Public Health Implications of Locally Femented Milk (Nono) and Antibiotic Susceptibility Testing of Pseudomonas Aeruginosa Isolated From The Product in Borokiri , Rivers State Nigeria

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Abstract*:- The study is to determine the PH and moisture content of Nono sold in Port Harcourt , the prevalence of Pseudomonas aeruginosa in Fura da nono and finally the antibiotic resistance pattern of Pseudomonas aeruginosa isolated from the fermented products. nono samples were purchased from Borikiri in portharcourt township. A total of 20 samples were assessed to determine their microbiological quality and to conduct antibiotic susceptibility test. Moisture content and pH of the samples were also assessed. Enumeration of the total viable bacterial count (TVBC), Total coliform count (TCC) and Total Pseudomonal count (TPC) were also assessed to determine the sanitary quality of the product. The PH ranges between 2.99 to 3.89 while the moisture content ranges between 80% to 88%. The result obtained from the microbial culture indicated that a wide array of microorganism were present in Fura da nono including species of Bacilu, klebsiella, Pseudomonas Staphylococcus aureus, Streptococcus, Lactobacillus and Escherichia coli.. The highest TVBC, TCC and TPC were $9.8x10^3$ cfu/ml, $10x10^3$ cfu/ml and $9.7x10^3$cfu/ml respectively. Antibiotic susceptibility was conducted using 12 broad spectrum antibiotics and compared against a standard provided by the Clinical laboratory standard institute (CLSI). Gentamycin, Ofloxacin and Levofloxacin recorded 100% resistance , while Cotrimoxazole, Ciprofloxacin, Vancomycin, Nitrofurantoin, Norfloxacin and Azithromycin recorded 100% susceptibility as indicated by the complete clear zone of inhibition.It was discovered that the absence of regulatory agencies like National Agency for Food Drug Administration and Control (NAFDAC) in the regulation of the quality of the product was the cause of the high contamination, since there were no quality control measures in its production line .It was recommended that NAFDAC should provide a standard operating procedure for local food producers and should include them in their scope for regulation.

I. INTRODUCTION

Nono is a spontaneously fermented products prepared from unpasteurized milk. It may be consumed on its own used to prepare the latter. Fura da nono a fermented milk and grains is a highly nutritious food drink which is a two in one product, consisting of a cereal called 'Fura', made from millet grains and 'nono' (milk) obtained from cow. Fura da nono is sold from calabashes while nono is sold from plastic bucket. Fura is mixed with nono in a bowl for customers. Mostly one bowl is used to mixed for all the consumers, often the bowl is not washed after each use. Depending on the consistency, the product is used as food, refreshing drink and a weaning food for children and adults. The product is in high demand in Port Harcourt, especially in the months of July to November (Umoh *et al*, 1988).

The unhygienic handling of Nono and fura da nono during processing and sale exposes these products to contamination. Contamination may occur even at the poin of milking the cow or goat. Fura is usually moulded into balls (fura balls) by hand during the production of fura da nono, and the hands of the producers could be a source of contamination. Houseflies are always found in large numbers at the production sites and at sale outlets. (Shehu & Adesiyun, 1990) reported that in order to increase the volume and improve colour of nono, the female hawkers, before sale, engage in the fraudulent act of adding stream water and a milky whitish supernatant of water-soaked baobab tree seeds to the products. This act could further lead to the contamination and spoilage of the product. Milk is the substrate for nono production and a major component in fura da nono. Milk is usually refer to as food for the facts that it posses proteins in casein form whey, lactose, lipids, multivitamins and minerals (Gaman and Sherington, 1990). Milk is a necessarily an initial food for babies, for so many generations, it has become a major sources of humans diet, including adults and children (Komorowski and Early, 1992). Milk is extracted from healthy cows, usually sterile and free from germs . However, it harbours low numbers of microorganisms called udder commensals. The commensals are usually of the genera of *Micrococci* ,*Streptococci*, and *Corynebacterium* (Ozer, 2000).

Locally produced milk in Nigeria is usually undertaken by local Fulani herdsmen who live especially in Northern Nigeria. Milk extraction is done by the able men the product is then distributed to the women in the farm. The Women processes the raw unpasteurized milk into various dairy products such as nono, manshanu and fura da nono (Belewu and Aina , 2000). Milk and its product create a conducive

atmosphere for microbial proliferation, which prompts it contamination and subsequent spoilage.

Untidy surrounding where milking is done attracts flies which latter serve as a source of contamination. Flies pick faecal materials from dirty environments, thus serving as a source of enteric harmful microorganism (Norman and Gravani, 2006).

Pseudomonas aeruginosa

Is a member of the Gamma Proteobacteria class of bacteria. It is a Gram-negative, aerobic rod belonging to the bacterial family Pseudomonadaceae. Since the revisionist taxonomy based on conserved macromolecules (e.g. 16S ribosomal RNA) the family includes only members of the genus Pseudomonas which are cleaved into eight groups. *Pseudomonas aeruginosa* is the type species of its group which contains 12 other members.

Like other members of the genus, *Pseudomonas aeruginosa* is a free-living bacterium, commonly found in soil and water. However, it occurs regularly on the surfaces of plants and occasionally on the surfaces of animals. Members of the genus are well known to plant microbiologists because they are one of the few groups of bacteria that are true pathogens of plants. In fact, *Pseudomonas aeruginosa* is occasionally a pathogen of plants. However, *Pseudomonas aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies track its occurrence as a nosocomial pathogen and indicate that antibiotic resistance is increasing in clinical isolates. *Pseudomonas aeruginosa* is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. In fact, *Pseudomonas aeruginosa* is the epitome of an opportunistic pathogen of humans.

II. MATERIALS AND METHODS

Description of Study Area

The study area is Borokiri in Port Harcourt metropolis. Port Harcourt is the capital of Rivers State. The state is located in the south-south geopolitical zone of Nigeria.Borokiri was selected due to the fact that it doubles as residential area domininated by the Fulani women who are the producer of the product

Source of Samples

The fermented products were purchased from Borokir Area of Portharcourt township. Nono samples were collected in sterile large screw capped bottles and transported in ice to the microbiology laboratory in Rivers State University, Port Harcourt.

Determination of pH

A pH meter (Metter Delta 340) equipped with glass electrode was used to measure the pH of the *fura da nono* samples. The pH meter was calibrated using buffer solution of pH 4 and another of pH 7 contained in a 100ml beaker. The electrode of the pH meter was placed in a 100ml beaker containing the sample to obtain **Moisture Content**

A crucible was washed and put in a hot air oven to dry. It was allowed to cool and then weighed. The sample (2ml) was added and the weight was taken. The crucible was then put in a hot air oven at 105^{o}C for about 1 hour. The weight was taken and it was put back into the oven. It was done repeatedly till a constant weight was obtained.

Sterilization

All media were sterilized using autoclave. Glassware were also sterilized using the autoclave. Wireloop and spreader were routinely sterilized by passing over burning flame.

Enumeration of Colonies

The aerobic bacterial count were conducted using 0.1ml of the serially diluted sample in nutrient agar using spread plates technique. For counting of coliforms, 0.1ml of the serially diluted sample was inoculated on MacConkey agar . The media were plated separately into their individual Petri-dishes that have been dried in hot air oven.

All plates were incubated for 24 - 48 hours at 37^{o}C. Pure cultures of each isolate were obtained by streaking the individual colonies on MSA, MacConkey agar *S.aureus*, *E.coli* and incubated at 37^{o}C, these were maintained in agar slants in McCarthney bottles.

Identification of Bacterial Isolates

The identification of bacteria colonies was based on classification procedures proposed by (Harrigan and McCance 1976), (Buchanan and Gibbons 1974) and Collins and Lyne, (1984). Identification was based on morphology and biochemical analysis.

Antibiotic Susceptibility Testing

Two methods of antibiotic susceptibility testing were use:

1. Agar well diffusion method (Antibiotics needed were available in powdering form)
2. Disk plate diffusion method (antibiotics needed were available in paper disk form)

Agar Well Diffusion Method

Agar well diffusion method was used to evaluate the antibiotics spectrum of four antibiotics namely Azithromycin (15µg), Vancomycin (30µg), Norfloxacin (10µg) and Nitrofurantoin (300 µg). The agar plate surface was inoculated by spreading a loopful of the inoculums on nutrent agar. Then, a hole with a diameter of 6 milimetre was punched aseptically with a sterile cork borer and a known volume of the various antibiotics were introduced into the wells. The, agar plates were incubated for 48hrs at 37^{o}C . The antibiotics diffuses in the agar medium and may inhibits the growth of the test organism. The various zones of inhibition were measured in millimeter using a transparent metre rule and it

was compared with a standard by Clinical Laboratory Standard institute (CLSI). Special charts which correlate the size of the zone of inhibition to susceptibility of bacteria to the drug were used to determine the susceptibility of the organism.

Disk Plate Diffusion Methods

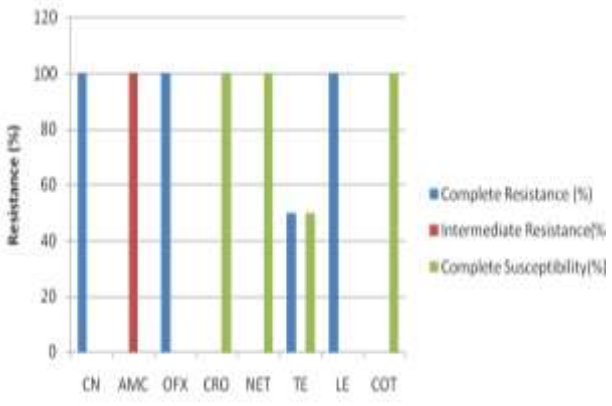
Standard amounts of antibiotics impregnated onto absorbent paper (paper disk) were placed onto nutrent which has been streaked with loopful of inoculums. These plates were then incubated for 24 hours at 37C. The antibiotic diffuses through the medium from the disk to the bacteria. Sensitivity to antibiotics was observed as clear zone of inhibition around the tested drug. The diameter of the zone were measured using a transparent rule and compared with CLSI guidelines for zone sizes to determine whether the test organism should be classified as sensitive, intermediately sensitive or completely resistant to the antibiotic.

Statistical Analysis

Statistical analysis was done using simple excel spread sheet and SPSS.

III. RESULTS

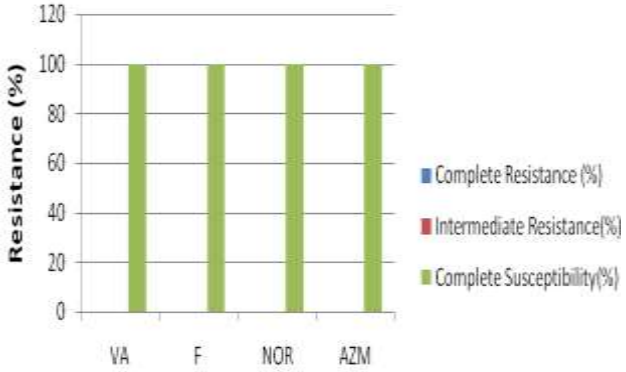
The pH values and moisture content of the nono samples are shown in Table 4:1. The pH values of the samples were similar and ranged from 2.99 to 3.89. The moisture contents of the samples ranged from 80-88%. Biochemical characterization shows the organisms of interest to be both gram negative. Molecular identification was done to confirm the isolates and a Phylogenetic tree showing the evolutionary distance betwen them.



Antibiotics Susceptibility of klebsiella pneumonia to Antibiotics

Key

AM (Amoxycilin) OFX (Ofloxacin) CN (Gentamycin) COT (Co-triomoxazole) LE (Levofloxacin) NET (Netilin) CRO (Ceftriaxone) TE (Tetracycline



Antibiotics Susceptibility of Pseudomonas aeruginosa against uncommonly used antibiotics in Nigeria

NOR (Norfloxacin) AZM (Azithromycin) VA(Vancomycin)F(Nitrofurantoin)

Microorganisms Associated Nono in Borokiri

SAMPLE	Food type	*S.aureus*	*E. coli*	*Klebsiela pneumonia*	*Pseudomonas aeruginosa*	Lactobacili species
BKR11		+	+	+	+	+
BKR 17						
		+	+	+	+	+
BKR 20						
		+	+	+	+	+

IV. DISCUSSION

The mean pH of nono ranges from 2.99 to 3.89. The result agrees with the report of (Umor *et al.*1988). The pH of nono in this report was however lower than that reported for Yoghurt by (Odunfa, 1988), which reported a ph of 4.0-4.50.

Proximate analysis involves the determination of the major components of food as moisture, ash, crude fat, crude protein, crude fiber, and carbohydrate. The results of the study, has an average composite of moisture content 84.92 , ash 0.48, carbohydrate 7.70, protein 3.32, lipid 3.09, and fibre 0.49 in percentage respectively, this result agrees with the work of

(Odunfa 1988) who reported lipid 3.00, carbohydrate 8.05, ash 0.50, and moisture content of 83.01.

The bacteria isolated from fura da nono includes *E.coli, Pseudomonas aeruginosa, Streptococcus sp, Klebsiella pneumonia Staphylococcus aureus, B. cereus, Lactobacillus* , the result agrees with that of (*Adebesin, et al,*. 2001). Results of the study shows the presence of *S aureus, B cereus,* and *E. coli, Klebsiella pneumonia,* and *Pseudomonas aeruginosa*, which have previously been linked with food contamination. The presence of *E. coli* and other enterobacteria in the samples also indicates that they are likely to contain other pathogens, as the presence of *E. coli* in foods is an indication of contamination of faecal origin . However, *Lactobacillus* is a *lactic* acid bacterium probably involved in fermentation of the product. The presence of *Pseudomonas aeruginosa* and *klebsiella pneumonia* in fura da nono is an indication of possible contact with discharge from wounds and excretory products respectively this agrees with (Bothast 1978) who confirmed that *Pseudomas aeruginosaand klebsiella* species are associated with wounds sour and body secretion. The probable sources of contamination associated wi1th the products were traced to the use of the old portion of previously fermented nono as starter culture and the use of non potable water for the preparation of the products. The contaminating microorganisms may also be through air microflora that attaches to the smoothening stick, calabashes, wooden spoons and bowls used for the sale of the products. Normal human flora on the customers body could also serve as contaminants especially when one bowl is used for mixing the product for all customers without washing after each use.

The result is in conformity with that of Shehn and Adesiynh (1990). *Klebsiella pneumonia* was susceptible to many broad spectrum antibiotics incluing Gentamycion, Amoxicillin, ciprofloxacin, Levofloxacin, cotrimoxazole while they were resistance to tetracycline, this result is in agreement with that of Odunta 1988, *Pseudomonas aeruginosa* recorded 100% susceptibility to the four major antibiotics namely vancomycin, Azithromycin Norfloxacin, Nitrofurantoin which is a perfect agreement with the result of Doyle *et al.*, (1997)Zone diameter greater than 18 MM was interpreted as susceptible in accordance with clinical laboratory Science institute specification.Zone diameter of less than 17 mm were interpreted as intermediate resistance. Zone inhabition of less than 14mm were interpreted as resistance. This result agrees with clinical laboratory science institute the bacterial count for total viable count (TVC) ranges from $1.1x10^5$ – $2.1x10^5$ cfu/ml, count for *Klebsiella pneumonias* ranges from 1.1×10^3 - 10×10^3 cfu/ml, while count for *pseudomonas aeruginosa* ranges from 7.5×10^3- 9.2×10^3 Cfu/ml which is in agreement with (Shehu and Adesiyuh 1990) who reported a Slightlyhigher count for *Klebsiella Pneumonia* 1.2×10^5- 2.2×10^5 cfu/ml, *Pseudomonas aeruginosa* 7.5×10^3 – 9.3×10^3 cfu/ml.Biochemical test were conducted and Isolates were identify as either gram positive or gram negative base on their reaction to Dyes, this is also in perfect agreement with the work of (George *et al.*,2006)

V. CONCLUSION

Pseudomonas aeruginosa isolated from fura da nono samples were resistant to Gentamycin, Ofloxacin,Levofloxacin while 50% resistance was observed in Tetracycline. The continuous abuse of antibiotics was probably responsible for the resistance of *Staphylococcus aureus* to these antibiotics. Gentamycin was observed to be the most abused antibiotics, as brands were available for both human and livestock. Resistance was also due to the facts that same antibiotics were use in treatment of livestock like cattle which diffuses to the milk which is used in the production of nono.

Pre-exposure to certain antibiotics was however the possible result of some *S.aureus* resistance. Vancomycin, Nitroforantoin and Azithromycin were among the list abused antibiotics, this was however due to their unavailability in most pharmaceutical outlets and also because they were prescription drugs as against the former which were over-counter drugs. The complete none implementation of local content laws were also responsible for high used of contaminated raw milk (Nono) in the production of locally produced foods (Fura da nono) , this law promotes indigenous products as against importation.. There is no quality assurance of locally fermented dairy product in Nigeria. Foods regulatory agencies never included locally fermented dairy products in their scope of quality assurance and regulation. The education of local foods producers were not encouraged encouraged. Foods agencies (i.e. NAFDAC) never provided any standard operating procedures (SOP) for the locally fermented dairy product producers thus the product has no standard, it is been produced as desired by individual producer thereby increasing more contamination.

VI. RECOMMENDATIONS

In order to overcome contamination of nono and reduce antibiotic resistance by *Pseudomonas aeruginosa* it is recommended that;

- Regulatory agencies like NAFDAC should regulate the production of locally made foods in Nigeria.
- Sanitary measures should be adopted during milk extraction as pathogens are often introduced during the process.
- nono should be packed in sachets and Cans.
- Only milk from healthy cattle should be used for the production of local fermented dairy products, since the milk is not pasteurized.

P^H value and Moisture Content of Nono from Borokiri

Location	Sample	Ph	Moisture Content (%)
Borokiri	1	3.00	87
	2	3.00	88
	3	3.23	84
	4	2.99	87
	5	3.50	83
	6	3.89	85
	7	3.38	84
	8	3.44	87
	9	3.25	82
	10	3.13	84
	11	3.00	81
	12	3.45	83
	13	3.23	85
	14	3.20	82
	15	3.25	84
	16	3.22	83
	17	3.27	82
	18	3.00	81
	19	3.33	88
	20	3.30	84

Bacterial Count for Nono Sample from Borokiri

Sample	TVBC cfu/ml	*klebsiella* cfu/ml	*Psedomonas* cfu/ml	TSC
BKR1	$8.8X10^3$	$8.7X10^3$	NILL	10X103
BKR2	$8.8X10^3$	$6.8.X10^3$	NILL	8.9X103
BKR3	$8.0X10^3$	$8.1X10^3$	$8.7X10^3$	NILL
BKR4	$8.2X10^3$	$7.2X10^3$	NILL	NILL
BKR5	$9.7X10^3$	$9.1X10^3$	NILL	8.7X193
BKR6	$8.5X10^3$	$6.1X10^3$	NILL	NILL
BKR7	$8.1X10^3$	$8.4X10^3$	NILL	NILL
BKR8	$7.4X10^3$	$8.0X10^3$	NILL	NILL
BKR9	$9.0X10^3$	$6.0X10^3$	NILL	NILL
BKR10	$9.2X10^3$	$7.1X10^3$	NILL	NILL
BKR11	$83X10^3$	$7.7X10^3$	$9.7x10^3$	NILL
BKR12	$7.9X10^3$	$8.2X10^3$	NILL	NILL
BKR13	$8.0X10^3$	$7.1X10^3$	$8.7x10^3$	$8.0X10^3$
BKR14	$8.3X10^3$	$8.2X10^3$	NILL	NILL
BKR15	$7.8X10^3$	$8.5X10^3$	NILL	NILL
BKR16	$8.4X10^3$	$6.0X10^3$	NILL	NILL
BKR17	$9.8X10^3$	$8.0X10^3$	$8.7x10^3$	NILL
BKR18	$8.5X10^3$	$9.4X10^3$	NILL	NILL
BKR19	$7.5X10^3$	$9.4X10^3$	NILL	NILL
BKR20	$9.1X10^3$	$9.0X10^3$	$7.7x10^3$	NILL

TVBC(Total Viable Bacterial Count)TCC(Total Coliform Count) TSC(Total Staphylococcal Count) BKR(Borokiri)

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