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Original Research Article

Bacteriological examination of fresh cow milk and *fura de nunu* using rapid dye reduction test

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Fresh cow milk often connotes an off-white nutritious liquid conjectured from the off-whitish natural fluid secreted by female mammals from the mammary gland and *nunu* is a fermented milk product consumed as a drink in parts of West Africa. The quality of raw milk and "*nunu*" was assessed by the rapid dye reduction method, comprising of methylene blue reduction (MBR) test and resazurin test. Standard microbiological methods were adopted to determine aerobic bacteria, coliforms and fungal characteristics. Total aerobic bacteria counts were 2.92×10^{10}-3.2×10^{9}; coliform count ranged between 7.0×10^{9}-1.0×10^{8}. Fungal count was 1.45×10^{10}- 4.0×10^{8}. The total microbial count was high, indicating gross contamination. The microorganism isolated includes species of *Escherichia coli, Enterococcus, Streptococcus, Staphylococcus, Micrococcus, Bacillus, Corynebacterium* and *Lactobacillus* for bacteria and, *Mucor, Saccharomyces, Rhizopus, Fusarium, Geotrichum, Aspergillus* and *Penicillium* species of fungi. The presence of faecal coliform and *Staphylococcus aureus* could account for food infection, whereas *Streptococcus agalatiae* suggest mastitis infection from the udder of the cow. Some of the fungal isolates have been implicated with serious mycotoxicosis of animals and man. Results obtained from the dye reduction test suggest absolute rejection of the samples. The sources of contamination could be from the milk producing animals, the milking utensils as well as the water used for '*nunu*' processing and suitable environmental factors that favours the proliferation of contaminating microorganisms. Efforts should be intensified on improving the sanitary condition of the product to avoid fatal health hazards.

Key words: Microbiological quality, rapid dye reduction, *nunu*.

INTRODUCTION

Milk of cattle, buffalo, goat, sheep, camel, yak, llama, mare etc., contains almost same but varying concentration of the chemical constituents. Milk obtained from healthy animal's udder is free from pathogenic bacteria, but some of the animals in field condition may be suffering from sub-clinical mastitis and are excreting the causative agent in milk (FAO, 2008). Such milk contaminates the bulk milk. Moreover, fresh milk may get microbial contamination from utensils, animal skin, the environment, or water used for adulteration (FAO, 2009). Milk is enrichment medium to support growth of contaminating microbes. During transportation of milk at ambient temperature, the contaminated microbes may multiply and deteriorate the quality of loose milk (Muhammad *et al*., 2009).

Nunu has been described as a locally fermented dairy product obtained from mixed culture fermentation of freshly drawn cow milk with sour milk or yoghurt like in taste (Nebedum *et al*., 2007). Untreated milk is highly vulnerable to microbial spoilage; therefore measures for preservation have to be taken to ensure conservation (Adebayo *et al*., 2013). The findings of earlier authors has, however, reveal poor hygiene being practiced by handlers of this product which may pose health risk to its consumers and hence the need to ensure its microbiological safety, (Adebayo et al., 2013). The poor hygiene practiced by *nunu* handlers discourages many people from buying and taken it from the Fulani's who are the major producers and custodians of *nunu* (Aernan *et al*., 2011).

This study assessed the bacteriological examination of fresh cow milk and *fura de nunu* using a rapid dye reduction test.

Materials and Methods

Description of Sampling Location

The research was carried out in Owerri, Imo State. Imo State lies in latitude 5^0 29'N, $7^0$2'E, South Eastern Nigeria and covered the informal mammy and cattle markets at Obinze, in

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Owerri, Imo State of Nigeria. The study area is dominated by small-scale traders, livestock traders and insignificant number of Fulani settlers.

Sample Collection

A total of one hundred (100) composite samples, comprising of fifty fresh cow milk and fifty *fura de nunu* was collected from the Fulani women between the hours of 10:30 – 11:30am were composited in five batches and transported in an iced bag to the laboratory immediately for analysis.

Microbiologycal Analysis

Ten milliliters of fresh cow milk were dispersed and swirled thoroughly in 90 ml of freshly prepared peptone water. Twenty grams of the bolus of *fura de nunu* were thoroughly mixed in a stomacher blender containing 180 ml of peptone water. The mixtures were serially diluted and aliquot portion inoculated onto bacteriological and mycological media, spread evenly and incubated at appropriate temperature and time (Cheesbrough, 2000; Beishir, 1987).

Enumeration and Characterization of Microbial Isolates

Bacteria and fungi isolated from the samples were counted by the neubauer colony counter and expressed as total colony forming units per gram/milliliters (Harrigan and McCance, 1990). Isolates were identified based on colonial, microscopic and biochemical characteristics using standard methods (Cheesbrough, 2000; Beishir, 1987). The identities of the isolates were confirmed with reference to standard manuals (Bannett and Hunter, 1987; Buchanan and Gibbon, 2000).

Methylene Blue Reduction (MBR) Test

Hygienic status of the raw cow milk and fura de nunu samples was determined using methylene blue reduction (MBR) test (Harrigan and McCance, 1990; Sharma, 2009). One milliliter of methylene blue solution (1:25000) was transferred into a sterilized 20 ml screw capped test tube containing 10 ml of each of the samples. The tube was capped and gently inverted three times to mix up the dye with milk sample. Each of the tubes was incubated at 370C, in a water bath and examined after every 30 minutes for a period of 4h. The time taken for the methylene blue in the milk to become colourless was recorded. The suggested classification is as follows: Class 1 – Excellent not decolourized in 8h; Class 2 – Good, decolourized in less than 8h but not less than 6h; Class 3 – Fair, decolourized in less than 6h but not less than 2h; and Class 4 – Poor, decolourized in less than 2h.

Resazurin Test

Sanitary status of the samples was also determined using resazurin test. One standard tablet of resazurin was added to 50ml of distilled water to give resazurin solution (0.0005%). One milliliter of resazurin solution was transferred into a sterilized 20 ml screw capped test tube containing 10 ml of each of the samples. The tube was capped and gently inverted three times to mix up the dye with samples. Each of the tubes was incubated at 37^0C, in a water bath and examined after 10 minutes. The time taken by the resazurin in the samples to become colourless was recorded. The following relationships of colour and quality are generally accepted (Al-Shamary and Abdalali, 2011).

Colour of sample	Quality of milk
Blue (no colour change)	Excellent
Blue to deep mauve	Good
Deep mauve to deep pink	Fair
Deep pink to whitish pink	Poor
White	Bad

Total Viable Aerobic Bacterial Counts

Total viable aerobic bacterial count and coliform count of the milk samples was determined as described by Collins *et al*., (1989). Each of the samples was diluted ten folds in properly labeled 20 ml glass tubes containing 9 ml sterile physiological saline as diluents. Separate sterilized glass pipette (1ml) was used to transfer the sample serially. One-tenth milliliter (0.1 ml) sample was inoculated on the surface of the media in triplicates and spread evenly with a sterile glass spreader. The plates were incubated at 370C for 48 hours. A total colony of aerobic bacteria, fungi and coliform were counted and expressed as colony forming units per milliliter/gram (Cfu/ml/g). Further characterization was done colonially, microscopically and biochemical methods (Cheesbrough, 2000).

Results

The total bacterial and fungal count is shown in Table 1. Table 2 shows colonial, microscopic and biochemical characteristics of bacteria isolated from the samples. The characteristics of the fungal isolates are shown in Table 3. Bacteria and fungi distribution across the samples is shown in Table 4. The result shows that species of *Micrococcus, Bacillus, Enterococcus* and *Staphylococcus* were isolated from all samples collected from the different locations. *Saccharomyces cerevisiae, Penicillum notatum, Saccharomyces ellipsoideus, Mucor spp* and *Rhizopus spp* were also identified (Barnnett and Hunter, 1987; Buchanan and Gibbon, 2000).

Table 1: Total bacteria and fungi counts on fresh cow milk and *Fura de nunu*

Sample code	Total coliform count	Total aerobic bacterial count	Total fungal count
COWA	2.85×10^{10}	4.0×10^{8}	1.45×10^{10}
COWE	3.2×10^{9}	3.6×10^{8}	6.0×10^{8}
COWS	2.54×10^{10}	3.2×10^{8}	7.0×10^{8}
COWO	2.98×10^{10}	1.0×10^{8}	8.0×10^{8}
COWU	1.74×10^{10}	5.6×10^{8}	4.0×10^{8}
FURA	2.92×10^{10}	7.0×10^{9}	3.9×10^{9}
FURE	2.73×10^{10}	2.0×10^{9}	5.0×10^{9}
FURS	1.94×10^{10}	1.0×10^{9}	4.8×10^{9}
FURO	1.83×10^{10}	3.1×10^{9}	9.0×10^{8}
FURU	1.22×10^{10}	2.2×10^{9}	4.0×10^{8}

Table 2: Colonial, Microscopic and Biochemical Characteristics of Bacterial Isolates

Bacterial isolates	Colonial characteristics	Mot	Grams reaction	Cat	Oxi	Coag	In	Cit	L	S	M	MN	G
Micrococcus luteus	small circular low convex yellow colonies	-	+S	+	-	-	-	+	-	-	-	-	-
Bacillus subtilis	mucoid and slimy rough cream colonies	+	+R	+	-	-	-	+	-	-	-	+	-
Bacillus cereus	dull and dry serrated flat cream colonies	+	+R	+	-	-	-	+	-	-	-	-	-
Staphylococcus aureus	large circular moist golden yellow colonies	-	+S	+	-	+	-	-	+	+	+	+	+
Escherichia coli	shiny metallic sheen	+	-R	+	-	-	+	-	+	+	+	+	+
Corynebacterium diptheriae	cream dull and dry umbonate colonies	-	+R	+	-	-	-	+	-	-	+	-	+
Streptococcus agalactiae	small cream moist colonies	-	+S	-	-	-	-	+	+	+	-	-	+
Bacillus polymyxa	cream medusa head colonies	+	+R	+	-	-	-	+	-	+	-	-	+
Lactobacillus sp	circular small yellow colonies	-	+R	-	-	-	-	+	+	+	-	+	+
Enterococcus faecalis	small circular cream	-	+S	-	-	-	-	+	+	+	+	+	+

Mot, motility; Cat, catalase, Oxi, oxidase; Coag, coagulase; In, indole; Cit, citrate, L, lactose; S, sucrose; M, maltose; Mn, mannitol; G, glucose; R, rod shaped; S, spherical shape

Table 3: Characteristics of Fungal isolates

Fungal isolates	colonial characteristics	Microscopic characteristics
Saccharomyces cerevisiae	moist shiny butyrous cream colonies	gram positive oval and spherical cells
Saccharomyces ellipsoideus	rough mucoid and slimy cream colonies	gram positive ellipsoidal cells
Rhizopus stolonifer	tall filamentous hyphae bearing black spores	hyphae non septate, spores enclosed in a sporangium
Penicillium notatum	dirty green spores enclosed in white hyphae	septate hyphae, conidia mop-head
Aspergillus flavus	black spores attached to short white hyphae	septate hyphae, conidia globosed
Mucor sp	short white hyphae serrated edges	non septate hyphae, sporangiophore Septate
Fusarium sp	white cotton wool like hyphae with yellow reverse	conidia sickle shaped
Geotrichum candidum (dairy moulds)	white creamy soft growth	hyphae septate and dichotomously branched into rectangular or oval cell
Rhizopus nigricans (bread moulds)	hyphae tall and filamentous bearing orange spores	non septate hyphae
Saccharomyces sp	large circular moist orange colonies	gram positive oval budding cells

Table 4: Distribution of bacterial and fungal isolates on fresh cow milk and *Fura de nunu*

Sample codes	Bacterial isolates	Fungal isolates
COWA	*Micrococcus luteus, Bacillus cereus Bacillus subtilis, Lactobacillus sp*	*Saccharomyces ellipsoideus, Sacch cerevisiae, Rhizopus stolonifer*
COWE	*Enterococcus faecalis, B. cereus, Streptococcus agalactiae*	*Sacch. cerevisiae, Sacch. ellipsoideus, Mucor sp*
COWS	*M. luteus, B. cereus, Ent. faecalis Strep. agalactiae, Lactobacillus sp*	*Rh. stolonifer, Sacch. Ellipsoideus*
COWO	*M. luteus, B. cereus, B. subtilis Staphylococcus aureus*	*Penicillium notatum, Rh. stolonifer Sacch. Ellipsoideus*
COWU	*Strep. agalactiae, S. aureus, M. luteus B. cereus, Lactobacillus sp*	*Rh. nigricans, Sacch. cerevisiae, Sacch. Ellipsoideus*
FURA	*Ent. faecalis, S. aureus, B. subtilis B. cereus, Corynebacterium diptheriae*	*Sacch. cerevisiae, P. notatum, Aspergillus flavus, Rh. Stolonifer Sacch. Ellipsoideus*
FURE	*Ent. faecalis, S. aureus, Escherichia coli, B. cereus*	*Sacch. cerevisiae, Mucor sp, Fusarium sp, Rh. Stolonifer*
FURS	*E. coli, Ent. faecalis, B. cereus, B. subtilis*	*Sacch. cerevisiae, P. notatum Sacch. ellipsoideus, Geotrichum candidum*
FURO	*B. polymyxa, B. subtilis, B. cereus, S. aureus, Ent. faecalis*	*Rh. stolonifer, P. notatum, Sacch. Sp Mucor sp, Sacch. Cerevisiae*
FURU	*E. coli, B. subtilis, B. polymyxa, B. cereus, S. aureus, Ent. faecalis*	*Rh. nigricans, P. notatum Sacch. cerevisiae, Rh. stolonifer, Sacch. ellipsoideus*

The percentage distribution of bacteria and fungi is shown in Table 5. *Bacillus* and *Saccharomyces* species are were *frequently* isolated from the samples*. Escherichia coli, Corynebacterium, Aspergillus, Geotrichum,* and *Fusarium* species are insignificant.

Table 5: Percentage Distribution of Bacterial and Fungal Isolates

Bacterial isolates	Percentage Distribution (%)	Fungal isolates	Percentage Distribution (%)
Bacillus polymyxa	2(4.4)	*Saccharomyces ellipsoideus*	8(22.2)
Bacillus cereus	10(22.2)	*Rhizopus stolonifer*	6(16.6)
Bacillus subtilis	6(13.3)	*Rhizopus nigricans*	2(5.54)
Staphylococcus aureus	6(13.3)	*Fusarium sp*	1(2.8)
Escherichia coli	3(6.7)	*Mucor sp*	3(8.3)
Enterococcus faecalis	7(15.5)	*Saccharomyces cerevisiae*	8(22.2)
Micrococcus luteus	4(8.9)	*Saccharomyces sp*	1(2.8)
Corynebacterium diptheriae	1(2.2)	*Penicillium notatum*	5(13.9)
Streptococcus agalactiae	3(6.7)	*Aspergillus flavus*	1(2.8)
Lactobacillus sp	3(6.7)	*Geotrichum candidum*	1(2.8)

Methylene blue reduction test result shown in Table 6 suggests that the samples are grossly contaminated and therefore translate to outright rejection of the milk. Tables 7 and 8 shows resazurin test on fresh cow milk and *fura de nunu* respectively. The test also rejects the samples which strongly indicate contamination.

Table 6: Methylene blue test for raw cow milk and *Fura de nunu*

Sample	Time (duration) and Colour change								
Code	5min	30min	60min	90min	120min	150min	180min	210min	240min
COWA	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
COWE	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
COWS	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
COWO	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
COWU	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
FURA	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
FURE	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
FURS	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
FURO	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White
FURU	Blue	Blue	Bluish	Bluish	Bluish	Creamy	Creamy	White	White

Table 7: Resazurin Test on Fresh Cow Milk

Resazurin disc no	Colour	Grade of Fura de Nunu	Action
COWA1	White	Very bad	Rejected
COWA2	White	Very bad	Rejected
COWA3	White	Very bad	Rejected
COWE1	White	Very bad	Rejected
COWE2	White	Very bad	Rejected
COWE3	White	Very bad	Rejected
COWS1	White	Very bad	Rejected
COWS2	White	Very bad	Rejected
COWS3	White	Very bad	Rejected
COWO1	White	Very bad	Rejected
COWO2	White	Very bad	Rejected
COWO3	White	Very bad	Rejected
COWU1	White	Very bad	Rejected
COWU2	White	Very bad	Rejected
COWU3	White	Very bad	Rejected

Samples analyzed in triplicates

Table 8: Resazurin Test on *Fura De Nunu*

Resazurin disc no.	Colour	Grade of Fura de Nunu	Action
FURAA	White	Very bad	Rejected
FURAB	White	Very bad	Rejected
FURAC	White	Very bad	Rejected
FUREA	White	Very bad	Rejected
FUREB	White	Very bad	Rejected
FUREC	White	Very bad	Rejected
FURSA	White	Very bad	Rejected
FURSB	White	Very bad	Rejected
FURSC	White	Very bad	Rejected
FUROA	White	Very bad	Rejected
FUROB	White	Very bad	Rejected
FUROC	White	Very bad	Rejected
FURUA	White	Very bad	Rejected
FURUB	White	Very bad	Rejected
FURUC	White	Very bad	Rejected

Samples analyzed in triplicates

Discussion

Dye reduction test using methylene blue and resazurin has been used as a rapid method in the detection of microbial contaminants in food, especially milk and other dairy products (Harrigan and McCance, 1990). Poor handling of *fura de nunu* during processing and marketing exposes it to microbial contamination, thereby making it a "source of microbial food poisoning". Houseflies are always found in large numbers at the production sites and at sale outlets. Aernan *et al.* (2011) reported that some female hawkers, prior to sale, in order to increase volume and improve colour of *nunu*, engage in sharp practices and fraudulent act by adding stream water and milky white supernatant obtained from water soaked with baobab tree seeds. The local Fulani's who are the major producers are not conscious of sanitary conditions during milking and preparation of the *nunu*. In most cases potable water is not available or in short supply thereby encouraging the use of water from the stream and local wells which may serve as a vehicle in the contamination by waterborne pathogens.

Raw milk drawn from a healthy udder normally will contain only a few hundred to a few thousands of bacteria per milliliter, mostly from the genus *Micrococcus* and *Streptococcus* (Frazier and Westhoff, 2000; Adams and Moss, 1995). From the results, samples collected from the informal markets recorded a high total viable aerobic count of 2.92×10^{10} cfu/ml - 3.2×10^{10}cfu/ml; a coliform count of 7.0×10^{9}cfu/ml – 1.0×10^{8}cfu/ml and total fungal count of 1.45×10^{10}cfu/ml – 4.0×10^{8} cfu/ml. The high microbial population in the samples suggests gross contamination. Results obtained

from the rapid dye reduction test confirm the routine microbiological enumeration of bacteria and fungi in food samples. The microbiological assessment of the raw milk and nunu samples revealed the bacteria and fungi associated with food spoilage and food poisoning. The presence of *Escherichia coli* and *Enterococcus faecalis* indicates faecal contamination while Staphylococcus aureus produce potent enterotoxin involved in food borne intoxication (Nester *et al*., 2001). Species of *Bacillus, Micrococcus* and *Corynebacterium* are soil borne bacteria that contaminate food and water. Bacillus species can resist environmental stress during spore formation and causes emetic syndrome and food borne intoxication leading to diarrhea and subsequently dehydration (Adams and Moss, 1995). *Streptococcus agalatiae* cause mastitis in the udder of a cow (Adams and Moss, 1995), whereas *Lactobacillus sp* ferments lactose in milk resulting in sour taste and spoilage (Harrigan and McCance, 1990). Fungal species such as *Aspergillus, Rhizopus, Penicillium* and *Fusarium* produce several mycotoxins resulting in mycotoxicosis in animals and humans (Efiuvwevwere, 2000; Uzeh *et al*., 2006.).

Dankor *et al*., (2007) had reported on bacterial contamination of raw milk. It has reported that fermented milk in regions with low temperatures supports the growth of mesophillic bacteria, such as *Lactococcus* and *Leuconostoc spp*, while areas having high temperatures (like the study area) favours the growth of thermophillic bacteria like *Lactobacillus* and *Streptococcus* (Savadogo *et al*., 2004).

It is therefore recommended that herdsmen wash teat of cows, hands and other milking equipment adequately with a suitable antiseptic to prevent contamination of raw milk. Subsequently, milk should be stored in the aluminum cans recommended by the FAO (which are easier to clean), and in the absence of refrigeration, milk should be transported during the cool of the day. In the markets, raw milk should be subjected to effective heat treatment (boiling or pasteurization) to eliminate organisms. Also, with the probability of post-process contamination, it is recommended that different vessels be used for dishing out different products during the sale.

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