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Identification and Characterization of *Lactobacillus* Isolates Recovered from Locally Fermented Milk (Nunu) consumed within Lagos Metropolis

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Abstract: **This work assessed the probiotic potential of "Nunu" using *Lactobacillus*, a genus of Lactic acid bacteria and a major probiotic organism as the indicator. The viable *Lactobacillus* counts of samples purchased from six different areas within Lagos Mainland were enumerated by serial dilution and pour plating in selective media, DeMann, Rogosa and Sharpe agar (MRS). Twelve strains of *Lactobacillus* were randomly selected from the samples and identified by standard morphological, physiological and biochemical methods. The isolates were identified as *L. casei, L. bulgaricus, L. acidophilus, L. plantarum, L. rhamnosus, L. thermophilus, L. reuteri, L. helviticus, L. delbrueckii, L .brevis. L. cellobiosus, L. fermentum* with *L. acidophilus* occurring the most and elicited the highest count of 421.2 x 10^6 cfu/ml while *L. reuteri* has the lowest count of 2.5 x 10^6 cfu/ml. This indicates that *L. reuteri* probiotic level is been compromised by count. The isolates were further examined for bacteriocin like activity against *E.coli* ATCC25922 using agar well diffusion method. After incubation, the zones of inhibition was observed and recorded. Locally fermented milk of probiotic value has bacteriocinogenic property against *E.coli* although it carries resistant plasmid which can serve as drug resistance when consumed.**

Keywords: **Characterization, Fermentation, Identification, Incubation, Lactic Acid Bacteria (LAB), *Lactobacillus*, Milk, Nunu.**

I. INTRODUCTION

Milk is an excellent source of vitamins and minerals, particularly calcium. Milk proteins increase the value of poorer quality cereal and vegetable proteins in the diet by providing the amino acids these proteins lack, protects against tooth decay, reduces oral acidity (which causes decay), stimulates saliva flow, and decreases plaque formation. Raw milk has low keeping quality and at room temperature, spontaneous microbial spoilage occurs turning the product sour few some days. This is brought about by the activity of lactic acid bacteria [1]. Locally fermented milk is a form of food processing where microbes, for example, lactic acid bacteria (LAB) are utilized for food production via the process known as fermentation [2]. Fermentation in food processing is the conversion of carbohydrates to alcohol and carbon dioxide or organic acids using yeast and/or bacteria, under anaerobic conditions [3]. Fermentation is one of the classic methods to preserve foods. The fermentation techniques are often a small scale and household basis, characterized by the use of simple non-sterile equipment, chance or natural inoculums, unregulated conditions, sensory fluctuations, poor durability and unattractive packing of the processed products resulting in food of unpredictable quality [4]. Locally fermented milk is processed by collecting fresh cow milk and allowing it to ferment for a day or two. The Fulanis ferment the milk in calabashes, or rubber buckets. Locally fermented milk (nunu) is yoghurt-like in taste (a sharp acid taste) and can be

consumed alone or with sugar and fura. Even when the milk is fermented, the fermentation process with the attendant drop in pH may not rid the product of these organisms and may be carried to consumers. *Salmonellae* and other microorganisms have been known to underscore the importance of milk and milk products as vehicle for human infections.

Lactobacillus is considered as part of the indigenous microflora of the mammalian gastrointestinal tract and of many other niches and fermented foods [5], [6], [7]. Locally fermented milk contains a good balance of protein, fat and carbohydrate and is a very important source of essential nutrients including: Calcium, Riboflavin, Phosphorous, Vitamins A and D, Pantothenic acid. There are claims that the digestibility of the milk proteins is improved by fermentation [8]. The *Lactobacillus* genus consists of a genetically and physiologically diverse group of rod-shaped, Gram-positive, non-spore forming, none pigmented catalase negative and microaerophilic to strictly anaerobic. Mainly, the ability to reduce serum cholesterol levels, antimicrobial substrate production and immune modulation are considered as effective properties, in which, *Lactobacillus* (Lactic Acid Bacteria, LAB) is a commercially important bacterium with wide variety of application, both in the food industry and as a probiotic agent for the improvement of human health [9], [10]. From the health point of view, ingestion of live cells of certain species and strains, the probiotic concept of lactobacilli in adequate amounts is believed to confer several beneficial physiological effects on the host such as maintaining a healthy and equilibrated intestinal microbiota and reducing incidence of intestinal infection [11]. The criteria for the in vitro selection of *lactobacilli* to be used as health-promoting, probiotic ingredients, in food and pharmaceutical preparations include antibiotic tolerance as well as the production of lactic acid that inhibits the growth of other microorganisms, which allow them to be established in the intestinal tract [12]. Most *Lactobacillus* strains regardless of their source harbour at least one indigenous plasmid and often more in which, some lactic acid bacteria may carry potentially transmissible plasmid encoded antibiotic resistance genes and any strains harbouring antibiotic resistance plasmids are considered unsuitable for use as human or animal probiotics. However, the importance of intrinsic antibiotic resistant strains which may benefit patients whose normal intestinal micro biota has become unbalanced or greatly reduced in numbers due to administration of various antimicrobial agents have also been reported [13], [14]. These health benefits have been attributed to an array of antimicrobial substances produced by *lactobacillus* especially bacteriocin.

"Nunu", as a locally fermented milk is being produced in limited daily consumable quantities due to its poor keeping quality. Deterioration of this locally fermented milk starts quickly during storage in terms of growth of microorganisms (lactic and non-lactic) present naturally or as contaminant. Poor hygiene, practiced by handlers of the products, may lead to introduction of pathogenic microorganisms into the products and since they do not undergo further processing before consumption, this food may pose risk to the consumer. Therefore, screening of locally fermented milk products for lactobacillus composition by species, cell density, and probiotic functions remain the standard measures of quality and marketability in human population to promote public health. Knowledge of the biochemical and microbial changes that are associated with its processing will obviously enhance the production and proper utilization on a larger scale.

Therefore, this work investigates the bacteriocin-like activity and probiotic value of locally fermented milk consumed in Yaba, Lagos, Nigeria. The objectives are to identify *Lactobacillus* isolates present in a selected sample of locally fermented milk consumed in Yaba community in Lagos State of Nigeria. To determine the total *Lactobacillus* bacteria, count in the locally fermented milk product and their percentage resistance to antibiotics by the isolated *Lactobacillus* strains. To determine the bacteriocinogenic activity of the *Lactobacillus* isolates against multi drug resistance *E. coli* isolates, and to isolate and characterize plasmids harbored by the *Lactobacillus* isolates.

II. MATERIALS AND METHODS

Sixty (60) non-replicate samples of locally made fermented milk were collected from six different locations at different times over one month (October to November, 2015) in Yaba, L.G.A of Lagos State. They were bought randomly and collected aseptically from sellers at the following locations: Yaba bus stop, Montogomery, Biney, Ojuelegba road, Railway and Empire. Samples were labelled and cultured within 2 hrs of collection at Microbiology Laboratory of Nigeria Institute for Medical Research (NIMR), Yaba, Lagos, Nigeria for analysis using several standardized methods.

A. *Lactobacillus* and Total Bacterial Count

Total *Lactobacillus* count was obtained using spread plate technique method. A volume of 10 µl from 10^{-4}, 10^{-6} dilutions of each fermented milk sample in sterile water was inoculated on de Man Rogosa Sharpe (MRS) agar and spread using a

glass spreader. For total bacterial count; 10µl aliquots of 10^{-4}, 10^{-6} dilutions were inoculated on to iso-Sensitest agar plate. Both inoculated MRS and iso-Sensitest agar plates were incubated anaerobically and aerobically at 37^0C for 48 hrs. The growth on each plate was examined at the end of incubation period and colonies were counted using coulter colony counter and reported as colony forming units per gram. (cfu/g).

B. Purification of Isolates

Colonies observed were repeatedly sub cultured in MRS agar plates to obtain a pure culture by employing streak plate method. After streaking on the MRS plate, it was incubated anaerobically at 37^oC for 24 – 48hrs. The pure culture obtained was stored in MRS broth in McCartney bottle and kept in the refrigerator at 4^oc until when required.

C. Identification and Characterization of Isolated Organisms

The identification of the bacteria isolates was done using appropriate methods. Each locally fermented milk sample was mixed and diluted 10-fold in sterile water. This was followed by direct inoculation on to MRS agar medium (pH 6.4 + 02). The inoculated plates were incubated anaerobically at 37^0C for 24 - 48 hrs. Suspected colonies of *Lactobacillus* were identified based on the following standard tests: morphology on MRS plate, gram staining, oxidase test, catalase test, methly red Voges Proskauer test, sugar fermentation including fructose, sucrose, maltose, mannose, sorbitol and cellulose at 1% concentration as substrates using methly red as indicator. Other tests carried out, were arginine hydrolysis test, growth at 15^0C, 30^oC, 37^oC and 45^oC. Subsequent confirmation of *Lactobacillus* isolates was done using API (50CHL) biochemical reactions (API, France).

D. Gram Reaction

Microscopy of isolates was carried out using Gram staining technique for all isolates.

Gram Staining: Under aseptic condition, a smear of fresh culture at 18-24hrs of the isolates was mixed with a drop of sterile water on a clean slide using a well flamed wire loop. The smears were air dried and heat fixed by passing the slides over flame. The slides were then flooded with crystal violet for 1 – 2mins each. They were rinsed with sterile water. The resulting smear was flooded with gram's iodine and left to stand for a minute and rinsed off with water. The smear was then decolorized with 75% alcohol. It was counterstained with safranin for 30 seconds. The slides were finally dried and examined under an oil immersion lens.

E. Plasmid Profile Analysis

A high pure plasmid isolation kit (Roche Applied Science, Mannheim, Germany) was used to extract and purify plasmid DNA from the bacterial isolates. Plasmids were separated by gel electrophoresis (Apelex, France) in 0.7% agarose gel in 1 x TAE buffer (Sambrook and Russell, 2001). The gels were run for 10minutes at 100 volts and then approximately 2 hours at 70 volts, stained with ethidium bromide, exposed to ultraviolet light and photographed (Visi-Doc-It system,UVP, UK). Commercial DNA ladders (Fermentas**,** Germany).

III. RESULTS AND DISCUSSION

The results of the experiments are presented in Tables. Table 1 shows the frequency distribution of *Lactobacillus* isolates and yeast recovered from locally made fermented milk (*nunu*) from six different locations within Lagos mainland area of Lagos state. Of the sixty (60) samples of locally fermented milk collected, a total of seventy (70) *Lactobacillus* were isolated, Yeast fourty-seven (47). Samples from Yaba bus stop yielded the highest rate of *Lactobacillus* isolates while samples from Biney yielded the least rate of *Lactobacillus* isolates. Of these seventy *Lactobacillus* isolates found, twelve species of *Lactobacillus* were recorded according to the number of their repetition. The *Lactobacillus* isolated were *L. casei, L. rhamnosus, L. acidophilus, L. plantarum, L. helviticus, L. brevis, L. reuteri, L.delbrueckii, L. fermentum, L. lactis, L. cellobiosus, L. bulgaricus.*

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Table 1: Frequency distribution of *Lactobacillus* isolates and Yeast at different locations.

Location	No. of Samples	No. of *Lactobacillus* Isolates screened	No. of Yeast found
Montgometry	13	14	10
Biney	5	6	5
Ojuelegba Rd.	7	9	5
Railway	6	7	6
Empire	7	10	5
Yaba Bus Stop	22	24	16
	60	**70**	**47**

Table 2 shows total *L.acidophilus* count of the samples. Further analysis revealed *L.acidophilus* to elicit highest total count of 421.2 x 10^6 cfu/ml being the most available in all the locations with high probiotic value unlike *L. reuteri*, which has the lowest total count of 2.5 x 10^6 cfu/ml. *L. reuteri* and *L. lactis* did not elicit probiotic level. Similar organisms were also isolated from locally processed "nunu" [15].

Table 2: Total *Lactobacillus* count.

Lactobacillus Strains	Locations						
	Ojuelegba	**Biney**	**Yaba Bus stop**	**Montgometry**	**Railway**	**Empire**	**Total**
L. brevis	2.5	0	19.8	4	0	6	32.3
L. delbrueckii	0	0	33	2	0	0	35
L. cellobiosus	5.8	0	13	5	3	0	26.8
L. plantarum	4.2	12.8	51.9	14	0	13	95.9
L.fermentum	3.7	5.2	70.2	6	0	0.2	85.3
L. acidophilus	97.8	26	192.5	80.1	1.2	23.6	421.2
L. casei	2.7	14.8	52.9	40.1	1.5	4	116
L. helviticus	0	0	2	0	3	5	10
L. rhamnosus	0	2.9	2	0	0	7.3	12.2
L. lactis	0	0	8	0	0	0	8
L.reuteri	0	0	2	0.5	0	0	2.5
L. bulgaricus	0	0	31	14	0	0	45
TLC	126.7	61.7	487.1	165.7	8.7	59.1	909

Key: TLC= Total *Lactobacillus* count.

Table 3 shows the species distribution of the *Lactobacillus* isolates with *L. acidophilus* occurring the most (14) at a percentage of 20. *L. brevis*, *L. helviticus* and *L. reuteri* has the lowest number of occurrence (2) with a percentage of 2.86 respectively. *L. acidophilus* and *L. delbrueckii* subs *bulgaricus* have been found to be among the predominant species involved in *nunu* production [16]. *Latococci*, *lactobacilli*, other predominant LAB and yeast species were also found in nyarmie, a Ghanaian fermented milk product [17].

Table 3: Species distribution of the *Lactobacillus* isolates.

Lactobacillus Isolates	No. of occurrence	Percentage (%) approx.	Lactobacillus Isolates	No. of occurrence	Percentage (%) approx.
L. brevis	2	2.86	***L. casei***	7	10
L. delbrueckii	4	5.71	***L. helviticus***	2	2.86
L. cellobiosus	7	10	***L. rhamnosus***	5	7.14
L.plantarum	10	14.26	***L. lactis***	3	4.29
L. fermentum	6	8.57	***L. reuteri***	2	2.86
L. acidophilus	14	20	***L. bulgaricus***	8	11.43
Total no. of occurrence =70, Total percentage (%) approx. = 99.98.					

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Table 4 shows the preliminary identification of *lactobacillus* isolates obtained from nunu samples.

Table 4: Preliminary identification of the *Lactobacillus* isolates.

Test	*L. brevis*	*L. debrueckii*	*L. cellobiosus*	*L. plantarum*	*L. fermentum*	*L. Acidophilus*	*L. casei*	*L. helveticus*	*L. rhamnosus*	*L. lactis*	*L. reuteri*	*L. bulgaricus*
Growth in MRS												
15°C	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
30°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
37°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
45°C	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve
Production of CO_2 from glucose (1%) in MRS broth												
Arginine hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Esculin hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Sugar fermentation												
Sucrose	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
Maltose	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
Mannose	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
Sorbitol	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fructose	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl red	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Voges proskeuar	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Growth in 10% skimmed milk	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Growth in skimmed milk indicates all the isolated *Lactobacillus* isolates can be used to produce fermented milk locally.

IV. PLASMID PROFILE

The plasmid profiles of twelve (12) strains are shown in Fig. 1 and Fig. 2. Plasmids ranging in size from 2-30.1kb were detected in all the examined strains, with the number of plasmids observed in each samples in which *L. casei* 027, *L. rhamnosus* 055 and *L. plantarum* 060 strains were observed with maximum number of plasmids while *L. helviticus* 011, 032 and *L. brevis* 017 strains had no plasmid.

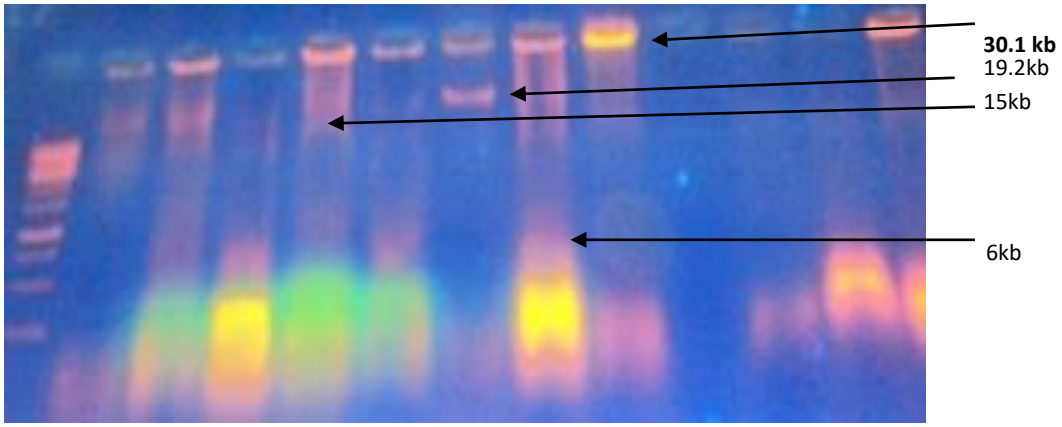

Fig 1: Plasmid profiles of lactobacillus isolates recovered from the fermented milk samples.

Lane 1 = 1`0 kb DNA markers; Lane 2 - 5 = *Lactobacillus acidophilus*; Lanes 6 – 8 = *L. casei*; Lanes 9 = *L. reuteri*; Lanes 10 - 11 = *L. helveticus*; Lane 12 = *L. delbrueckii*.

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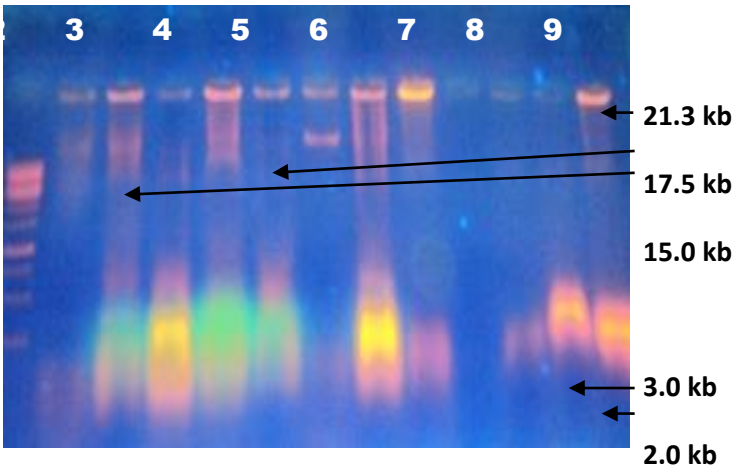

Fig. 2: Plasmid profiles of *Lactobacillus* isolates recovered from the fermented milk samples.

Lane 1 = 1`0 kb DNA markers; Lane 2&3 = *Lactobacillus carmosus*; Lanes 4 – 6 = *L. fermentum*; Lanes 7-9 = *L. plantarum*; Lanes 10 - 13 = *L. rhamnosus*

V. BACTERIOCIN ACTIVITY

The 12 isolates were examined for bacteriocin like activity against *E. coli* ATCC25922. Table 5 indicates that the supernatant of *L.acidophilus* possessed the highest inhibitory activity (12mm zone of inhibition) against *E. coli* while *L. rhamnosus, L. cellobiosus,* and *L. delbrueckii* showed no zone of inhibition. This result leads to the assumption that these 12 isolates except *L.rhamnosus, L.cellobiosus,* and *L.delbrueckii* are capable of producing different levels of bacteriocin compounds; however, this needs further substantiation.

Table 5: Bacteriocin production and growth inhibition of *E. coli* ATCC 25922 strain.

***Lactobacillus* isolate**	**No.**	**Bac+**	**Zone of inhibition, mm**								
L. brevis	4	1	8								
L debruecki	2	0	0								
L cellobios	7	0	0								
L. plantarum	12	2	7	10							
L. casei	5	2	6	8							
L. acidophilus	17	9	10	6	8	12	9	7	10	8	9
L helveticus	9	2	8	9							
L. rhamnosus	2	0	0								
L. lactis	6	2	10	8							
L. reuteri	1	1	8								
L. bulgaricus	5	1	8								
Total	**70**	**20**									

VI. INDICATOR STRAIN AND BACTERIOCIN ANTIMICROBIAL ACTIVITY

E. coli ATCC25922 was used as an indicator strain to test for bacteriocinogenic activity of the *Lactobacillus* isolates recovered from the locally fermented milk. A loopful of pure culture of each *lactobacillus* isolate was grown in MRS broth (5 ml) under anaerobic condition at 30^0C for 24 hrs. The overnight culture was centrifuged at 8000 rpm for 10 mins and the resulting supernatant was transferred into a new tube. This was followed by neutralization to pH 6.5 by the addition of NaOH (5N), and inhibition of hydrogen peroxide by the addition of bovine catalase (65 U/ml) and subsequent

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filteration by passage through 0.22 µm millipore filter. The filtered supernatant was used to soak 6 mm sterile filter paper (1 mm) disk by incubation at 40^{o}C for 2hrs. Molten agar was first seeded with indicator organism (110 µl of overnight culture per 20 ml of 1% agar) in sterile Petri dishes, and after solidification, dried for 15 min under flow hood. The bacteriocin soaked disks were then mounted into the indicator strain agar plate, followed by incubation at 37^{0}C for 24 hrs under anaerobic condition. Disks producing zone of inhibition were observed and measured to the nearest 1 mm. Positive antimicrobial activity was indicated by zone of inhibition ≥ 5mm.

VII. ANTIBIOTIC SUSCEPTIBILITY

Result of the sensitivity pattern of *Lactobacillus* isolates from locally fermented milk (Nunu) was also obtained as the minimum inhibitory concentration (MIC) of the twelve *Lactobacillus* isolates was tested with different antibiotics of different modes of action. The antibiotics used were: Ampiclox, Cefuroxcine, Amoxacillin, Rocephin, Ciprofloxin, Streptomycin, Cotrimazole, Erythromycin,Perfloxacin, and Gentamycin. Of the *Lactobacillus* isolates, *L. cellobiosus* shows the highest sensitivity while *L. fermentum* and *L. plantarum* exhibits the lowest sensitivity. Isolates showing MIC values higher than MIC breakpoint established by European Safety Authority (EFSA 2005), were resistant to Amoxacillin. All the isolates were sensitive to Perfloxacin except *L. rhamnosus* (5B2). Regarding the DNA interfering antibiotic (Ciprofloxacin), ten out of the twelve isolates were susceptible, only *L. plantarum* and *L. fermentum* were resistant. Six resistant patterns were also recorded. From this result it is difficult to judge whether resistance of probiotics to antibiotics is desirable or not depending upon what these probiotic formulations are used for.

Generally, the results obtained from the isolated organisms in nunu which include *Lactobacillus brevis L. casei, L. fermentum, L. acidophilus, L. reuteri, L.* and *bulgaricus, L.* agree with results of previous work [18]. Following laboratory screening of different *nunu* samples, seventy (70) was found to be Gram positive rods, catalase and oxidase negative, non-spore forming bacteria. These isolates were assigned as members of the genus *Lactobacillus*. With the use of API 50CH kits, API fermentation profile varied with the different species and this is in tandem with similar results reported by [19], [20]. All the isolates grew optimally at 30^{o}C and 37^{o}C. However, variations of growth at 15^{o}C and 45^{o}C were observed. *L. fermentum* and *L. cellobiosus* were able to grow at 45^{o}C but not at 15^{o}C. The same trend was observed as isolated *L.fermentum* which grew well at 45^{o}C grew poorly at 15^{o}C. The results of antibiotic susceptibility of the isolates studied by using ten antibiotics indicated resistance to Amoxacillin. Streptomycin resistance was also common except *L. delbrueckii* and *L. acidophilus*. This is also in harmony with a previous work which reported resistance of *Lactobacillus* to ciprofloxacin, gentamycin and other aminoglycosides antibiotics [21].

VIII. CONCLUSION

In this study lactic acid bacteria (LAB) were isolated from traditionally fermented nunu samples purchased from local producers within Lagos metropolis of Lagos state, Nigeria. The LAB were identified as belonging to the genus *Lactobacillus.* Twelve (12) strains of *Lactobacillus* were randomly selected from the samples and identified by standard morphological, physiological and biochemical methods. The isolates were identified as *L. casei, L. bulgaricus, L. acidophilus, L. plantarum, L. rhamnosus, L. thermophilus, L. reuteri, L. helviticus, L. delbrueckii, L .brevis. L. cellobiosus, L. fermentum* with *L. acidophilus* occurring the most and elicited the highest count of 421.2 x 10^{6} cfu/ml while *L. reuteri* has the lowest count of 2.5 x 10^{6} cfu/ml. This study would enable a process flow diagram for the traditional processing of *nunu* and other naturally fermented milk products to be developed. There are no standardized methods of processing *nunu* and this seems to result in a product of varying quality and stability. The possible sources of contaminating organisms associated with these products could be traced to the use of the old portion of previously fermented *nunu* as starter and the use of well and stream water for processing. The contaminating organisms could also be through rmicroflora which sticks to the smoothening stick, calabash spoons and bowls used for the sale of the products. Moreover, normal human flora of the customers could also serve as contaminants especially when one bowl is used for mixing the product for all customers without cleaning between use. Due to the poor hygienic practices observed during processing, the quality of the final product appears to be compromised. Locally fermented milk of probiotic value has bacteriocinogenic property against *E. coli* although carries resistant plasmid. Unfortunately, when it is consumed it can serve as drug resistance. Therefore, there is need to cure these *Lactobacillus* of resistant plasmid before they can be used as starter cultures. In addition, there is also need to standardize the cottage industry because most of the *Lactobacillus* in the industry are multi drug resistant and that is because of the plasmids they carry.

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