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FULL PAPER

The survival rate of virulent coagulase-negative staphylococci in pasteurized spiced *kunu* (a Nigerian non-alcoholic beverage)

*Fowoyo, P.T.

Microbiology Department, Baze University, Abuja, Nigeria

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Abstract

Kunu is a non-alcoholic fermented beverage popularly consumed in Nigeria and attributed to possess immense nutritional and antimicrobial properties. The objective of this research was to determine the survival of virulent and antibiotic resistant coagulase-negative staphylococci (CoNS) in pasteurized spiced *kunu*. *Kunu* was prepared using the spices; clove and ginger and then pasteurized. The survival of five virulent and antibiotic resistant CoNS species; *Staphylococcus simulans*, *Staphylococcus kloosi*, *Staphylococcus epidermidis*, *Staphylococcus caprae* and *Staphylococcus xylosus* in pasteurized spiced *kunu* samples was determined at intervals of 0 hr, 24 hrs, 48 hrs, 72 hrs and 96 hrs. It was observed that there was a significant decrease of virulent and antibiotic resistant CoNS species in *kunu* samples prepared with high concentrations of the spice either alone or in combination. The virulent strains were below detectable limit from 48 hrs especially for those prepared with a combination of spices at high concentration and that with clove alone. The un-spiced *kunu* (control) also inhibited the virulent species although the rate of decline was not as pronounced like the spiced *kunu* even at 96 hrs. This study had shown that *kunu* prepared with a combination of clove and ginger at high concentration of 30 mg/mL exhibits antimicrobial activity against CoNS. *Kunu* may be used as an antimicrobial against gastrointestinal infections caused by these organisms. Further studies may be carried out using other gastrointestinal pathogens to fully explore and lend credence to the antimicrobial activity of spiced *kunu*.

1. Introduction

Food is a vital requirement for man; however, it also serves as a vehicle for transmission of pathogenic organisms to man. Food is rich with all the necessary nutrients therefore it is a rich medium that supports the growth of microbes. Recently, the isolation of pathogenic and antibiotic resistant microorganisms from food suggests that food could become a reservoir for the transmission of antibiotic resistance to man. Therefore, it is necessary for foods to be prepared, in such a way that they can act as antimicrobials themselves capable of limiting or killing pathogenic organisms present in food.

Microbial pathogens in food cause a variety of food-borne related diseases. Some common food-borne pathogens that have been reported to cause several diseases and also exhibit antibiotic resistance include *Salmonella enterica* serovar Typhimurium, *Salmonella Salmonella enterica* serovar Typhi, *Vibrio cholerae*, *Shigella dysentariae*, *Campylobacter* species, *Staphylococcus aureus* and more recently coagulase-negative staphylococci which is an emerging food-borne pathogen.

Coagulase-negative staphylococci were previously recognized as non-pathogenic organisms and commensals of the skin although, could become opportunistic. Recently, these organisms are not only associated as causes of nosocomial infections but have also been isolated in food.

It has been isolated from some Nigerian foods and discovered to exhibit virulent traits and antibiotic resistance (Fowoyo and Ogunbanwo, 2016a; Fowoyo and Ogunbanwo, 2016b; Fowoyo and Ogunbanwo, 2017). Coagulase negative staphylococci isolated from food samples exhibited the ability to produce biofilms, superantigenic enterotoxins, DNAse, TNAse, hyaluronidase, haemolysins and were resistant to broad spectrum antibiotics. Their ability to resist antibiotics was further demonstrated with genotypic analysis that indicated the presence of *blaz* and *mecA* genes in their genome. Their occurrence in food and especially their ability to produce enterotoxins similar to their coagulase-positive counterpart i.e. *Staphylococcus aureus* makes

*Corresponding author.
Email: *patience.fowoyo@bazeuniversity.edu.ng*

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their incidence in food worrisome. It is estimated that over 200 types of diseases are caused or spread by food, sometimes causing long term health problems in vulnerable groups of people such as the elderly, pregnant women and infants (WHO, 2015). The majority of morbidity and mortality related to foodborne infections are caused by bacterial agents (CDC, 2011). Food-borne diseases remain a challenge globally, with a higher incidence rate in developing countries (Biranjia-Hurdoyal and Latouche, 2016). The World Health Organization's Food-borne Disease Burden Epidemiology Reference Group estimated 582 million cases of food-borne diseases and 351,000 associated deaths worldwide (WHO, 2015). Food-borne pathogens are developing resistances to antibiotics which is an increasing health and economic problem worldwide. Many of these organisms are becoming resistant to routinely used antibiotics thereby making therapy ineffective. This continual concern about evolving antibiotic resistance and declining food safety especially in developing countries requires new strategies to be developed to combat this impending menace. The emerging antibiotic resistance in food-borne pathogens has paved the way for the use of natural antimicrobials such as spices in the food industry.

Kunu is a cereal food drink prepared using additives like spices and has become a popular refreshing non-alcoholic beverage in Nigeria. *Kunu* also protects the body against cholesterol and bile acid metabolism related diseases such as gallstones and certain forms of heart diseases (Olaitan *et al*., 2012). *Kunu* has been reported to elevate lymphocyte counts in the blood serum of albino rats fed with *kunu* which is indicative of its medicinal attributes, a concept widely believed by its numerous consumers (Akoma *et al*., 2006). Spices have long been added to enhance flavor and serve as preservatives in food however, more recently, attention has been drawn to the medicinal value of spices. It has also been reported that spices such as ginger and clove used in *kunu* preparation demonstrate antimicrobial activity against food pathogens (Lai and Roy, 2004; Joe *et al*., 2009, Sethi *et al*., 2013; Mane *et al*., 2014; Liu *et al*., 2017). The aim of this study is to determine the survival of CoNS in spiced pasteurized *kunu* samples.

2. Materials and methods

2.1. Test organisms used for this study

The virulent coagulase-negative staphylococci used were *S. kloosii* KIL 4, *S. xylosus* KIL 2, *S. caprae* NOMA 6, *S. simulans* NOJ 6, *S. epidermidis* IRIL 5. The organisms were identified from a previous study using phenotypic and genotypic methods. They were virulent and antibiotic resistant species of coagulase-negative staphylococci isolated from Nigerian fermented foods (Fowoyo and Ogunbanwo, 2016a, Fowoyo and Ogunbanwo, 2016b, Fowoyo and Ogunbanwo, 2017).

2.2 Preparation of Spiced Kunu

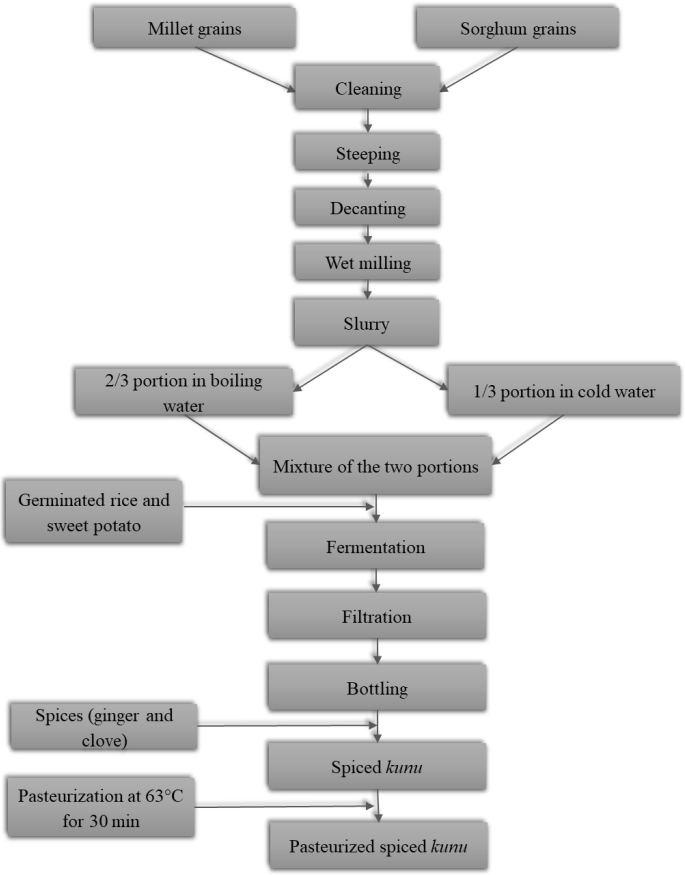

Figure 1. Flow chart for the preparation of spiced pasteurized *kunu*

Kunu was prepared according to the method described by Odunfa and Adeyele (1985) with slight modifications as shown in Figure 1. The raw materials for the preparation of *kunu* were purchased from the International Market, Lokoja, Nigeria. The raw materials were millet (500 g) and sorghum (500 g), spices (ginger and clove), sweet potato (150 g) and germinated rice (100 g). They were cleaned of extraneous materials and dirt by winnowing and washing. The millet and sorghum grains were mixed together and steeped in 15 L of sterile distilled water for 12 hrs. The grains were washed with sterile distilled water and wet milled hygienically to obtain a smooth, fine paste. The spices were dry milled separately not with the grains. The ground mixture of millet and sorghum were divided into 3 equal parts; 1/3 was mixed with 5 L of sterile cold water and homogenized while 2/3 was mixed with 8 L of boiling water to give a gel-like consistency. The 1/3 part (cold suspension) was added to the gel-like part and mixed thoroughly. Sweet potatoes and germinated rice were cleaned and wet milled then, added to the mixture. This was covered with aluminum foil and fermented overnight at 30^{o}C during which the slurry was allowed to

settle and sediment. The slurry was sieved using a sterile and fine cloth. The resulting mixture was divided into ten portions. *Kunu* of 200 mL was dispensed into tight, sterile screw cap bottles to which the respective spices were added. A total of ten different *kunu* samples were prepared and labeled as KCG1: *kunu* spiced with 10 mg/mL of each of clove and ginger, KCG2: *kunu* spiced with 20 mg/mL of each of clove and ginger, KCG3: *kunu* spiced with 30 mg/mL of each of clove and ginger, KC1: *kunu* spiced with 10 mg/mL of clove, KC2: *kunu* spiced with 20 mg/mL of clove, KC3: *kunu* spiced with 30 mg/mL of clove, KG1: *kunu* spiced with 10 mg/mL of ginger, KG2: *kunu* spiced with 20 mg/mL of ginger, KG3: *kunu* spiced with 30 mg/mL of ginger, C: Control (un-spiced *kunu*).

The *kunu* samples were pasteurized using low temperature holding method in a hot water bath at a temperature of 63ºC for 30 mins. The survival of virulent CoNS was determined in the spiced, pasteurized *kunu* samples to elucidate the efficacy and antimicrobial potential of spiced *kunu* against these pathogens that are antibiotic resistant that occur in food.

2.3 Survival of virulent coagulase - negative staphylococci in laboratory prepared pasteurized spiced kunu

The modification of the method by Simango and Rukure (1992) was used to determine the survival of virulent CoNS in laboratory prepared pasteurized and spiced *kunu*. The sterility of each spiced kunu sample was checked by performing total plate count. The virulent CoNS species were each inoculated into brain heart infusion broth and incubated at 37°C for 18 hrs. The 18-hour-old broths were standardized and the turbidity adjusted to correspond to 0.5 MacFarland standard, and this was used as the inoculum. The standardized inoculum (1 mL) was plated out on nutrient agar plates using the spread plate technique and this directly correlates to the initial microbial count in 1 mL of each spiced, pasteurized *kunu* sample. The cell suspension or inoculum (1 mL) was dispensed into 50 mL of the sterile spiced *kunu* sample in 100-mL bottles and the content was thoroughly mixed. The inoculated samples were kept at 25^{o}C. Surviving bacterial cells were enumerated by plating out at different time intervals of 0 h, 24 hrs, 48 hrs, 72 hrs and 96 hrs on duplicate Baird Parker agar. The agar plates were incubated at 37°C for 24 hrs. Colonies morphologically representative of each pathogenic CoNS were counted on the plates using a colony counter and the values were multiplied by the corresponding dilution factor to get the total bacterial count.

3. Results

The *kunu* samples that were spiced with clove alone at high concentration and that spiced with a combination of ginger and clove had the highest inhibitory effect on the strains.

At 48 hrs, there was a steady decline in microbial count from 5.49 $\log_{10}$ CFU/mL – 0 $\log_{10}$ CFU/mL for all the virulent species in the *kunu* (KCG3 and KC3) samples. The microbial count of *S. kloosii* and *S. simulans* also decreased to 0 $\log_{10}$ CFU/mL at 48 hrs in KCG2 and KC3 samples. It was also observed that the *kunu* samples prepared with higher concentrations of the spice either alone or in combination rapidly reduced the microbial count of the virulent CoNS in the spiced *kunu* samples.

The inhibitory effect of KCG2, KC2, KG3 and KC1 kunu samples against the virulent species was very high at 72 hrs and were no longer detectable. It was observed that KCGI, KG2 and KG1 decreased the microbial count of *S. simulans* while KC3 decreased the microbial count for *S. kloosii* to 0 $\log_{10}$ CFU/mL at 72 hrs. At 96 hrs, KG2 and KG1 inhibited *S. xylosus* and *S. caprae* while KCG1, KC2 and KG1 inhibited *S. epidermidis* although, KCG1, KC2 and KG1 inhibited *S. kloosii*.

S. simulans was the most inhibited because at 72 hrs, the microbial count had dropped to 0 $\log_{10}$ CFU/mL in all the *kunu* samples irrespective of the concentration or combinative effect of spices. *S. simulans* was not detected between 48 and 72 hrs showing that the organisms were greatly inhibited in the spiced *kunu* samples irrespective of the concentration used. At 24 hrs, *S. xylosus* was nearly undetectable in the *kunu* samples and this demonstrates the antagonistic effect of the spiced *kunu* on the microorganisms. *S. kloosii*, *S. caprae* and *S. epidermidis* showed more resistance as compared to the other species, that were not detectable at 48 hrs, 72 hrs and 96 hrs. They were inhibited at 48 hrs in the *kunu* sample prepared with a combination of the spices at higher concentrations

The potency of KCG3, KC3, KCG2 and KG3 was the highest decreasing the microbial counts to 0 $\log_{10}$ CFU/mL at 48 hrs for all the virulent strains excluding KG3 that had its profound effect only on *S. simulans*. The *kunu* samples that were spiced with clove alone and that spiced with a combination of ginger and clove had the highest inhibitory effect on the strains. There was a steady decline in microbial count from 5.49 $\log_{10}$ CFU/mL – 0 $\log_{10}$ CFU/mL at 48 hrs for all the CoNS species in *kunu* samples prepared using only one spice or a combination of spice at higher concentrations.

The virulent strains were below detectable limit from

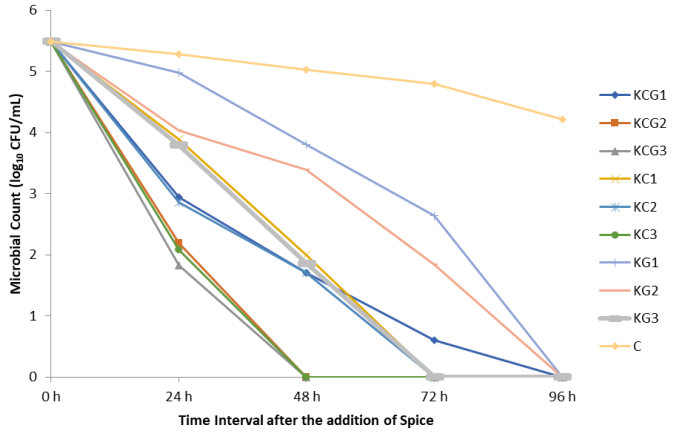

Figure 2. Survival pattern of *S. kloosii* KIL 4 in pasteurized *Kunu* at different spice concentration

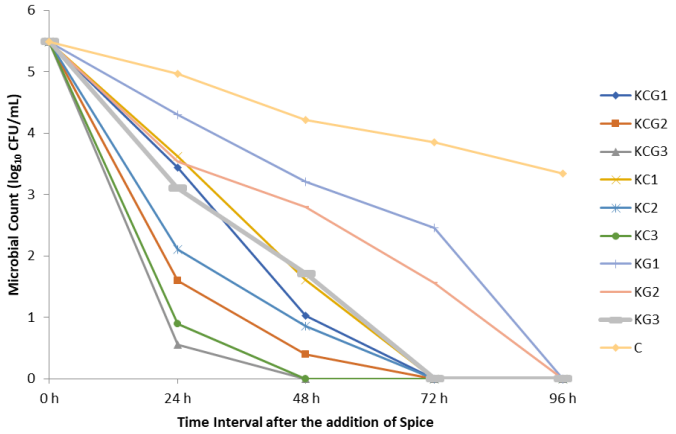

Figure 3. Survival pattern of *S. xylosus* KIL 2 in pasteurized *Kunu* at different spice concentration

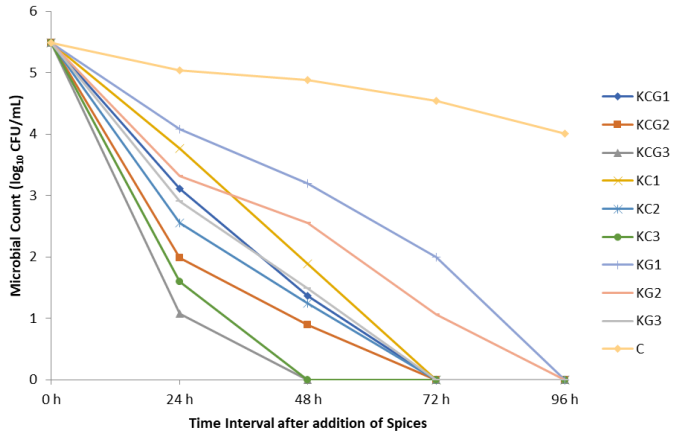

Figure 4. Survival pattern of *S. caprae* NOMA 6 in pasteurized *Kunu* at different spice concentration

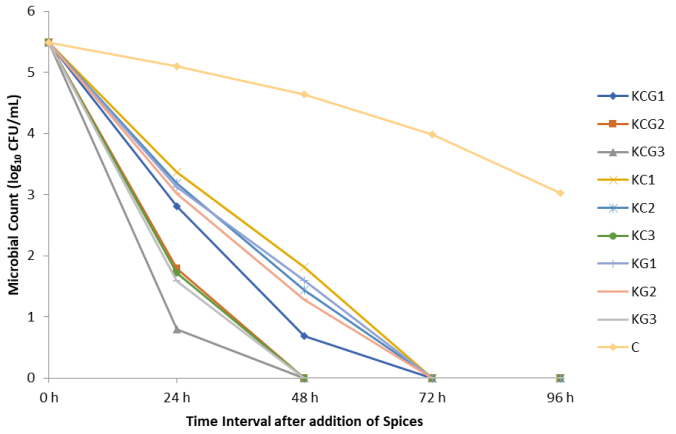

Figure 5. Survival pattern of *S. simulans* NOJ 6 in pasteurized *Kunu* at different spice concentration

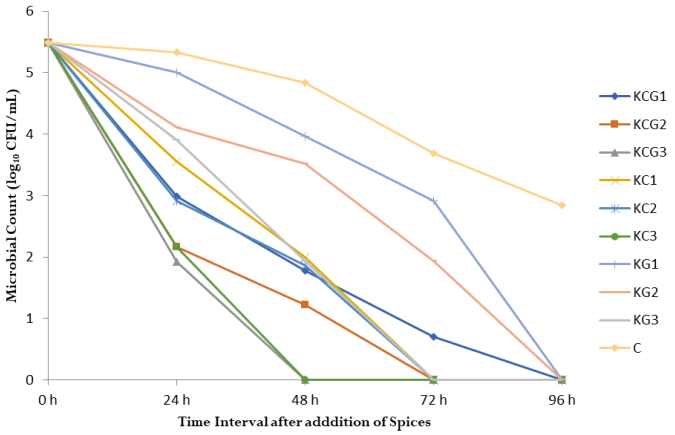

Figure 6. Survival pattern of *S. epidermidis* IRIL 5 in pasteurized *Kunu* at different spice concentration

KCG1, KCG2, KCG3: *Kunu* prepared with clove and ginger at concentrations of 10 mg, 20 mg and 30 mg respectively; KC1, KC2, KC3: *Kunu* prepared with only clove at concentrations of 10 mg, 20 mg and 30 mg respectively; KG1, KG2, KG3: *Kunu* prepared with only ginger at concentrations of 10 mg, 20 mg and 30 mg respectively; C: control without spice.

48 h especially for those prepared with combined spices at higher concentration and that with clove alone. The un -spiced *kunu* (control) also inhibited the virulent species though the rate of decline was not as pronounced like the spiced *kunu* even at 96 hrs. (Figures 2 - 6).

4. Discussion

The test organisms were below detectable limit in the pasteurized spiced *kunu* samples between 48 and 72 hrs especially for those prepared with high concentration of clove alone and, a combination of ginger and clove within a short time. The *kunu* samples; KCG3 and KC3 demonstrated high antimicrobial activity against *S. kloosi*, *S. simulans*, *S. epidermidis*, *S. caprae* and *S. xylosus* within 48 hrs and this may be attributed to the high amounts of the phytochemicals thus within a short

time they can elucidate their effect and lower the microbial count. Thus, a combination of the spices and the quantity of the spices used in preparing the *kunu* has a significant effect on the strains. A similar study carried out by Kong *et al*. (2007) on the combinatory effect of spices on vacuum packaged chilled pork also confirms that the presence of multiple active compounds in mixed spices could illicit stronger antimicrobial effects as compared to when the spices were used alone and this explains their synergistic bacteriostatic actions. This phenomenon apparently contributes to the antimicrobial potential of the drink to lower microbial count. Also, the high quantity of clove and ginger used in *kunu* preparation may have also contributed significantly to the decline in microbial count.

Clove has been reported to be used in treating infectious diseases and to protect food because they have been experimentally proven to possess antimicrobial activities against pathogenic and spoilage bacteria and fungi (Liu *et al*., 2017). Clove is also commonly applied in the food industry as a natural additive to increase shelf -life and also act as an antimicrobial against food-borne pathogens (Liu *et al*., 2017). A study carried out by (Liu *et al*., 2012) demonstrates the antimicrobial potential of clove in food in which the extract and essential oil of clove had the highest inhibitory effect against the microbial populations in chilled pork. Ginger also used in *kunu* preparation contributed to the inhibitory activity exhibited by the *kunu* samples. Ginger owes its antibacterial activity to its main component sesquiterpenoids (Gull *et al*., 2012). The antimicrobial activity of ginger against several pathogenic organisms was also demonstrated in a study by Goswami *et al*. (2014) against *Clostridium perfringes*, *Escherichia coli* and *Clostridium sporogenes* in minced chicken meat. The ginger was incorporated into the minced chicken meat by kneading and the pathogenic organisms were then inoculated into the meat samples. The microbial count was lowered considerably in the meat samples. The antimicrobial activity of the *kunu* samples was enhanced due to the antimicrobial activity derived from the spices (Olaitan *et al.,* 2012).

S. simulans was the most inhibited of all the test organisms. It was inhibited greatly in four different *kunu* samples which was noticeable at 24 hrs and this indicates the susceptibility of *S. simulans* to the spiced *kunu* samples. All the strains were slightly inhibited in un-spiced *kunu* and this may be explained using the study carried out by Olaitan *et al*. (2012) which reported that *kunu* is self-sanitizing and had antimicrobial potential because of the presence of phenol and polyphenols present in the millet and sorghum grain used for its preparation.

5. Conclusion

The combination and quantity of spices, the phytochemical -phenol and polyphenols present in the raw materials used in preparing *kunu* plays an apparent role in inhibiting virulent CoNS. Foods prepared with spices possessing antimicrobial property could represent a likely alternative to antibiotics against food-borne pathogens. Combinatory or synergistic effect and use of high quantity of spices in food preparation may be a likely method of inhibiting food- borne pathogens.

Conflict of Interest

Author declares no conflict of interest**.**

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