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Comparative Evaluation of Enterotoxigenic Bacteria in Kunu and Zobo Drinks in Bayelsa

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Abstract

Background: Kunu and zobo are non-alcoholic drinks made from grains and calyx of hibiscus. This study aimed to isolate, identify potentially pathogenic bacteria strains and determine the enterotoxin production abilities of some bacteria strains.

Methods: A cross-sectional sampling of 150 bottles each of kunu and zobo respectively. The beverages were serially diluted, cultured on appropriate media and incubated at 37°C for 18-24 hrs and examined for growth. Isolates were identified and enterotoxin production was determined.

Results: The percentage occurrences rods isolated from kunu were *E. coli* 150 (27.8%) and Bacillus sp. were 150 (27.8%), while isolates from zobo were Salmonella sp. 90 (18.6%). and Bacillus sp. were 150(31.3%). The percentage of cocci isolated from kunu were *S. aureus* 150(27.8%) and S. aureus from zobo were 120(25.0%); coagulase-negative *Staphylococci sp*. were 120(25.0%) from zobo respectively. *Streptococci sp* isolated from kunu were 90(16.7%). The percentage of enterotoxins producing *E. coli* isolated from kunu was 19(12.7%), while the percentage of enterotoxins producing S. aureus from kunu was 25(16.7%) and 18(15.0%) from zobo respectively. The counts of bacteria in refrigerated kunu was 7.5 x 105 to 8.7 x 105 CFU/ml and 7.5 x 105 to 1.3 x 106 CFU/ml at ambient temperature while in zobo refrigerated the count was 1.1 x 103 to 1.6 x 103 CFU/ml and 1.1 x 103 to 2.5 x 103 CFU/ml at ambient temperature.

Conclusion: The study identified some enterotoxin-producing *E. coli* and *S. aureus* from the beverages.

Keywords: Non-Alcoholic Drinks; Enterotoxigenic Bacteria; Microbiological Quality

Introduction

Kunu (kununzaki) is a beverage made from grains of millet, sorghum and maize and combinations. It's popular in northern parts of Nigeria. Kunu produced from sorghum is milky light-brown coloured, while the product from maize is whitish [1]. The grain seeds used for the production of kunu drink were steeped for few days, allowed to germinate and blended with sweet potatoes, ginger and pepper to form a paste. The paste is divided into two, one part is placed in a vessel boiled water is added to form a thick mixture. The unheated half is added to the previous and stirred to give a thick mixture. The mixture is left for 1-2 days for it to settle. The husk and other sediments are filtered off the mixture and the filtrate is boiled for consumption.

Zobo, a non-alcoholic beverage is made from dried petals, acid-succulent calyxes of Hibiscus sabdariffa by boiling and filtrating [2,3]. Hibiscus sabdariffa is an annual erect, herbaceous shrub with smooth cylindrical and a typically red

stem. The flowers are mostly cultivated in Northern Nigeria. The beverage is popular in West Africa, sold in schools and served in social gatherings as an alternative, cheap, relaxing non-alcoholic [4,5]. The beverage has a soured taste but is often sweetened. The name zobo is derived from zoborodo in Hausa, goneura in Hindi, krajeab in Thailand, bissap in Senegal and sorrel in the Caribbean [6]. Zobo beverage is more popular in Northern Nigeria [7]. It was reported that zobo is a traditional medicine for the treatment of several diseases such as hypertension and urinary tract infection [8].

Kunu beverage is marketed in several public places such as offices, markets, schools, motor parks and commonly is consumed during festivities such as weddings, naming ceremonies, birthday celebrations etc. [9]. Kunu is an appetizer, food complement and refresher to quench thirst [10-12]. The proximate composition of Kunu includes; protein 2.3 – 3.6%, fats 3.4 – 3.7%, ash content 1.2 – 1.2% and carbohydrates 82.9 – 83.6% [1].

The poor bacterial quality of kunu is an indication of poor hygiene, poor quality cereals and poor water qualities used in preparation and packaging processes [13]. The bacteria isolated from kunu were *E. coli* 33.3%, *S. aureus* 26.7%, Streptococcus sp. 23.3%, Pseudomonas sp. 10% and Bacillus sp. 6.7% [13,14] investigating the microbial quality of kunu isolated Bacillus sp. 15%, *E. coli* 15%, Salmonella sp. 12.5%, Streptococcus sp.10%, Pseudomonas sp. 7.5%, Proteus sp. 7.5%, Lactobacillus 22.5% and *S. aureus* 10% respectively. Some bacterial isolates from kunu might be responsible for food spoilage, food infections and poisons [15].

Bacteria isolates from zobo include *S.aureus*, Bacillus sp, Lactobacillus sp, Escherichia coli, Pseudomonas sp Enterobacter sp. [16,17] isolated *S.aureus*, Streptococci sp. and Proteus sp. Others isolated *S.aureus*, Bacillus sp. Micrococcus, Proteus sp. Streptococci sp, *E. coli* [18]. The studies aims to determine the bacteriological quality of kunu and zobo marketed in Bayelsa State, the percentages occurrence of isolated bacteria, enterotoxin producing *E. coli* and *S. aureus* and the beverages shelf life.

Materials and Methods

Study Area

The research was carried out in Bayelsa State, Nigeria. Samples of zobo and kunu drinks were procured from Yenagoa, Sagbama and Ogbia town. Bayelsa State is situated in latitude 4° 15' North, latitude 5°23' South and longitude 5°22' West and longitude 6°45' East (Alagoa, 2009). It is bound by Delta State on the North, River State on the East and the Atlantic Ocean on the West and South. Bayelsa has the largest wetland in the West African sub-region. Its population is about 1.7 million people [19].

Collection of Samples

A total of 150 bottles of kunu and 150 bottles of Zobo were purchased from Yenegoa, Sagbama and Ogbia town respectively. The samples were transported to the laboratory in a cold chain.

Bacteriological Examination of Samples

A total of 150 bottles of kunu and 150 bottles of Zobo were purchased from Yenegoa, Sagbama and Ogbia town respectively. The samples were transported to the laboratory in a cold chain. Kunu and Zobo drinks were sold in recycled bottles.

Bacterial Counts at Different Temperature

A set of freshly prepared kunu and zobo were stored at ambient temperature and another set was refrigerated at about 4°C after the initial bacteria counts. The counts from the preserved Kunu and Zobo at room and refrigeration temperature were re-determined on the second and third day of storage.

Identification of Isolates

The bacterial isolates were identified using morphology, culture, Gram's stain reaction, chemical and biochemical reactions such as citrate, VP, Methyl red, indole, catalase, coagulase and carbohydrate fermentation.

Detection of Enterotoxin Producing *E. coli* and *S. aureus*

Enterotoxin-producing *E. coli* and *S. aureus* isolates were detected using PROTM 0157 and Prolex™ Staph Latex kits respectively.

Statistical Analysis

Data analysis was done using Graph Pad Prism 6.

Results

Percentage Occurrences of Bacteria Isolated from Kunu and Zobo

Table 1 showed percentage occurrences of rods shaped bacteria isolated from kunu and zobo. A total of 150 samples of kunu were examined out of which 50 samples were

examined from each location. The total number of *E. coli* isolated from kunu purchased from Yenagoa was 50 (27.8%), Sagbama 50(27.0%) and Ogbia 50(27.8%) respectively. *E. coli* were not isolated from zobo 0(0.0%). The number of Salmonella sp. isolated from kunu was 0(0.0%) and Salmonella from zobo isolated from Yenagoa was 25(16.7%), Sagbama 35(20.0%) and 30(19.4%) respectively but no Salmonella sp. was isolated from kunu 0(0.00). Bacillus sp. recovered from kunu in Yenagoa was 50(27.8%), Sagbama 50(27.0%) and Ogbia 50(28.6%) while from zobo bought in Yenagoa was 50(33.3%), Sagbama 50(28.6%) and Ogbia 50(32.3%) respectively.

	Bacteria								
	E. coli			***Salmonella* sp.**			***Bacillus* sp.**		
Location	Kunu	Zobo	*P*-value	Kunu	Zobo	*P*-value	Kunu	Zobo	*P*-value
Yenegoa	50(27.8)	0(0.0)	<0.0001	0(0.0)	25(16.7)	<0.0001	50(27.8)	50(33.3)	<0.0001
Sagbama	50(27.0)	0(0.0)	<0.0001	0(0.0)	35(20.0)	<0.0001	50(27.0)	50(28.6)	<0.0001
Ogbia	50(28.6)	0(0.0)	<0.0001	0(0.0)	30(19.4)	<0.0001	50(28.6)	50(32.3)	<0.0001
Total	150(27.8)	0(0.0)		0(0.0)	90(18.6)		150(27.8)	150(31.3)	

Numbers in Parenthesis = Percentages

Table 1: Percentage Occurrences of Rods Shaped Bacteria Isolated from Kunu and Zobo.

Percentage Occurrences of Cocci Bacteria Isolated from Kunu and Zobo

Table 2 depicts the percentage occurrences of cocci bacteria isolated from kunu and zobo. The percentage occurrences of *S. aureus* from kunu in Yenagoa was 50(27.8%), Sagbama 50(27.8%) and Ogbia 50(27.8%), and from zobo purchased in Yenagoa was 40(26.7%), Sagbama 40(25.7%) and Ogbia 35 (22.6%) respectively. Coagulase-negative Staphylococci from zobo were Yenagoa 33(23.3%), Sagbama 45(25.7%) and Ogbia 40(25.8%) respectively. Coagulase-negative Staphylococci were not isolated from kunu. Streptococci sp. isolated from kunu bought in Yenagoa was 30 (16.7%), Sagbama 35(18.9%) and Ogbia 25(14.3%) respectively.

Organism	***S. aureus***			**Coagulase –ve *Staphylococci***			***Streptococci* sp.**		
Location	**Kunu**	**Zobo**	***P*-value**	**Kunu**	**Zobo**	***P*-value**	**Kunu**	**Zobo**	***P*-value**
Yenegoa	50(27.8)	40(26.7)	>0.9999	0(0.0)	33(23.3)	<0.0001	30(16.7)	0(0.0)	<0.0001
Sagbama	50(27.8)	40(25.7)	>0.9999	0(0.0)	45(25.7)	<0.0001	35 (18.9)	0(0.0)	<0.0001
Ogbia	50(27.8)	35(22.6)	0.4639	0(0.0)	40(25.8)	<0.0001	25 (14.3)	0(0.0)	<0.0001
Total	150(27.8)	120(25.0)		0(0.0)	120(25.0)		90 (16.7)	0(0.0)	

Table 2: Percentage Occurrences of Cocci Bacteria Isolated from Kunu and Zobo.

Percentages of Enterotoxin Producing *S. aureus* and *E. coli* from Kunu and Zobo

Table 3 represents the percentages of enterotoxin producing *S. aureus* and *E. coli* from kunu and zobo. A total of 50 *S. aureus* were isolated from kunu in Yenegoa out of which 7(28%) were enterotoxin producing strains and 50 *S. aureus* were isolated from Sagbama, 10(40%) produced enterotoxin, while in Ogbia Town 50 *S. aureus* were isolated, 8(32%) were positive for enterotoxin production respectively. The overall percentage of *S. aureus* that produced enterotoxin was 25(33.2%). The number of *E. coli* isolated were 150, 50 each from Yenegoa, Sagbama and Ogbia, 7(37%) produced enterotoxin from kunu bought from Yenegoa and Ogbia respectively, while 5 (26%) were from kunu purchased from Sagbama.

Out of the 40 isolates of *S. aureus* obtained from zobo drinks, 4(22%) produced enterotoxin from the Yenegoa zone, out of 45 *S. aureus* isolated from zobo purchase in Sagbama, 6(33%) produced enterotoxin, while in Ogbia, 35 *S. aureus* were isolated and 8(37%) produced enterotoxin respectively.

Organisms	*S. aureus*			*E. coli*		
Location	**Kunu**	**Zobo**	***p*-value**	**Kunu**	**Zobo**	***p*-value**
Yenegoa	7(28)	4 (22)	0.5818	7(37)	0(0.00)	0.0165
Sagbama	10(40)	6 (33)	0.5644	5(26)	0(0.00)	0.0435
Ogbia	8(32)	8 (45)	0.6447	7(37)	0(0.00)	0.0165
Total	25(16.7)	18(15.0)		19(12.7)	0 (0.00)	

Numbers in Parenthesis = Percentages

Table 3: Percentages of Enterotoxin Producing *S. aureus* and *E. coli* from Kunu and Zobo.

Bacterial counts in preserved Kunu and Zobo

The count of bacteria isolated from kunu after preparation (ready to drink) at 0 hr was 7.5 x 10^5 CFU/ml, at 24 hrs it was 8.4 x 10^5 CFU/ml and at 48 hrs 8.7 x 10^5 CFU/ml respectively. The count bacteria from kunu preserved at ambient temperature was 7.5 x 10^5 CFU/ml at 0 hr, 12.0 x 10^5 CFU/ml at 24 hrs and 13 x 10^5 CFU/ml at 48 hrs respectively. The total counts of bacteria isolated from preserved zobo at refrigeration temperature were; 1.1×10^3 CFU/ml at 0 hr, 1.6×10^3 CFU/ml at 24 hrs and 1.6×10^3 CFU/ml at 48 hrs respectively. The counts obtained from the set preserved at ambient temperature were 1.14×10^3 CFU/ml at 0 hr, 2.34×10^3 CFU/ml at 24 hrs and 2.54×10^3 CFU/ml at 48 hrs respectively (Figure 1).

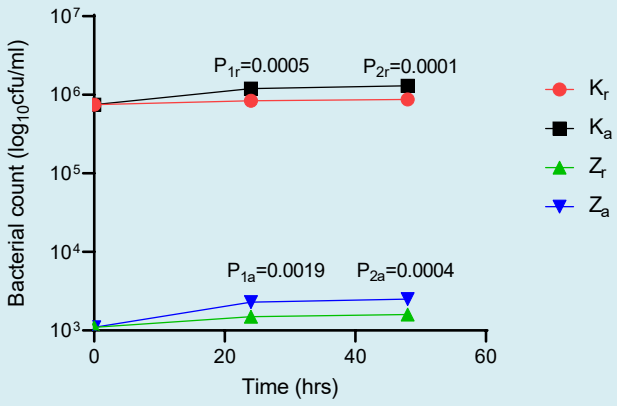

Figure 1: Time-dependent growth response of bacteria in Kunu and Zobo at refrigerated and ambient temperature. K= Kunu, Ka=Ambient, Kr=Refrigerated, Z= Zobo, Za=Ambient, Zr= Refrigerated.

Post-hoc Analyses of the Effect of Time on the Growth Response of Bacteria

Turkey's multiple tests at 95% Cl showed there was no significant difference between the growth of bacteria in kunu at 0 hr vs 24 hrs, 0 hrs and 48 hrs, 24 vs 48 hrs respectively. In zobo at 0 hrs vs 24 hrs, 0 hrs vs 48 hrs, and 24 hrs vs 48 hrs respectively. The other comparisons were significant (Table 4 & Figure 2).

Tukey's multiple comparisons	95.00% CI of diff.	*p*-value
0k vs 24k	-911247 to 1461247	0.6073
0k vs 48k	-931247 to 1441247	0.4181
0k vs 0z	-432397 to 1940097	0.0250
0k vs 24z	-433147 to 1939347	0.0251
0k vs 48z	-433297 to 1939197	0.0252
24k vs 48k	-1206247 to 1166247	0.9979
24k vs 0z	-707397 to 1665097	0.0057
24k vs 24z	-708147 to 1664347	0.0057
24k vs 48z	-708297 to 1664197	0.0058
48k vs 0z	-687397 to 1685097	0.0042
48k vs 24z	-688147 to 1684347	0.0042
48k vs 48z	-688297 to 1684197	0.0042
0z vs 24z	-1186997 to 1185497	>0.9999
0z vs 48z	-1187147 to 1185347	>0.9999
24z vs 48z	-1186397 to 1186097	>0.9999

Keys: z represents zobo and k represents kunu. ANOVA p=<0.0001.

Table 4: Post-hoc analyses of the effect of time on growth response of bacteria in Kunu and Zobo

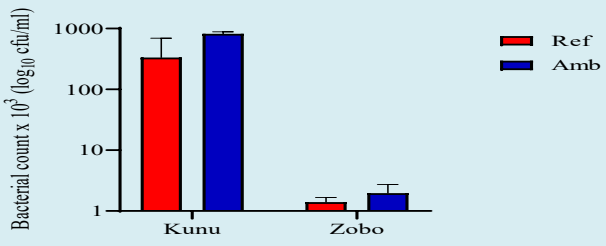

Figure 2: Effect of temperature on growth response of bacteria in Kunu and Zobo.

Post-hoc Analyses of the Effect of Temperature on Growth Response of Bacteria

Turkey's multiple comparison showed at 95% CI, Rk vs Ak and Rz vs Az were not significant respectively while the other comparisons were all significant (Table 5).

Tukey's multiple comparisons	95.00% CI of diff.	*p*-value
Rk vs Ak	7.857 to 965.5	0.2159
Rk vs Rz	339.8 to 1297	0.0007
Rk vs Az	339.2 to 1297	0.0007
Ak vs Rz	-146.9 to 810.7	<0.0001
Ak vs Az	-147.4 to 810.2	<0.0001
Rz vs Az	-479.4 to 478.2	>0.9999

Keys: z = Zobo and k = Kunu, R = refrigeration temperature, A = ambient temperature. ANOVA p= <0.0001

Table 5: Post-hoc analyses of the effect of temperature on growth response of bacteria in Kunu and Zobo.

Discussion

The rod-shaped bacterium isolated from kunu was *E. coli* and Salmonella sp from zobo while Bacillus sp. was isolated from both kunu and zobo non-alcoholic beverages. There was a significant difference among the isolates using chi-square at > 0.05. *E. coli* was isolated from kunu only, while Salmonella sp. was isolated from zobo only. The presence of *E. coli* and Salmonella sp. indicates contamination with human or animal faeces during processing. Similar rod-shaped bacteria were isolated by [15,20,21]. The spices used to boost the nutritional and sensory qualities of kunu and zobo drinks such as ginger, garlic etc. may have some level exhibit inhibitory effects on some bacteria. Zobo and kunu drinks are always spiced with ginger, garlic or a mixture of ginger and garlic and other spices [22]. These spices are also known to improve the pH level to slightly alkaline which may be hostile to some bacteria especially with the garlic and ginger combined as spices [22]. The spices apart from improving the nutritional properties of kunu or zobo, have been shown to possess antimicrobial properties [20,21,23]. Zobo drink has high phenolic and flavonoids, mainly polyphenol which has been identified to possess antimicrobial activities [24]. Their proximate composition includes alkaloids, saponins and tannins at different concentrations [25]. The extract from Hibiscus sabdariffa used for zobo preparation may possess minimal antimicrobial properties but when combined with spices such as ginger, garlic etc. might demonstrate good antimicrobial activities. The addition of spices contributes to the nutritional quality of kunu and zobo and on the other hand, might influence the development of resistance to the spices and possibly trigger resistance of bacteria to some therapeutic agents. The presence of Bacillus sp. in kunu and zobo concomitantly suggest contamination with soil because Bacillus sp. are geophilic and the spores can survive boiling (sterilization) at 100oC and germinate later to cause spoilage [26]. All the rods isolated from kunu and zobo were of public health importance and it calls for caution to consumers of the beverages. Bacillus is associated with food poisoning and infections.

The most predominant bacterium isolated from kunu and zobo was Staphylococci sp. Staphylococci are normal human flora carried on the palms, skin, nostrils etc. transmission to food can occur via human contacts, nasal droplets, desquamated skin dropping. The heavy growth observed in zobo and kunu may portray laxity in hygiene and deficiencies in the knowledge of food processing and preservation techniques [27,28]. Streptococci sp. is associated with health conditions such as impetigo, erysipelas, cellulitis, puerperal sepsis, acute rheumatic fever etc [6].

There was a significant difference in the counts of bacteria isolated from kunu and zobo preserved at ambient temperature compared to those refrigerated. This suggests the rapid proliferation of bacteria in both beverages at ambient temperature which accounts for poor keeping quality. The isolation of *S. aureus*, Salmonella sp., *E. coli* and Bacillus sp. might predict that the beverages are good media for the growth of entero-pathogens capable of causing gastroenteritis. Therefore, consumers of the beverages should bear in mind the risks that could be associated with its consumption due to the poor microbiological quality. Other workers also remarked that local produced kunu and zobo preserved at ambient temperature for 24hrs and beyond may pose a public health risk to the consumers. Both Zumbes & Shimelis [27,29] noted that the shelf life of kunu depends on the temperature of storage. The microbiological quality of locally produced drinks is determined by total microbial counts and the pathogenic bacteria present in them.

The ability of a strain of bacterium to produce toxin enhances its pathogenic ability, for example, the production of enterotoxins are associated with gastroenteritis. Foodborne infections are of public health concern worldwide. One of the commonest foodborne illnesses is staphylococcal food poisoning due to enterotoxins pre-formed in food by enterotoxigenic strains of coagulase-positive *S. aureus*. Staphylococci can survive desiccation and halophilic conditions but are destroyed by heat. The already produced enterotoxin in food can withstand approved irradiation and some recommended heat treatments including pasteurization [30]. Kunu and zobo non- alcoholic beverages are favourable for the growth of *S. aureus* and can be implicated in food poisoning. The isolation of 25(16.7%) and 18(15%) of

enterotoxins producing *S. aureus* from kunu and zobo concomitantly was an indication of serious health risks that may be associated with consumers of products, for example, food-borne intoxication. Staphylococcal enterotoxin (SEs) are secreted proteins that use super-antigenic and emetic activities, they are resistant to proteolytic enzymes which enables them to maintain their activity in the digestive tract. The property enables staphylococcal SEs to cause severe gastroenteritis with nausea, emesis, and diarrhoea.

Enterotoxigenic *E. coli* (ETEC) are *E. coli* strains that produce toxins that stimulate the intestine by causing it to produce excess fluid (diarrhoea). The main source of ETEC is human and or animal wastes. Infection ensues when a person eats food, drink water or ice contaminated with ETEC. ETEC is a major cause of traveller's diarrhoea and diarrhoea in children mostly in developing countries like Nigeria. Other researchers have isolated *E. coli* from kunu [15,29]. The percentage of ETEC producing *E. coli* 19(27.7%) isolated from kunu calls for caution in the consumption of these non-alcoholic beverages.

Kunu is produced from the combinations of millet, sorghum, maize etc., they were steeped for few days and allowed to sprout and blend with sweet potatoes, after which spices such as ginger, pepper, garlic etc. were added. The multiple raw materials used to compound kunu make it a more nutritious and good medium for bacterial growth. The multiple steps involved in kunu preparation and indigenous microbiota from the different raw materials exposes the final product to microbial contamination, compared to zobo which uses only the calyx of Hibiscus sabdariffa and spices. The reasons might account for the higher counts observed in kunu at 0Hrs, 24Hrs and 48Hrs respectively, coupled with the deficiency in food processing and preservative methods of producers. The result of Post-hoc analysis of the effect of time on the growth response of bacteria was significant for kunu and zobo. Post-hoc analyses of the effect of temperature on the growth response of bacteria in kunu and zobo were also significant.

Conclusion

The microbiological quality of locally produced kunu and zobo in Bayelsa State are poor. The isolation of bacteria capable of producing toxins calls for caution in the consumption of both beverages. The shelf life of both kunu and zobo at ambient temperature are less than 24 hours.

Limitation Of Study

The limitation of this study was the non assay for the presence of enterotoxins produced by the *E. coli* and *S. aureus* in the beverages.

Declaration of Conflict of Interest

The authors which to declare that there was no conflict of interest during and after the development of the manuscript.

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