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Molecular Studies of Prevalent Bacteria Isolated from Unpasteurized Traditional Fermented Dairy Products Sold in Parts of Imo State, Nigeria

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Abstract

Fura, Nunu and *Fura de Nunu* are locally fermented dairy product processed from raw cow milk and corn paste. The product is very popular in the Northern part of Nigeria and is mostly processed in an unhygienic environment. A total of one hundred samples from two locations (Obinze and Owerri municipal) were assayed for microbial contaminants using standard methods. The identities of the isolates were confirmed by molecular characterization and sequencing. Mean Total Viable Counts (TVC), Total Heterotrophic Bacterial Counts (THBC), Total Faecal Coliform Count (TFCC), Total Salmonella/ Shigella Count (TSSC), Total Campylobacter Count (TCC) and Total Staphylococcus Count (TSC) were in the progression $THBC > TFCC > TCC > TSC > TSSC$. Generally, higher counts were recorded from the *Fura* samples (Logarithms of counts of 9.19 and 8.47) than *Nunu* (Logarithms of counts of 9.10 and 8.44) in both locations. Three hundred isolates were recovered with *Micrococcus* sp having the highest predominance (16%) while *Lactobacillus* sp, *Enterobacter* sp, *Salmonella* sp, *Providencia* sp and *Shigella* sp had least with a percentage of 2% each. *Staphylococcus* sp, *Enterococcus* sp and *Campylobacter* sp had a 15% predominance each. *Escherichia coli* and *Bacillus* sp had a predominance of 10% each; and *Klebsiella* sp with a 9% predominance. The 16S rRNA of the strains SSASL, GYCAMP, EMBEC and LABSY showed that they were *Providencia* strain UAF-03 (MH924823), *Enterobacter* sp Strain AB55 (MF407145), *Escherichia coli* strain AR_0067 (CPO32261) and *Lactobacillus fermentum* strain K2 (KT183368) respectively. Most of the bacterial isolates are predominant in food, soil, water and faecal materials. Isolation of *Escherichia coli, Salmonella, Enterococcus, Staphylococcus, Klebsiella, Shigella, Campylobacter, Providencia* and *Enterobacter* species from the fermented dairy products portends serious health risk to consumers. *Lactobacillus fermentum* is a probiotic involved in the fermentation whereas *Bacillus* is a notorious contaminant. Good Manufacturing Practices (GMP) are therefore recommended for wholesome and acceptable product.

Introduction

Milk is one of the highest consumed animal products in Nigeria. It can be obtained from cattle, buffalo, goat, sheep, camel, cow, yak, llama, mare etc. It has been described as a nearly perfect food because it contains the essential nutrients required by the body in appropriate proportions [1]. It is an aqueous colloidal suspension of proteins, fats, and carbohydrates that contains numerous vitamins and minerals [2]. Milk has distinct physical, chemical and biological characteristics, which justify its high quality for consumption [3]. Milk from healthy animal's udder is free from pathogenic bacteria, but some of the animals in field condition may be suffering from a range of infectious diseases. As a result, various contaminants can occur in milk [4] causing Food-borne infections which are common and constitute an important health and economic burden globally (Nazzal et al., 2012).

According to [5], fresh milk may get microbial contamination from utensils, animal skin, the environment or water used for adulteration. Such contaminated milk product cannot be safe for consumption only if is properly pasteurized to kill microorganisms that may be in association with it. *Nunu* has been described by Nebedum et al. (2007), as a locally fermented diary product obtained from mixed culture fermentation of freshly drawn cow milk with sour milk or yoghurt-like in taste. It is always consumed unpasteurized by most consumers and may contain high load of microbial contaminants. These contaminants may be introduced from different sources. Most consumers of this locally fermented milk "*Nunu*" consume it when mixed or mashed with *Fura* which are millet dough balls that gives the cow milk more flavour and

taste. The combination of *Fura de Nunu*, mainly sold by Fulani's is a very nutritious and delicious meal but may poses a high risk of contamination due to poor preparation procedure, improper handling by food hawkers, use of contaminated utensils and lack of proper preservative techniques. Braide et al. (2015) [5] has reported that untreated/unpasteurized milk is highly vulnerable to microbial spoilage and is of public health concern as the consumption of such food may cause health risk to the consumers and the ailment caused by this contaminants (bacteria, yeasts, moulds) can cause infection that are resistant to antibiotics thus causing more harm than good in the health of the consumers.

This study reports on the molecular characterization of prevalent strains of bacteria isolated from locally fermented dairy products.

Materials and Methods

Description of Sampling Location

The research was carried out in Owerri, Imo State which lies in latitude 50 29'N, 702'E, South Eastern Nigeria. There are two marked seasons in the state; dry windy season (Harmattan) and the wet rainy season. It is located within the humid tropical zone of Nigeria with high amount of rainfall in most parts of the year which ranges from 343cm^3 to 440cm^3. The mean temperature is between 21 ^{0}C and 28 ^{0}C with relative humidity of about 98% during the dry season (Onu, 2011). The state is dominated with Igbo's dealing on small-scale businesses and few Hausa/Fulani settlement (dealing on livestock) in the Owerri capital territory. The sampling covered the informal mammy and Cattle markets at Obinze-Mgbirichi and Ama-Hausa in Owerri municipal council, Imo State, Nigeria.

Sample Collection

A total of one hundred (100) samples were collected randomly at different sales point in a pre-sterilized polyethylene bags, and transported immediately to the laboratory in an ice-chest. *Nunu, Fura de nunu* and *Fura* were collected from the Fulani women between the hours of 12:30am - 4:25pm.

Microbiological Analysis

Ten milliliters of fresh *Nunu* and *Fura de nunu* were dispersed and swirled thoroughly in 90 ml of freshly prepared peptone water respectively. Also, ten grams of the ball of *Fura* were thoroughly mixed in a stomacher blender containing 90 ml of peptone water. The 10-fold dilutions were carried out serially on the mixtures and 0.1 ml was inoculated into selective, differential and general purpose medium in duplicate and spread evenly. Culture plates were incubated at 37 ^{0}C for 24 h [6].

Enumeration and Characterization of Microbial Isolates

Bacteria isolated from the samples were counted using the neubauer colony counter and expressed as total colony forming units per gram/milliliters [7]. Preliminary studies on the isolates was done based on colonial, microscopic and biochemical characteristics using standard methods [6].

Molecular Identification

DNA extraction (Boiling method)

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95 ^{0}C for 20 min. The heated bacteria suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5 ml microcentrifuge tube and stored at -20 ^{o}C for other downstream reactions [8].

DNA Quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2µl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button [9].

16S rRNA Amplification

The 16SrRNA region of the rRNA genes of the isolates were amplified using the 27F:5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R:5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Bio systems thermal cycler at a final volume of 25 microliters for 35 cycles. The PCR mixtures included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95 ^{0}C for 5 min; denaturation, 95 ^{0}C for 30 seconds; annealing, 52 ^{0}C for 30 seconds; extension, 72 ^{0}C for 30 seconds for 35 cycles and final extension, 72 ^{0}C for 5 minutes. The product was resolved on a 1% agarose gel at 120 V for 20 min and visualized on a blue light transilluminator [10].

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria, South Africa. The sequencing was done at a final volume of 10ul; the components included 0.25 ulBigDye® terminator v1.1/v3.1, 2.25ul of 5 x Big Dye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96 ^{0}C for 10s, 55 ^{0}C for 5s and 60 ^{0}C for 4min (Inqaba Biotechnological, Pretoria, South Africa).

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit. Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [11].

Results

The colonial characteristics of the bacteria isolated from *Fura de nunu, Fura* and *Nunu*on the basis of morphological characteristics is shown in Table 1. Microscopic and biochemical characteristics gave a presumptive identity as species of *Micrococcus*, *Enterococcus*, *Staphylococcus*, *Campylobacter*, *Escherichia coli*, *Bacillus*, *Klebsiella*, *Lactobacillus*, *Enterobacter*, *Salmonella*, *Providencia* and *Shigella* (Table 2).

Colonial characteristics	Mot	Spo	Cap	Gram morphology	Identity of isolates
Smooth golden yellow colonies on nutrient agar, light yellow on mannitol salt agar	-	-	-	Gram positive cocci predominantly in clusters, few in pairs	*Staphylococcus* sp
Small circular moist and shiny cream colonies on nutrient agar	-	-	-	Gram positive cocci predominantly in chains, few in pairs	*Enterococcus* sp
Creamy white butyrous colonies on Campylobacter Blood Free agar	-	-	-	Small slender gram negative rods	*Campylobacter* sp
Small circular moist colonies on De Mann Rogosa and Sharpe agar	+	-	-	Long slender gram positive rods in chains	*Lactobacillus* sp
Grey and creamy colonies on MacConkey agar	-	-	-	Small gram negative rods	*Providencia* sp
Pink and slimy mucoid colonies on MacConkey agar	+	-	+	Small thick gram negative rods in short chains	*Klebsiella* sp
Light pink moist and shiny colonies on Salmonella Shigella agar	-	-	-	Gram negative rods in short chains	*Shigella* sp
Black fish eye colonies on Salmonella Shigella agar	+	-	-	Gram negative rods in singles and pairs	*Salmonella* sp
Greenish metallic sheen on Eosin Methylene Blue agar	+	-	-	Gram negative rods predominantly in singles and few in pairs and short chains	*Escherichia coli*
Smooth moist and shiny yellow colonies on nutrient agar	-	-	-	Gram positive cocci predominantly in tetrads, few in pairs and clusters	*Micrococcus* sp
Rough slimy cream colonies on nutrient agar	+	+	-	Large gram positive rods with central spores	*Bacillus* sp
Small moist and shiny pink colonies on Eosin Methylene Blue agar	+	-	-	Gram negative rods in short chains and pairs	*Enterobacter* sp
motility (Mot), capsule formation (Cap), spore formation (Spo)					

Table 1: Colonial characteristics of Bacteria isolated from locally fermented Dairy Products.

Bacterial isolates	Cat	Coag	Oxi	In	MR	VP	Cit	Glu	Suc
Enterococcus sp	-	-	-	-	-	+	-	+	+
Klebsiella sp	+	-	-	-	-	+	+	+	+
Staphylococcus sp	+	+	-	-	+	-	+	+	+
Micrococcus sp	+	-	+	-	-	+	+	+	-
Providencia sp	+	-	-	+	+	-	+	+	+
Bacillus sp	+	-	-	-	-	+	+	+	+
Enterobacter sp	+	-	-	-	-	+	+	+	+
Escherichia coli	+	-	-	+	+	-	-	+	+
Salmonella sp	+	-	-	-	+	-	-	+	-
Shigella sp	+	-	-	-	+	-	-	+	-
Lactobacillus sp	-	-	-	-	+	-	-	+	+
Campylobacter sp	+	-	+	-	-	+	-	-	+
Key: Catalase (cat), Citrate (Cit), Coagulase (Coag), Oxidase (Oxi), Indole (In), Methyl Red (MR), Voges Proskauer (VP), Glucose (Glu), Sucrose (Suc).									

Table 2: Biochemical and sugar Fermentation of Bacterial isolates.

The percentage growth of bacteria according to the media used is presented in Table 3. The five media used for recovery of isolates include Nutrient Agar (NA), Eosin Methylene Blue Agar (EMB), Mannitol Salt Agar (MSA), Campylobacter Agar Base (CAMPY) and Salmonella-Shigella Agar (SSA). Results recorded according to the recovery of isolates by the media used follows the progression $NA > EMB > CAMPY > MSA > SSA.$ This indicated that NA had the highest recovery (100%) while SSA had the least Recovery (8%).

Media	Sample number	No with growth	No without growth	Total
NA	100	100(100%)	0	100(100%)
EMB	100	91(91%)	9(9%)	100(100%)
CAMPY	100	73(73%)	27(27%)	100(100%)
MSA	100	45(45%)	55(55%)	100(100%)
SSA	100	8(8%)	92(92%)	100(100%)

Table 3: percentage Recovery of Isolates by different Media used.

Table 4 shows the predominance of isolates recovered from the milk samples. A total of 300 isolates were recovered. Results recorded in Table 4 showed that *Micrococcus* sp had the highest predominance (16%) while *Lactobacillus* sp, *Enterobacter* sp, *Salmonella* sp, *Providencia* sp and *Shigella* sp had least predominance with a percentage of 2% each. Others include *Staphylococcus* sp, *Enterococcus* sp and *Campylobacter* sp which had 15% predominance each. *Escherichia coli* and *Bacillus* sp which had a predominance of 10% each, and *Klebsiella* sp with a 9% predominance.

Bacterial isolates	Frequency	Percentage (%)
Micrococcus sp	49	16
Enterococcus sp	44	15
Staphylococcus sp	44	15
Campylobacter sp	44	15
E. coli	32	10
Bacillus sp	32	10
Klebsiella sp	27	9
Lactobacillus sp	6	2
Enterobacter sp	6	2
Salmonella sp	6	2
Providencia sp	6	2
Shigella sp	6	2
Total	300	100

Table 4: Predominance of Isolates recovered from analyzed samples.

Mean Total viable counts of Total Heterotrophic Bacterial Counts (THBC), Total Faecal Coliform Count (TFCC), Total Salmonella/Shigella Count (TSSC), Total Campylobacter Count (TCC) and Total Staphylococcus Count (TSC) are presented in Figure 1, using their logarithm values respectively. Counts recovered are in the progression $THBC > TFCC > TCC > TSC > TSSC$. Considering the THBC, Higher counts were recorded from the *Fura* samples than the *Nunu* samples in both sampling location. Similarly counts of samples from Obinze had relatively higher values for Logarithms of counts of 9.19 and 8.47, compared to the samples from Owerri municipal which had values of 9.10 and 8.44 for *Fura* and *Nunu* Samples respectively. Similar results were recorded for the THFC which also had values for Logarithms of counts of 7.18 and 7.68 in Obinze samples, as well as 6.75 and 7.33 in Owerri municipal samples for *Fura* and *Nunu* respectively.

Log (TSSC) and Log (TCC) recorded values of 0.701349 and 6.245717; as well as 0.835368 and 5.631061 for samples from Obinze while recording 0.453562 and 4.606851; as well as 0.440668 and 5.612874 for samples from Owerri municipal for *Fura* and *Nunu* respectively. This also indicated that higher counts were recorded from the *Fura* samples than the *Nunu* samples in both sampling location and the counts of samples from Obinze had relatively higher values than that from Owerri Municipal. Conversely, Log (TSC) recorded relatively higher counts from samples obtained from Owerri municipal than those from Obinze for both *Fura* and *Nunu* samples with values of 4.081355 and 3.575185; as well as 3.491146 and 2.803797 for *Fura* and *Nunu* Samples respectively. However, generally, higher counts were recorded in *Fura* Samples than in *Nunu* Samples (Figure 1).

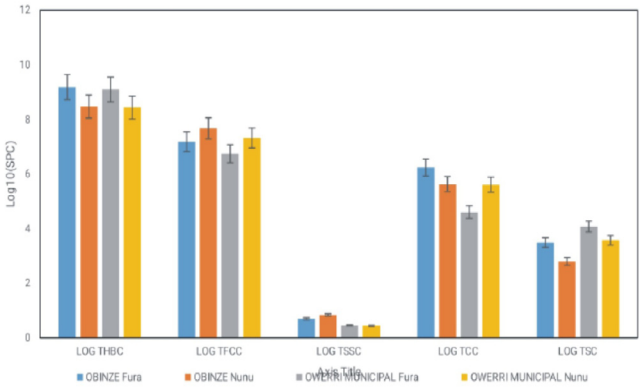

Figure 1: Mean Total Viable Counts of Samples.

The obtained 16SrRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolate SSASL, GYCAMP, EMBEC and LABSY showed a percentage similarity to other species: *Providencia* sp at 99%, *Enterobacter* sp at 72.4%, *Escherichia coli* at 100% and *Lactobacillus fermentum* at 86.4% respectively. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates SSASL, GYCAMP, EMBEC and LABSY within the *Providencia* sp and *Enterobacter* sp, *Escherichia coli* and *Lactobacillus fermentum* revealed a closely relatedness to *Providencia* strain UAF-03(MH924823), *Enterobacter* sp Strain AB55 (MF407145), *Escherichia coli* strain AR_0067 (CPO32261) and *Lactobacillus fermentum* strain K2 (KT183368). The detailed dendogram is shown in Figure 2. Prior to characterization, the Genes were separated using the agarose gel. The Agarose gel electrophoresis of the 16S rRNA gene of the selected bacterial isolates is shown in Plate 1.

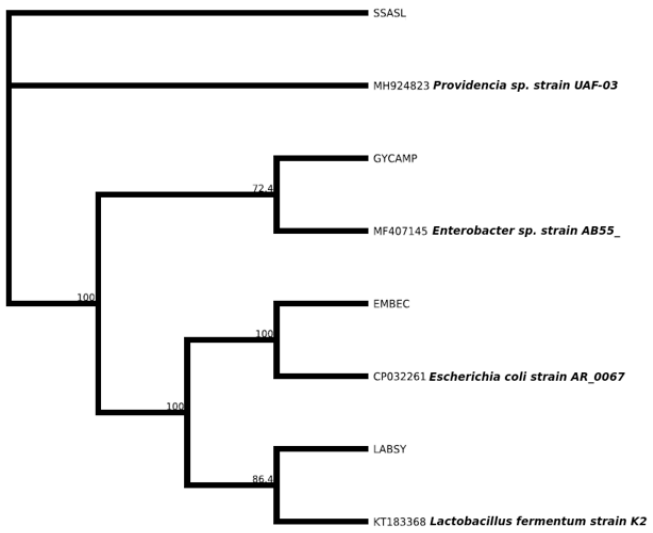

Figure 2: Phylogenetic tree of chatacterized isolates showing *Providencia* strain UAF-03(MH924823), *Enterobacter* sp Strain AB55 (MF407145), *Escherichia coli* strain AR_0067 (CPO32261) and *Lactobacillus fermentum* strain K2 (KT183368).

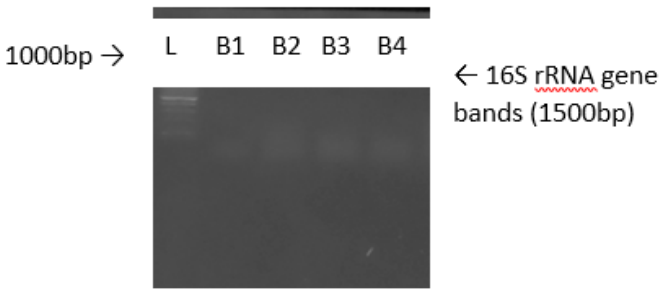

Plate 1: Agarose gel electrophoresis of the 16S rRNA gene of some selected bacterial isolates. Lanes B1-B4 represent the 16SrRNA gene bands (1500bp), lane L represents the 100bp molecular ladder.

Discussions

Fura, an indigenous fermented cereal-based product is a very nutritious food mostly consumed in Northern Nigeria contains microorganisms of public health concern as a result of methods used in its preparation, storage and marketing [12]. *Nunu*, on the other hand, is processed by collecting fresh cow milk and allowing it to ferment for a day or two [13]. This information indicates that contaminants found in the milk and the fermented cereal could have occurred during the production line thereby increasing counts and species diversity recovered from the food. Indeed, Abid et al. (2009) [14] reported that counts greater than $Log_{10}3$cfu/ml for raw milk or milk product indicates a serious problem in hygiene. This implies that all counts recovered from this work are indicative of poor processing conditions. Comparatively, milk and other samples obtained from municipal area were relatively processed in a better environment than those from Obinze. Similarly, the counts were not within the limit and specifications' standards supplied by APHA (2001). Since raw milk is a potentially hazardous food, when collected from a normal and healthy udder, it records counts of a few hundred to a few thousands of bacteria per milliliter, mostly from the genus of *Micrococcus* and *Corynebacterium* [13]. Counts recorded from previous researchers also indicated that relatively higher counts than specified standards were recovered in Nigeria and Ghana respectively [13,15].

The possible sources of microbial contaminants associated with the products in this study could be the use of previously fermented *Nunu* as starter culture (back slopping), unhygienic milking area, improper handling and processing of the millet for *Fura* production and unclean water for processing. The contaminating organisms could also be introduced through microbial adherence to the utensils such as spoons, and bowls used during production of the *Fura, Nunu* or both. In addition, the high microbial contamination of the samples used in this study could be due to the health status of the milked animals. According to Harrigan (1998) [7], high microbial contamination in milk may be due to clinical and subclinical state of the cattle as organisms of milk and milk products may get into milk either directly from udder and or indirectly through infected body discharge of the animal which may find its way into the milk. The high counts of microorganisms in *Fura* and *Fura de Nunu* in the present study also indicated that those microorganisms not only contaminate milk but also multiply and grow on it. This might be due to the fact that the milk and millet, used in producing *Fura de Nunu* contains a lot of nutrients which made *Fura de Nunu* a good substrate for the growth of the microorganisms, especially with poor sanitary procedures and the lack of the cooling facilities and pasteurization. According to Pourhassan and Taravat (2011) [16], the growth and multiplication of microorganism in milk conditions is further aggravated by the tropical hot climate.

Microbiology in the dairy industry is an important issue, as recent outbreaks of food-borne illness were recorded as a result of consumption of milk and dairy products that had been contaminated with pathogenic organisms or their toxins. As a result, huge attention has been paid on the microbiological analysis of milk and dairy products to evaluate the quality and also to ensure that there are no public health hazards. Some of the organisms identified in the samples belong to the family Enterobacteriaceae including *E. coli, Providencia* sp*, Enterococcus* sp and *Salmonella* sp. Enterobacteriaceae are common inhabitants of the intestinal tract of various domestic animals including cow, and are commonly found in cow dung which are abundant at the milking area. Therefore, poor sanitation of the milking environments can

predispose the milk to contamination and by extension the other products. *Staphylococcus* and *Bacillus* which are the predominant isolates are known to be associated with food borne intoxication through production of enterotoxins. The main species involved in food borne intoxication are *Bacillus cereus* and *Staphylococcus aureus* [17]. *Staphylococcus* sp. which is a normal flora of the skin could be introduced into the *Fura de Nunu* from the skin of the humans and animals during production. Poor sanitary practices adopted during production, storage and marketing also exposes the final product to contamination. According to Ledenbach and Marshal (2009) [18], the enterotoxin produced by *Staphylococcus aureus* and some coagulase negative Staphylococci that affects the Central Nervous System (CNS) are heat stable and can survive pasteurization. The isolation of *Staphylococcus aureus* is of public health significance since it is said to be a commonly recovered pathogen in outbreaks of food poisoning from milk and milk products [19]. *Bacillus* sp. on the other hand may have been introduced into the milk product from the soil, animal feed, milking equipment, milking environment, and the udder of the animal. Brown (2000) [20] reported that *Bacillus* sp has been implicated as the main cause for significant economic losses in the dairy industry. *Bacillus* sp such as *B. licheniformis, B. cereus, B. subtilis, B. Mycoides,* and *B. megaterium* are said to be able to produce spores which can survive pasteurization [18].

Other bacteria species isolated from the samples includes, *Enterobacter, Providencia, Klebsiella*, *Enterococcus, Micrococcus, Campylobacter, E. coli, Klebsiella, Lactobacillus, Salmonella* and *Shigella* species. In addition, the primitive system of transportation and marketing practice may also be predisposing factors for all kinds of microorganisms to grow and multiply in the milk product. *Micrococcus* may originate from the environment or human sources [21,22]. *Enterococcus* and *Providencia* are both Enterobacteriaceae commonly found in the intestine of animals. Their presence in the samples could be from the animal that produced the milk. *Campylobacter* on other hand is a pathogenic organism that can infect both humans and animals, especially from poultry and meat [5]. It has been recently reported that beef and vegetables were potential sources of *Campylobacter* [23]. Therefore, possible contamination of milk could be from forages and water samples consumed by the cattle or used during processing, as was reported researchers [23,24]. *Escherichia coli* and *Salmonella* are both faecal coliforms. The presence of *Salmonella* in *Nunu* is in close agreement with that of report of Junaidu et al. (2011) [19] who isolated 2(2.17%) in clinically mastitic cow and 2.6% overall prevalence reported by Van-Kessel et al. (2004) [25] from bulk tank milk. The presence of this organism may indicate faecal contamination of milk but more importantly an indicator for poor sanitary practice during milking. De- Buyser *et al*. (2001) [26] had reported that *Salmonella* is one of the most etiological agents implicated in several outbreaks associated with the consumption of raw milk and milk products. All *Salmonella* species also are of public health concern having the ability to produce infection ranging from a mild self-limiting form of gastroenteritis to septicemia and life threatening typhoid fever [22]. *Lactobacillus* sp isolated could be beneficial to consumers because they are mostly probiotics and can be found in fermented foods, yoghurt and supplements. Although, some lactic acid bacteria cause spoilage of dairy products when conditions for their growth becomes unfavourable [13].

Lactobacillus fermentum, which is a lactic acid bacterium is considered probiotics or a friendly bacterium in animals, they can be used for food and feed fermentation and are probably involved in fermentation of the product (*Fura* de *Nunu*) as reported by Akabanda et al. (2010) [13].

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