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ASSESSMENT OF THE PHYSICOCHEMICAL AND BACTERIOLOGICAL QUALITIES OF NONO – A FERMENTED COW MILK

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ABSTRACT

Nono is a spontaneously fermented yoghurt-like milk product consumed is a staple food commodity in parts of the Sub-Saharan West Africa. Nono is usually consumed along with 'Fura' as 'Fura da Nono' in Nigeria. Studies on physicochemical and bacteriological qualities were carried out on samples of Nono obtained from 5 different sources in Ado-Ekiti, Nigeria. The Nono samples were found to be nutritious, containing moderate levels of ash, crude fat, crude protein and carbohydrate. The pH of the Nono samples was relatively low (4.04 ±0.04), while the density and specific density were close to that of distilled water at room temperature. Total aerobic plate count of Nono samples was 1.8 ±0.02 × 106 CFU.mL^{-1}. A total of 15 bacteria species namely *Eubacterium nodatum*, *Bacillus subtilis*, *Chromobacterium violaceum*, *Propionibacterium acnes*, *Amycolatopsis benzotilytica*, *Tropheryma whipplei, Moraxella catarrhalis, Campylobacter gracilis, Neisseria sicca, Vibrio natiensis, Photobacterium damselae, Corynebacterium kutsceri, Corynebacterium xerosis, Lactobacillus fermentum* and *Lactobacillus casei* were isolated from the Nono samples. The gram-positive bacterial isolates were resistant to all antibiotics tested with the exception of Erythromycin where 40% susceptibility was obtained, while the gram-negative bacteria showed high resistance to the tested antibiotics, but with 80% susceptibility to Ofloxacin. The nono samples were observed to exhibit antibacterial activity against cultures of *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 29929 and *Staphylococcus aureus* ATCC 29293. Most of the bacteria isolated were of less public health importance, but the high prevalence of multi-drug resistance is of great concern.

Keywords: Nono; fermented milk; nutrition; antibiotics resistance

INTRODUCTION

Milk has been preserved since early times by fermentation techniques. Asia, Africa, the Middle East, Northern and Eastern Europe are known for the production of traditionally fermented milk products **(Savadogo et al., 2004)**. Nono is a spontaneously fermented yoghurt-like milk product consumed as a staple food commodity in parts of the Sub-Saharan West Africa. Its production and consumption gives economic benefits such as food security to the rural people in the region **(Akabanda et al., 2010)**.

Nono is produced by Fulani women in Nigeria. The Fulani is an ethnic group in northern Nigeria with the tradition of earning a living from cattle rearing and sales, but they are found all over the country. Cattle rearing is an integral part of the culture of this group of people. They produce their fermented milk, 'Nono', for sales by traditional methods. Fresh cow milk is collected in the morning in calabashes, sieved and left to ferment for a period of 24 to 48 hours under the ambient temperature (28 ±2 °C). Fermentation of milk is spontaneous (with its own natural bacteria). The curd separates from the whey, the curd is removed and used in the preparation of local cheese or butter, while the milk (whey) is left to ferment further for some few hours thereby converting it to yogurt-like **(Akabanda et al., 2010)**. Fermentation span varies from one producer to another resulting in products of variable quality and stability. The finished product – nono is sold in beautiful calabashes along with Fura (dough of boiled ground millet mixed with a host of other ingredients and spices) as 'Fura da Nono' (Figure 1).

The milking process, production and marketing of nono is not appealing to some people, especially the elites, because of the the quality of the water commonly used, the feeding methods of the cows, and above all the processing environment. Sanitary practices are essential in milk processing so as to minimize the risk of infection to people through consumption of milk products **(Chan et al., 2007)**.

Figure 1. Fulani woman displaying nono **(Dobby's Signature.com, 2014)**.

Common bacterial flora of fermented milk are the Lactic Acid Bacteria (LAB) which among others comprises of *Lactobacillus lactis*, *L. bulgarius*, *L. helvericus*, *Leuconostoc* species, *Streptococcus thermophilus*, *S. lactis* and *S. cremoris*; as well as the Propionic Acid Bacteria (PAB). Most coliforms inhabit the intestines of warm-blooded animals and are shed along with their faeces to living environments. Many other food borne pathogens equally originated from faecal contaminations **(Congan, 1995)**.

Pathogenic bacteria are not supposed to be present in any successfully fermented dairy product because of heat treatment and low acidity of the product. Their presence in these products is sign of re-contamination. The occurrence of *Enterococcus* species, coliforms, *Salmonella* species, *Clostridium* species *and Bacillus* species is a sign of re-contamination **(Ledenbach and Marshall, 2009)**.

Milk products have been frequently implicated in the transmission of diseases such as tuberculosis, brucellosis, diphtheria, scarlet fever and gastroenteritis. This is as a result of contaminations by the implicated human pathogens **(Bryan et al., 1988)**. The contamination could be attributed to three reasons. Firstly, the wide distribution of coliform in nature which predisposes products to contamination before and after pasteurization; secondly, conditions of storage and thirdly, some enteric pathogen may be disseminated by dairy products gotten from an infected animal **(Ledenbach and Marshall, 2009)**.

Quality and safety or raw cow's milk have to be regularly controlled **(Zajác et al., 2012)**.

Scientific hypothesis

There has been many contradicting reports on the safety of Nono consumption. But nono is believed to be safe for human consumption, hence the popular demand for this fermented milk product. The present study investigates the quality and safety of nono with the following objectives of the study:

i. Determining the physicochemical qualities of the nono
ii. Assessing for bacteria of public health importance in nono samples
iii. Evaluating the probiotic potential of nono.

MATERIAL AND METHODOLOGY

Collection of Samples

Samples of nono were purchased from 5 different locations in Ado-Ekiti, Nigeria, and brought in sterile bottles to the Microbiology Laboratory, Afe Babalola University, Ado-Ekiti, Nigeria, for analysis.

Physicochemical analyses of nono

Proximate nutrient composition and some other physico-chemical properties such as pH, density and specific density was carried out according to the **Association of Official Analytical Chemists (2000)**.

Isolation of aerobic organisms from nono

The Pour-plate technique was used for primary isolation of aerobic bacteria. Following serial dilution of the nono samples, 1 mL each of 10^{-4} and 10^{-5} dilutions were evenly spread on freshly prepared nutrient agar (Oxoid, England), in du regulatory microbial criteria for raw cow milk plicates and incubated at a temperature of 37 $^{\circ}$C for 24 hours. Bacterial load were estimated, and distinct colonies were picked and sub-cultured on nutrient agar plates.

Isolation of lactic acid bacteria from nono

This was carried out as described by **Ibrahim and Nural (2016)**, 1 mL each of 10^{-4} and 10^{-5} dilutions of Nono was evenly spread on freshly prepared deMan, Rogosa and Sharpe agar, MRS (Oxoid, England), distinct colonies were picked and sub-cultured on nutrient agar plates.

Characterization and identification of isolates

All isolates were characterized using standard morphological and biochemical tests as described by **Barrow and Feltham (1993)**. Bacterial isolates were identified using online bacterial identification application **(Gideon informatics, 1994-2017)**, with reference to Bergey's Manuals **(Brenner, et al., 2005a,b, Vos et al., 2009, and Krieg et al., 2010)**.

Antibiotic susceptibility test of bacterial isolates from nono

The susceptibility of the isolated aerobic bacteria to antibiotics was determined by Kirby-Bauer disc diffusion method on Mueller-Hinton agar **(CLSI, 2016)**. This test was carried out by making an even spread of the pure isolates on prepared Mueller-Hinton agar (Oxoid, England) using sterile swab sticks and aseptic placement of the antibiotic disks (Oxoid, England) using sterile forceps. The plates were incubated at 37 °C for 24 hours after which the zone of inhibition was measured and interpreted according to Clinical Laboratory Standards Instituite **(CLSI, 2016)**. Antibiotics used are; Augumentin, AUG (30 µg), Ofloxacin, OFL (5 µg), Ampicillin, AMP (30 µg), Ciprofloxacin, CIP (5 µg), Gentamicin, GEN (10 µg), Ceftazidime, CAZ (30 µg), Nitrofurantoin, NIT (300 µg), and Cefuroxime, CXM (30 µg), for gram negative isolates; and Streptomycin, STP (10 µg), Ampicillin (30 µg), Cloxacillin, CLX (5 µg), Tetracycline, TET (10 µg), Chloramphenicol, CHL (10 µg), Erythromycin, ERY (5 µg), Penicillin, PEN (10 µg), and Gentamicin (10 µg), for gram positive isolates.

In-vitro antibacterial activities of nono

The in-vitro antibacterial of nono was carried out by agar well diffusing assay with Mueller-Hinton agar (Oxoid, England) as described by **Clinical and Laboratory Standards (2016)**. Nono was centrifuged and the supernatant was tested against Salmonella typhimurium ATCC 14028, Escherichia coli ATCC 29929 and Staphylococcus aureus ATCC 29293. Each test was performed in triplicate.

Statisic analysis

All data were expressed as mean ± Standard deviation.

RESULTS AND DISCUSSION

Nutritional quality of nono

The Nono samples were found to be nutritious, containing moderate levels of ash, crude fat, crude protein and carbohydrate (Table 1). The pH of the nono products was relatively low (4.04 ±0.04), while the density and specific density were close to that of distilled water at room temperature (Table 1). The low pH value of 4.04 has the ability to inhibit spoilage microbes, as well as giving Nono its sharp taste. The pH values obtained in this study is in consistent with pH values of 4.1 – 4.4 reported earlier by **Bankole et al. (1992)** from Nono samples obtained from Zaria, Northern Nigeria.

Bacteriological quality of Nono

Aerobic plate count (bacterial load) of 1.8 ±0.02 x 10^6 CFU.mL^{-1} (6.26 $\log_{10}$ CFU.mL^{-1}) was recorded in Nono in this study. The bacterial load reported is slightly higher

Table 1 Physicochemical qualities of Nono.

Analyte	Value ±SD
pH	4.04 ±0.03
Density (g.mL^{-1})	0.96 ±0.01
Specific density (g.mL^{-1})	0.97 ±0.01
Moisture (%)	90.88 ±0.04
Ash (%)	3.81 ±3.67
Crude Fiber (%)	ND
Crude Fat (%)	1.35 ±0.07
Crude Protein (%)	2.88 ±0.78
Carbohydrate (%)	0.92 ±0.16
Energy (Kcal.kg^{-1})	230.25 ±3.04

SD – Standard Deviation.
ND – Not Detected.

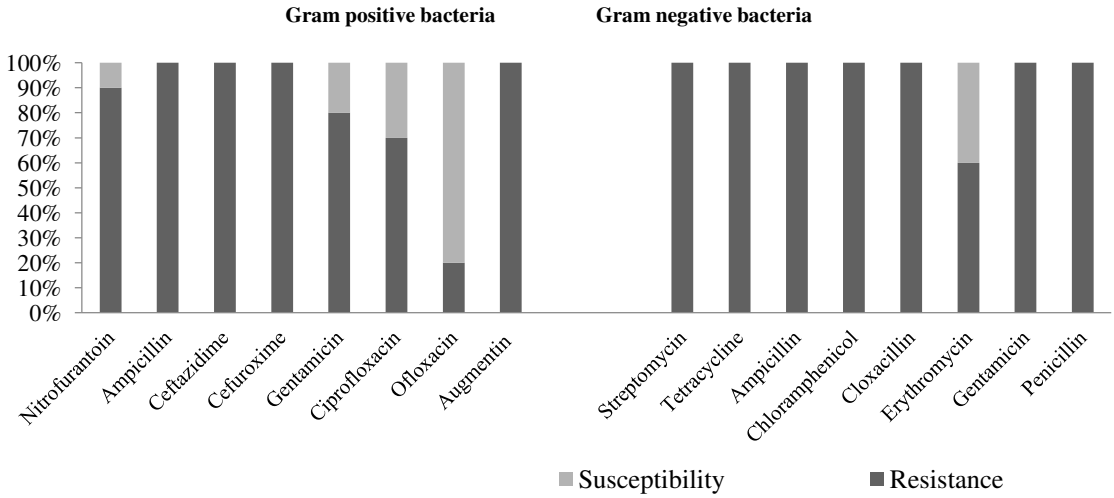

Figure 2 Antibiogram of bacteria isolated from Nono.

than the values of 4.1, 4.9 ±0.11 and 4.5 ±0.4 $\log_{10}$ CFU.mL^{-1} for raw whole milk reported by **Asaminew and Eyassu (2007)**, **Abebe et al. (2012)** and **Kunová et al (2017)**, respectively. The bacterial load of 6.26 $\log_{10}$ CFU.mL^{-1} compared favourable with the value of 6.68 ±1.19 $\log_{10}$ CFU.mL^{-1} and 5.59 ±0.69 $\log_{10}$ CFU.mL^{-1} of Nono produced in 24 and 48 hr respectively in Ghana **(Akabanda et al., 2010)**. However earlier studies by **Bankole et al. (1992)** reported an average aerobic plate count of 1.6 x 10^9 from Nono samples obtained from Zaria, Northern Nigeria.

Fifteen (15) different bacteria species were isolated. The aerobic bacteria isolated from Nono were *Eubacterium nodatum, Bacillus subtilis, Chromobacterium violaceum, Propionibacterium acnes, Amycolatopsis benzotilytica, Tropheryma whipplei, Moraxella catarrhalis, Campylobacter gracilis, Neisseria sicca, Vibrio natiensis, Photobacterium damselae, Corynebacterium kutsceri* and *Corynebacterium xerosis.* The isolated lactic acid bacteria were *Lactobacillus fermentum,* and *Lactobacillus casei.*

Most of thebacteria species isolated from nono in this study are not commonly associated with food related illnesses. However, other species of some of the genera, especially *Campylobacter, Bacillus,* and *Vibrio*, have been implicated in food related illnesses **(Donkor et al., 2007)**. *Campylobacter* species such as *C. jejuni, C. coli* and *C. fetus* are considered pathogenic while *B. cereus a*nd *B. anthrax* are of clinical importance and V. *cholerae* is the causative agent of cholera **(Shah et al., 1998; Logan, 1988 and Julian, 1997)**. Although these species were not reported in the study, the species reported are indicators of potential contamination by the organisms. This is an indication of inadequate hygienic practices during production **(Theodore et al., 2016 and Zajác et al., 2012)**.

According to **Harrigan (1998)**, raw milk drawn from a healthy udder normally will contain only a few hundreds to a few thousands of bacteria per milliliter, mostly from the genus *Micrococcus* and the udder diphtheriod, *Corynebacterium bovis*. Microbial contamination of milk often arises from the udder surface, bovine faeces, soil, bedding, feed, as well as milk-handling equipment. Lack of potable water and use of detergent was a major constraint to hygienic practices on the farm **(Theodore et al., 2016; Solomon et al., 2013 Galton et al., 1989)**.

The gram-positive bacteria isolated from Nono were resistant to all antibiotics tested with the exception of Erythromycin where 40% susceptibility was obtained, while the gram-negative bacteria showed high resistance to the tested antibiotics, but with 80% susceptibility to Ofloxacin (Figure 2).

All the bacteria were resistant to multiple drugs showing cluster of resistance to 5 – 8 antibacterial drugs (Table 2). High prevalence of multi-drug resistant bacteria among

Table 2 Antibiotics resistance cluster of bacteria isolated from Nono.

Bacteria	Drug cluster
Vibrio natiensis	AMP/CAZ/CXM/GEN/CIP/OFL/AUG
Photobacterium damselae	NIT/AMP/CAZ/CXM/GEN/CIP/OFL/AUG
Campylobacter gracilis	NIT/AMP/CAZ/CXM/GEN
Neisseria spp	NIT/AMP/CAZ/CXM
Moraxella catarrhalis	NIT/AMP/CAZ/CXM
Tropheryma whipplei	AMP/CAZ/CXM/GEN/CIP/OFL/AUG
Propionibacterium acnes	STR/TET/AMP/CHL/CLX/ERM/GEN/PEN
Amycolatopsis benzoatilytica	STR/TET/AMP/CHL/CLX/ERM/GEN/PEN
Eubacterium nodatum	STR/TET/AMP/CHL/CLX/ERM/GEN/PEN
Bacillus subtilis	STR/TET/AMP/CHL/CLX/ERM/GEN/PEN
Chromobacterium violaceum	STR/TET/AMP/CHL /CLX/ERM/GEN/PEN

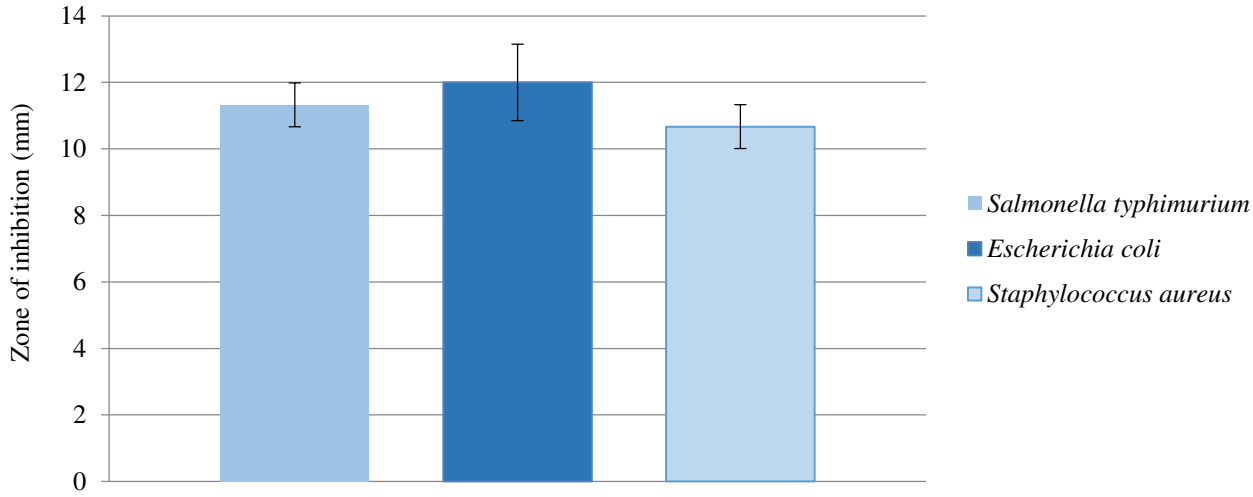

Figure 3 Antibacterial activity of nono against selected bacterial isolates.

food animals and meat processing equipment in Nigeria, has been well documented **(Osibote et al., 2014; Okiki et al., 2013)**. The high prevalence of bacteria that are resistant to multiple drugs in Nono is of great public health concern, because many people in Nigeria especially those from the Northern part of the country, have preference for nono to industrially produced yogurts.

Enterococci and members of the Enterobacteriaceae were not detected in all the samples (Nono) analyzed as against the report of **Fabianova (2010)** who reported the presence of Enterococci in raw cow milk. This may reflect the inhibitory effect of metabolites (acetic acid and lactic acid) and reduced pH produced by the mixed culture lactic acid bacteria thereby contributing to the quality and safety of the fermented milk product. Their presence, even at the initial fermentation period, suggested that they were probably introduced from the external environment, i.e. from the udder surface, bovine faeces, soil, beddings, human skin, and they survived because of the high pH **(Bezkorovainy, 2001; Harrigan, 1998)**.

Antimicrobial quality of Nono

The nono samples were also observed to exhibit antibacterial activity against cultures of *Salmonella typhimurium* ATCC 14028*, Escherichia coli* ATCC 29929 and *Staphylococcus aureus* ATCC 29293 (Figure 3).

According to the work of **Krishnendra et al. (2013)** selected lactic acid bacteria (*L. casei, L. fermentum* and *L. plantarum*) showed inhibitory properties against *Escherichia coli* and *Staphylococcus aureus*. The report of the author agrees with the findings of the study as the nono samples containing *L. casei* and *L. fermentum* showed inhibition of *S. aureus* and *E. coli.* The study is further established by the **Coconnier et al. (1997)** and **Bernet Camard (1997)** who reported the inhibition of *Salmonella typhymurium* by *L. fermentum* in their separate works.

Lactobacillus fermentum is a potent probiotic. Strains of *Lactobacillus fermentum* have been found to be pH and bile tolerant, strong enough to survive the stresses of the digestive system, the stomach pH between 1.5 and 3, and the upper intestine 3 – 5 gL^{-1} of bile **(Pan et al., 2011)**. *Lactobacillus fermentum* has the ability to reduce cholesterol levels. *L. fermentum* removes cholesterol *in vivo* by the absorption of cholesterol, which as a result accelerates cholesterol metabolism, as well as by the incorporation of cholesterol from the host into its cell membrane or walls **(Pan et al., 2011)**. **Marika and Zilmer (2009)** work on experimental introduction of the strain ME-3 of *Lactobacillus fermentum* into dairy products as a probiotic ingredient, revealed that the organism (*L. fermentum*) was able to suppress reputable contaminants of food such as pathogenic *Salmonella* species, *Shigella* species, and bacterial agents of urinary tract infections such as *E. coli* and *Staphylococcus* species.

Some *L. casei* strains are considered to be probiotic, and may be effective in alleviation of gastrointestinal pathogenic bacterial diseases **(WHO, 2002)**. According to **Ridwan et al (2008)** *Lactobacillus* species are safe and effective in treatment of acute and infectious diarrhoea. *L. casei* has been found to have a wide spectrum of coverage against pathogenic organisms that translocate from the gastrointestinal tract, thereby demonstrating therapeutic benefit in pancreatic necrosis **(Gratino and Alvarez, 2006)**. *L. casei* bacteria can also be used in the natural fermentation of beans to lower levels of the compounds causing flatulence upon digestion **(Gratino and Alvarez, 2006)**.

CONCLUSION

Since there are no standardized methods of processing nono, the products are of varying quality and stability, and if adequate hygienic practices are not ensured during processing, the quality of the final product may be compromised. Standard processing method that will ensure the production of Nono that meets International regulatory standards for safety should be employed. Thus microbial process technology can be transferred to the local producers. Education of producers on good manufacturing practices including basic hygienic principles will equally be crucial in achieving standard products.

In conclusion, Nono as a fermented milk product is of high nutritional value and a good probiotic food.

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