ANTIBIOTIC SUSCEPTIBILITY PATTERN OF AEROBIC MESOPHILIC BACTERIA ISOLATED FROM KUNU SAMPLES SOLD IN ILE-IFE, OSUN STATE, NIGERIA.

BY

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BEING A PROJECT WRITE-UP SUBMITTED TO:
DEPARTMENT OF MICROBIOLOGY,
FACULTY OF SCIENCE,
FEDERAL UNIVERSITY OYE-EKITI,
EKITI STATE, NIGERIA.

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF A BACHELOR OF SCIENCE (B.Sc) DEGREE IN MICROBIOLOGY.

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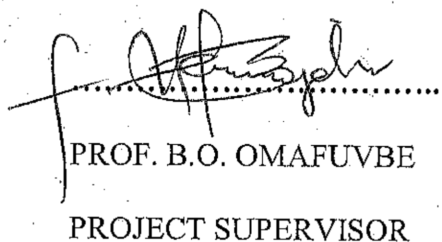
MARCH, 2019.

CERTIFICATION

This is to certify that this project work was carried out by AFOLABI, DAMILOLA ELIZABETH with the matriculation number MCB/14/2314 of the Department of Microbiology, Faculty of Science, Federal University, Oye-Ekiti, Ekiti State under the supervision of Prof. B.O. Omafuvbe.



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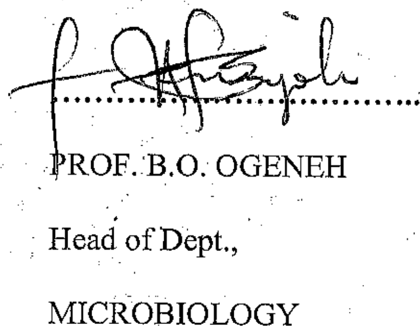

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DEDICATION

I dedicate this project to the All Powerful and Mighty God whose abundant grace, love and mercy has seen me through and to my loving parent; Mr and Mrs Afolabi for their all-round support.

ACKNOWLEDGEMENT

I appreciate the all-sufficient God, the doer of all things for giving me the golden opportunity and grace to succeed in this chosen field of mine.

I am greatly indebted to my parent, Mr and Mrs Afolabi for their all-round support, most especially, for planting my feet on the path of education and praying towards the success of my aspirations. No doubt, you have secured a better future for me; it is thus left for me to reciprocate the love and care you have shown towards me. I specially acknowledge my aunt, Kike James (Mrs) for her support in cash and kind, I wish to, appreciate Mrs Ayeni Adebukola for her useful words of advice.

I want to thank my wonderful H.O.D, Prof. Bryan O. Ogeneh and all my lecturers in the Department of Microbiology, namely: Prof. B.O. Omafuvbe, Dr (Mrs) Ajobiewe, Dr. Adegoke, Dr Okoroh, Dr. Ajayi, Dr Akinyele, Dr. Ojo, Mrs Adesemoye, Mr Osanyinlusi, Mrs Osamoka and all others, for their contribution towards my academic career.

The unrelenting effort of my eminent supervisor, Prof. B.O. Omafuvbe remains germane. I appreciate her motherly love, kindness, patience, understanding, advice, guidance, ideas, rich and robust contributions helped in nourishing this work. My wish is for her to continue soar higher in all areas of her life.

I also want to appreciate my colleague Oladayo Ayomide Toluwanimi aka steady for his moral and financial support throughout this project work.

I acknowledge my siblings, Afolabi Taiwo and Afolabi Idowu for their cooperation,. leading to my success academically and in all areas of life. I do wish you all the very best in your future undertakings. I also wish to acknowledge my special friend for his all-round support towards my success, Comrade Adewumi, Idowu Peter. I do pledge my everlasting loyalty to you.

I wish to appreciate the leadership and members of Livingstone Church, Oye-Ekiti, Ife for their support both physically and spiritually. May the church of God continue to flourish. I specially acknowledge my project colleague Adeyemo Nurudeen and my departmental mates Adebisi, Ayomide Blessing, Akinduntire Funmi, Owolabi Jumoke, Quarshie Doris, Olowomeye Bunmi and other Microbiology class of 2018 for their impactful contributions. I hope to see you all at the top.

I wish to acknowledge the pioneer executives of FUOYE Student Union, (2017/2018) led by Fasakin Taiwo, where I served as Treasurer and FESSU FUOYE Chapter 2016/2017 executives, where I served as Vice President which was led by Akomolafe Idowu.

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ABSTRACT

The antibiotic susceptibility pattern of aerobic mesophilic bacteria isolated from kunu samples sold in Ile-Ife, Osun State, Nigeria was studied following standard microbiological methods. Seven ready-to-drink kunu samples were purchased from different hawkers at Ile-Ife. The samples were transported immediately in ice bucket to the laboratory for analysis. The aerobic mesophilic bacteria associated with kunu samples were isolated, labeled and identified by phenotypic and biochemical tests. The antibiotic susceptibility pattern of the isolates against ceftazine, ceftazidine, cefuroxime, gentamicin, ceftriaxone, erythromycin, cloxacillin, ofloxacin and amoxicillin, cefixime, augumentin and nitrofurantion was determined using the kirby-bauer disc diffusion method. The isolates identified include: *Staphylococcus saprophyticus, Enterobacter intermidis, E. aerogenes, Bacillus sphaericus, Corynebacterium spp., Staphylococcus aureus, B. brevis, Citrobacter spp., Shigella spp.* and *Escherichia coli.* The percentage occurence pattern of *E. intermidis* was 85.71%, present in six out of the seven samples except Sample C while *Staphylococcus saprophyticus and Shigella spp* were 71.43%, present in five out of the seven samples, studied. E. coli has the least occurrence pattern with 14.29%. The Gram positive organisms were resistant to ceftazidime, cefuroxime, ceftriaxone and cloxacillin but susceptible to gentamicin, erythromycin, ofloxacin and amoxycillin. The Gram negative organisms were resistant to cefixime, cefuroxime and augumentin but susceptible to gentamicin, nitrofurantion, ofloxacin and ciprofloxacin. The results obtained from this study indicated that kunu was contaminated by poor handling and lack of good manufacturing procedures. The microorganisms isolated from this study are of public health significance and their resistance to commonly used antibiotics such as ceftazidime, cefuroxime, ceftriaxone, augumentin and cloxacillin could constitute an alarming development with significant public health consequences.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Cereals are the most significant source of the world's food and have major impact in human diet throughout the world (Adebayo *et al.*, 2010). A majority of traditional fermented products consumed in Africa are processed by natural fermentation of cereals and are particularly important as weaning foods for infants and dietary staples for adults (Viéra-Dalodé *et al.*, 2007; Tou *et al.*, 2006). A wide range of cereal based fermented foods exist, and these include *ogi* and *mawè* in Benin, *kenkey* in Ghana, *injera* in Ethiopia, *poto-poto* in Congo, *ogi* and *kunu* in Nigeria, *uji* and *togwa* in Tanzania, *kisra* in Sudan (Soro Yao *et al.*, 2013).

Kunu is an African traditional non-alcoholic beverage that is produced by conventional fermentation of cereal grains by beneficial microbes, and has been widely known for its high acceptability, palatability and energy replenishing potentials, which is claimed to be associated to its rich content of various functional components. Kunu, a product of lactic acid fermentation [Akoma *et al.*, 2006], is commonly prepared locally by women, and taken mainly by low and middle income earners for its thirst quenching and energy-giving properties. Its cheapness is owed to the ready availability of cereals and additives locally sourced as they grow throughout the savannah belt of West Africa (Elmahood and Doughari, 2007). It is also used locally for entertainment at homes and during festivities such as Sallah and Christmas ceremonies (Ayo *et al.*, 2014).

Mbachu *et al.* (2014) reported that *kunun* zaki contains 0.3% protein, 1.0% fat, 1.52% ash, 12.2% carbohydrate and 8.9 mg of vitamin C per 100 ml. More so, the health benefits of kunu include but not limited to reduction of risk associated with diabetes, lowering of blood cholesterol, prevention of formation of blood clot and help to fight against cancer (Ofudje *et al.,* 2016).

Traditionally, the production procedure of kunu varies depending on the taste and cultural habits of the consumers, thus leading to variation in quality and stability. Aboh and Oladosu (2014) reported that production methods of kunu-zaki are crude, ingredient concentrations are neither quantified nor standardized and the preparation is largely traditional with the art passed down from family.

Depending on the major cereal used in kunu production, the common types of the beverage include kunu zaki, kunu gyada, kunu akamu, kunu tsamiya and kunu baule. Soghum (*Sorghum bicolor*) and millet (*Pennisetum americanum*) grains are the most commonly used raw materials (Ayo *et al.*, 2014). However, maize (*Zea mays*), rice, acha (*Digitaria exilis),* guinea corn and other grains may also be used [Akoma *et al.,* 2006].

The variety of the drink made from sorghum is a milky light brown colour, while that made from millet and maize is whitish in colour. Spices such as ginger, black pepper, garlic, red pepper and cloves are commonly added as flavor and taste enhancers. Sugar is also added to act as a sweetener, honey together with small quantity of sweet potatoes, malted rice, malted sorghum and cadaba farinose crude extract can also be used. The grains are used singly or combined; sorghum/millet has been reported as the most common combination in the ratio 1:2 (w/w) (Ahmed *et al.,* 2003).

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 pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* spp. among others (Bibek, 2001). 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 (Osuntogun and Aboaba, 2006). *Staphylococcus* spp., *Candida spp.* and *Trichoderma* spp could cause spoilage of the drink when present in large amount (Osuntogun and Aboaba, 2006). 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 (UFDA, 2009).

1.2 Statement of Problem

Kunu zaki has high moisture content; its method of production is crude and is packaged under unhygienic condition. It is also highly consumed because is cheap compared to other non alcoholic drinks. The drink is hawked in the motor parks, school premises and in market places where it is exposed to contamination. 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 subjected 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 (Oranusi *et al.*, 2003). This research was conducted to evaluate the microbiological qualities of kunu-zaki and antibiotics susceptibility patterns of its associated mesophilic bacteria in Ile-Ife, Osun State.

1.3 Aim and Objectives

This study was designed to determine the bacteriological quality and antibiotic susceptibility pattern of aerobic mesophilic bacteria isolated from kunu sold in Ile-Ife, Osun state, Nigeria. This was with a view to ascertain whether the product contain pathogenic bacteria and to determine their susceptibility to antibiotics.

The specific objectives of this research include:

i. The isolation and identification of aerobic mesophilic bacteria from kunu

ii. Study the occurrence pattern of the bacteria isolated

iii. Determine the antibiotic susceptibility pattern of the identified isolates.

CHAPTER TWO

LITERATURE REVIEW

Cereals which include maize (*Zea mays*), Sorghum (*Sorghum bicolor*), millet (*Peninselum americarum*) are used in the production of gruels which are used as complementary food for babies and serves as breakfast for adults. Maize, millet, rice and sorghum cereals provide mainly carbohydrates and low quality protein. The generation and fermentation of cereals enhance the availability of elemental iron, the deficiency of which is responsible for the high incidence of anaemia in tropical countries. It is estimated that about 50% of perishable food commodities including fruits, vegetables, roots and tubers and about 30% of food commodities including maize, sorghum millet, rice and cowpeas are lost after harvest in Nigeria (FDA, 2013).

Apart from their poor nutritional qualities, traditional Nigerian cereal based gruels used as complementary foods have high paste viscosity and require considerable dilution before feeding; a factor that further reduces energy and nutrient density. Although nutritious and safe complementary foods produced by food multinationals are available in Nigeria, they are far, too expensive for most families (Ayo *et al.*, 2014). The economic situation in Nigeria necessitate the adoption of simple, inexpressive processing techniques that result in quality improvement and that can be carried out at household and community levels for the production of nutritious, safe and affordable complementary foods which is the leading cause of protein-energy malnutrition in infants and preschool children in Nigeria (Musa and Hamza, 2013).

2.1 Nutrient Composition of Cereals

The major cereal crops in Nigeria are rice, maize, sorghum, wheat, pearl and millet with rice ranking as the sixth major crop in terms of the land area while sorghum account for 50% of

the total cereal production and occupies about 45% of the total land area devoted to cereal production in Nigeria (Ismaila *et al.*, 2010).

Cereal grains and their diverse products, form the most important source of dietary nutrients for many people, especially those in the developing countries of the world. However, it has been reported that the nutritional quality of cereal grains and their products are substandard due to lower protein content, deficiency of certain essential amino acids, presence of certain anti-nutrients and the coarse nature of the grains (Kahlon, 2009). In view of the above report, kunu-zaki has been noted to have a gross chemical composition of 87.85 – 89% moisture, 9.84 – 12% carbohydrate, 1.56 – 3% protein, 0.10 – 0.30% fat and 0.61 – 0.75% ash, indicating that the drink is low in protein (Ayo *et al.,* 2006). Anti-nutrients found in cereals, such as phytate, can decrease the absorption of minerals such as Zn, Ca, Fe and Mn, and high intake of these anti-nutrients might lead to mineral deficiency. Zinc and iron are essential trace elements in human nutrition, whose deficiencies are among the common nutritional problems affecting the world. Their deficiencies are of major concern because of the serious health consequences they have, as well as the large number of people afflicted; especially in developing countries of the world. These deficiencies may be caused by the presence of certain anti-nutrients, especially the ability of phytate to reduce dietary Zn and Fe bioavailability by formation of insoluble mineral chelates at physiological pH. The formation and stability of these chelates depend on the relative concentrations of Zn, Fe and phytate as well as on the levels of dietary Ca present (Afify *et al.*, 2011; Igwe *et al.*, 2013). However, certain traditional food processing techniques, either individually or in combination, such as germination, steeping, boiling and fermentation of grains for a limited period have been reported to cause increased activities of hydrolytic enzymes, certain essential amino acids,

total sugars, B-group vitamins, and a decrease in dry matter, starch and anti-nutrients (Akinhanmi *et al.*, 2008).

Cereals are the major dietary energy suppliers and provide significant amount of protein, minerals (potassium and calcium) and vitamins A and C (Idem and Showemimo, 2004). Cereals are consumed in a variety of forms, including pastes, noodles, cakes, breads, drinks etc. depending on the ethnic or religious affiliation. The bran, husk, plant parts and other residues (after processing) are useful as animal feeds and in the culture of micro-organism. Wax syrup and gum are extracted from cereals for industrial purposes. Different Nigeria ethnic groups use cereal crops residues for different purposes (Ismaila *et al.*, 2010).

2.2 Food Fermentation

Fermentation is the biochemical process of using microorganisms, such as bacteria or yeast, to convert carbohydrates to alcohol or organic acids under anaerobic conditions. This is a process used to produce the finest wine; many of our basic staples, such as bread and cheese; and pleasurable delights, including beer, chocolate, coffee and yoghurt. Fermentation is an easy process, enjoyed and done by anyone and anywhere with the most basic tools. Cultures around the world have been fermenting longer than we've been cultivating soil or writing books, benefiting from the countless delicacies as a result (Willey *et al.*, 2008).

Fermentation is not only a way to preserve certain foods, in some cases it actually adds to the nutrient value of the food. Fermented vegetables contain more vitamin C and fermented milk products have ample amounts of B vitamins. The bioavailability of these vitamins also increases with fermentation. Probiotics, or "good bacteria" are also formed through the process of fermentation. Fermented soy products contain more vitamin B12 (Chung *et al.*, 2010).

2.3 Benefits of Food Fermentation

The primary benefit of fermentation is the conversion of sugars and other carbohydrates to usable end products. According to Steinkraus (1995), the traditional fermentation of foods serves several functions, which includes: enhancement of diet through development of flavour, aroma, and texture in food substrates, preservation and shelf-life extension through lactic acid, alcohol, acetic acid and alkaline fermentation, enhancement of food quality with protein, essential amino acids, essential fatty acids and vitamins, improving digestibility and nutrient availability, detoxification of anti-nutrient through food fermentation processes, and a decrease in cooking time and fuel requirement.

1. **Nutritional value**: Fermentation makes foods more edible by changing chemical compounds and can produce important nutrients or eliminate anti- nutrients. There are extreme examples of poisonous plants like cassava that are converted to edible products by fermentation. Some coffee beans are dehulled by a wet fermenting process, as opposed to a dry process (Battcock and Aza-Ali, 1998). Fermentation also helps in the reduction of anti-nutritional and toxic components in plant foods. E.g Cereals, legumes, and tubers that are used for the production of fermented foods may contain significant amounts of antinutritional or toxic components such as phytates, tannins, cyanogenic glycosides, oxalates, saponins, lectins, and inhibitors of enzymes such as alpha-amylase, trypsin, and chymotrypsin. The fermentation process reduces the anti-nutritional value of foods by interfering with the mineral bioavailability and digestibility of proteins and carbohydrates. In natural or pure mixed-culture fermentations of plant foods by yeasts, molds, and bacteria, anti-nutritional components (e.g. phytate in whole wheat breads) can be reduced by up to 50%; toxic components, such as lectins in tempeh and other fermented foods made from beans, can be reduced up to 95% (Larsson and Sandberg, 1991). Fermentation increases

nutritional values of foods, and allows a healthier life, e.g the sprouting of grains, seeds, and nuts, multiplies the amino acid, vitamin, and mineral content and antioxidant qualities of the starting product (Wigmore, 1986).

2. **Preservation of Food**: Fermentation uses up food energy and create conditions unsuitable for spoilage microorganisms. For instance, in pickling, the acid produced by the dominant organism inhibits the growth of all other microorganisms.
3. **Digestibility**: Fermented beans are easier to digest, like the proteins found in soy beans that are nearly indigestible until fermented (Katz, 2003). Fermented dairy products, like, cheese, yoghurt, and kefir, can be consumed by those not able to digest the raw milk, and aid the digestion and well-being for those with lactose intolerance and autism. Porridge made from grains allowed to ferment increases the nutritional values so much that it reduces the risk of disease in children (Battcock and Aza-Ali, 1998). Probiotic supplements (beneficial bacterial cultures for microbial balance in the body) are capable of fighting cancer and other diseases. Vinegar is used to leach out certain flavours and compounds from plant materials to make healthy and tasty additions to the meals.

2.4 Nigeria fermented foods

Scientist speculates that our ancestors possibly discovered fermentation by accident and continued to use the process out of preference or necessity. Preserving by fermentation did not only make foods available for future use and nutritious, but more digestible and flavorful. Fermented foods, whether from plant or animal origin, in combination with fungi or bacteria are an intricate part of the diet of people in all parts of the world. Fermented food plays a very important role in the socioeconomics of developing countries. Nigeria has its own types of fermented food, representing the staple diet and the raw ingredients available. The

preparation of many indigenous or "traditional" fermented foods and beverages remains a household art today.

Fermented foods enjoyed across the globe, conveys health benefits through lactic acid fermentation. The fermentation process can transform the flavour of food from the plain and mundane to a mouth-puckering sourness enlivened by colonies of beneficial bacteria and enhanced micronutrients (Tamang *et al.*, 2016).

2.5 Nutritional Composition of Kunu

The nutritional composition of kunu has been reported to consist of 2.31 – 3.63% (protein), 3.55 – 3.63% (fat), 1.16 – 1.21% (ash), 82.92 – 83.55% (carbohydrate) (Amusa and Odunbaku, 2009). The most abundant amino acid in kunu is glutamic acid (4.49-11.66g/100g) with the least being cysteine (0.34-1.45g/100g) (FAO, 1997). The lowest amount of amino acid except for tryptophan was reported to occur when rice was used as substrate to produce kunu beverage (0.44-1.40 g/100g) [Gaffa *et al.*, 2002b]. Also, among the amino acids, cysteine, valine, isoleucine and methionine are present in trace amounts when compared with FAO/WHO reference protein values (FAO, 1997).

2.6 Preparation of Kunu

The production of kunu is still at village technology level where procedures and materials or ingredients used are always not standardized. The traditional production process involves steeping of the chosen cereal or mixture of the cereals in water for 24 – 72 hours and wet milling with the aid of a local grinding machine. The steeped and washed grains are usually ground with spices such as ginger (*Zingiber officimals*) and alligator pepper (*Afromonium melegueta*) or red pepper (*Capsicum annuum*), or black pepper (*Piper guineense*) depending on the taste of the local producer. The slurry obtained is sieved and divided into two unequal portions. A potion (two-third volume) of the slurry is gelatinized with boiling hot water,

while to the remaining portion (one-third volume), about an equal volume of sweet potato (*Ipomonea batatas*) tuber paste, malted rice paste or extract of *Cadaba farinose* stem is added and mixed. The latter mixture is mixed vigorously and thoroughly with the former (the gelatinized portion while still hot) and allowed to ferment for 24 hours at room temperature, and then filtered using a local sieve. The filtrate (kunu-zaki) is consumed as a beverage with or without the addition of a sweetener, usually sugar. The beverage can be consumed fresh or bottled and stored at refrigeration temperature (Ayo *et al.*, 2014, Akoma 2006, Oluwajoba *et al.*, 2013). The flowchart for kunu-zaki is shown in Figure 1.

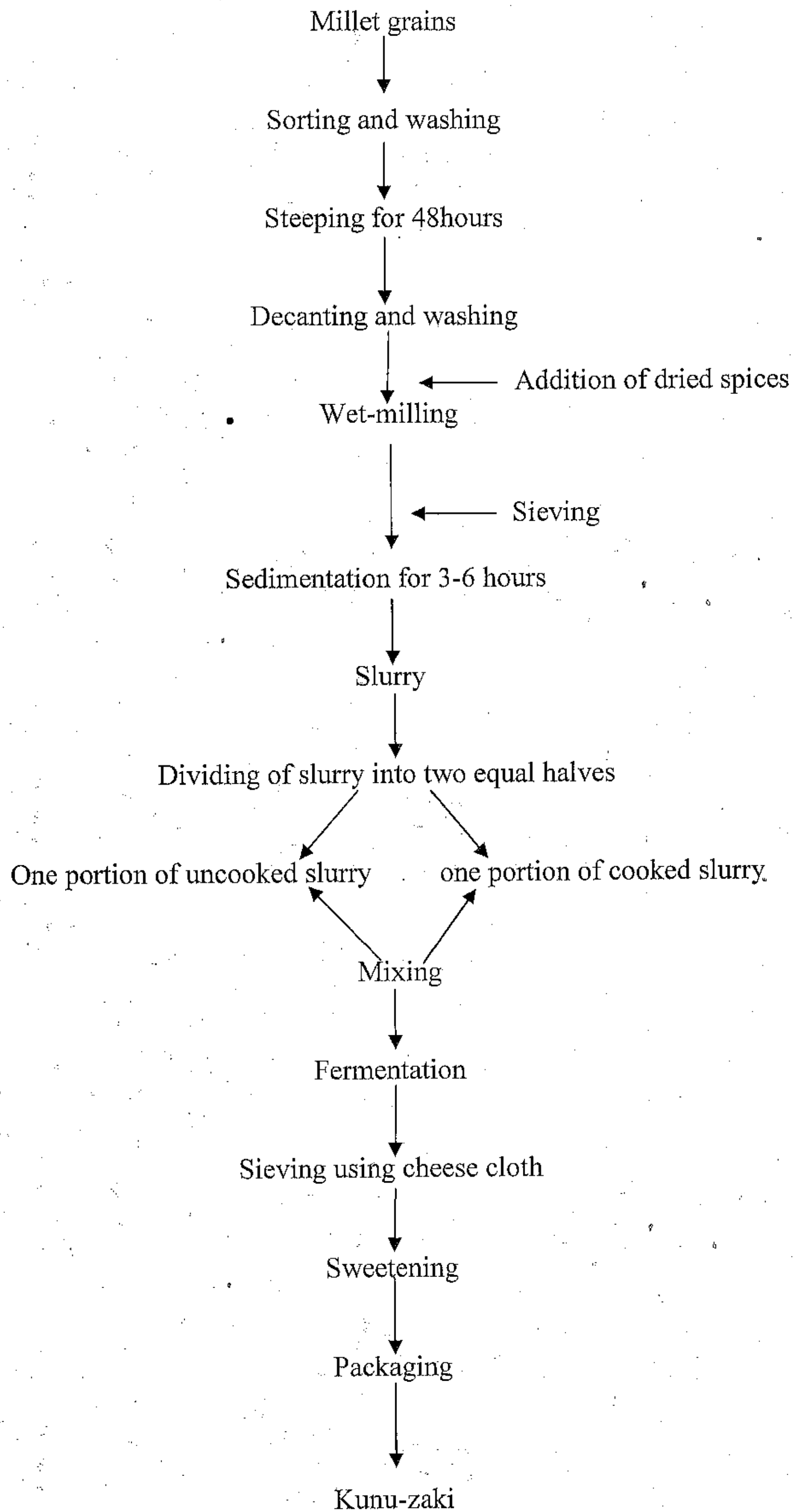

Figure 1: Flowchart for kunu-zaki production

Source: Agarry *et al.* (2010).

2.7 Micrognism in ready-to-drink Kunu

Microorganisms have been used to produce food for thousands of years. Fermented foods are not only perceived as pleasant-tasting but the acids produced as a by-product of microbial metabolism inhibit the growth of many spoilage organisms as well as food- borne pathogen (Nester *et al.*, 2004).

The microbial isolates found in Kunu Zaki sold include *Enterobacter, Shigella, Escherichia coli, Klebsilla, Micrococcus, Proteus, Leuconostoc, Bacillus, Citrobacter* and *Staphylococcus* (Ayo *et al.*, 2004). Amusa and Odunbaku (2009) reported *Lactobacillus plantarum, Bacillus sutilis, B. cereus, Streptococus feaceaum, Streptococus lactis, Staphylococcus aureus, Micrococcus acidiophilis, Escherishia coli, Pseudomonas aureginosa, Saccharomysces cerevisiae, Candida mycoderma, Aspergillus niger, Penicillium oxalicum* and *Fusarium oxysporum* as microbial diversity found in freshly processed and hawked kunun drinks sold in southwestern (Oyo, Ogun, Lagos and Osun State) Nigeria. Mbachu *et al.*, (2014) reported microbial isolates found in Kunun drink sold in Calabar, Nigeria as *Staphylococcus, Streptococcus, Bacillus, Pseudomonas* species and *Escherichia coli* (bacteria), and *Fusarium, Aspergillus, Penicillium* and yeast (fungi). Edward and Ohaegbu (2012) reported microbial diversity of kunu drink as *Lactobacillus, Bacillus, Staphylococcus, Aspergillus, Penicillum, Fusarium and Saccharomyces* species. Musa and Hamza (2013) *reported Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Rhizopus nigircans* and *Penicilium* species in Kunun aya sold in Kaduna State University, main campus.

The presence of some of these microbes in the kunu drinks could be from contaminated water used in processing, feedstock (whole cereal used in production of the kunu) and empty plastic bottles used in packaging of the kunu. The plastic bottles used by the processor to package the kunu drink are mostly picked from ceremony environment where they are found in large quantity. Occasionally, it can be gotten from individual who drink bottled water.

2.8 Spoilage of Kunu

Kunu has relatively short shelf-life storage. Adeyemi and Umar (1994) reported that the product has a shelf life of 24 hours at ambient temperature. The shelf life could be extended to days by pasteurization at 60^0C for 1 hours and storage under refrigeration conditions (Agarry *et al.,* 2010). Kunu can undergo spoilage as a result of some factors such as microorganisms involved in the fermentation process such as *Lactobacillus sp., Streptococcus sp.* and *Leuconostoc sp.* and these organisms have been reported to cause the spoilage of the beverage. Other organisms such as *Staphylococcus sp, Bacillus sp, Pseudomonas sp, Penicillium sp, Aspergillus sp, Trichoderma sp,* and *Candida sp* could cause the spoilage of kunu beverage if found in large quantity (Osuntogun and Aboaba, 2006).

Other factors such as temperature, light, time and the activities of insects, rodents or pests in the environment during the preparation could contribute to the spoilage of kunu and also, its high moisture content may encourage growth of strains to hazardous levels during storage at ambient temperature (Olasupo *et al.*, 2002).

2.9 Health Benefits of Kunu

Kunu has been reported to have the following benefits;

1) Kunu elevates lymphocyte counts which are indicative of its medicinal attributes, a concept widely believed by its numerous consumers (Akoma *et al.*, 2006).
2) It helps to stimulate the flow of breast milk in nursing mother (Adebayo *et al.*, 2010).
3) The kunu drink is rich in fiber, thus helping to promote bowel emptying and prevention of constipation (Essien *et al.*, 2009).
4) The presence of ginger in kunu helps to stimulate bile and saliva production; relieve gastrointestinal irritation and suppress gastric contractions (Essien *et al*, 2009).

5) Kunu drinks help in the prevention of chronic inflammatory diseases such as rheumatoid arthritis (Gaffa and Ayo, 2002).
6) It is good for women that have reached the stage of menopause as it helps in relaxation of muscle (Adebayo *et al.*, 2010).

2.9.0 Importance of Nigerian Cereal Fermented Beverages

Socio-cultural and economic aspect

Nigerian cereal beverages are empirically derived from the spontaneous fermentation of wort from germinated cereals (sorghum, maize, millet). They are produced and consumed in most parts of Africa where sorghum, maize and millet grow. The preparation of many traditional fermented foods and beverages remains as a household art. They are produced in homes, villages and small scale industries. Their production is deeply rooted in the tradition of people in African countries like Nigeria where they play a significant socio-cultural and economic role. The importance of traditional fermented beverages has been reviewed (Chelule *et al.*, 2010). Indeed, they are often attached to the traditions of hospitality and friendliness and are part of the etiquette of most families. They serve to seal harmonious relationships between individuals.

Traditional Nigeria beverages are generally consumed during fieldwork, popular festivities (marriage, naming, and initiation), and funerals. The fermented beverages serve as food supplement such as weaning food to supplement breastfeeding. Beverages are sometimes consumed in an environment and a specific social setting. This consumption is most popular in cabaret and used together in a "community of consumers', governed by rules of conviviality, sociability and sociality that induce a perception of product quality and its consumption patterns. They are usually consumed when they are fermenting and near the place of production. All social strata consume fermented beverages.

Today, traditional African beverages production has became a very important economic activity carried out by women. The sale of these products allows them to generate income for their families. Despite the technology that differs from one country to another and from one region to another, traditional African beverages have almost the same characteristics: a short shelf-life, a non or low alcohol, an sour aspect, solids and microorganisms in suspension, as well as taste and color characteristics with the low cost and the widespread availability in some populations (FAO, 1995).

2.9.1 Antibiotics

The importance and value of antibiotics cannot be overestimated; humans are totally dependent on them for the treatment of infectious diseases (Aminov and Mackie, 2007). In addition to their use in the treatment of infectious diseases, antibiotics are critical to the success of advanced surgical procedures, including organ and prosthetic transplants (Bush and Jacoby, 2010). There is a close association between the quantities of antimicrobials being used and the rate of development of resistance to these substances and thus the misuse of antibiotics in human medicine is believed to be the principal cause of the antibiotic resistance problem (Singer *et al.*, 2003). Resistance against a certain antimicrobial agent can be inherent in a bacterial species, this being referred to as intrinsic resistance, or "natural resistance". In this case, the resistance is typical for all of the strains of that particular species. In contrast, the resistance is considered as acquired, when a strain of a normally susceptible species becomes resistant to an antimicrobial drug (European Commission, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

Seven ready-to-drink kunu samples were purchased from different hawkers at Ile-Ife, Osun State, Nigeria. The samples were transported immediately in ice bucket to the laboratory for analysis.

3.2 Preparation of Reagents/Media

All chemicals used in this study were of analytical grade. The reagents and culture media used were prepared following standard methods as shown in the appendix.

3.3 Sterilization of Media and Apparatus

3.3.1 Autoclaving

The culture media used for the isolation, cultivation and identification of the bacterial isolates were sterilized by autoclaving at 121^0C for 15 minutes under pressure.

3.3.2 Flaming

The mouth of the conical flasks, test tubes, McCartney bottles and Bijou bottles were sterilized by flaming with a Bunsen burner before and after taking/pouring media into them. Inoculating loops and needles were sterilized using the Bunsen burner flame before and after inoculation. Streaking, inoculation and pouring of molten agar into Petri dishes were done close to the Bunsen burner flame in order to avoid contamination and maintain sterility.

3.3.3 Disinfection

The work tops were swabbed with 70% ethanol before any activities to provide a sterile condition and also after analysis in order to prevent contamination.

3.3.4 Hot Air Oven

Glassware's used in this study were thoroughly washed with detergent, rinsed with running tap water and set to drain out before sterilization in the hot air oven at a temperature of 160^0C for two hours. Pipettes were always wrapped with foil paper before sterilizing in the hot air oven.

3.4 Isolation and Identification of Bacterial Isolates

3.4.1 Serial dilution

Exactly 5 mL of kunu sample was aseptically transferred into 45 mL of distilled water in a 100ml capacity flask. It was then mixed with a vortex mixer to ensure uniformity of the sample. The resulting suspension was further diluted in sterile distilled water up to 10^7 dilutions.

3.4.2 Plating and Isolation of Bacteria

Exactly 1.0 mL of appropriately diluted sample was plated in nutrient agar and Eosin methylene blue agar using the pour plate technique. The inoculated plates were allowed to set, inverted and incubated at 35°C for 24-48 hours. After the incubation period, the colonies were studied and representative of the respective types were isolated and purified by streaking repeatedly on fresh nutrient agar. Pure cultures were then stored on nutrient agar slant in the refrigerator until required for further use.

3.5 Identification of Bacterial Isolates

The bacterial isolates were identified according to the scheme of Harrigan and McCance (1998) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1976).

3.6 Cultural Characteristics of Bacterial Isolates

Each of the bacterial isolate was grown on nutrient agar plate and examined for their growth pattern such as elevation, shape, opacity, edge, surface and size. The isolates were also grown in nutrient broth and examined for their growth pattern such as turbidity, amount of growth, surface growth, sedimentation and deposit.

3.7 Morphological Characteristics of Bacterial Isolates

3.7.1 Gram Staining

A thin film of each isolates (18-24 hour old) was made on a grease free microscope slide. The smear was heat fixed by passing it lightly through a Bunsen flame. The fixed smear was then flooded with basic crystal violet dye and left for a minute before rinsing off with tap water. The smear was flooded with Gram's iodine solution for a minute and rinsed off with tap water. The smear was decolorized with 70% ethanol and rinsed immediately with tap water. The smear was then counter stained with safranin for 30 seconds, rinsed with tap water, allowed to dry and examined under oil immersion objective of a microscope. Gram positive cells stained purple while Gram negative cells stained pink. The shape and the arrangement of the cells were also observed.

3.7.2 Spore Staining

Spore staining was performed for Gram positive and catalase positive rods using Bartholomew and Mittwers method (Harrigan and McCance, 1998). A heat fixed smear of 5 days old culture of the isolate was prepared on a grease free microscopic slide. The smear was stained with malachite green solution and steamed for 5-10 minutes ensuring that the stain does not dry out. The stain was carefully rinsed off with clean water and counter stained with safranin solution for 15 seconds, washed with water, blotted dry and examined under oil immersion objective of a microscope. Spores stained green while vegetative cells stained red.

3.7.3 Motility test

Sterile motility test medium was allowed to solidified, stab inoculated with 24 hours old broth culture of the test organism and incubated at 35°C for 48 hours. A diffuse zone of growth spreading from the line of inoculation indicated a positive reaction to the test medium. This was carried out following the scheme of Harrigan and McCance (1998).

3.8 Biochemical Characteristics of Bacterial Isolates

The biochemical test was carried out following the scheme of Harrigan and McCance (1998).

3.8.1 Catalase test

A loop full of 18-24 hours old broth culture of each isolate was emulsified with one or two drops of 3% hydrogen peroxide on a clean slide. Effervescence caused by the liberation of free oxygen as gas bubbles indicated catalase production.

3.8.2 Citrate utilization test

Sterile Koser's citrate medium in Bijou bottles were stab inoculated with 18-24 hours old broth culture of each isolate and incubated at 35°C for 5 days. A change in colour from green to blue indicated the utilization of citrate as the sole carbon source.

3.8.3 Gelatin Liquefaction

Sterile nutrient gelatin in test tubes were kept in ice bath to solidified, stab inoculated with 24 hours old broth culture of each isolate and incubated at 30°C for 14 days. Liquefaction of gelatin indicated positive results, while solidification of gelatin indicated negative result. This was carried out following the scheme of Harrigan and McCance (1998).

3.8.4 Indole/Ammonia Production Test

Sterile peptone water in test tubes was inoculated with 0.1 mL of 24 hours old broth culture of each isolate and incubated at 37°C for 7 days. 1 mL of Nessler's reagent was added to the

peptone water culture for ammonia production, development of orange to brown colour indicated a positive result while pale yellow or no colour change indicated a negative result when compared to the the control. 0.5 mL of Kovac's reagent was added to the peptone water culture and shaken; development of a deep red colour indicated the presence of indole. This was carried out following the scheme of Harrigan and McCance (1998).

3.8.5 Methyl red and Voges-Proskauer Test

Sterile MRVP medium in test tubes were inoculated with 0.1mL of 24 hours old broth culture of each isolates and incubated at 35°C for 5 days. The MRVP broth culture was aseptically divided into two portions labeled MR and VP. 5 drops of methyl red reagent was added to the MR test tubes and examined for colour change, a red colouration denoting a pH of 4.5 or less indicated a positive result while yellow colouration denoted a negative result. 0.5mL of 6% α-naphthol solution and 0.5mL of 16% of potassium hydroxide solution was added to the VP test tubes, shaken vigorously and left for 5-10 minutes. Development of red colouration indicated a positive reaction while a negative reaction was indicated by no colour change. This was carried out following the scheme of Harrigan and McCance (1998).

3.8.6 Nitrate reduction test

Sterile peptone water in test tubes was inoculated with 0.1mL of 24 hours old broth culture of each isolates and incubated at 37°C for 5 days. Reduction of nitrate was detected by development of red colouration within 5 minutes of adding 0.5mL of Griess-Illosvay's reagent to the inoculated and control test tubes. The test tubes that remained unchanged i.e show negative result were further confirmed by the addition of a pinch of zinc dust, development of pink colouration indicated a negative result while a positive result remains colourless indicating the reduction of nitrate beyond the nitrite stage.. This was carried out following the scheme of Harrigan and McCance (1998).

3.8.7 Oxidative/Fermentation test

Hugh and Leifson's medium was used to differentiate between bacterial isolates which have the ability to ferment carbohydrates either aerobically (oxidative) or anaerobically (fermentative). Duplicate tubes of Hugh and Leifson's medium were stab inoculated with 24 hours old broth culture of each isolates and incubated at 35°C for 5 days. A set of the duplicate tubes were left uncovered while the other set were covered with 2.0 mL of sterile liquid paraffin oil. The tubes were observed, fermentative organisms were indicated by colour change from blue to yellow in both tubes while oxidative organisms were indicated by colour change from blue to yellow in the open tubes only. This was carried out following the scheme of Harrigan and McCance (1998).

3.8.8 Starch Hydrolysis

Test organisms were streaked once across the surface of starch agar plate and incubated at 35°C for 5 days. The plates were flooded with Gram's iodine solution and examined. Hydrolysis of starch was indicated by a clear zone of the medium seen around or under the colony isolates while non-hydrolysis of the starch was indicated by blue back colouration around the isolates. This was carried out following the scheme of Harrigan and McCance (1998).

3.8.9 Sugar fermentation test

Sugar fermentation medium (composed of peptone water, 1% fermentable substrate (glucose, D-mannitol, sucrose, lactose) and Bromocresol purple indicator (with inverted Durham tubes) in test tubes were inoculated with 0.1mL of 24 hours old broth culture of each isolates and incubated at 35°C for 5 days. A change in the colour of indicator from purple to yellow indicated acid production while gas production was indicated by downward displacement of medium in inverted Durham tubes. This was carried out following the scheme of Harrigan and McCance (1998).

3.8.10 Triple sugar iron reaction test

TSI agar slope were prepared with a deep butt. The butt was stab inoculated and the slope was streaked with 24 hours old broth culture of each isolates and incubated at 35°C for 5 days. A pink colouration indicated alkaline production; blackening of the medium indicated hydrogen sulphide production while displacement of the butt indicated gas production. This was carried out following the scheme of Harrigan and McCance (1998).

3.9 Antibiotics sensitivity test

The bacteria isolated were sub cultured from stock culture into sterile nutrient broth and incubated at 37°C for 24 hours. The broth culture of the isolated organism was spread inoculated on Mueller Hinton agar (MHA) plates with the aid of a sterile cotton swab stick. The antibiotic sensitivity test disc (Gram positive disc was used for Gram positive bacteria and Gram negative disc was used for Gram negative bacteria) was picked and placed on the surface of the MHA plates using a pair of sterile forceps on the inoculated MHA plates. The MHA plates were then incubated at 37°C for 24 hours and observed for clear zone of inhibition (sensitivity) or resistance to the antibiotics. The antibiotics used were ceftazidime(30µg), erythromycin(5µg), cloxacillin(5µg), cefuroxime(30µg), ofloxacin(5µg), ceftriaxone(30µg), gentamicin(10µg) and amoxicillin(30µg) for Gram positive isolates while the Gram negative disc consisted of ceftazidime(30µg), cefuroxime(30µg), cefixime(5µg), augumentin(30µg), ofloxacin(5µg), gentamicin(10µg), nitrofurantion(300µg) and ciprofloxacin(5µg). This was carried out following the scheme of CLSI (2018).

CHAPTER FOUR

RESULTS

Table 4.1 shows the morphological and cultural characteristics (on nutrient agar) of aerobic mesophilic bacteria isolated from kunu samples on nutrient agar (NA) and Eosin methylene blue agar (EMB). Eleven organisms were isolated; six were Gram positive while five were Gram negative. Both Gram positive and Gram negative rods and cocci were isolated; seven were rods while four were cocci. The three Gram positive rods produced endospores.

Table 4.2 shows the cultural characteristics (in nutrient broth) of aerobic mesophilic bacteria isolated from kunu samples. Each isolates showed distinct characteristics that differentiate it from other isolates.

Table 4.3a shows the biochemical characteristics of aerobic mesophilic bacteria isolated from kunu samples. It is significant to note that all the isolates were catalase positive, nine isolates produce ammonia and three were able to reduced nitrate. Five of the isolates were fermentative and six were oxidative. Motility test and triple iron sugar test was carried out for the five Gram negative organisms.

Table 4.3b shows the probable identities of aerobic mesophilic bacteria isolated from kunu samples. The isolates were identified as *Staphylococcus saprophyticus*, *Enterobacter intermidis*, *E. aerogenes*, *Bacillus sphaericus*, *Corynebacterium spp.*, *Staphylococcus aureus*, *B. brevis*, *Citrobacter spp.*, *Shigella spp.* and *Escherichia coli.*

Table 4.1: Morphological and Cultural* Characteristics of Bacterial Isolates from Kunu.

ISOLATE CODE	GRAM REACTION	SPORE FORMATION	SHAPE	COLOUR	OPACITY	ELEVATION	SURFACE	EDGE
N1	Positive cocci	ND	Circular	Cream	Opaque	Flat	Smooth	Entire
N2	Negative cocci	ND	Punctiform	Cream	Opaque	Flat	Smooth	Entire
N3	Positive rod	+	Irregular	Cream	Translucent	Raised	Dull	Tentate
N4	Negative rod	ND	Irregular	Cream	Opaque	Raised	Dull	Undulate
N5	Positive rod	+	Rhizoid	Cream	Opaque	Flat	Glittering	Rhizoid
N6	Positive rod	+	Irregular	Cream	Opaque	Flat	Glittering	Fimbrate
N7	Positive cocci	ND	Circular	Cream	Opaque	Raised	Smooth	Entire
N8	Positive cocci	ND	Irregular	Colourless	Translucent	Raised	Dull	Entire
E1	Negative rod	ND	Circular	Purple**	Opaque	Low convex	Dull	Entire
E2	Negative rod	ND	Punctiform	White**	Opaque	Flat	Smooth	Entire
E3	Negative rod	ND	Circular	Metallic Green Sheen**	Opaque	Flat	Glittering	Entire

*Cultural characteristics of the isolates were described on nutrient agar

*ND- Not determined

**Colour on Eosin Methylene Blue Agar

Table 4.2 Cultural Characteristics of Bacterial Isolates in Broth Medium.

ISOLATE CODE	AMOUNT OF GROWTH	SURFACE GROWTH	TURBIDITY	DEPOSIT	SEDIMENTATION
N1	Scanty	Absent	Absent	Disintegrates on shaking	Pellicle
N2	Scanty	Absent	Absent	Absent	Uniform
N3	Profuse	Pellicle	Uniform	Disintegrates on shaking	Deposit of pellicle
N4	Moderate	Absent	Turbid	Granular	Uniform with deposit
N5	Scanty	Absent	Absent	Absent	Uniform
N6	Scanty	Absent	Turbid	Absent	Uniform
N7	Scanty	Present	Uniform	Viscoid	Uniform with deposit
N8	Scanty	Absent	Absent	Present	Pellicle
E1	Scanty	Absent	Uniform	Viscoid	Uniform
E2	Scanty	Absent	Absent	Absent	Uniform
E3	Moderate	Absent	Turbid	Granular	Uniform with deposit

Table 4.3a Biochemical Characteristics of Bacterial Isolates from Kunu.

BIOCHEMICAL TEST	ISOLATE CODES										
	N1	N2	N3	N4	N5	N6	N7	N8	E1	E2	E3
Nitrate reduction	-	-	-	-	-	-	-	-	-	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	+
Ammonia production	-	+	+	+	+	+	+	-	-	+	+
Methyl Red	-	+	+	+	-	-	+	-	-	-	+
Voges-Proskauer test	-	+	+	-	+	-	+	-	-	+	-
Citrate utilization	+	-	-	-	+	+	+	+	-	+	-
Catalase test	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	-	+	-	+	-	+	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-
Motility test	ND	-	ND	+	ND	ND	+	ND	-	-	-
Oxidative/Fermentative test	F	O	O	O	O	O	O	F	F	F	F
TSI test	ND	ND	ND	A	ND	ND	ND	ND	AG	-	AG
Sugar fermentation test											
Glucose	AG	A	A	A	A	A	A	AG	A	AG	-
Mannitol	A	A	-	-	A	A	A	A	-	AG	A
Lactose	-	A	-	-	-	-	-	-	-	AG	AG
Sucrose	AG	A	A	A	A	A	A	AG	-	A	AG

KEYS: O = Oxidative, F = Fermentative, A = Acid only, G = Gas only, AG = Acid and gas, ND = Not determined, - = Negative, P = Positive.

Table 4.3b Probable Identity of Aerobic Mesophilic Bacteria Isolated from Kunu.

ISOLATE CODES	PROBABLE IDENTITY
N1	*Staphylococcus saprophyticus*
N2	*Enterobacter intermidis*
N3	*Bacillus sphaericus*
N4	*Enterobacter aerogenes*
N5	*Corynebacterium spp*
N6	*Bacillus brevis*
N7	*Staphylococcus aureus*
N8	*Staphylococcus saprophyticus*
E1	*Citrobacter spp*
E2	*Shigella spp*
E3	*Escherichia coli*

Table 4.4 depicts the occurrence pattern of aerobic mesophilic bacteria isolated from kunu samples. It is significant to note that, *Enterobacter intermidis* was present in six out of the seven samples except Sample C while *Staphylococcus saprophyticus and Shigella spp* were present in five of the seven samples studied. *Bacillus sphaericus*, *Citrobacter spp.*, and *Corynebacterium spp.* were present in three samples while *S. aureus, E. aerogenes* and *B. brevis* were present in two out of the seven samples studied. *Escherichia coli* were only present in Sample B.

Table 4.5a shows the antibiotic susceptibility pattern of Gram positive bacteria isolated from kunu. All the Gram positive organisms were resistant to Ceftazidime, Cefuroxime, Ceftriaxone and Cloxacillin. *Bacillus sphaericus*, *Corynebacterium spp., Staphylococcus aureus* and *B. brevis* were all susceptible to Gentamicin, Erythromycin and Ofloxacin except *B. sphaericus* that is intermediate to Erythromycin. *Staphylococcus aureus* is also susceptible to Amoxycillin.

Table 4.5b shows the antibiotic susceptibility pattern of Gram negative bacteria isolated from kunu. All the Gram negative organisms were resistant to Cefixime, Cefuroxime and Augumentin but susceptible to Ofloxacin, ciprofloxacin and nitrofurantoin.

Table 4.4 Occurrence Pattern of Aerobic Mesophilic Bacteria Isolated from Kunu.

Organism Isolated	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F	Sample G	% Occurence Pattern
Staphylococcus saprophyticus	+	+	-	-	+	+	+	71.43
Enterobacter intermidis	+	+	-	+	+	+	+	85.71
Bacillus sphaericus	-	+	-	-	+	-	+	42.86
Enterobacter aerogenes	-	-	+	+	-	-	-	28.57
Corynebacterium spp	-	-	+	+	+	-	-	42.86
Bacillus brevis	-	-	+	-	-	-	+	28.57
Staphylococcus aureus	-	+	+	-	-	-	-	28.57
Staphylococcus saprophyticus	-	+	+	-	+	+	+	71.43
Citrobacter spp	-	+	-	-	-	+	+	42.86
Shigella spp	-	+	+	-	+	+	+	71.43
Escherichia coli	-	+	-	-	-	-	-	14.29

KEYS: - = Absent, + = Present

Table 4.5a Antibiotic Susceptibility Pattern of Gram Positive Bacteria Isolated from Kunu.

ISOLATES	ANTIBIOTICS/SUSCEPTIBILITY PATTERN							
	CAZ	CRX	GEN	CTR	ERY	CXC	OFL	AUG
Staphylococcus saprophyticus	R	R	R	R	R	R	R	R
Bacillus sphaericus	R	R	S	R	I	R	S	R
Corynebacterium spp	R	R	S	R	S	R	S	R
Bacillus brevis	R	R	S	R	S	R	S	R
Staphylococcus saprophyticus	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	S	R	S	R	S	S

KEYS: CAZ = Ceftazidime (30µg), CRX = Cefuroxime(30µg), GEN = Gentamicin (10µg), CTR = Ceftriaxone (30µg), ERY = Erythromycin (5µg), CXC = Cloxacillin (5µg), OFL = Ofloxacin (5µg), AUG = Amoxycillin (30µg)

S = Susceptibility, R = Resistance, I = Intermediate.

Table 4.5b Antibiotic Susceptibility Pattern of Gram Negative Bacteria Isolated from Kunu Samples.

ISOLATES	ANTIBIOTICS/ SUSCEPTIBILITY PATTERN							
	CAZ	CRX	GEN	CXM	OFL	AUG	NIT	CPR
Enterobacter intermidis	R	S	S	R	S	R	S	S
Enterobacter aerogenes	R	R	S	R	S	R	S	I
Citrobacter spp	I	R	S	R	S	R	S	S
Shigella spp	S	R	R	R	R	R	R	R
Escherichia coli	I	R	S	S	S	R	S	S

KEYS: CAZ = Ceftazidime (30µg), CRX = Cefuroxime(30µg), GEN = Gentamicin (10µg), CXM = Cefixime (5µg), NIT = Nitrofurantoin (300µg), CPR = Ciprofloxacin (5µg), OFL = Ofloxacin (5µg), AUG = Augumentin (30µg)

S = Susceptibility, R = Resistance, I = Intermediate.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The kunu samples obtained from different retailers in Ile-Ife, Osun state, Nigeria contained a variety of bacteria which were Gram positive and Gram negative. Most of the bacteria were resistant to wide range of antibiotics while others were either sensitive or intermediate.

The presence of aerobic mesophilic bacteria reported in this study may be attributed to several factors which include lack of effective precautions on good manufacturing practice in handling procedures during processing of the beverage, the practice of addition of some quantity of water to dilute Kunu after fermentation may also be a source of microbial contaminants which may have come from the water itself or from the utensils used for such purposes. The spices used as additives to add flavour to kunu may be an additional source of contaminants (Essien *et al.*, 2009).

The presence of coliforms such as *Enterobacter aerogenes, E. intermidis, Citrobacter spp,* in hawked Kunu was as a result of contaminated water, containers, as well as dirty environment where the Kunun-zaki were being processed and hawked. The percentage occurrence of *Shigella spp*, *Staphylococcus saprophyticus, S. aureus* and *Enterobacter intermidis* in the samples analyzed is a pointer to the fact that the Kunu samples studied are contaminated with potentially pathogenic bacteria and this may have come from the water used for domestic purposes, or human handlers during processing and sales of the product. This is in agreement with Amusa and Ashaye (2009) and Akoma *et al.* (2013), who had reported that water used for production coupled with the crude method of production and packaging under improper sanitary conditions predisposes Kunu drink to microbial contamination by an array of both Gram negative and Gram positive bacteria. There is therefore need for surveillance by Public

Health officials to ensure safety of the Kunun-zaki being sold for public consumption in Ile-Ife.

Staphylococcus aureus is a Gram positive bacteria found in smaller amounts in the nose and on the skin of clinically healthy people. Higher amounts can be found in lesions of skin such as infected eczema, psoriasis or any other pus draining lesion. The presence of *S. aureus* in the samples could therefore be from human handling. The presence of Staphylococcus in food has been reported to cause food poisoning resulting from heat resistant staphylotoxin (Wonang *et al.*, 2001). The symptoms of staphylococcal poison include diarrhea, vomiting, cramps and fever which starts suddenly but usually disappears within 24 hours.

Shigella spp are Gram negative rods found in humans and primate. Its main mode of transmission is person to person contact due to its low infectious dose; it can also be transmitted through infected food and water. The clinical manifestation of shigellosis are fever, watery diarrhea and can also manifest as a dysenteric syndrome which includes fever, abdominal cramps and bloody stools containing mucous (Umaru *et al.*, 2014).

Bacillus brevis is a Gram positive, aerobic, motile spore forming bacillus commonly found in water, soil, air, and decaying matter. It is implicated in peritonitis on individual having heptocellular carcinoma due probably from ingestion of fermented foods containing *Brevibacillus brevis* spores yet it's rarely associated with infectious disease (Van der Woude and Baumler, 2004).

Citrobacter spp is a genus of Gram negative coliform bacteria in the Enterobacteriaceae family. They are occasional inhabitants of the gastrointestinal tract and are responsible for

disease in debilitated or immunocompromised patients. Citrobacter infections can be fatal, with 33-48% overall death rates and 30% for neonates. They are found in soil, water, wastewater etc (Murray *et al.*, 2010).

Food pathogens such as *Escherichia coli* could cause food poisoning resulting from consumption of contaminated product or food (Mbachu *et al.*, 2014). The presence of *Escherichia coli* and *Enterobacter aerogenes* is indication of contaminants from fecal origins. The presence of *E.coli, Staphylococcus aureus, Streptococcus sp.* and *Shigella sp.* in the beverage is of public health significance as they are considered the leading cause of food-borne toxicosis outbreak worldwide (Karagozlu *et al.,* 2007). The presence of *E. coli* (14.29%) in *kunun* is an indication of faecal and environmental contaminations (Umaru *et al.*, 2014; Aboh and Oladosu, 2014), probably through the use of water or directly during handling. *E. coli* is capable of causing gastroenteritis, diarrhea, and urinary tract Infection (UTI) if ingested by humans.

Most of the antimicrobial resistance in the treatment of infectious diseases is due to the extensive use and misuse of antimicrobial drugs which have favoured the emergence and survival of resistant strains of microorganisms (Singer *et al.,* 2003). The prevalence of resistant strains of *E. coli*, *Enterobacter aerogenes*, *Streptococcus* spp and *Staphylococcus aureus* in Kunun-zaki is a reflection of the use and misuse of the antibiotics in the society. This is not surprising because outside the hospital environment, the general populace have access to various kinds of antibiotics at any drug store even without prescription from a medical practitioner.

The Public Health implication of this study is that antimicrobial resistant strains of pathogenic bacteria may colonize the human population through consumption of

contaminated Kunu and this would lead to chemotherapeutic failures among the human consumers of this popular beverage in the environment under study.

5.2 CONCLUSION

The presence of antibiotic resistant strains of *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus* and *Streptococcus spp* in Kunun-zaki studied suggests that consumption of this beverage has potential health hazard to the consumers in Ile-Ife, Osun State, Nigeria. The consumers of this popular drink may therefore be placed at health risk which may culminate into failures of commonly used clinical antibiotics for the treatment of infections.

From the result obtained, it could be seen that organisms associated with kunun-zaki were *Escherichia coli, Staphylococcus species, Corynebacterium species, Enterobacter species and Citrobacter species* and the activity of these organisms in kunun –zaki may rendered it unfit for human consumption.

5.3 RECOMMENDATIONS

To protect the healthy consumers, government and regulatory authorities such as National Agency for Food Drug Administration Control (NAFDAC) should screen food handlers and inspect the production environment in order to reduce health hazards and mortality rate, intervene by setting standards in acquisition of raw material, production techniques as well as health status of personnel involved in the production process.

The high counts of spoilage and pathogenic microorganisms in Kunun-zaki could be reduced if starter cultures are employed in its fermentation process as done in the developed world. Food handlers should be encouraged to strictly adhere to Good Manufacturing Practices

(GMPs) to minimize the spread of infections from the drink and adequate amount of heat should be used during beverage processing to kill pathogenic organisms.

Treated water or clean water should be used during processing and in dilution of the processed drinks to avoid contamination with pathogenic microorganisms. The packaging materials should also be sterilized.

Health education training should be organized regularly for those involved in the producrion of kunu zaki by the health workers on the importance of cleanness of their environment.

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APPENDIX I

PREPARATION AND COMPOSITION OF MEDIA USED FOR MICROBIAL ISOLATION AND IDENTIFICATION.

Nutrient Agar (NA)

Composition g/L

Lab. Lemco powder	1.0g
Yeast extracts	2.0g
Peptone	5.0g
Agar	15.0g
Sodium chloride	5.0g
Distilled water	1000 mL
pH	7.4

Powdered NA (22.4g) was dissolved in 800ml of distilled water by heating on a hot plate for homogenization, and thereafter dispensed into McCartney bottles and sterilized in the autoclave at 121°C for 15 minutes.

Nutrient Broth (NB)

Composition g/L

Lab. Lemco powder	1.0g
Yeast extracts	2.0g
Peptone	5.0g
Sodium chloride	5.0g
Distilled water	1000 mL
pH	7.4

Powdered NB (5.2g) was dissolved in 400ml of distilled water by heating in a microwave, and thereafter the rehydrated broth was dispensed into test tubes and sterilized in the autoclave at 121°C for 15 minutes under pressure.

Mueller Hinton agar

Composition g/L

Beef extract	2.0g
Acid hydrolysate of casein	17.0g
Starch	1.5g
Agar	17.0g
Distilled water	1000 mL
pH	7.3±0.1

38g of the powered medium was dissolved in 1000mL of distilled water, mixed thoroughly and heated in a microwave to dissolve completely, and thereafter dispensed into McCartney bottles and sterilized in the autoclave at 121°C for 15 minutes under pressure.

Starch Agar

Composition g/L

Nutrient agar	400 mL
Starch	4g

1.0g of soluble starch was added in 400ml of molten nutrient agar, mixed thoroughly and heated in a microwave to dissolve completely, and thereafter dispensed into McCartney bottles and sterilized in the autoclave at 121°C for 15 minutes under pressure.

Eosin Methylene Blue Agar (EMBA)

Composition g/L

Peptone	10.0g
Lactose	10.0g

Agar	1.50g
Dipotassium hydrogen phosphate	2.0
Methylene blue	5.0g
Eosine	0.4g
Distilled water	1000 mL
pH	6.8±0.2

Powdered EMBA (7.5g) was dissolved in 1000ml of distilled water by heating on a hot plate for homogenization, and thereafter dispensed into McCartney bottles and sterilized in the autoclave at 121°C for 15 minutes under pressure.

Peptone Water

Composition g/L

Peptone	10.0g
Sodium chloride	5.0g
Distilled water	1000 mL
pH	7.4

7.5g of peptone powder was added in 500ml distilled water, mixed and dispensed into McCartney bottles and sterilized in the autoclave at 121°C for 15 minutes under pressure.

Nutrient Gelatin

Composition g/L

Nutrient broth	2.6g
Gelatin	20.0g
Distilled water	1000 mL

The mixture was dissolved by microwaving and dispensed in test tubes for sterilization in the autoclave at 121°C for 15 minutes under pressure.

Koser's Citrate Medium

Composition g/L

Sodium ammonium hydrogen phosphate	1.5g
Potassium dihydrogen phosphate	1.0g
Magnesium sulphate	0.2g
Sodium citrate	3.0g
Bromothymol blue	0.016g
Distilled water	1000 mL
pH	6.8

The mixture was dissolved completely and dispensed in bijou bottles for sterilization in the autoclave at 121°C for 15 minutes under pressure.

Sugar Fermentation Basal Medium

Composition g/L

Peptone	10.0g
Sodium chloride	5.0g
Bromocresol purple	0.025g
Distilled water	1000ml

The mixture was dissolved completely and divided into four portions of 200ml each; 2g of each sugar (Lactose, Sucrose, Mannitol and Glucose) was weighed into each of the flasks and labeled accordingly. Each of the flasks was heated in the microwave to dissolve the sugars, dispensed into test tubes and autoclaved at 121°C for 15 minutes under pressure.

Motility Agar

Composition g/L

Bacteriological tryptone	0.5g
Sodium chloride	0.5g
Agar	0.25g
Distilled water	50ml

The mixture was completely dissolved by microwaving and dispensing in 8 test tubes for sterilization in the autoclave at 121°C for 15 minutes under pressure.

Methyl Red and Voges-Proskauer Medium (MRVP)

Composition g/100 mL

Peptone	0.5g
Dipotassium hydrogen phosphate	0.5g
Dextrose	0.5g
Distilled water	100 mL
pH	7.4

The component was mixed well and dissolved completely before dispensing in 40 test tubes for sterilization in the autoclave at 121°C for 15 minutes under pressure.

Nitrate Peptone Water

Composition g/L

Peptone water	100ml
Potassium nitrate	0.2g

0.1g of potassium nitrate was added to 100ml of peptone water, the mixture was dissolved completely by steaming and dispensed into test tubes for sterilization in the autoclave at 121°C for 15 minutes under pressure.

Triple Sugar Iron Agar

Composition g/L

Peptone from casein	15.0g
Peptone from meat extracts	5.0g
Meat extracts	3.0g
Yeast extracts	3.0g
Sodium chloride	5.0g
Lactose	10.0g
D(+) Glucose	1.0g
Sucrose	10.0g
Ammonium Iron (iii) citrate	0.5g
Sodium thiosulphate	0.5g
Phenol red	0.024g
Agar	12g
Distilled water	1000 mL

The components were dissolved completely by steaming and dispensed into test tubes for sterilization in the autoclave at 121°C for 15 minutes under pressure. They were then placed in slant positions to set.

Hugh and Leifson's Medium

Composition g/L

Peptone	1.6g
Sodium chloride	4.0g
Potassium hydrogen phosphate	0.24g
Bromothymol blue	0.064g
Agar	2.4g

Glucose	8.0g
Distilled water	800ml

The components were mixed together and dissolved completely before dispensing into test tubes for sterilization in the autoclave at 121°C for 15 minutes under pressure.

APPENDIX II

PREPARATION OF REAGENTS

Greiss Illosvay's Reagents

Composition

Reagent A: Sulphanilic acid	0.8g
Acetic acid (5N)	100ml
Reagent B: Naphthyamine	0.5g
Acetic acid (5N)	100ml

Voges-Proskauer Test Reagents

(Barrit's Modification)

Reagent A: α-Naphthol	6.0g
Ethanol 95%	100ml
Reagent B: Potassium hydroxide	16.0g
Distilled water	100ml

GRAM'S STAINING REAGENTS

Gram's iodine solution

Composition

Potassium iodide	2.0g
Iodine	1.0g
Distilled water	300ml

Crystal violet solution

Composition

Crystal violet	0.5g
Distilled water	100ml

Safranin solution

Composition

Safranin O powder	4.0g
Ethanol (Absolute)	200ml
Distilled water	800ml

Bartholomew and Mittwer's spore stain reagents

Malachite green

Composition

Malachite green	5.0g
Distilled water	100ml

Counterstain - safranin

Composition

Safranin	0.25g
Distilled water	100ml

Methyl Red Reagent

Composition

Methyl Red	0.1g
Ethanol	300ml
Distilled water	500ml

Nessler's Reagents

Composition

Potassium iodide	7.0g
Mercuric iodide	10.0g
Potassium hydroxide	10.0g
Distilled water	100ml

Kovac's indole Reagents

Composition

Amyl alcohol	150ml
p-dimethylaminobenzaldehyde	10.0g
Concentrated hydrochloric acid	50mL