much more if not properly managed could contribute to the spoilage of “kunu-zaki”. The presence of the amount of sanitary indicator organisms in foods are of importance in the assessment of the quality and safety of foods. Even though, epidemiological evidence on outbreaks of food borne disease as a result of taking “kunu-zaki” is scarce, there are indications that it could still be contaminated to unsafe level at the point of consumption with air flora and other microorganisms from handlers, equipment serving containers, raw materials and lack of portable water for processing. Poor hygiene and preparation practices in which the production process is subjected can also introduce microbial pathogens in foods and have been implicated in causing food-borne illnesses (Amusa and Odunbaku, 2009). In developing countries like Nigeria, it has not been possible to have safety control over the processing of hawked foods, because most of the vendors lack the adequate knowledge of food processing and handling practices as well as lack of portable water, proper storage and waste disposal facilities at preparation and service points have resulted in poor unsanitary conditions and thus served as potential microbial contaminants and increased risk to public health (Omemu and Aderoju, 2008; Sperber, 2003). Though there are a lot of literatures on the microbiological and nutritive quality of kunu drinks, there is scarce or no information on the safety and nutritional status of kunu drinks sold in Awka metropolis, Anambra state, where this study was carried out. Hence, the study intends to evaluate the proximate composition and microbial quality of kunu drinks sold in Awka Metropolis as this will serve as a comparative indices as to what is obtainable in other parts of the country and help take holistic and enduring decision towards the production of this food drink.

Materials and Methods

Study Location: Awka is a city in Anambra State, South-East, Nigeria. Its area is approximately 120 km². The city's population is about 301,657. It lies below 300meters above sea in a valley on the plains of the Mamu River.

Methods

Sterilization of Materials: The glasswares (Test tubes, pipettes, conical flasks, beakers, petri-dishes and universal bottles) were washed with soapy water and rinsed with distilled water; they were allowed to dry and wrapped with kraft paper and further sterilized in a hot air oven at 180°C for 1hour and stored at 4°C. The media used was also sterilized.

Collection of Samples

Samples of freshly prepared “kunu-zaki” were collected from different hawkers at 3 different locations all within Awka metropolis. The samples were labeled and transferred immediately to the microbiology laboratory of Nnamdi Azikiwe University, Awka in their original package and the contents aseptically withdrawn from the bottles for pH analysis, isolation and enumeration of micro organisms

Preparation of Media: The three media (Nutrient Agar, NA; MacConkey agar, MAC; Potato Dextrose Agar, PDA) used were prepared according to the manufactures’ procedures (Titan Biotech Ltd. BHIWADI-301019, Rajasthan, India).

Preparation of Nutrient Agar (NA): Twenty eight grams (28.0g) of nutrient agar powder was dissolved in 1000 ml of distilled water. The medium was gently heated to dissolve and completely sterilize by autoclaving at 15psi (121°C) for 15minutes. The autoclaved media was allowed to cool before pouring 15ml each onto sterile Petri dishes and allowed to gel.

Preparation of MacConkey agar (MAC) Agar: This was done by suspending 47grams of the powder in 1000ml of distilled water. The suspended was mixed very well and heated with frequent agitation to dissolve the powder completely. The suspended media was sterilized by autoclaving at 121°C and 15psi, for 15minutes. The autoclaved media was allowed to cool before pouring 15ml each onto sterile Petri dishes and allowed to gel.

Preparation of Potato Dextrose Agar (PDA): This was done by suspending 39g of the media in 1000ml of distilled water. The suspended was mixed very well and heated with frequent agitation to dissolve the powder completely. The suspended media was sterilized by autoclaving at 121°C and 15psi, for 15minutes. The autoclaved media was allowed to cool before pouring 15ml each onto sterile Petri dishes and allowed to gel.

Microbiological Analysis: One milliliter of each sampled kunu drink was put in 9ml of sterile distilled water in sterile test tubes, shaken and then serially diluted. From the appropriate dilution, 0.1ml was inoculated separately on to MacConkey agar, Nutrient Agar and Potato Dextrose Agar plates and spread evenly using sterile bent glass rod. Each experiment was carried out in duplicates to get a mean standard value of the colony forming units (cfu/ml) on the plates. The inoculated MacConkey agar, Nutrient agar and Potato Dextrose agar plates were incubated at 30°C and 35°C for 24 and 48 hours respectively. After the period of incubation, the colonies on the plates were counted and recorded as colony forming unit per milliliter (cfu/ml) and coliform respectively (Harrigan and McCance, 1976).

Each of the bacterial colonies on the agar plates was sub-cultured and the pure culture obtained. Isolates were identified by carrying out tests which include Gram staining, spore staining and biochemical tests such as catalase, coagulase, oxidase, citrate utilization, indole, methyl red, urease, VogesProskauer and sugar fermentation (Onyeagba, 2004; Cheesbrough, 2006).

The bacteria isolates were characterized using microscopic techniques and biochemical tests. The identities of the isolates were determined by comparing their characteristics with those of known taxa as described by Bergey's manual of Determinative Bacteriology (Holt *et al.*, 2004) and Cheesbrough, (2006). The cultures used for biochemical test were between 18 to 24 hours old. The biochemical tests used include: indole test, oxidase test, citrate test, catalase test, urease test, glucose test etc

Fungal identification and enumeration was based on their colony elevation, colour, texture, shape and arrangement of conidia (spherical or elliptical, unicellular or multicellular), branched or unbranched mycelia, presence or absence of cross walls (whether septate or non-septate) and others. They were enumerated by reference to illustrated manual on identification of some seed borne fungi (Kulwant *et al.*, 2001) and illustrated genera of imperfect fungi (Barnet and Hunter 2002).

Biochemical Tests

Catalase Test (Cheesbrough, 2006)

Catalase is an enzyme that catalyzes the reaction by which hydrogen peroxide (H_2O_2) is decomposed to water and oxygen. It was used to differentiate *Staphylococcus* (catalase-positive) from *Streptococcus* (catalase negative).

A loopful inoculation of a 24 hour culture of the isolate was placed on a clean grease-free glass slide using a sterile wire loop. It was smeared using a drop of normal saline. A drop of hydrogen peroxide (H_2O_2) was placed on the smear. Catalase positive organisms produced effervescence reaction immediately.

Coagulase Test (Bound Coagulase) (Cheesbrough, 2006)

A drop of normal saline was placed on two separate slides. Colony of the test organism was emulsified in each of the drops to make two suspensions and mixed gently. No plasma (human plasma) was added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase clumping.

Clumping of the organisms within 10 seconds indicated positive result. No clumping within 10 seconds indicated no bound coagulase ie negative coagulase.

Motility Test (Cheesbrough, 2006)

10ml of nutrient agar (LabM) was poured into each of the test tubes aseptically. The test tubes were clogged and sealed using cotton wool and slanted to solidify. The test organisms were each inoculated into the nutrient agar slant using a straight wire loop and incubated for 24 hours. Growth along the stab is negative. Growth moving into the medium away from the stab is positive.

Indole Production Test (Cheesbrough, 2006)

The test organisms were each inoculated into test tubes containing 5ml each of sterile peptone water. The test tubes were clogged using cotton wool and incubated at 37⁰C for 48 hours. Kovacs reagent (0.5ml) was thereafter added and the culture shaken for 1min. a red color in the reagent layer indicated the presence of indole. A yellow color in the reagent layer indicated the absence of indole.

Methyl Red Test (Cheesbrough, 2006)

The test organisms were introduced into different test tubes containing 5ml of sterile peptone water. The test organisms were incubated at 37⁰C for 48 hours. 0.5ml or 5 drops of methyl red (indicator) was added to the culture. Then, it was mixed gently to disperse the methyl red and was allowed to settle. Red coloration is positive while a yellow coloration is negative.

Voges-Proskauer Test (Cheesbrough, 2006)

The test organisms were inoculated into 5ml of sterile peptone water in corresponding test tubes. The test tubes were clogged using cotton wool and incubated at 37⁰C at 48 hours. 0.5ml or 5 drops of alpha-naphthol (Baird's reagent A) and 0.5ml or 5 drops of potassium hydroxide (Baird's reagents B) were added to the tubes containing the test organisms. The culture were shaken gently and allowed to settle for 15 minutes for color development. A positive reaction for Voges-Proskauer was indicated by red coloration. A negative reaction for Voges-Proskauer was indicated by yellow coloration.

Citrate Utilization Test (Cheesbrough, 2006)

The test organisms were introduced into prepared slopes of Simmons citrate medium in test tubes using a sterile wire loop the slope was inoculated with the test organism aseptically, then it was covered with cotton wool lightly. The test tubes containing the test organisms were incubated for 48 hours at a temperature of 35 – 37⁰C. Bright blue color indicated positive citrate test. Green color indicated negative citrate test

Sugar Fermentation Test (Cheesbrough, 2006)

0.5g of sugar (glucose, lactose and sucrose) each was mixed with 100ml of peptone water and 3-5 drops of pH indicator, bromothymol blue was added. 5ml of medium each were dispensed in test tubes with the Durham tubes. The whole set up was sterilized at 115⁰C for 10 minutes and was allowed to cool. Thereafter, the test organisms was introduced into the medium and incubated at 37⁰C for 24 hours. Then the culture was examined for evidence of acid or gas production which is an indication that the organism fermented the sugar.

Gas production was indicated by bubbles in the Durham tubes while acid production was indicated by a change in color of the medium.

Gram Staining ((Cheesbrough, 2006)

A thin film of the bacterial isolate was made on a clean grease free slide. It was fixed by passing the slide over a flame 3 times. The fixed smear was flooded with 0.5% aqueous solution of crystal violet and left for thirty (30) seconds. The stain was washed off in clean

running water and then the smear was covered with Lugols iodine for 30 – 60 seconds. The iodine was washed off in clean running water. The smear was decolorized rapidly with acetone-alcohol for 2-5 seconds, and then the slide was washed in clean running water. The smear was then counterstained with 0.5% safranin solution, left for 30 seconds and washed off with clean running water. The slide was then air dried and examined microscopically under the oil immersion lens (100x) without a cover slip for either a Gram- positive or Gram-negative bacteria.

Spore Staining Test (Cheesbrough, 2006)

Bacteria film was made on a slide, dried and heat fixed with minimal flaming. The slide was placed on the rim of beaker of boiling water, with the bacterial film uppermost. The film was flooded with 0.5% aqueous solution of malachite green when large droplets have condensed on the underside of the slide and left to act for one minute while the water continued to boil. The slide was washed with cold water and treated with 0.5% of safranin solution and left for 30 seconds. The slide was washed, dried and examined microscopically under the oil immersion lens (100x). Red color indicated a negative result.

Urea Test (Cheesbrough, 2006)

The test organisms were inoculated onto test tubes with solidified urea medium. Urea medium was dispensed into six test tubes and after inoculation with the organisms, the test tubes were sealed with cotton wool and incubated at 24 hours at 37°C. Positive result was indicated by pinkish color. Negative result was indicated by yellow color.

Results and Discussion

Colony counts of bacteria isolated from fresh kunu samples are presented (Table 1). The counts ranged from 0.5×10^5 to 3.2×10^5 for total coliform bacteria, 2.5×10^5 to 4.2×10^5 for total heterotrophic bacteria and 5.2×10^5 to 8.0×10^5 for total heterotrophic fungi. The results indicate that fresh kunu presented a high bacteria count after 24 hr of incubation. Efiuwewere and Akoma, (2015) also reported similar abnormality of high bacterial populations in kunuzaki prepared and sold in Jos metropolis (Hatchers *et al.*, 2002). The high colony count is an indication of spoilage as a consequence of either poor hygiene or poor quality of cereals and the water used. Ten microbial isolates including six species of bacteria and four species of fungi were isolated from the kunu samples. The bacterial isolates include; *Lactobacillus* sp. *Staphylococcus* sp., *Streptococcus* sp., *Salmonella* sp., *Escherichia coli* and *Pseudomonas* sp (Table 2) while the fungal isolates were the species of *Fusarium*, *Aspergillus*, *Penicillium* and *Saccharomyces* sp

LOCATIONS	KUNU		
	MAC (cfu/ml)	NA(cfu/ml)	PDA(cfu/ml)
LOCATION P	2.3×10^5	2.5×10^5	5.2×10^5
LOCATION Q	0.5×10^5	4.2×10^5	8.0×10^5
LOCATION R	3.2×10^5	3.7×10^5	5.3×10^5

Where; MAC = MacConkey Agar, NA = Nutrient Agar, PDA= Potato Dextrose Agar; CfU/ml = Colony forming unit per millimetres

Table 1: Total Microbial Count of Kunu Drinks Sold at AwkaaMetropolis at Three Different Locations

Isolated Strain	Morphology	Gram's	Catalase	Oxidase	Motility	Citrate	Spore	Indole	Methyl Red	Voges-Proskauer	H ₂ S	Product	Urease	Glucose	Sucrose	Lactose	Mannito	Probable	Organism
A	R	-	+	-	-	-	-	-	+	+	-	-	-	A	-	-	A	<i>Lactobacillus</i>	<i>spp</i>
B	R	-	+	-	+	-	-	-	+	-	+	-	-	A	-	-	A	<i>Salmonella</i>	<i>lla spp</i>
C	C	+	-	+	-	+	-	-	-	-	+	-	-	A	-	A	A	<i>Streptococcus</i>	<i>spp</i>
D	R	-	+	+	+	-	-	-	-	-	+	-	+	+	+	-	-	<i>Pseudomonas</i>	<i>spp</i>
E	C	+	+	-	+	-	-	-	-	-	+	-	+	+	+	-	+	<i>Staphylococcus</i>	<i>spp</i>
F	R	-	+	-	+	-	-	+	-	-	-	-	A/G	A/G	A/G	-	-	<i>E. coli</i>	

Key: A/G=Acid and Gas production; A= Acid production, R = Rod, C = Cocci

Table 2: Morphological and Biochemical Characteristics of the Bacterial Isolates

All the Kunu samples were acidic in nature with pH range of 3.20 to 4.85. Various researchers have attributed this to the presence of fermentative microorganisms in kunu which causes spoilage of the beverage by fermentation of its carbohydrate content and producing undesirable changes in them, altering their aroma and taste thus making them unpalatable for human consumption. Osuntogun and Abiola (2004) isolated lactic acid bacteria such as *Lactobacilli*, *Leuconostoc* and *Streptococcus* which were reported to possess the ability to ferment carbohydrates to produce lactic acid thus lowering the pH. *Lactobacilli* have also been isolated from other indigenous nonalcoholic beverages. Olasupo *et al.* (2002)

reported the isolation of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiellasp.* And *Enterococcus faecalis* from kunu drink. Akinrele *et al.* (2008) reported that the yeasts *Saccharomyce scerevisiae*, *Candida mycoderma* and molds *Cephalosporium*, *Fusarium*, *Aspergillus* and *Penicillium* are the major organisms responsible for the fermentation and nutritional improvement of cereal based fermented foods (ogi and kunu-zaki). These organisms can cause the spoilage of the beverage if not eliminated during the heating process. The high bacterial and fungal loads in all the Kunu samples can be attributed to the poor hygienic practices of the handlers and possible contamination from utensils and water used for processing the beverage as well as packages used in its distribution. The bacterial isolates with their frequency and percentage occurrences in each sample is as presented (Table 3). The result showed that *Escherichia coli*, *Staphylococcus* sp and *Salmonella* sp had a hundred percent frequency in all the three samples analysed .the percentage occurrence in each of the samples are; Sample A (67.0%), Sample B (100%) and Sample C(50%).

Sample	E.coli	Staph	Pseud	Salm	Lact	Strep	Total(%)
P	+	+	-	+	+	-	67
Q	+	+	+	+	+	+	100
R	+	+	-	+	-	-	50

Key: E. coli = *Escherichia coli*, *Staph.sp* = *Staphylococcus* specie; *Pseu. sp* = *Pseudomonas* specie, *Lact. sp* = *Lactobacillus* specie

Table 3: Percentage Occurrence of the Isolates in each sample

Isolates	Cultural characteristics	Morphological Features	Microscopy	Identity
F1	Yellowish-green mycelium	Conidia in long chains, branched cells	Branched smooth conidiophores, brush-like	<i>Penicillium</i> sp

F2	Woolly black domains	white,	Conidia chains	in	conidia head Non-septate hyphae with many branches	<i>Aspergillus</i> sp
F3	White With colony	cottony felty	Macro-conidia in light periphery in chains.	with	Septate hyphae with branched conidiophore	<i>Fusarium</i> sp
F4	Colourless		Nil		Egg shaped	<i>Saccharomyces</i> sp

Key: *E. coli* = *Escherichia coli*, *Staph.sp* = *Staphylococcus* specie; *Pseu. sp* = *Pseudomonas* specie, *Lact. sp* = *Lactobacillus* specie

Table 4: Morphological and Cultural Characteristics of Fungi Isolates

Conclusion

From the result obtained, it could be seen that the probable organisms associated with kunu-zaki were *Escherichia coli* *Staphylococcus* species, *Streptococcus* species, *Lactobacillus* species *Candida* species, *Penicillium* sp, *Fusarium* sp and *Aspergillums* species despite the nutritional values of Kunu-zaki, the presence of these organisms rendered it unfit for human consumption.

Recommendations

The presence of these isolated organisms in “kunu-zaki” samples analyzed could serve as indicator for need to promote awareness about the possible health hazards that could arise due to handling and processing of the beverage. To safeguard public health, Governments and regulatory authorities should intervene by setting standards in acquisition of raw materials, production procedures. Sick persons should not be allowed to handle or process beverage. Proper hygienic measures should be taken by persons processing the beverage to ensure that his/her body or bodily secretions does not get in contact with the beverage during processing. The general public should be informed on the importance of clean water in the preparation of food beverage or other foods. Processing water should be boiled before using.

Addition of spices to the kunu drink has been shown to inhibit microbial growth. Processed “kunu-zaki” beverage should be stored properly and refrigerated

Producers and vendors of “kunu-zaki” should be encouraged to utilize the technical assistance of National Agency of Food and Drugs Administration and Control (NAFDAC) towards attaining quality standards.

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