Scientific Research Publishing

Open Journal of Medical Microbiology, 2023, 13, 91-100
https://www.scirp.org/journal/ojmm
ISSN Online: 2165-3380
ISSN Print: 2165-3372

Detection, Biochemical and Molecular Characterization of *Clostridium sporogens* in *Nono*: A Nigerian Traditionally Fermented Yoghurt Drink

Nofisat Olaide Oyedokun[1*], Gbadebo Barnabas Olukotun[1], Uchenna Iman Igwegbe[2], Binta Buba Adamu[1], Roseline Rumi Ideh[1], Olaitan Comfort Shekoni[3], Amin Oderaa Igwegbe[4*]

[1]Department of Food and Industrial Biotechnology, National Biotechnology Development Agency, Musa Yar'adua Express Way, Abuja, Nigeria
[2]Department of Medical Microbiology, University of Maiduguri Teaching Hospital (UMTH), Maiduguri, Nigeria
[3]Department of Veterinary Medicine, College of Food, Agricultural and Environmental Science (CFAES), The Ohio State University, Colombos, USA
[4]Department of Food Science and Technology, University of Maiduguri, Maiduguri, Nigeria
Email: *nofisatoyedokun@gmail.com, *amanjide1964@yahoo.com

How to cite this paper: Oyedokun, N.O., Olukotun, G.B., Igwegbe, U.I., Adamu, B.B., Ideh, R.R., Shekoni, O.C. and Igwegbe, A.O. (2023) Detection, Biochemical and Molecular Characterization of *Clostridium sporogens* in *Nono*: A Nigerian Traditionally Fermented Yoghurt Drink. *Open Journal of Medical Microbiology*, **13**, 91-100.
https://doi.org/10.4236/ojmm.2023.131008

Received: November 21, 2022
Accepted: March 20, 2023
Published: March 23, 2023

Abstract

Nono is a traditionally fermented milk drink commonly consumed in the Northern parts of Nigeria. It is produced through the spontaneous fermentation of raw cow milk by Lactic Acid Bacteria (LAB), a process that could result to the contamination of the product with such pathogenic organisms as *Clostridia spp.* The aim of this research was therefore to determine the incidence of *Clostridia* species in thirty-two (32) ready-to-drink *nono* samples collected directly from a number of Fulani vendors in randomly selected locations within the Federal Capital Territory (FCT), Abuja, Nigeria. Isolated organisms were further subjected to some morphological and biochemical characterizations using standard microbiological procedures. The results obtained indicate that fourteen (14) isolates were putatively identified to be *Clostridium sp.,* out of which five (5) isolates were confirmed to be *Clostridium sporogens* by a BLAST analysis of their respective 16SrRNA nucleotide sequence. It was concluded that, the detection of these pathogenic strains in frequently consumed product like *nono* could pose a public health risk and proactive measures to prevent an outbreak of food borne illness from *nono* consumption, were recommended.

Keywords

Fermentation, *Nono*, *Clostridium sporogens*, Pathogenic, Food Borne Illness

1. Introduction

Foods which contain essential nutrients such as fats, proteins, carbohydrates, vitamins and minerals, are the most indispensible part of human life as well as microorganisms. They are the basic necessity without which the survivals of all living things are not feasible. Consequently, milk is an ideal medium for the growth of many organisms [1], having a high water content and abundant nutrients, and being nearly neutral pH (6.4 - 6.8) [2] [3]. It contains a plentiful of energy in the form of milk sugar or lactose, milk fat, citrate, and nitrogenous compounds (proteins, amino acids, ammonia, urea and other non-protein nitrogenous compounds) [3] [4] [5]. In addition, the electron potential (Eh) of milk is above 0.3 volts, thus enabling aerobic organisms to grow readily [5].

The relative ease with which milk can be converted into a wide variety of products makes it an extremely useful base material. In some cases, milk undergoes relatively limited processing, consisting of heat treatment to increase the bacterial shelf life of the product [6] and homogenization to increase the physical shelf life through retarding fat separation. Other well-known processes involve the acid-induced coagulation of milk to produce yoghurt, or the enzymatic coagulation of milk to manufacture cheese. In addition, milk may be spray-dried or be used as a base from which constituents, e.g. proteins or fats, are isolated [7]. As a result of the widespread applications and use of milk, and products derived there from, in human nutrition, it has been the subject of scientific study for over a century [2] [3] [4] [8] [9] [10].

Throughout the world, it has become established that fermented dairy products provide nutrients in the diet of both young and the elderly people. This occurs as a result of the fact that some of the microorganisms involved in the processing of these products are capable of producing several metabolites that not only add value in the form of taste, aroma, and firm consistency that are unique and attractive to the consumers during fermentation, but also have positive health implications [11] [12] [13] [14].

Consumption of local spontaneously fermented milk drinks has increased over time, due in part, to the dietary benefits and also the cheap cost of the products; the ingestion of which is considered to be the main cause of several outbreaks of diseases posing a major public health concern [15]. According to Anyanwu [14], locally fermented products offer a great alternative to mainstream food drinks. Raw cow milk forms the basis for most of the commonly sold local milk products in Nigeria and in order to extend the shelf life and add value, it is processed into various products like *Nono*, *Kindirmo*, *Mayin Shanu* and *Wara* [11] [15]. In order to prevent spoilage and disease transmission via these highly nutritious foods, milk should be adequately pasteurized [6] [12]. This is essential because at the level of processing of these products, they can be easily populated by a diverse array of both pathogenic and spoilage microorganisms, capable of imparting undesirable organoleptic and physicochemical characteristics that could be detrimental to health [13] [15] [16] [17]. Due to the spontaneous nature of the fermentation process associated with the dairy fer-

mentation, it is exposed to massive contamination regarding the presence of undesirable microorganisms in the final products. The contamination may be as a result of a cow's dirty exterior, the milking environment, and/or poorly cleaned equipment [18]. Especially remarkable is the incidence of spore-formers which are contaminants of public health significance in the food and dairy processing industries [7] [16] [19]. For instance, a case of food poisoning caused by *Clostridium perfringes* which resulted in the death of thirteen (13) individuals and ten (10) survivors was reported to have occurred in Saburi community, Gwagwa Ward of Abuja Municipal Area Council (AMAC), Nigeria in 2016 (Premium Times Online: https://www.premiumtimesng.com/regional/north-central/199091-mystery-deaths-abuja-community-caused-food-poisoning-health-board.html).

For these reasons, there is renewed interest in the possible presence of some *Clostridia* species in the raw milk and milk products, particularly traditionally manufactured products—including *nono*, in Nigeria. Isolation and molecular characterization of this group of microorganisms will offer knowledge of proper identification for effective control measures to be taken to ensure the wholesomeness and integrity of *nono* and other locally made dairy products. The main objective of this study therefore, was to determine the incidence of *clostridium* specie in *nono* (local yoghurt drink) vended in some parts of the FCT, Abuja, Nigeria.

2. Materials and Methods

2.1. The Study Area

Figure 1 shows the physical map of the Federal Capital Territory (FCT) Abuja, indicating the areas covered in this study.

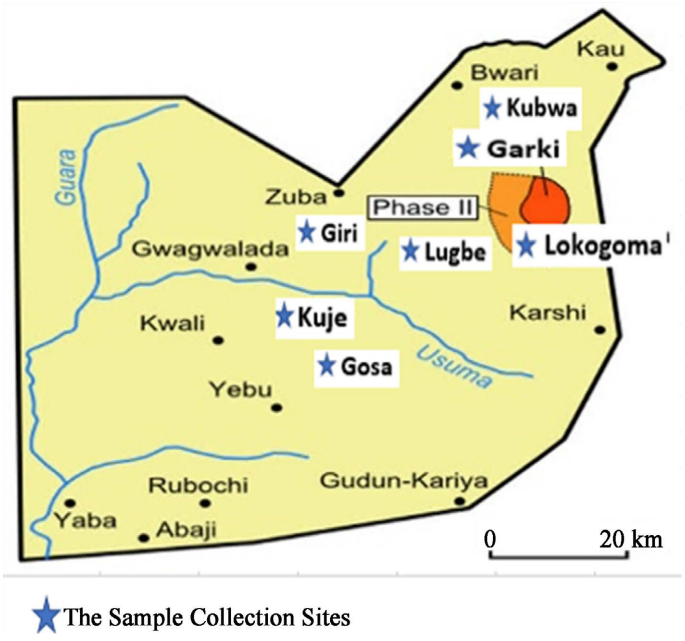

Figure 1. Physical map of the Federal Capital Territory (FCT) Abuja, indicating the areas covered in this study.

2.2. Collection of Samples

Thirty-two (32) samples of locally fermented cow milk, *nono* were randomly purchased in some markets within Abuja metropolis namely, Kuje (KJ03), Gosa (GS26A), Lokogoma (LK23), Giri (GR41), Lugbe (LG04), Garki (GK21) and Kubwa (KB06) respectively. The samples were collected in previously sterilzed capped bottles, labeled and transported to the microbiology laboratory of The Biotechnology Center, Abuja, for the analysis.

2.3. Isolation of Bacteria from the *Nono* Samples

The Isolation of *Clostridium* species was performed by serially diluting the *nono* samples up to 10^{-8} fold and inoculated on Reinforced *Clostridial* Agar media which is selective for *Clostridium* species. The plates were incubated anaerobically at 37˚C for 24 hours [20].

2.4. Identification of the *Clostridium* Isolates

Cultural characteristics of the isolates were identified on the basis of different colony characteristics including color, shape and size of colony on the plates. The isolates were equally identified on the basis of their morphological and biochemical characteristics as described in [17] [21]. The morphological characteristics like gram staining of isolated bacteria was carried out to determine the gram status while the different biochemical tests such as Methyl Red (MR) test, Voges-Proskauer (VP), Oxidase, Indole, Catalase, Motility, Starch hydrolysis, Esculin Hydrolysis, and Lipase tests were equally performed on the pure overnight-grown cultures as described Allameh *et al.* [22].

2.5. Molecular Confirmation of Isolates

2.5.1. Extraction of Bacterial DNA for 16S rRNA Gene Sequencing

The 16S rRNA gene sequencing is used as a tool to identify bacteria at the species level and assist in differentiating between closely related bacterial species [23]. It also enhances the analytical methods for detection and enumeration of C*lostridia* species. In this study, the process was carried out using the technique described by Kennedy *et al.* [24]. In this method, aliquots of 80 µl of NaOH (0.05 M) were added to 20 µl of bacterial cells suspended in distilled water and the mixture was incubated at 60˚C for 45 minutes, followed by the addition of 6 µl of Tris/HCl (pH 7.0), to achieve a final pH of 8.0. The resultant mixture was then diluted × 100 and 5 µl of the diluted extract was used for the Polymerase Chain Reaction (PCR).

2.5.2. PCR, Gel Electrophoresis, and 16S rRNA Gene Sequencing

The bacterial DNA extracts and control were amplified with 0.5 µM primers (LPW58, 5'-AGGCCCGGGAACGTATTCAC-3' and LPW81, 5'-TGGCGAAC GGGTGAGTAA-3'. After which, 10 µl aliquot of each amplified product was electrophoresed in 1.0% (wt/vol) agarose gel, with a molecular size marker (λ DNA AvaII digest; Boehringer Mannheim) in parallel. Electrophoresis in

Tris/borate/EDTA buffer was performed at 100 V for 1.5 hours. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 minutes, rinsed, and photographed under ultraviolet light illumination. The PCR products were gel purified using the QIAquick PCR purification kit and then sequences of the PCR products were compared with known 16S rRNA gene sequences in the GenBank (http://www.ncbi.nlm.nih.gov) by BLAST analysis on the NCBI [23].

3. Results and Discussions

Milk is usually sterile when secreted into the alveoli of the udder. Microbial contamination occurs mainly during and after milking processes. Vissers and Driehuis [18] have summarized the main sources of microorganisms occurring in milk and associated spoilage and safety issues in the dairy products. The summary is provided here in **Table 1** for more readerships. While the types of organisms present in raw milk and the products there from are influenced by many factors including temperatures and time of storage as well as the methods of handling during and after milking, the prevalence of these organisms vary considerably depending on geographical area, season, farm size, number of animals, on farm hygiene and farm management practices [5]. These factors, particularly the prevailing tropical temperatures, lack of adequate knowledge of milking, unhygienic handling and storage of raw and the processed milk products of the local milk producers, may also be regarded as potential factors for the proliferation of diverse forms of microorganisms in *nono* and other similar dairy

Table 1. Main sources of microorganisms occurring in milk and associated spoilage and safety issues in dairy products*.

Microbial Species	Associated Problem	Contamination source (Main Pathway[1])
Bacillus cereus (spores)	Spoilage of pasteurized dairy products	Environment[1] (feeds, faeces and soil), milking equipment
Bacillus sporothermadurans (spores)	Spoilage of UHT-treated dairy products	Environment (feeds and faeces)
Butyric acid bacteria (spores)	Spoilage of Gouda and Emmenthal cheeses	Environment (feeds and faeces)
Campylobacter jejuni	Food safety (products made of raw milk)	Environment (faeces)
Escherichia coli	Spoilage and food safety (products made of raw milk	Environment (faeces and deddind),
Listeria monocytogenes	Food safety (products made of raw milk and soft or surface ripened cheeses)	Environment[1] (faeces)
Mycobacterium paratuberculosis	Food safety (products made of raw milk)[2]	Environment[1] (faeces)
Pseudomonas spp.	Spoilage	Environment[1] (bedding, soil), milking equipment
Salmonella spp.	Food safety (products made of raw milk	Environment[1] (faeces)
Staphylococcus thermophilus	Spoilage	Environment[1] (faeces, bedding, soil), milking equipment
Staphylococcus aureus	Food safety (products made of raw milk	Interior of teats

[1]For species having the environment as the major source of contamination and are the main microbial carriers indicated between brackets; [2]Relevance for human health in unclear; * Vissers and Driehuis [18].

products in Nigeria.

The results of this present study indicate positive growth for *Clostridium* species on the Reinforced Clostridial Agar media (Table 2). Out of the 32 samples collected and analyzed, the percentage occurrence of the target bacteria was: KJ03 (27%), GS26A (0%), LK24 (33%), GR41 (0%), LG04 (21%), GK21 (14%) and KB06 (5%). The occurrence ranged from 0 (None) at locations GS26A and GR41 to the highest in the products obtained from LK24 (33%) which were therefore found to be highly contaminated with *Clostridium* species as compared to samples from the other locations investigated; and the difference was found to be statistically significant ($P < 0.05$).

Also, the results of the biochemical characterization of the identified *Clostridium* isolates with 16S ribosomal RNA (rRNA) gene sequencing showed that the species had a percentage nucleotide identity of >99. Given the present trend towards the love for natural products by consumers, there has been an increased preference for yogurts and fermented yogurt-like products, due to their several perceived and proven health benefits; *nono* is one of such fermented yogurt-like milk products that is gaining increasing acceptance by many consumers, particularly in Nigeria and other parts of West Africa. Due to the fact that *nono* is produced using either pasteurized or unpasteurized cow's milk, the lack of pasteurization is presumed to be one of the reasons for the proliferation of diverse forms of microorganisms in the finished product; in addition to the factors mentioned earlier in this section and in Table 1.

This study has adjudged the traditional yoghurt drinks (*nono*) sold by the vendors in the locations investigated, to be of very poor quality owing to the high incidence of *Clostridium sporogens* in the finished products. Taylor *et al.* [25], reported high level of contamination in a similar study on the conditions associated with *Clostridium sporogens* growth as a surrogate for *Clostridium botulinum* in non-thermally processed canned butter. In this study, five out of the seven locations investigated, had incidence of the spore-forming bacterial contaminants, which represents 71.4% of the study areas (Figure 2). The massive

Table 2. Incidence of *C. sporogens* in Selected *Nono* Samples from the Seven Locations.

Location (Code)*	Occurrence of Isolate (%)
KJ03	27
GS26A	-
LK24	33
GR41	-
LG04	21
GK21	14
KBO6	05

*KJO3 = Kuje03; GS26A = Gosa26A; LK24 = Lokogoma24; GR41 = Giri21; LG04 = Lugbe04; Gk21 = Garki; KB06 = Kubwa06.

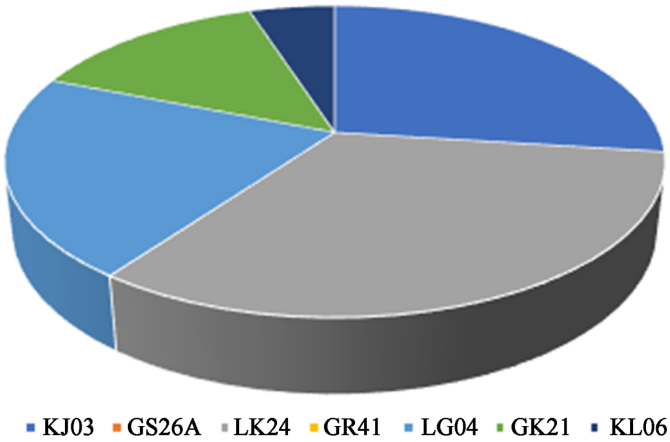

Figure 2. Pie-Chart Presentation of the occurrence of *Clostridium sporogens* in the studied locations.

occurrence of *Clostridium* species of this nature in milk and milk products is an indication of the potentially hazardous product which should be of food safety concern, due to the close relationship between *Clostridium sporogenes* and the dangerous and proteolytic strains of *Clostridium botulinum* [7].

This observed incidence of these health-threatening organisms in *nono* and other similar products could be due to either inadequate pasteurization (boiling) of the milk or the post-processing contamination of the products as also indicated by Eluchie *et al.* [26]. Other human microbial pathogens have been reported in raw milk and/or products made from it include *Listeria monocytogenes, Salmonella* spp. and *Campylobacter jejuni* [27]. In addition to their significance for public health, a very good microbial quality of raw milk is also important to prevent production losses and to achieve an optimal shelf life of dairy products.

The findings of this study have underscored the importance of adequate adherence to basic hygienic practice during and after processing of dairy products. There is also need for further studies on good manufacturing procedures in order to completely eliminate the occurrence of toxins produced by *Clostridium* species and other harmful microorganisms in the human food supply chain. The use of molecular tool for the identification of bacteria was confirmed in this work to be simple, comprehensive and reliable which is in line with the findings of Lim *et al.* [28] in a similar study.

4. Conclusion

Knowledge of the microbiology of raw milk and of milk after different heat treatments is essential for ensuring the safety and quality of milk and it's product at consumption. The preliminary handling, storage and treatment conditions of raw milk will have a major effect on the type of bacteria present and their effects on the final product. This study concludes that the naturally fermented milk products, *nono,* as presently vended in the sampled locations within and around Abuja Capital Territory (FCT), are obviously contaminated with the dangerous

strains of Clostridium species. The study recommends adequate sensitization of all those involved: the producers, vendors and consumers on the best milk handling and processing practices to prevent adverse health consequences.

Acknowledgements

The authors highly acknowledge the management of the National Biotechnology Development Agency, Abuja, and that of all the Institutions that provided the suitable research environments for this study.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Worku, T., Negera, E., Nurfeta, A. and Welearegay, H. (2012) Microbiological Quality and Safety of Raw Milk Collected from Borana Pastoral Community, Oroma Regional State. *African Journal of Food Science and Technology*, **3**, 213-222.

[2] Huppertz, T. and Kelly, A.L. (2009) Properties and Constituents of Cow's Milk. In: Tamime, A.Y., Ed., *Milk Processing and Quality Management*, Blackwell Publishing Limited, UK, 23-47. https://doi.org/10.1002/9781444301649.ch2

[3] Yusuf, H.L., Igwegbe, A.O. and Idakwo, P.Y. (2016) Comparison of Some Quality Characteristics of Milk from White Fulani Cow, Ouda Ewe, and Kano Brown Doe Reared under the Same Environment. *Agriculture and Food Sciences Research*, **3**, 19-24. https://doi.org/10.20448/journal.512/2016.3.1/512.1.19.24

[4] Hassan, A.N. and Frank, J.F. (2011) Microorganisms Associated with Milk. In: Roginski, H., Fuquay, J.W. and Fox, P.F., Eds., *Encyclopedia of Dairy Sciences*, 2nd Edition, Academic Press, London, 447-557.

[5] Touch, V. and Deeth, H.C. (2009) Microbiology of Raw and Market Milks. In: Tamime, A.Y., Ed., *Milk Processing and Quality Management*, Blackwell Publishing Limited, UK, 48-71. https://doi.org/10.1002/9781444301649.ch3

[6] Juffs, H.S. and Deeth, H.C. (2007) Scientific Evaluation of Pasteurization for Pathogen Reduction in Milk and Milk Products. Food Standards Australia New Zealand, Canberra.

[7] McHugh, A.J, Feehily, C., Hill, C. and Cotter, P.D. (2017) Detection and Enumeration of Spore-Forming Bacteria in Powdered Dairy Products. *Frontiers in Microbiology*, **8**, Article 105. https://doi.org/10.3389/fmicb.2017.00109

[8] Cook, G.M. and Sandeman, R.M. (2000) Sources and Characteristics of Spore-Forming Bacteria in Raw Milk. *Australian Journal of Dairy Technology*, **55**, 119-126.

[9] Chambers, J.V. (2002) The Microbiology of Raw Milk. In: Robinson, R.K., Ed., *Dairy Microbiology Handbook*, John Wiley & Sons, New York, 39-90. https://doi.org/10.1002/0471723959.ch2

[10] Kelly, A., Datta, N. and Deeth, H.C. (2005) Thermal Processing of Dairy Products. In: Sun, D.W., Ed., *Thermal Food Processing*: *New Technologies and Quality Issues*, Taylor & Francis Group, Oxfordshire, 265-298. https://doi.org/10.1201/9781420027372.ch9

[11] Adesokan, O.B., Ekanola, B.Y. and Avanrenren, R.E. (2011) Production of Nigerian

Nono Using Lactic Starter Cultures. *Pakistan Journal of Nutrition*, **10**, 203-207. https://doi.org/10.3923/pjn.2011.203.207

[12] Igwegbe, A.O., Maina, F.J., Kassum, A.L., Agbara, G.I., Chibuzo, E.C. and Badau, M.H. (2015) Evaluation and Acceptability of Yoghurt Drink Processed from Goat Milk and a Combination of Goat and Cow Milks. *International Journal of Biotechnology and Food Science*, **3**, 41-48.

[13] Agunwa, I.M. and Odimegwu, E.N. (2019) Microbiological Quality of a Locally Fermented Milk-Cereals Mixture (*fura de nunu*) Sold in Owerri Metropolis, Imo State, Nigeria. *Advances in Food Science and Engineering*, **3**, 214-421.

[14] Anyanwu, N. (2019) Microbiological and Comparative Analysis of Indigenous and Semi-Industrial Fermented Milk Drinks (Fura Da Nono and Fura Da Yoghurt) Sold in Nigeria's Capital. *International Journal of Bioassays*, **8**, 5716-5723.

[15] Omola, E.M., Kawo, A.H. and Bukar, A. (2019) Microbiological Quality of Traditionally Fermented Fresh Cow Milk (Nono) Retailed in Selected Local Government Areas of Kano State, Nigeria. *Journal of Microbiology Research*, **4**, 45-52.

[16] Edema, M.O. and Akingbade, A.A. (2007) Incidence of Spore-Forming Bacteria in Unsweetened Evaporated Milk Brands in Nigeria. *Nigerian Food Journal*, **25**, 137-144. https://doi.org/10.4314/nifoj.v25i1.33662

[17] Nduko, J.M., Joseph, W.M., Zacchaeus, O.N. and Moses, B.S. (2017) Spontaneously fermented Kenyan Milk Products: A Review of the Current State and Future Perspectives. *African Journal of Food Science*, **1**, 1-11. https://doi.org/10.5897/AJFS2016.1516

[18] Vissers, M.M.M and Driehuis, F. (2009) On-Farm Hygienic Milk Production. In: Tamime, A.Y., Ed., *Milk Processing and Quality Management*, Blackwell Publishing Limited, UK, 1-22. https://doi.org/10.1002/9781444301649.ch1

[19] Wanjala, G.W., Mathooko, F.M., Kutima, P.M. and Mathara, J.M. (2009) Milk Production. In: Tamime, A.Y., Ed., *Gateway to Dairy Production and Products*, Blackwell Publishing Limited, UK, 48-71.

[20] Wanjala, G.W., Mathooko, F.M., Kutima, P.M. and Mathara, J.M. (2017) Microbiological Quality and Safety of Raw and Pasteurized Milk Marketed in and Around Nairobi Region. *African Journal of Food, Agriculture, Nutrition and Development*, **17**, 11518-11532. https://doi.org/10.18697/ajfand.77.15320

[21] Barrow, G.I. and Felthman, R.K.A. (2003) Cowan and Steel's for the Identifiction of of Medical Bacteria. Cambridge University Press, Cambridge.

[22] Allameh, S.K., Daud, H., Yusoff, F.M., Saad, C.R. and Ideris, A. (2012) Isolation, Identification and Characterization of *Leuconostoc Mesenteroides* as a New Probiotic from Intestine of Snakehead Fish (Channa Striatus). *African Journal of Biotechnology*, **11**, 3810-3816. https://doi.org/10.5897/AJB11.1871

[23] Iliyasu, M.Y., Bamanga, R.A., Umar, A.F., Agbo, E.B. and Uba, A. (2019) 16S rDNA Sequencing Analysis in Identification of Some Multidrug Resistant (MDR) Bacterial Isolates from Clinical Specimens. *Nigerian Journal of Biotechnology*, **36**, 158-166. https://doi.org/10.4314/njb.v36i2.16

[24] Kennedy, K., Hall, M.W., Lynch, M.D., Moreno-Hagelsieb, G. and Neufeld, J.D. (2014) Evaluating Bias of Illumina-Based Bacterial 16S rRNA Gene Profiles. *Applied and Environmental Microbiology*, **80**, 5717-5722. https://doi.org/10.1128/AEM.01451-14

[25] Taylor, R.H., Dunn, M.L., Ogden, L.V., Jefferies, L.K., Eggett, D.L. and Steele, F.M. (2013) Conditions Associated with *Clostridium sporogenes* Growth as a Surrogate for *Clostridium botulinum* in Non-Thermally Processed Canned Butter. *Journal of*

Dairy Science, **96**, 2754-2764. https://doi.org/10.3168/jds.2012-6209

[26] Eluchie, C.N., Chukwu, M.N., Amandikwa, C., Umelo, M.C., Alagbaoso, S.O., Njoku, E.N., Agunwa, I.M. and Odimegwu, E.N. (2019) Microbiological Quality of a Locally Fermented Milk-Cereals Mixture (*fura de nunu*) Sold in Owerri Metropolis, Imo State, Nigeria. *Advances in Food Science and Engineering*, **3**, 5608-5613.

[27] Jayaroa, B.M. and Henning, D.R. (2001) Prevalence of Foodborne Pathogens in Bulk Tank Milk. *Journal of Dairy Science*, **84**, 2157-2162. https://doi.org/10.3168/jds.S0022-0302(01)74661-9

[28] Lim, Y., Totsika, M., Morrison, M. and Punyadeera, C. (2017) The Saliva Microbiome Profiles Are Minimally Affected by Collection Method or DNA Extraction Protocols. *Science Repository*, **7**, 8523-8530. https://doi.org/10.1038/s41598-017-07885-3