The Public Health Significance of Pathogens Isolated from "Kunun-Zaki", Sold in Retail Outlets in Zaria, Nigeria

Umoh,* V.J.; Oranusi, S. U. and Kwaga, [1]J. K. P
Department of Microbiology, Veterinary Public Health and Preventive Medicine,
Ahmadu Bello University, Zaria, Kaduna-State

*Corresponding Author: veroumoh@hotmail.com

ABSTRACT

This study assesses the presence and antimicrobial sensitivity of some pathogens and the enterotoxigenicity of *Bacillus cereus* isolated from "Kunun-zaki". Counts of *B. cereus* and *S. aureus*, the isolation of *Escherichia coli, Salmonella* and *Shigella* and the antimicrobial sensitivity of the pathogens were carried out using standard procedures. The processing of kunun-zaki by one producer was studied to note the possible points of contamination. The ability of *B. cereus* to produce toxin was tested using ileal loop technique. Out of 240 samples of "kunun-zaki" purchased from retail outlets, 105 had *B. cereus* counts of less than 10^4 cells ml^{-1}. *S. aureus* counts ranged from 68 to 7.2×10^2 cells ml^{-1}. All the samples were contaminated with *B. cereus*, 221(92.1%) had *E. coli* and 216(90.0%) had *S. aureus*. *Salmonella* and *Shigella* were not present in any of the products. The pathogenic bacteria isolated were alpha and beta haemolytic *S. aureus*, faecal strains of *E. coli* and enterotoxin producing *B. cereus*. The pathogens were highly sensitive to Floxacin, Gentamycin and Tetracycline. These agents are however, not in common use for the treatment of infections caused by the pathogens isolated. The high frequency of contamination and antimicrobial resistant patterns observed, calls for hygienic handling and control processing of kunun-zaki, to reduce hazards to health.

Key Words: Pathogens, "Kunun-zaki", Retail outlets

INTRODUCTION

"Kunun-zaki" is one of the indigenous non-alcoholic beverages prepared from guinea corn (*Sorghum bicolor*), millet (*Pennisetum typhoides*), maize (*zea mays*), rice (*Oryza sativa*) or wheat (*Triticum aestivum*). Its wide acceptance has extended beyond the savannah region of Nigeria. It is consumed at anytime of the day by both adults and children as breakfast drink, food complement, refreshing drink for visitors, appetizer and is commonly served in social gatherings.

"Kunun-zaki" is a fermented drink either packed for sale in polyvinyl chloride (PVC) bags, bottled or in bulk in large containers and distributed under ambient temperature or cooled in refrigerator where available. Fermentation in food processing can improve the hygienic quality of food. However, such practices as frequent handling ssof food with hands, addition of raw and contaminated ingredients, use of contaminated water and preparation of foods in a polluted environment can expose the food to contamination with pathogens which could result in health hazards (Ehiri *et al.*, 2001). "Kunun-zaki" has been shown to be contaminated with staphylococci but more information on the presence of other pathogens of public health importance is essential (Onuorah *et al.*, 1985). For instance, *B. cereus* is a spore former and can survive various food-processing methods (Yusuf *et al.*, 1992). It is often implicated in food borne gastroenteritis, a potential cause of infections in humans and animals and may give rise to food spoilage (Borchardt *et al.*, 1982, Fossum *et al.*, 1986, Yusuf, *et al.*, 1992 Umoh *et al.*, 1995, Faille *et al.*, 2001). *S. aureus* too has been estimated to cause 185,060 illness, 1,753 hospitalizations, and 2 deaths per year in the United States, all of which were via consumption of contaminated foods (Mead *et al.*, 1999). Enterotoxigenic and enteropathogenic *E. coli* presently the leading cause of diarrhoea is associated with significant mortality rates (Rappelli *et al.*, 2001). Based on

this, it was necessary to assess "kunun-zaki" for the presence of such pathogens as *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella* and *Shigella*. The enterotoxigenicity of *B. cereus* and sensitivity of the pathogens isolated to antimicrobial agents were studied. The processing of Kunun-zaki by one producer was studied to note the possible points of contamination.

MATERIALS AND METHODS

Sample Collection

Two hundred and forty samples comprising 130 PVC packed, 90 bottled and 20 bulk packed samples were purchased from the retail outlets around Zaria. The samples were purchased into sterile containers and transported in ice packs to the laboratory for analysis,

Traditional "Kunun-zaki" Production

One of the producers was visited to observe the method of processing and handling. The method of preparation involved the use of a mixture of millet (*Pennisetum typhoides*) and Guinea corn (*Sorghum bicolor*). The grains were steeped for 12 hours (overnight), wet milled with spices, which included ginger (*Zingiber officinale*), red pepper (*Capsicum amum*), cloves (*Eugenia calophylloides*) and black pepper (*Pipper nigrum*). About $^1/_4$ quantity of the wet milled mixture was removed and mixed with wet milled sweet potato (*Ipomoea batatas*) for use in pitching. The remaining ¾ quantity was gelled with hot water, allowed to cool before pitching with the $^1/_4$ mixture. The final mixture was allowed to ferment overnight before sieving. The supernatant fluid was diluted with water to required consistency, sweetened with sugar to taste and packed in PVC bags ready for sale (Fig.1). The remnants were sundried and used as animal feed.

Enumeration of Microorganisms

The PVC packed "kunun-zaki" was cleaned externally with 70% alcohol and the content poured into sterile containers. An aliquot of 100ml of each sample was centrifuged at 5000 rpm for 5 min and the supernatant decanted leaving 5.0ml in each tube. After mixing thoroughly 0.2ml was spread inoculated on Baird Parker agar (BPA) (Oxoid) for isolation of *S. aureus* and tryptone dextrose agar (TDA) (Oxoid) for *B. cereus*. One milliliter was inoculated into 9ml lactose broth for enrichment and streaked on eosine methylene blue (EMB) agar for isolation of *E. coli. Salmonella* and *Shigella* isolation was by pre-enrichment in peptone water, enrichment in selenite F and streaking on salmonella-shigella agar (SSA) (Oxoid). All the inoculated tubes and plates were incubated at 37^{0}C for 24hrs except lactose broth, which was incubated at 44^{0}C for 24 hrs.

Characterization of isolates

Typical colonies on EMB plates appearing bluish black with greenish metallic sheen characteristic of *E. coli* were stored on nutrient agar slants for further characterization. The confirmatory tests included indole, methylred, Voges-Proskauer (VP), citrate, carbohydrate utilization, and reaction on Triple Sugar Iron (TSI), gelatin liquefaction, nitrate reduction, urease production and motility (Speck 1976). All Gram negative isolates from EMB that were methylred and nitrate positive but VP, citrate and urease negative, ferment carbohydrates with or without gas and produced acid and gas on TSI were confirmed as *E. coli*. Colonies on TDA that appeared large, flat, irregular, wrinkled or smooth, 4-6mm in diameter were counted as *B. cereus* and confirmed as described by Yusuf *et al.*, (1992). Typical colonies on BPA were counted as *S. aureus* and confirmed on the bases of the result of catalase, coagulase, phosphates, nitrate and carbohydrate utilization. *S. aureus* isolates were inoculated on blood agar base (Oxoid) containing 10% sheep blood. The plates were incubated at 37^{0}C for 24 hours and zones of haemolysis around colonies were observed and recorded as alpha (á), beta (â) or gamma (ã) (Umoh *et al.*, 1990a). For the confirmation of *Salmonella* and *Shigella* procedures recommended by Speck (1976) were followed.

Susceptibility of *B. cereus, E. coli* and *S. aureus* to antimicrobial agents

The disc diffusion method of Bauer *et al.*, (1966) using Muller Hinton agar (Oxoid) and the following disc concentrations were employed: ampicillin (25µg), cephalexin (25µg), cefuroxime

(30μg), cotrimoxazole (50μg),floxaxin (10μg), gentamycin (10μg), nitrofurantoin (200ug)and tetracycline (50μg) (Brodisks) ampicillin (2μg), chloramphenicol (10μg), cloxacillin (5μg), erythromycin (10μg), penicillin G (1.5iu), sulphafurazole (100μg), streptomycin (10μg) and amoxicillin (2μg) (Oxoid Multodisks). *S. aureus* (NCTC 6571) and *E. coli* (K. 1218Nx) lactose positive, nalidisic acid resistant were used as controls. A test organism was considered susceptible to an agent when the difference in the zone of inhibition in the test organisms were not more than 3mm as compared to the zone of inhibition of the control (Umoh *et al.*, 1990b).

Enterotoxin Production, Extraction and Toxicity Test

Ten *B. cereus* isolates from "kunun-zaki" were tested for their ability to produce enterotoxin. The method of Kramer *et al.*, (1982) was used in the production and extraction of enterotoxin in brain heart infusion broth (Oxoid) with 0.1%($^{w}/_{v}$) glucose supplement (BHIG). The ligated New Zealand white rabbit ileal loop test was used (Yusuf *et al.*, 1992). The extracts and sterile BHIG as control were injected intraluminally into the test loop. The fluid length ratios ranging from 0.2 to 0.4 were considered to be mild, 0.5 to 0.7 as moderate and 0.8 to1.5 as severe.

Pathogenicity of *E. coli* was based on ability to grow at elevated temperature (44.5^{0}C) in 24hours (Speck 1976). Pathogenicity testing of *S. aureus* was based on coagulase and haemolusin production (Umoh *et al.*, 1990a).

RESULTS AND DISCUSSION

Figure 1 is the observed method of "kunun-zaki" preparation. The possible sources of contamination with pathogens observed during "kunun" preparation were such practices as adding raw milled grains to gelled mixture as starter, sieving after fermentation, dilution with water, preparation in an unsanitary environment and presence of animals in processing area.

Out of 240 samples of "kunun-zaki" purchased from the retail outlet, 105 had *B. cereus* counts of less than 10^4 cells ml^{-1} and 35 had counts of 10^4 cells ml^{-1}. The mean *S. aureus* counts ranged from 68 to 7.2×10^2 cfu ml^{-1}. The mean *B. cereus* and *S. aureus* counts were lower than the 10^5 to 10^7 cells required to present a risk of intoxication (ICMSF 1974, Bergdoll 1979). However, it was noted that all the samples were contaminated with *B. cereus*, 216(90%) with *S. aureus* and 221(92.1%) with *E. coli* (Table 1). The *B. cereus* tested produced beta haemolysis on sheep blood agar, five were enterotoxigenic with fluid volume length ratio ranging from 0.31 to 0.89. Of the enterotoxigenic *B. cereus*, one gave severe reaction with bloody fluid (Table 2). The ten *S. aureus* isolates tested were both coagulase and hemolysis positive and the *E. coli* were able to grow at elevated temperature.

The antimicrobial susceptibility test carried out on 11 *B. cereus*, 15 *E. coli* and 10 *S. aureus* strains is presented on Table 3. All the *B. cereus* isolates tested were sensitive to floxacin and tetracycline, *E. coli* to floxacin and *S. aureus* to floxacin , chloramphenicol and gentamycin. The percentage resistance of *B. cereus* to all the drugs ranged from 0 to 27%, *E. coli* from 0 to 87% and *S. aureus* from 0 to 70%. It is important to also note that the strains were sensitive to the uncommonly used drugs for the treatment of infections caused by any of the pathogens tested.

The study revealed that all the samples of "kunun-zaki" contained *B. cereus*, 90% had *S. aureus* and 92% had *E. coli*. The high frequency of occurrence of these pathogens is of concern and is attributed to the method of processing and handling. The critical areas likely to be sources of contamination in "kunun-zaki" are the raw materials and ingredients, improperly washed equipment and utensils, processors and contaminated environment. Earlier studies in this environment have confirmed the presence of *B. cereus* in cereals, spices, sweetening agents, refuse dumps and enteric organisms in water and water vessels (Yusuf *et al.*, 1992, Inabo *et al.*, 2000, Oranusi *et al.*, 2003).

The level of 10^4 cells ml^{-1} for *B. cereus* and 10^2 cells ml^{-1} for *S. aureus* in this study are below the hazardous level of 10^7 cells ml^{-1}for these organisms in food (ICMSF, 1974). However, the Standards Organization of Nigeria (SON),

recommends that pathogenic microorganisms should be absent from soft drinks (SON, 1985). *B. cereus* as an endospore former may be activated during gelling and made to germinate and grow when holding at ambient temperature for sale. *E. coli* too have been shown to survive high temperature (Braocett *et al.*, 1994) and acidic conditions (Donald and John, 1995).

Staphylococcal food poisoning frequently occurs when food is contaminated after cooking by person carrying the organism. Subsequently if the food is exposed to temperature abuse for several hours toxin production may be encouraged before consumption. The ingestion of the toxin causes foodborne illness. *S. aureus* especially coagulase positive strains are considered the most virulent of the staphylococci (Kloos and Bannerman, 1999). Therefore, the presence in "kunun-zaki" of enterotoxigenic strains of *B. cereus*, coagulase and haemolysin positive strains of *S. aureus* and enteropathogenic *E. coli* and the holding at ambient temperature for sale may encourage growth to a level that can cause food borne illness and/or gastroenteric infection.

However, "kunun-zaki" is a fermented product with pH ranging from 4.06 to 4.80 and a pH of less than 4.5 is not suitable for growth and toxin production by *B. cereus* and *S. aureus* (Bergdoll, 1979;Umoh, 1989; Yusuf *et al.*, 1992). One must note here that acid formation in an uncontrolled fermented product like "kunun-zaki" is dependent on the quality of raw materials and starter, the type and number of fermenting strains present and the prevailing conditions during fermentation. Secondly, most of the pathogens encountered are likely to be due to post-processing contamination. It is not uncommon during social gatherings to find people refuse "kunun-zaki" because they are not sure of the source. Some refuse because of a previous experience of stomach upset after drinking "kunun-zaki". The high frequency of occurrence of these pathogens in the retail products is of serious concern because children are more at risk.

Based on the fact that "kunun-zaki" could be hazardous if improperly processed, the resistant patterns of some of the isolates to antimicrobial agents was studied. Secondly, bacteria are increasingly developing resistance to many classes of antibiotics and strains that were previously considered to be harmless are posing significant health threats and need to be examined. The isolates tested in this study showed varying degree of resistance (up to 70%) for some agents but all were susceptible to floxacin. The high resistance is attributed to antibiotic overused in humans, animals and agriculture resulting in the emergence of resistant phenotypes of bacteria. This findings collaborates the findings by other researchers elsewhere on bacterial resistance to antimicrobial agents (DeBoer *et al.*, 2001, Gomi *et al.*, 2001) and in this environment (Umoh *et al.*, 1990b, 1995).

CONCLUSION

The study revealed that the high frequency of contamination of "kunun-zaki" with pathogenic organisms could be due to faulty fermentation techniques. This may possibly include the addition of raw milled grains and potatoes as starter, sieving after fermentation, addition of poor quality water to obtain the required consistency and holding of product at ambient temperature (>25°C) for sale. The faulty practices, in addition, to the resistance of pathogens to drugs of choice showed that consumption of contaminated products could be of serious health risk. Education of processors on these hazards and the control measures to reduce the hazards is imperative. Such control measures include frequent washing of hands with soap during processing, use of properly washed equipment and utensils especially grinding machine before and after use, checking indicator of heat treatment by use of colour change, sieving before gelling, use of boiled and cooled water to dilute to required consistency and preparing "Kunun-zaki" in quantities that could be sold off the same day especially in cases of lack of refrigeration system.

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Table 1: Mean count of B. *cereus* and S. *aureus* isolated from retailed "Kunun-Zaki"

Sample Type	No. Tested	No. positive for B. *cereus*	No. of sample with counts (cfuml^{-1}) <10^4	10^4	Mean	Mean S.*aureus* counts (cfum1^{-1})
Bulk	20	20	10	10	$1.0x10^4$	$7.3x10^2$
PVC	130	130	111	19	$5.2x10^3$	$4.6x10^2$
Bottled	90	90	84	6	1.0x10	$6.8x10^1$
Total	240	240	105	35	—	—

PVC (PolyVinyl Chloride), No. (Number)

Table 2: Pathogenicity testing of isolates from "kunun-zaki"

Isolates		**Source**	**Vol/Length ratio**
B. cereus			
No. Tested	No. Positive		
4	2	Bulk	0.51 and 0.61
3	1	PVC	0.31
3	2	Bottled	0.39 and 0.86*
S. aureus			
No. Tested	**No. Positive**	**Haemolysin Production**	**Coagulase Production**
10	6	alpha (60%)	Human and Sheep 10 (100%)
	4	beta (40%)	
E. coli			
No. tested	**Growth at 44^{0}C**		
40	40 (100%)		

(PVC) Polyvinyl Chloride, * bloody fluid, No. Number

Table 3: Antibiogram of *B. cereus, E. coli* and *S. aureus* isolated from "kunun-zaki"

Antimicrobial agent (conc.)	**Number (%) Resistance**		
	***B. cereus*(n=11)**	***E. coli*(n=15)**	***S. aureus*(n=10)**
Chloramphenicol (10μg)	1(9.1)	5(33.3)	0(0.0)
Erythromycin (10μg)	3(27.3)	7(46.6)	3(30.0)
Sulphafurazole (100μg)	1(9.1)	6(40.0)	6(60.0)
Cloxacillin (5μg)	2(18.2)	—	2(20.0)
Penicillin (1.51μg)	3(27.3)	—	7(70.0)
Amoxicillin(2μg)	2(18.2)	8(53.3)	3(30.0)
Streptomycin (10μg)	2(18.2)	8(53.3)	7(70.0)
Tetracycline(50μg)	0(0.0)	4(26.6)	1(10.0)
Gentamycin(10μg)	1(9.1)	3(20.0)	0(0.0)
Floxacin(10μg)	0(0.0)	0(0.0)	0(0.0)
Cephalexin(25μg)	—	13(86.6)	—
Cefuroxime(30μg)	—	12(79.9)	—
Nitrofurantoin(200μg)	—	2(13.3)	—
Co-Trimoxazole(50μg)	—	4(26.6)	—
Ampicillin(25μg)	—	6(40.0)	—

— Not tested. Neither *Salmonella* nor *Shigella* was isolated

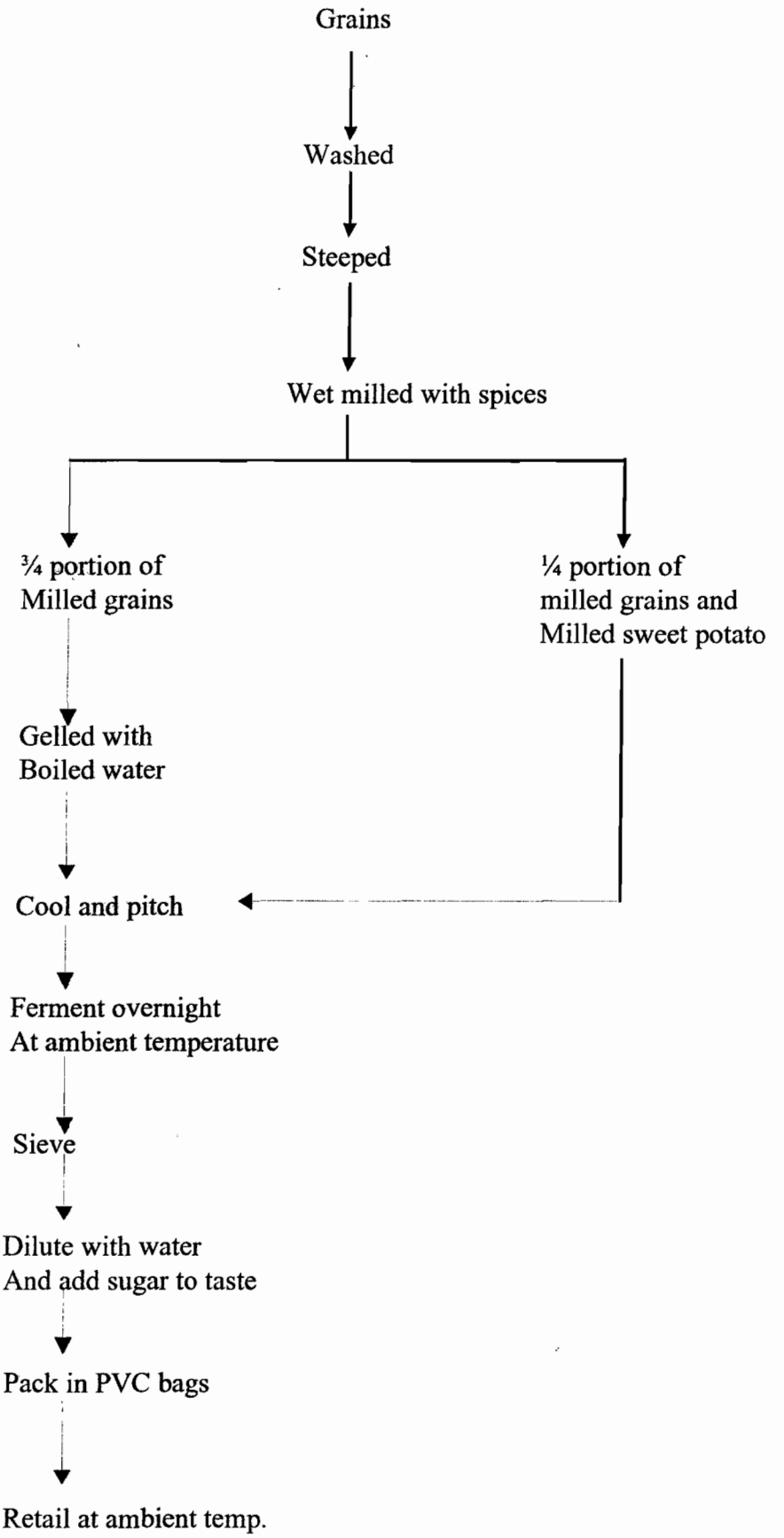

Fig.1 ""Kunun-zaki" production by one producer

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