Microorganisms and Antibiotic Resistance of *Lactobacillus* Species from Fermented and Dewatered Maize Slurry *(Akamu)* Sold In Port Harcourt Metropolis, Nigeria

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ABSTRACT

The microorganisms and antibiotic resistance of *Lactobacillus* species from fermented and dewatered maize slurry (*Akamu*) sold in Port Harcourt Metropolis was investigated. This was carried out by using standard microbiological techniques. The antibiotic sensitivity test was carried out using antibiotic impregnated multi disc containing ten different antibiotics. The total heterotrophic bacteria count ranged from 1.3 x 10^{9}cfug^{-1} to 7.1 x 10^{6}cfug^{-1} of *Akamu* and total lactic acid bacteria count ranged from 1.56 x 10^{5} to 2.27 x 10^{5}cfug^{-1}. The mean value of fungal count ranged from 1.50 x 10^{3} to 2.50 x 10^{6}cfu/g^{-1}, total coliform count ranged from 34 to ≥1600 coliform (MPN) 100ml^{-1} while the thermotolerant coliform and facecal coliform ranged from 27 to ≥1600 coliform (MPN) 100ml^{-1}. The incidence (%) of bacteria was; *Lactobacillus acidophilus* (28%), *L. brevis* (14%) *L. casei* (18%), *L. dextrinicus* (4%). *L. fermentum* (4%), *L. pentosus* (8%), *L. plantarum* (4%), *L. rhamnosus* (10%) and *Pseudomonas aeruginosa* (10%). *L. acidopilus* had the highest incidence. The fungi isolated were *Aspergillus niger*, *Aspergillus versicola*, *Fusarium solani*, *Mucor pusillus*, and *Rhizopus oligosporus*. The fungi all had incidence of 20% each. Generally, microbial counts were highest in samples purchased from hawkers and lowest in the samples from producers. The antimicrobial susceptibility profile indicated that *Lactobacillus rhamnosus* exhibited the highest resistance (70%) to antibiotics while *L. plantarum* was the least resistant (10%). The bacteria were most resistant (71.43%) to Amoxil® and Ampiclox®, and least resistant (14.28%) to Norfloxacin. Generally, with the exception of *L. plantarum* which was resistant only to streptomycin, all the bacteria isolates exhibited multiple resistance to the antibiotics used. Antimicrobial resistance is a global problem and the emergence of multidrug resistance will hinder the therapeutic options. The presence of thermotolerant coliform and facecal coliform bacteria must have been implicated in the diseases or illnesses associated with the consumption of maize slurry (*Akamu*). It is highly recommended that fermented and dewatered maize slurry (*Akamu*) should be prepared and handled under good sanitary and hygienic practices to avoid contaminating it with faecal materials especially from hawkers are essential to maintaining the quality of *Akamu* products.

Key words: Maize slurry, fermentation, "*Akamu*", coliform, *Lactobacillus*, antibiotic resistance.

INTRODUCTION

Maize (*Zea mays*) also known as corn is a cereal grain that is one of the staple widely grown and consumed in Nigeria. Maize is the third most widely cultivated food crop

in Nigeria Maize as a crop is highly yielded, easy to process and readily digested. It is a versatile crop that grows on a cross range of agroecological zones (Jay, 1998).
There are principally two types of maize (white and yellow variety) that are produced in Nigeria. Maize is planted for its grains that are used for making flour or eaten as vegetable Maize can be eaten as whole grain when boiled or roasted. Maize can also be used in its prepared form as pap (maize porridge) and Eko (Agidi) which is an extracted starch meal obtained after the cooking (boiling) of a prolonged soaking and fermentation of maize slurry.
Maize slurry (*Akamu*) is a product of fermentation of maize (corn), millet or sorghum (Kocchar, 1991). Maize slurry *(Akamu*) can be processed into a whitish or yellow-like custard depending on the variety of maize used for the fermentation. *Akamu* is classified among the breakfast foods (Adeyola *et al.*, 1988). It is the first native food given to babies at weaning (Banigo and Muller, 1997). Preparation of *Akamu* involves the steeping of the maize grains for two days and wet milling thereafter. Water is added to the mash and is sieved through a clean cloth. The filtrate is allowed to sediment for a day and dewatered with a clean cloth sac. These are the typical stages of *Akumu* and do not include some of the stages of Eka, 1999. Fermented foods are largely consumed in Africa where they constitute a bulk of diet. Majority of Nigerian fermented foods are products obtained though lactic acid fermentation such as Eko, fufu, Iru, Fermented cabbage, cucumber, pumpkin as well as Yoghurt, Palm Wine, Burukutu, Kununzaki etc (Ogunbanwo *et al*., 2004).
Lactic acid bacteria are usually found in decomposition of plants and lactic products and produces lactic acid as the major metabolic end product of carbohydrate fermentation. In other words, lactic acid bacteria are found in carbohydrate rich materials especially fermented foods (Kenneth, 2004). Lactic acid bacteria and other metabolic process contribute to the organoleptic and textural profile of a food item. The industrial importance of lactic acid bacteria is further evinced by their generally recognized as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces.
Bacteriocins are proteinaceous toxin produced by lactic acid bacteria are of global interest to the food fermentation industry because they inhibit the growth of many spoilage and pathogenic bacteria and thus extend the shelf life of foods (Herrero *et al.*, 1996). Bacteriocins are typically considered to be narrow spectrum antibiotics, though this has been debated (Farkas-Himsley, 1980).
Fermentation improves the nutritive content of some of the fermented foods by the presence of microorganism. Fermented foods are more easily digestible over unfermented food. This is because there is a partial or complete hydrolysis of some substances present in the substrate which will enhance their digestion (Savadogo *et al.*, 2004). Fermented foods generally enrich the human dietary through development of a wide diversity of aromas, flavours, and textures in foods (Soomro *et al*., 2002). Fermented foods usually have a better nutritional quality than the confirmed substrates. It has been reported that pap contains 82.26% carbohydrate. Proteins, amino acids, lipids and vitamins can also be derived from the microorganisms involved in the fermentation process (Saito *et al*., 1979).
However, in as much as microorganisms are beneficial in most fermentation process, some may pose high risk of food contamination and cause food-borne illness.
The topics of relevance to agricultural biotechnology in developing countries are options that are applicable to the study and improvement of microorganisms which offer potential for improving the quality, safety and consistency of fermented foods

among others and finally, improving diagnostics and identification systems applicable to foods (FAO, 1996).
It is common knowledge in Nigeria that some persons including children and adult fall ill after the consumption of *Akamu*. The aim of this study therefore was to determine the microbial load and types of microorganisms with emphasis on *Lactobacillus* species and coliforms in fermented and dewatered maize slurry *(Akamu)*; to isolate and characterize *Lactobacillus* species and to determine the antibiotic susceptibility of the *Lactobacillus* species isolated because most bacteria are potential pathogens.
This investigation will help to ascertain the health hazards associated with the improper (unsanitary) processing, cooking and consumption of such maize product.

MATERIALS AND METHOD

Collection and Processing of *"Akamu"* Samples

Samples of dewatered maize slurry *(Akamu)* were purchased from different locations in Port Harcourt metropolis and immediately taken to the laboratory for analyzes. Samples were collected weekly for a period of three weeks from different producers, sellers and hawkers.
Sample A was purchased directly from Producers of *Akamu* in Mile 2 Diobu area. Sample B was purchased from Sellers in Mile 3 Market in Diobu area. These are persons who have bought from the producers and are usually seated in a location to sell the *Akamu*. Sample C was purchased from Hawkers or Vendors in Eagle Island area. These are persons who hawk the *Akamu* all about as to sell it.

Microbiological Media and Antibiotics and their concentrations

The common media used for the study were Nutrient agar, Sabouraud Dextrose Agar (SDA), MacConkey agar, and Lactose broth. A total of ten different antibiotics were contained in each Gram negative and gram positive disc used. These antibiotic discs are disc of filter paper that has been impregnated with common antibiotics manufactured by Optun laboratories Rivers State, Nigeria. The antibiotics and their concentrations are as follows: Positive disc - Ciproflox 10µg, Norfloxacin 10µg, Amoxil® 20µg, Streptomycin 30µg, Gentamycin 10µg, Rifampicin 20µg, Erythromycin 30µg, Chlorampenicol 30µg, Ampiclox® 20µg, Levofloxacin 20µg. Nagative disc - Tarivid® 10µg, Reflacine® 10µg, Ciproflox® 10µg, Augmentin® 30µg, Gentamycin 10µg, Streptomycin 30µg, Ceporex® 10µg, Nalidixic acid 30µg, Septrin® 30µg, Amplicin 30µg.

Microbiological Analysis

Cultivation and Enumeration of Total Heterotrophic Bacteria and Fungi

Enumeration of Viable Microbial count of microorganisms, the total viable count of bacteria and fungi in the Maize slurry *(Akamu)* were estimated using the spread plate method.
Serial dilution was carried out on each sample of fermented and dewatered maize slurry (*Akamu*). The dilution factor for the isolation of bacteria was 10^{-3} while the dilution factor for the isolation of fungi was 10^{-2}. This was done so as to obtain discrete colonics when plated on the medium. One gram (1.0g) each of maize slurry (*Akamu*) samples was added to separate 9.0ml of normal saline (diluent) and further dilution was made up to 10^{-2} and 10^{-3}.

An aliquot (0.1ml) of the appropriately diluted sample was then inoculated onto nutrient agar plates for the isolation of bacteria and onto Sabouraud dextrose agar plates for the isolation of fungi. The spread plate method was done using sterile bent glass spreader to spread the sample evenly on the agar plates. Cultures were prepared in duplicates. Cultured Nutrient agar plates were incubated at 37^0C for 24 hours while the cultured SDA plates were incubated on the laboratory bench for 3 to 5 days. Discrete colonies that developed on the plates (overnight culture) were counted, the average taken and recorded as total heterotrophic counts of bacteria.
Discrete colonies were collected aseptically and streaked onto nutrient agar plates (for bacteria purification) and incubated at 37^0C overnight. Pure colonies were later stored in Mac Cartney bottles containing nutrient agar slants and put into the fridge as stocks cultures for further biochemical tests. A total of nine (9) pure cultures were stored and regarded as the bacteria isolates.
Colonies which developed after 5 days on SDA plates were counted and the average count for the duplicate cultures were recorded as total viable fungi of each sample. The colour and colonial morphologies or characteristics were also recorded. Discrete colonies were subcultured onto freshly prepared SDA to obtain pure cultures.

Estimation of Coliforms
Estimation of the coliform bacteria was done using the most probable number technique (MPN technique). Reaction to MPN technique and thermotolerant coliform bacteria MPN index 100ml of each maize slurry *(Akamu)* sample was done using double strength MacConkey broth for 10ml of sample and single strength MacConkey broth for 0.1ml and 1ml of the sample.
The test for the estimation of coliforms involves the following steps: presumptive, confirmatory and completed test. It was performed as described by Verma *et al*., (1999).

Enumeration of Faecal Coliform Test
The test for coliform does not distinguish coliform of animal origin and from others (Doyle and Erickson, 2006). In this test, the test tube with the production of gas in the presumptive test were streaked with the aid of a sterile wire loop onto MacConkey agar plates, and incubated at 37^0C for 24 hours.

Isolation, Characterization and Identification of Bacteria in Maize Slurry
Pure cultures of bacteria were obtained by aseptically streaking representative colonies of different morphological types which appeared on the cultured plates onto freshly prepared nutrient agar plates which were incubated at 28^0C for 24 hours. The isolates which developed were further sub cultured onto agar slopes/slants and incubated at 28^0C for 24 hours. These served as pure stock cultures used for subsequent characterization tests. The following characterization tests were performed in duplicates. Gram staining, catalase test, coagulase test, sugar fermentation test, methyl red test, indole test and acid gas test. The pure cultures were identified on the basis of their cultural, morphological and physiological characteristics in accordance with methods described by Cruikshank *et al.,* (1975) and with reference to Holt (1977).

Isolation, Characterization and Identification of Fungi in Maize Slurry
Pure cultures of fungi were obtained by sub culturing discrete colonies onto freshly prepared Sabouraud dextrose agar plates and incubated at 28^0C for 5 to 7 days. The

colonies which developed were further subcultured onto agar slopes or slants and incubated at 28^0C for 5 to 7 days. The following standard characterization tests were performed in duplicate; macroscopic examination of fungal growth was carried out by observing the colony morphology-diameter, colour (pigmentation), texture and surface appearance. Microscopic examination was done by needle mount or wet mount method and observing sexual and asexual reproductive structures.

Microscopic examination of fungi

A wet mount was carried out for the fungi isolated. A drop of sterile distilled water was aseptically dropped on a grease free clean slide. A piece of fungal hyphae under test was teased into it using two sterile needles. The teasing was done carefully and slowly so as to make good spread of the fungal hyphae. Each prepared slide was gently covered with a cover slip to avoid air bubble. The slides were observed under low and high power objective, and observation recorded as the cultural characteristics, sporangia, conidia, arthrospores, and vegetative mycelium, septate and non-septate hyphae according to Barnett and Hunter (1972).

Antibiotic susceptibility Testing

The *Lactobacillus* species and *Pseudomonas* specie isolated from *Akamu* were examined for resistance to several common antibiotics using Gram positive disc of Ciproflox® (CPX), Norfloxacin (NON), Amoxil® (AML), Streptomycin (STN), Gentamycin (CIN), Rifampicin (RIN), Erythromycin (ERN), Levofloxacin (LEV), Ampiclox® (APX), Chlorampenicol (CHL) and gram negative disc of Tarivid® (OFX), Gentamycin (CIN), Augmentin® (AUG), Septrin® (SXT), Streptomycin (STN), Ciprofloxacin (CPX), Nalidixic acid (NLA), Ceporex® (CEP), Amplicin® (AMN), Reflacine® (PEF). The antibiotic susceptibility testing was performed by plating each bacterium on the Petri plate containing nutrient agar routinely used in clinical setting for performing antibiotic susceptibility test. Culture of pure bacterial isolate grown overnight was inoculated into a MacCartney bottle containing peptone water and was mixed thoroughly and poured onto the nutrient agar plate to spread the bacteria all over the agar. Either the gram positive or gram negative disc containing the 10 antibiotics was carefully placed on the cultured plate with the aid of a sterile forceps. Cultured plates with the antibiotic discs were then incubated at 37^0C over night. Plates were thereafter observed for sensitivity or resistance of the organism to each of the 10 antibiotics in each plate. These were indicated by zones of inhibition (sensitive) or no zone of inhibition (resistant) to the antimicrobial drug.

RESULTS

Total Viable Count for bacteria and fungi in fermented and dewatered maize slurry (*Akamu*)

The mean value of the total viable bacteria count for the maize slurry (*Akamu*) samples from the producers, sellers and hawkers ranged from 1.03×10^5 to 2.10×10^5 cfu/g, from 1.40×10^5 to 2.20×10^5 cfu/g, and from 1.88×10^5 to 2.50×10^5 cfu/g respectively. The mean value of the total fungal count for the maize slurry (*Akamu*) samples from the producers, sellers and hawkers ranged from 1.50×10^3 to 1.0×10^4 cfu/g, from 2.0×10^3 to 1.50×10^6 cfu/g, and from 3.0×10^5 to 2.50×10^6 cfu/g respectively.

The result of the total coliform and of the thermotolerant coliform and facecal coliform is shown in Table 1 and Table 2 respectively. The total coliform count ranged from 34

to ≥1600 coliform (MPN) 100ml^{-1} while the thermotolerant coliform and facecal coliform ranged from 27 to ≥1600 coliform (MPN) 100ml^{-1}.

Generally, the total bacterial and fungal counts, and the total coliform and of the thermotolerant coliform and facecal coliform MPN index were highest in *Akamu* purchased from hawkers and lowest in *Akamu* purchased from the producers.

The bacteria isolated and their frequency (%) of isolation or incidence were; *L. acidophilus* (28%), *L. brevis* (14%) *L. casei* (18%), *L. dextrinicus* (4%). *L. fermentum* (4), *L. pentosus* (8%), *L. plantarum* (4%), *L. rhamnosus* (10%), and *Pseudomonas aeruginosa* (10%). The fungi isolated were *Aspergillus niger*, *Aspergillus versicola*, *Fusarium solani*, *Mucor pusillus*, and *Rhizopus oligosporus*. The fungi all have equal incidence of 20%.

Table 1: Total coliform bacteria Count of maize slurry *(Akamu)*

Location and source of sample	**TCB MPN (37°C) INDEX/100ml of maize slurry (*Akamu*)**		
	Week 1	**Week 2**	**Week 3**
Mile 2 producers	80	140	34
Mile 3 sellers	1600	220	220
Eagle Island hawkers	≥1600	900	≥1600

Table 2: Thermotolerant Coliform Bacteria and Facecal Coliform Bacteria Count of maize slurry *(Akamu)*

Location and source of sample	**TTCB/FCB (44.5^{0}C) MPN INDEX/100ml of maize slurry (*Akamu*)**		
	Week 1	**Week 2**	**Week 3**
Mile 2 producers	33	27	34
Mile 3 sellers	80	90	110
Eagle Island hawkers	≥1600	≥1600	900

Antibiotic Susceptibility Testing

The antibiotic susceptibility result showed the bacteria and their resistance to the antibiotics as *L. acidophilus* (50%), *L. brevis* (30%) *L. casei* (60%), *L. dextrinicus* (50%). *L. fermentum* (40), *L. pentosus* (20%), *L. plantarum* (10%), *L. rhamnosus* (70%), and *Pseudomonas aeruginosa* (20%). The resistance of the bacteria to each antibiotic was Ciproflox® (42.86%), Norfloxacin (14.28%), Amoxil® (71.43%), Streptomycin (66.66%), Gentamycin (33.33%), Rifampicin (57.14%), Erythromycin (28.57%), Levofloxacin (42.86%), Ampiclox® (71.43%), Chlorampenicol (28.57%), Tarivid® (0%), Augmentin® (0%), Septrin® (50%), Ciprofloxacin (0%), Nalidixic acid (0%), Ceporex® (0%), Amplicin® (0%), Reflacine® (0%). Note, only *Lactobacillus pentosus* and *L. plantarum* were tested for the all the antibiotics that recorded 0%.

DISCUSSION

This study has revealed the population of bacteria, fungi and of coliforms in samples of fermented and dewatered maize slurry (*Akamu*). Generally, samples purchased from the hawkers recorded the highest population of microorganisms while the samples from the producers had the lowest counts. The presence of various Lactic acid bacteria

and fungi was also revealed. Of the lactic acid bacteria isolated *Lactobacillus acidophilus* had the highest incidence of 28% while, *L. dextrinicus*, *L. fermentum*, and *L. plantarum* recorded the lowest incidence of 4% each. The persistent Pseudomonas *aeruginosa* had an incidence of 10%. The fungi isolated all had an equal incidence of 20%.

The presence of coliform bacteria especially of the thermotolerant coliform and facecal coliform bacteria in the maize slurry (*Akamu*) calls for concern. The presence of these coliform is attributed to the unhygienic behavior of the individuals that produce the maize slurry and the environment where it is prepared. The use of water of questionable quality in the absence of potable water for the production of *Akamu* is also a source for concern. The presence of the coliform bacteria and not of lactic acid bacteria must have been implicated in the diseases or illnesses associated with the consumption of maize slurry (*Akamu*). It has been stated that, variation in the type and number of the lactic acid bacteria present would influence the hygienic quality and organoleptic property of maize slurry which means that isolated lactic acid bacteria has antimicrobial activity creating a positive impact on their use as starter culture of traditional fermented food with a view to improving the hygiene and safety of food products (Zhao *et al.,* 2001) while many gram negative bacteria have been implicated in food borne disease (Mead *et al.,* 1999).

The present study has also revealed the antibiotic susceptibility profile of *Lactobacillus* and *Pseudomonas* species isolated from samples of fermented and dewatered maize slurry (*Akamu*). The antimicrobial susceptibility profile indicated that generally, all the bacteria isolates with the exception of *Lactobacillus plantarum* exhibited multiple resistance to the antibiotics used. *Lactobacillus rhamnosus* exhibited the highest resistance (70%) while *Lactobacillus plantarum* was the least resistant (10%). *L. plantarum* was resistant only to streptomycin. The bacteria isolates were most resistant to Amoxil and Ampiclox (71.43% each), and least resistant to Norfloxacin (14.28%).

These data sound a warning because the indiscriminate use of antibiotics, along with poor hygiene and infection control (risk factors for antibiotics resistance in bacteria), are highly prevalent in Nigeria and other developing countries (Obire *et al*., 2009). The source of resistance exhibited by organisms in the present study is not known, but possible source is water transfer. Suboptimal sanitary conditions and overcrowding in cities must have contributed to the spread of these resistant *Lactobacillus* species.

Antimicrobial resistance monitoring will help to review the current status of antimicrobial resistance locally, nationally and globally and helpful in minimizing the consequence of drug resistance and limit the emergence and spread of drug resistant pathogens (Obire *et al*., 2009).

Microorganisms are beneficial in most fermentation process. However, some microorganisms may pose high risk of food contamination and cause food-borne illness. The presence of high population of lactic acid bacteria and fungi reported in maize slurry (*Akamu*) of this study indicates the importance and involvement of these microorganisms in the fermentation process. The higher prevalence of rod-shaped lactic acid bacteria in this study corroborated the study of Ogunbanwo *et al.,* (2004) who reported that the genus *Lactobacillaceae* is commonly predominant during food fermentation. This is because they are the most aciduric of all lactic acid bacteria.

Lactic acid bacteria have been reported to produce bacteriocins -proteinaceous toxin (Herrero *et al.,* 1996). Bacteriocins are typically considered to be narrow spectrum antibiotics, though this has been debated (Farkas-Himsley, 1980). Bacteriocin from the producer organism has no inhibitory effect on the organism producing it (Ogunbanwo

and Johnson, 1998). It was also detected that maize slurry *(Akamu)* may be useful in preventing gastroenteritis caused by intestinal pathogens (Gilland and Speck, 1977). The presence of thermotolerant coliform and facecal coliform bacteria must have been implicated in the diseases or illnesses associated with the consumption of maize slurry (*Akamu*). The production of bacteriocins -proteinaceous toxin by lactic acid bacteriain foods may also be implicated in food–borne illnesses.

It is advocated that individuals that prepare maize slurry should practice good health hygiene to reduce the microbial load of organisms found in maize slurry. They should prepare it in clean environment and wash their hands properly before and after using the toilet to avoid contaminating the maize slurry with faecal materials especially hawkers. Maize slurry (*Akamu*) should be stored under appropriate temperature after production and the materials used during the preparation should be washed and kept properly to avoid contamination and excessive fermentation by the fermenters present in the maize slurry and also to avoid outbreaks of cholera, typhoid fever, gastrointestinal tract diseases, for the high death of infants and young adults.

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