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Microbiological Analyses of Kunu Drinks Locally Produced and Sold in Calabar, Southern Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author UEE designed the study, wrote the protocol and the first draft of the manuscript. Author GMI managed the literature searches and performed statistical analyses. Authors SMU and ANU managed laboratory analyses of the samples. Authors EEU, FZU and PIO managed the sample collection. All the authors read and approved the final manuscript.

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ABSTRACT

Kunu is a non-alcoholic Nigerian beverage produced locally from cereals. The method of preparation, as well as the key ingredients and spices added as flavoring agents are always not standardized. This study was carried out to investigate the microbial quality of kunu drinks locally produced and sold in Calabar and to determine the antimicrobial susceptibility profile of the isolates. A total of 9 kunu samples were collected and analyzed by standard microbiological methods. The pH of the samples, microbial colony counts and antimicrobial susceptibility profile were determined by standard methods. The pH values of the samples ranged from 3.80-4.20. The total viable counts for mesophilic bacteria, coliform and fungi ranged from 3.0-5.7x10^3 cfu/ml, 1.0-2-2x10^3 cfu/ml and 5.1-8.6x10^4cfu/ml respectively. The microorganisms isolated include bacteria; *Staphylococcus aureus* 4(10%), *Lactobacillus sp.* 9(22.5%), *Proteus sp.* 3(7.5%), *Pseudomonas aeruginosa*

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3(7.5%), *Streptococcus* sp. 4(10%), *Salmonella* sp. 5(12.5%), *Bacillus* sp. 6(15%) and *Escherichia coli* 6(15%), and fungi; *Saccharomyces* sp. 6(28.5%), *Fusarium* sp. 2(9.5%), *Aspergillus* sp. 4(19%), *Penicillium* sp. 4(19%) and *Rhizopus* sp. 5(24%). The results of antimicrobial susceptibility test showed 100% resistance of the bacterial isolates to Clindamycin, Erythromycin and Cefuroxime and 100% sensitivity to Imipenem, Ofloxacin and Amoxicillin. The fungal isolates, *Aspergillus* sp. and *Saccharomyces* sp. showed 100% sensitivity to Griseofulvin, Ketoconazole and Fluconazole at varying concentrations while the rest were resistant. The results of this study revealed contamination of kunu drinks from many sources, most commonly microorganisms which could present a significant health risk to the consuming population. Therefore, it is recommended that good hygiene and sanitation practices should be enforced at all levels during preparation and processing of kunu drinks to mitigate microbial contamination of the finished product and curtail the incidence of food-borne illnesses.

Keywords: Antimicrobial; cereals; food-borne pathogens; kunu drinks; microbial contamination.

1. INTRODUCTION

In Nigeria, kunu is regarded as the most popular indigenous drink produced mostly in the North [1]. It is a traditional, cereal-based and fermented non-alcoholic beverage renowned for its thirst quenching properties [2]. Kunu is consumed almost by all people because it is relatively cheap and nutritious when compared to carbonated drinks [3]. Kunu is sold in all parts of the country including Calabar and the cereals used in its production are similar ranging from millet (*Pennisetum typhoideum*), to guinea corn (*Sorghum bicolor*) and maize (*Zea mays*) in decreasing order of preference [1]. It can also be prepared from either rice (*Oryza sativa*) or wheat (*Triticum assesstivum*) [4].

The production of kunu drink is still at village technology level where procedures and materials or ingredients used are always not standardized. In general, the process of production involves steeping the grains for 6-24 hours, wet milling with spices, wet sieving and partial gelatinization of the slurry [4]. This is followed by addition of sugar and spices depending on the producer, such as ginger (*Zingiber officimals*) and alligator pepper (*Afromonium melegueta*) or red pepper (*Capsicum species*), or black pepper (*Piper guineense*). These serve as flavor and taste improver which are not quantified [5].

The nutritional composition of kunu produced locally consists of 87-92% moisture, 3.19-7.86% crude protein, 0.37-0.75% crude fat, 0.93-1.20% ash and 2.69-5.84% carbohydrate [5]. The most abundant amino acid is glutamic acid (4.49-11.66 g/100 g) with the least being cysteine (0.34-1.45 g/100 g) [6]. Research has shown that the lowest amount of amino acid except for tryptophan occurred when rice is used as substrate to produce kunu beverage (0.44-1.40 g/100 g) [5]. Also, among the amino acids, cysteine, valine, isoleucine and methionine are present in trace amounts when compared with FAO/WHO reference protein values [6]. Considering the method of preparation of kunu which normally does not conform to standard preparation protocol and its nutritional constituents, kunu provides an ideal environment for the growth of food-borne microbial pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* spp. among others [7]. Kunu can also undergo spoilage by fermentation processes carried out by indigenous microorganisms. Studies have shown that kunu contains lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Streptococcus* spp. and *Leuconostoc* spp. that could cause spoilage [8]. Other organisms that could cause spoilage of the food drink when present in large amount include *Staphylococcus* spp., *Candida spp*. and *Trichoderma* spp. [8]. Poor hygiene and preparation practices also introduce microbial pathogens in foods and have been implicated in causing food-borne illnesses. This constitutes an alarming development with significant public health consequences [9].

The consumption of locally made kunu drinks by both old and young people including market women and children at home pose serious health threats because these products are subject to microbial contamination as producers do not adhere to standard methods of preparation. They also lack information on the microbiological safety of ready-to-eat beverages and their health implications [4]. The presence of *E. coli* indicates fecal contamination of finished products mainly at the point of preparation where untreated water is used. Other enteric and non-enteric pathogens may also thrive causing salmonellosis, *E. coli* infections, shigellosis, botulism, brucellosis, and

other food-borne diseases [9]. In a developing country like Nigeria, the food safety, control and regulation agencies are plagued with myriad challenges that hinder their performance. As such, effective control on processing of hawked foods including locally made kunu drinks is not possible. This means that, the health implication of consuming these kunu beverages remain very high. Therefore, this study was carried out to investigate the microbiological quality of kunu drinks and also to determine the antimicrobial pattern of the isolated microbes from kunu drinks locally produced and sold in Calabar.

2. MATERIALS AND METHODS

2.1 Study Area

This study was conducted in Calabar, Cross River State located in the Southern part of Nigeria. The city of Calabar is divided administratively into Calabar Municipal and Calabar South LGAs. It has a GPS coordinates of $4^{o}57'0"N$ $8^{o}19'30"E$ and a population of 371, 022 at the 2006 census. Calabar features a tropical monsoon climate with a lengthy wet season spanning 10 months and a short dry season covering the remaining two months. The people of Calabar are mostly the Efik speaking people. The city has an area of 406 square kilometers (157 sq mi) with many internationally recognized landmarks such as museum, Tinapa resort, and Calabar free trade zone among others.

2.2 Sample Size and Collection of Samples

A total of 9 samples of freshly prepared kunu drinks were purchased randomly from three different markets in Calabar, Cross River State. These markets were Watt Market, Goldie Market and Abasi-Obori Market. The samples were collected in 500 ml capacity sterile specimen containers in batches from 9 different producers, 3 samples from each market weekly. This was done to prevent spread of contamination from one source and its influence on the microbial content of the kunu samples. These samples were taken to Microbiology Laboratory of the University of Calabar, Calabar on Ice Park for microbiological analyses.

2.3 Determination of pH of the Samples

The pH of the various samples was determined using sterile probes of the pH meter (JenWay 3505).

2.4 Determination of Total Bacterial and Fungal Counts

Samples were serially diluted aseptically using 1ml of kunu samples with 9ml of sterile distilled water to reduce the microbial load. After dilution, about 0.1ml of appropriate dilution was used to inoculate Nutrient agar (NA), Mannitol salt agar (MSA) and MacConkey agar (MAC) plates in triplicates for isolation of bacteria; and potato dextrose agar (PDA) plates in triplicates for isolation of fungi. The culture plates for isolation of bacteria were incubated at 37°C for 24 hours while the PDA plates were incubated at 30°C for 96 hours for enumeration of colonies. The mean triplicate results on NA, MAC and PDA plates were then enumerated for total mesophilic bacteria, coliform and fungi respectively and recorded as colony forming unit per millimeter (cfu/ml) of kunu samples.

2.5 Purification of Microbial Isolates

Isolated colonies were subcultured on NA, MAC and MSA and incubated at 37°C for 24-48 hours to obtain pure isolates of bacteria. Pure fungal isolates were obtained by subculturing isolated fungal colonies on freshly prepared PDA plates. The plates were incubated at 30°C for 96 hours. After incubation, pure bacterial and fungal isolates were stored on NA and PDA slant prepared in Bijou bottles respectively. The stock cultures were then preserved in a refrigerator at $4^{o}C$ until used for further microbiological analyses.

2.6 Characterization and Identification of Bacterial and Fungal Isolates

Bacterial isolates were characterized and identified by observation of colonial, and morphological characteristics, Gram reaction and biochemical tests. The various biochemical test used for identification were the citrate utilization, catalase, methyl red, Voges Proskauer, coagulase, triple sugar iron, oxidase and motility-indole-ornithine tests [10]. Fungal isolates were characterized and identified based on colonial appearance and microscopic characteristics by wet mount preparation using Lacto-phenol in cotton blue. This was accomplished using appropriate identification scheme for yeast and molds respectively [11].

2.7 Antimicrobial Susceptibility Testing (AST)

This was carried out using the Kirby Bauer disc diffusion method. For bacterial isolates, inoculum

was prepared appropriately and adjusted to match the 0.5 McFarland turbidity standard. This was used to inoculate Mueller Hinton Agar (MHA) plates. A combination of six different antibiotics was used on a 90 mm plate each. These include standard antibiotic concentration of Ofloxacin, Ceftriaxone, Amoxicillin, Ampicillin, Gentamicin, Cloxacillin, Clindamycin, Imipenem, Cotrimoxazole and Erythromycin (Oxoid, UK). Standard strains used for quality control were *E. coli* ATCC 29522 and *S. aureus* ATCC 29523 for Gram positive and Gram negative isolates. The plates were incubated at 37°C for 24 hours and then examined for zones of inhibition. Results were interpreted in accordance with the CLSI guidelines and interpretive criteria [12].

Antimicrobial susceptibility testing for fungal isolates was done using antifungal discs containing standard concentrations of Griseofulvin, Ketoconazole and Fluconazole. Yeasts cultured on potato dextrose broth overnight were used to inoculate fresh PDA plates and antifungal discs were then placed at equidistant point on the plates. For molds, a small cut was made at the sub cultured plates onto the center of the fresh plates and allowed to stay for 6-10 hours before antifungal discs were placed. The plates were incubated at 25°C for 48 hours and the zone of inhibition (if any) was measured and interpreted by standard methods. Fungal isolates were quality controlled using *C. albican* (ATCC 90028) strain.

3. RESULTS

Table 1 shows the pH values and total microbial count of kunu drinks sold at three major markets in Calabar. The pH values range from 3.80-4.20. The total mesophilic bacteria counts range from 3.0-5.7x10^3, the coliform counts range from 2.1-4.1x10^3 while the fungal counts range from 5.1-8.6x10^4.

Table 2 shows the antimicrobial susceptibility patterns of bacteria isolated from kunu drinks. Of the 8 different antibiotics each used for susceptibility testing against the bacterial isolates. Gentamicin and Imipenem had 100% sensitivity to all the Gram positive bacteria while Clindamycin, Erythromycin and Cloxacillin showed 100% resistance to the test organisms. Also, Amoxicillin, Imipenem and Ofloxacin showed 100% sensitivity to all the Gram negative bacteria while, the highest resistance was seen with Cotrimoxazole (75%).

Table 3 shows the antimicrobial susceptibility patterns of fungal isolates from kunu drinks. Of the 5 fugal genera tested against 3 different antifungal agents viz: Griseofulvin (500 µg), Ketoconazole (400 µg) and Fluconazole (50 µg); yeast and *Aspergillus* sp. were sensitive to all the antifungal agents (100%) while *Fusarium* sp., *Penicillium* sp. and *Rhizopus* sp. resisted all the antifungal agents used (100%).

Figs. 1 and 2 shows the percentage occurrence of microbial isolates in kunu drinks sold in Calabar. A total of 40 bacterial isolates and 21 fungal isolates were obtained. Of the 40 bacterial isolates, *Lactobacillus* sp. had the highest percentage occurrence 9(22.5%) followed by *Bacillus* sp. and *E. coli* 6(15%). Whereas, for fungal isolates, yeast occurred most 6(28.5%) while *Fusarium* sp. had the least percentage occurrence 2(9.5%).

Table 1. The Mean pH values and total microbial count (cfu/ml) of Kunu drinks sold in three major markets in Calabar

Location	Sample	NA	(Mean)	MAC	(Mean)	PDA	(Mean)	pH
Watt market	01	3.2x10^3		2.5x10^3		9.0x10^4		
	02	5.0x10^3	(3.1x10^3)	1.5x10^3	(2.1x10^3)	2.5x10^4	(5.8x10^4)	4.10
	03	1.0x10^3		2.2x10^3		6.0x10^4		
Goldie market	04	4.0x10^3		1.0x10^3		6.0x10^4		
	05	3.5x10^3	(3.0x10^3)	0.5x10^3	(1.0x10^3)	7.2x10^4	(5.1x10^4)	4.20
	06	1.5x10^3		1.5x10^3		2.0x10^4		
Abasi-Obori market	07	5.5x10^3		2.0x10^3		8.2x10^4		
	08	10.0x10^3	(5.7x10^3)	3.0x10^3	(2.2x10^3)	10.0x10^4	(8.6x10^4)	3.80
	09	1.5x10^3		1.6x10		7.5x10^4		

Keys: NA=Nutrient Agar, MAC=MacConkey Agar, PDA=Potato Dextrose Agar

Table 2. Antimicrobial susceptibility patterns of bacterial isolates from Kunu drinks

Gram isolates	Antibiotic discs used							
	GEN	**IMP**	**OFX**	**CLN**	**CFX**	**E**	**COX**	**CLX**
S. aureus	S	S	S	R	R	R	S	R
Lactobacillus sp.	S	S	S	R	R	R	R	R
Streptococcus sp.	S	S	S	R	S	R	R	R
Bacillus sp.	S	S	R	R	R	R	R	R
% of Resistance	**0**	**0**	**25**	**100**	**75**	**100**	**75**	**100**
Gram Isolates	**AMX**	**AMP**	**IMP**	**GEN**	**OFX**	**COX**	**CFX**	**E**
E. coli	S	S	S	S	S	S	S	S
Proteus sp.	S	S	S	R	S	R	R	R
Pseudomonas sp.	S	R	S	R	S	R	R	R
Salmonella sp.	S	S	S	S	S	R	S	S
% of Resistance	**0**	**25**	**0**	**50**	**0**	**75**	**50**	**50**

Keys: S= Sensitive, R=Resistance, GEN=Gentamicin, IMP=Imipenem, OFX=Ofloxacin, CLN=Clindamycin, CFX=Cefuroxime, E=Erythromycin, COX=Cotrimoxazole, CLX=Cloxacillin, AMX=Amoxicillin, AMP=Ampicillin

Table 3. Antimicrobial susceptibility patterns of fungal isolates from Kunu drinks

Isolates	Antifungal agents			
	Griseofulvin (500 µg)	**Ketoconazole (400 µg)**	**Fluconazole (50 µg)**	**% of resistance**
Saccharomyces sp.	+	+	+	0
Fusarium sp.	-	-	-	100
Aspergillus sp.	+	+	+	0
Penicillium sp.	-	-	-	100
Rhizopus sp.	-	-	-	100

Keys: - = Resistance, + = Sensitive

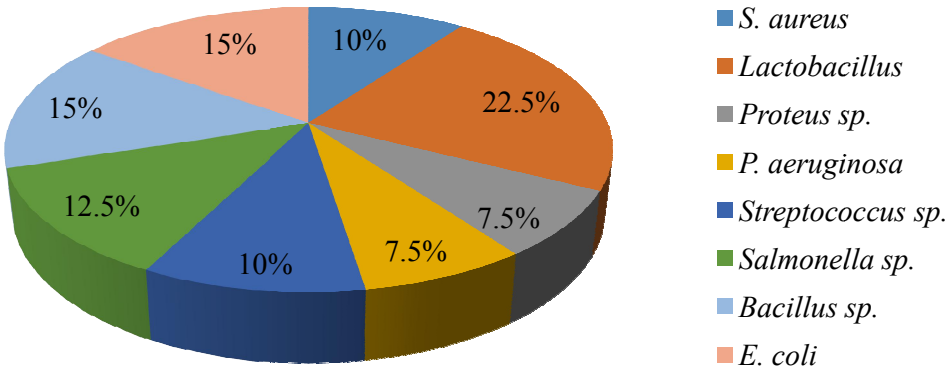

Fig. 1. Percentage of isolated bacteria in Kunu

4. DISCUSSION

Kunu drink is one of the most commonly consumed locally made non-alcoholic beverage drinks in Calabar and its environs. This drink is widely accepted by the community dwellers and is being produced in large quantities as substitutes and complements to carbonated drinks. The production is usually done under unhygienic conditions by the local producers devoid of supervision and control by food safety and regulatory bodies. Of the 9 kunu samples analysed to ascertain their microbial quality, the results showed that they were grossly contaminated with bacteria and fungi many of which could be pathogenic to humans. The presence of this high microbial load may be due to poor personal hygiene of the producers,

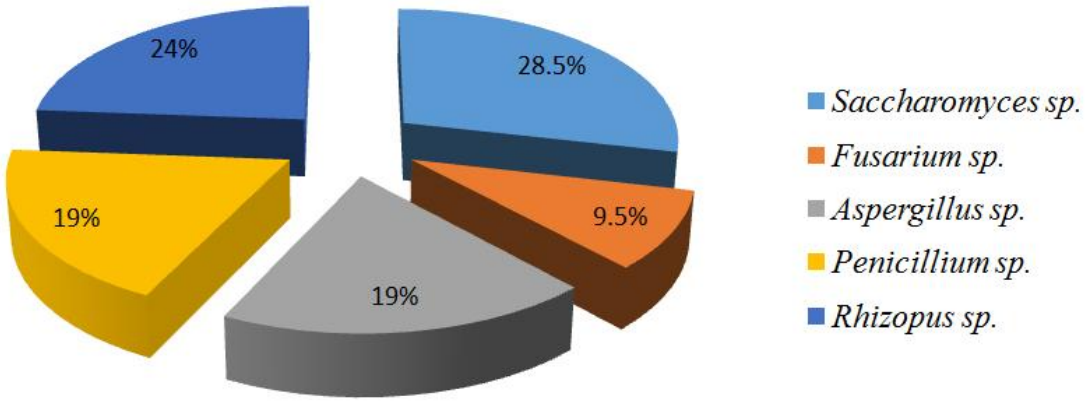

Fig. 2. Percentage of Isolated fungi in Kunu

spoilage of the substrates before use and improper handling and preparation of the product. These results of the microbial counts fall within the range reported by earlier studies [13, 14]. However, much higher microbial counts have been reported by other authors. For instance, total viable counts of 1.02-8.0x10^5 cfu/ml have been reported in kunu drinks sold in Port Harcourt Metropolis, Southern Nigeria [15], while in Oyo, Ogun, Lagos and Osun State, Southwestern Nigeria, total viable counts of 6.03-8.8x10^6 cfu/ml, total coliform counts of 2.2-5.6x10^6 cfu/ml, yeast counts of 2.054-2.323x10^7 cfu/ml and lactic bacteria counts of 4.965-5.725x10^7 cfu/ml have been reported in hawked kunu drinks [16].

It has been stated that the standard limit of <10^5 cfu/ml is permissible for aerobic mesophilic bacteria counts in food [17]. However, according to the revised guidelines for the assessment of microbiological quality of processed foods (cereals/cereals products) by the Food and Drug Administration (FDA), the set down maximum level of permissible microbial load for finished cereal products have been stated to include 10^2 cells/g for aerobic mesophilic bacteria, 5.0x10^2 cells/g for molds, 0.5x10^2 cells/g for coliforms and 0 mg/ml for *Escherichia coli* above which, the food is unfit for human consumption [18].

The microbial diversity in fresh kunu drinks locally produced and sold in Calabar is shown in Figs. 1 and 2. This result is consistent with that obtained by [13] from the same study area but differ slightly from that reported by [15] who isolated *Staphylococcus aureus, Enterobacter aerogenes, E. coli, Bacillus* sp., *Streptococcus* sp., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Saccharomyces* sp., *Candida* sp., *Salmonella* sp. and *Micrococcus* sp. from kunu drinks sold in major markets of Yenagoa Metropolis, Southern Nigeria. These contaminating organisms originate from different sources. The dominance of *Lactobacillus* as seen in this study showed that kunu drink is a lactic acid bacteria fermented beverage. This also account for the acidic nature of the drink. The mean hydrogen ion concentration (pH) of the various kunu samples ranged from 3.80-4.20. It has been shown that this acidity tends to increase during fermentation period resulting in spoilage. Consequently, the low pH values may have encouraged the growth of fungi as evident in this study. The isolation of enteric bacteria in kunu drinks such as *E. coli* is an indication of fecal contamination of the water used, *S. aureus* may be introduced by producers/handlers during and after packaging, while isolation of yeast and fungi have been linked to contamination of the cereals with soil/air-borne pathogens, packaging and processing environment [14].

The traditional preparation of kunu drinks involve cooking, a process which eliminates almost all these microorganisms except those that form spores such as *Bacillus sp.* The presence of these organisms in ready-to-eat kunu beverages may be due to contamination after cooking and cooling of the food [19]. These microorganisms have the potential to cause food borne illnesses such as food poisoning and food intoxication when consumed by humans. For instance, *Bacillus cereus* have been implicated in food poisoning, *S. aureus* can cause food poisoning and food intoxication by producing *Staphylococci* enterotoxin, the major cause of toxic shock syndrome (TSS) in humans, while *E. coli* and

Salmonella sp. could cause *E. coli* infections, enteric fever and bacillary dysentery respectively [20]. Fungi like *Penicillium*, *Fusarium* and *Aspergillus* produce potent mycotoxins capable of causing mycotoxocosis in humans. The type of mycotoxin produced depends on the type of fungi. For example, *Aspergillus sp.* produces aflatoxins which cause aflatoxicosis in humans [21].

The antimicrobial susceptibility test results showed 100% resistance of the Gram positive bacterial isolates to Clindamycin, Erythromycin and Cefuroxime, while the Gram negative ones showed high resistance (75%) to Cotrimoxazole and moderate resistances (50%) to Erythromycin and Cefuroxime. However, increase sensitivity (100%) was recorded with Gentamicin, Imipenem, Amoxicillin and Ofloxacin against the test bacterial isolates. Also, fungal isolates such as *Saccharomyces* sp. and *Aspergillus* sp. showed 100% sensitivity to different concentrations of Griseofulvin, Ketoconazole and Fluconazole used while, *Penicillium sp*., *Rhizopus* sp. and *Fusarium* sp. showed 100% resistance to the same antifungal agents. This result was in agreement with that obtained in a similar study carried out at Ogbomosho, Oyo State, Southwestern Nigeria by [22] who reported 100% resistance of the bacterial isolates from kunu drinks to Clindamycin, Cloxacillin, Ceftriaxone and Erythromycin, as well as Griseofulvin and Ketoconazole against *Aspergillus* and *Penicillium* isolates at different concentrations. The reason for this resistance may be due to abuse by people and widespread use of these antimicrobial agents in the society for purposes other than therapeutic use by humans [23].

5. CONCLUSION

The results of this study showed that the microbial content of kunu drinks locally prepared and sold in Calabar is high due to gross microbial contamination. The most predominant microbial pathogens were *Rhizopus* sp. (24%), *Lactobacillus* sp. (22.5%), *Bacillus* sp. and *E. coli* (15%), followed by *Salmonella* sp. (12.5%). Some antimicrobial agents used were highly effective in eliminating the isolates in vitro, whereas others showed marked resistance. The results of this study also showed that although, the microbial counts in these drinks are within the standard limit of $<10^5$ cfu/ml for aerobic mesophilic bacteria in food, they do not conform to that stipulated by FDA for permissible microbial load in cereals/finished cereal products. This implies that, consumption of these products may constitute a serious challenge to public health. Hence, there is need for regular monitoring of the local production protocol by the food safety agencies and sensitization on the importance of good hygiene and sanitation during preparation and processing of kunu and other drinks in order to reduce microbial contamination to acceptable level and improve the final product quality.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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