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Occurrence and Characterisation of Coagulase-Negative Staphylococci from Nigerian Traditional Fermented Foods

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Abstract
Coagulase-negative staphylococci (CoNS) were believed to be avirulent although recent studies have implicated them as causative agents of diseases. The lack of standardization and poor personal hygiene in the production of traditional fermented foods may introduce these organisms into food. This study was designed to investigate the occurrence of CoNS in Nigerian traditional fermented foods. In this study, the occurrence of CoNS in a total of 324 fermented food samples comprising of six traditional fermented food samples namely *ogi*, *iru*, *nono*, *wara*, *kindirmo*, *kunu* from North Central Nigeria was investigated. CoNS were isolated from the fermented food samples employing serial dilution technique and plating out on Mannitol salt agar (Oxoid, Germany). The identification of pure isolates was carried out using standard methods for phenotypic characterisation and genotypically characterised by PCR and 16S rRNA sequencing employing the primers; MSQ-F (5′- TGA AGA GTT TGA TCA TGG CTC AG-3′) and MSQ-R (5′-ACC GCG GCT GCT GGC AC-3′). A total of 255 (79.3%) CoNS were isolated and identified. The isolates were identified as *S. epidermidis*, *S. xylosus*, *S. simulans*, *S. kloosii* and *S. caprae* showing 90-95% homology with reference strains from NCBI database. *S. epidermidis* (50%) was the highest occurring CoNS species in the food samples examined. *S. epidermidis* (30.5%) was predominant in *iru*, *S. kloosii* (28.6%), *S. simulans* (32%) and *S. xylosus* (33.3%) were the highest occurring CoNS species in *kindirmo* and *S. caprae* (38.5%) was predominant in *nono* samples. This study established the occurrence of CoNS in Nigerian traditional fermented foods. Personal hygiene especially by food handlers and producers should be encouraged. The education on food safety should be taught to local traditional food producers and legal actions taken against non-compliance to food safety standards. Milk produced locally should be pasteurized or heat treated before use in food production.
Keywords: 16S rRNA sequencing, Characterization, Nigerian fermented foods, Coagulase-negative staphylococci, Occurrence, Food safety.

1. Introduction

Safe, nutritious foods are vital to human health and well-being, however, food-borne diseases pose a significant problem worldwide (Dilbaghi and Sharma, 2007). Fermented foods whether from plants or animals are an intricate part of the diet of people in all parts of the world. Each nation has its own types of fermented food, representing the staple food and the raw ingredients available in that particular place (Evans *et al*., 2013). In Nigeria, there are quite a number of fermented foods produced from raw materials such as cereals (*ogi, masa, burukutu, kunu, pito*); tubers (*gari, fufu, lafun*); fruits (*ogiri*); legumes (*iru* or *dcawadawa*, *ugba*, *okpehe*) and animal products such as milk and meat (*nono*, *wara*, *kindirmo*) (Wood, 1998).

Iru is prepared from the seeds of African locust beans (*Parkia biglobosa*) (Achi, 2005).In Nigeria and some other parts of the world, *ogi* is used as weaning food for infants and breakfast food for adults (Antai and Nzeribe, 1992). *Nono* is a Nigerian locally fermented milk product similar to sour yoghurt commonly prepared by nomadic fulanis (Yahuza, 2001). *Nono* is a fermented food drink gotten from defatted or skimmed cow milk. *Nono* is local uncontrolled fermented cow milk which forms a major part of the staple food in Northern Nigeria. The fresh milk is directly obtained from a cow into a properly washed semi-dried calabash and kept wide open in the sun for approximately two hours to facilitate isolation of the fat layer. Some quantity of overnight fermented milk is added to serve as starter culture and then is allowed to ferment for twenty four hours at room temperature. At the end of the fermentation period, the milk butter is removed by churning for further use and the remaining sour milk is the *nono* (Lawal *et al*., 2014). *Kindirmo* is a traditional, viscous full-fat fermented or partially skimmed cow milk product (Igwe and Yakubu, 2000). It is popular in Northern Nigeria and usually produced at the household level especially by women of the Fulani tribe herdsmen. It is prepared by boiling full fat milk; cooling and inoculation with starter culture (previously fermented or overnight left over *kindirmo*) after which it is left to ferment overnight. There are wide variations in the quality of the commercial finished products as a result of variation in the nature of the starter culture and fermentation conditions (Igwe *et al.,* 2014). *Kunu* is a non-alcoholic fermented beverage widely consumed in Northern part of Nigeria. It is consumed anytime of the day by both adult and children as breakfast drink, food supplement. It is a refreshing drink usually used to entertain visitors, appetizers and is commonly used / served at social gathering. The traditional process for the

manufacture of *kunu* involves the steeping of millet grains, wet milling with spices (ginger, cloves and pepper), wet sieving and partial gelatinization of the slurry, followed by the addition of sugar and bottling. The fermentation occurs briefly during steeping of the grains in water for 8 - 48 h period (Evans *et al*., 2013). *Iru* is one of the most important food condiments in Nigeria and many countries in West and Central Africa. It is used in much the same way bouillon cubes are used in the western world as nutritious flavouring additives along with cereal grains sauce and may serve as meat substitute. *Iru* is prepared from the seeds of African locust beans (*Parkia biglobosa*) (Achi, 2005). *Iru* is produced by a natural un-inoculated solid substrate fermentation of the boiled and dehulled cotyledon. Traditionally, fermentation of African locust beans involves boiling the beans for 12 h in excess water, until they are very soft to allow for hand dehulling after which the separated cotyledon is boiled for another 2 h to soften it. The cotyledon is then wrapped with enough banana leaves (*Musa saplentum*) and covered to ferment at room temperature (Evans *et al*., 2013). *Wara* is a white, soft, un-ripened cheese made by the addition of vegetable rennet made from a native plant extract (*Calotropis procera*) or pawpaw (*Carica papaya*) to the non-pasteurized whole milk from cattle which coagulates the milk (Adeyemi and Umar, 1994). It is consumed in several parts of West Africa. *Wara* processing involves the use of rudimentary equipments, in many cases, the starter culture used for processing are not normally standardized or optimized (Oladipo and Jadesimi, 2012). The soft wara cheese produced in Nigerian farms especially in the Northern part makes use of local ingredients. The number of microorganisms present at the time of milking has been reported to range between several hundreds and several thousand per milliliter (IDF, 1981). The West African soft cheese which is the typical type of cheese found in Nigeria has a shelf life of 2-3 days when immersed in the whey. The preparation of most traditional fermented foods and beverages remains a household art till today. The lack of standardization and quality control measures in the production of traditional foods present challenges regarding the safety of these foods (Evans *et al*., 2013). CoNS which are known to be commensals on the skin of man and animals could be introduced into food when food producers lack personal hygiene and there are no quality control checks during food processing or production. Coagulase- negative staphylococci were long considered non pathogenic and having few virulence factors however, this notion has been corrected as most studies have shown that just like the known pathogenic *S.aureus*, they also possess virulent factors and have been indicated as pathogens of diverse diseases (Akinkunmi and Lamikanra, 2012). CoNS have been recognized as etiologic agents of a wide variety of infections ranging from wound and urinary tract infection, neonatal sepsis, central nervous shunt and endocarditis. The incidence of CoNS in any food could therefore raise questions about its safety.

This study is designed to investigate the occurrence of CoNS in traditional fermented foods in Nigeria and to fully characterize them to species level using both phenotypic and genotypic methods.

2. Materials and Methods

2.1. Study area

The study area covered North Central Nigeria comprising of six states (Benue, Kogi, Nassarawa, Niger, Plateau and Kwara States) and FCT situated geographically in the middle belt region of the country.

2.2. Sample collection

A total of 324 fermented food samples namely *Nono* (54), *Iru* (54), *Kindirmo* (54), *Ogi* (54), *Wara* (54) and *Kunu* (54) were collected from traditional production sites using stratified random sampling technique in which the samples were purchased from nineteen (19) senatorial districts of the six states and FCT. The samples were transported in sterile containers placed in ice chest to the laboratory for immediate analysis.

2.3. Isolation of coagulase-negative staphylococci (CoNS) from fermented food samples

Serial dilutions of the traditional fermented food samples were prepared and 1ml of the aliquots of the fourth dilution was used to inoculate mannitol salt agar (MSA) plates in duplicates and incubated aerobically at 37ºC for 24 h.

2.4. Phenotypic characterisation of test isolates

The morphological characteristics of the isolates such as pigmentation, colony edge, elevation, shape, surface, consistency and opacity were examined. Gram staining, spore staining, catalase test, capsule test, coagulase test, oxidase test, indole test, novobiocin susceptibity test, gelatin hydrolysis, urease test and carbohydrate fermentation test were the biochemical tests used to further characterize the organisms. Distinct colonies that were Gram positive, cocci shaped occurring either in clusters or singly, coagulase negative and catalase positive were selected to be identified to species level using polymerase chain reaction (PCR) analysis and 16S rRNA sequencing.

2.5.Genotypic characterisation of test isolates

2.5.1. DNA extraction of test isolates

The presumptively identified CoNS were grown overnight in brain heart infusion broth, the cultured broth were centrifuged at 1000 rpm for 60 seconds and the supernatant discarded the pellets were then suspended in 500 µℓ of sterile distilled water and the DNA was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Qiagen, 2015).

2.5.2. Polymerase chain reaction (PCR) conditions

The method as described by Kim *et al*. (2008) was employed. Species specific PCR was carried out by targeting 500 bp amplicon of the 16S rRNA gene for CoNS species using the primers; MSQ-F (5'-TGA AGA GTT TGA TCA TGG CTC AG-3') and MSQ-F (5'-ACC GCG GCT GCT GGC AC-3'). The PCR mixture contained a total volume of 20 µℓ consisting of additional 0.1µℓ of AmpiTaq Gold (Bioneer, Daejeon, Korea), Master Mix consisting of PCR buffer and deoxynucleoside Triphosphate (dNTP), $MgCl_2$ Taq DNA polymerase) 4 µℓ primer concentration of 0.05 for each of the primers, 5 µℓ of template DNA and nuclease free water to make up the volume. The thermal cycling profile were as follows:

the PCR mixtures were denatured for 2 min at 94°C, and subjected to 30 cycles of amplification (94°C for 30 s, 55°C for 45 s, and 72°C for 60 s) with final extension for 10 min at 72°C. Each primer pair was added to a template and PCR premix (Bioneer, Daejeon, Korea). After PCR amplification, all products were resolved by electrophoresis on 2% agarose gel stained with ethidium bromide. All precautions to prevent carryover of amplified DNA were used. The positive control used was *S. epidermidis* (ATCC 43300) while the negative control used was *S. aureus* (ATCC 49230).

2.5.3. Visualization of PCR products in agarose gels and fragment analysis

The PCR product was electrophoresed by fragment analysis. The separated DNA fragments were visualized by staining the gel with ethidium bromide for 15 min and then de-stained in water for 15 min. The DNA bands were viewed by illumination with UV light and images recorded by transillumination photography.

2.5.4. Sequencing of PCR products

Both strands of the purified DNA from PCR were sequenced directly using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 3130 genetic analyzer (Applied Biosystems) at Inqaba Biotech, South Africa. The sequences were analyzed using CLC – bio Genomics Workbench software (Inqaba Biotech, South Africa). The sequences gotten were blasted using the BLASTn alignment software in order to determine the homology with sequences of reference strains in the GENBANK (Genome Database of the National Center for Biotechnology Information); www.ncbi.nlm.nih.gov.

3. Results

A total of 255 coagulase-negative staphylococci (CoNS) were isolated and identified using presumptive phenotypic and sugar fermentation tests to identify the strains to species level. Cultural plates that show typical small colonies with a characteristic whitish, creamy or yellowish pigmentation , entire edges, butyrid or dry consistency and a smooth to glistening surface were presumptively identified as CoNS. Gram positive, coagulase negative, catalase positive, indole negative was identified as CoNS. Their reaction to sugar utilization and novobiocin susceptibility differed and this was used to further screen and classify them into species level. The identities of the strains were further confirmed by molecular technique using PCR analysis (Plate 1) and 16S rRNA sequencing. Gene Bank published sequences of coagulase-negative staphylococci species revealed an alignment of 90% sequence similarity of isolate code KIL 2 to EU266748.1 *Staphylococcus xylosus* NY-5. IRUJ 3 to 1.37605.1 *Staphylococcus epidermidis* (90%), NOJ 4 to *S. simulans* AY0266056.1 (95%), however, IRUJ 4 showed 90% similarity to R_036906.1 *Staphylococcus simulans* MK 148; NOMA 6 revealed 92% similarity to DQ197962.1 *S. caprae,* KIM 5 was identical to KC849411.1 *S. simulans QTR-33* at 92% similarity. Isolate IRIL 5 was also identical to AY308046.1 STARGDI *S. epidermidis* and NOJ 4 sequence was identical to AY126233 *S. simulans* CM4. Then isolate code KUM 2 sequence revealed a 95% similarity to JQ660155.1 *S. kloosii* S4-437 and KIL 4 has a 90% similarity to JQ660231.1 *Staphylococcus kloosii* S7- 447 while IRUL 3 is 95% identical to JX646769.1 *S. kloosii* WD 16-1 (Fig. 1).

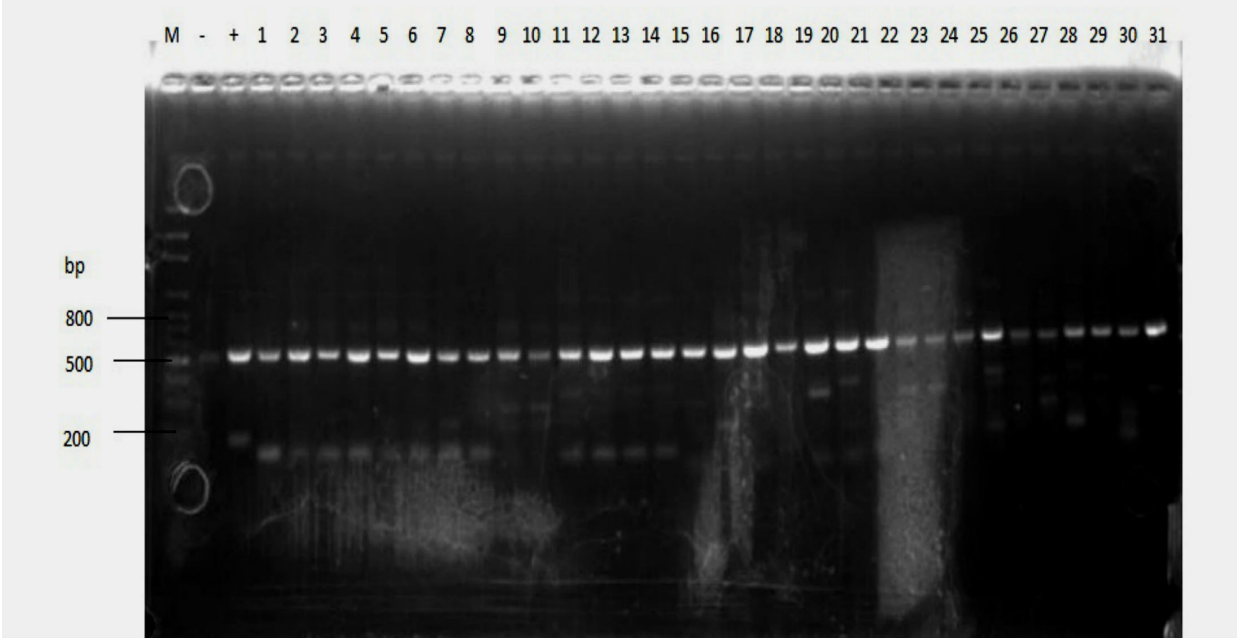

Plate 1: Amplification of the 16S rRNA gene. Lane 1 (M): O'GeneRuler 100 bp plus molecular weight marker (Thermo Scientific Fermentas™); Lane 2: *S. aureus* (negative control) ATCC 49230 (MRSA); lane 3: *S. epidermidis* (positive control) ATCC 43300; lane 4 - 31: positive isolates

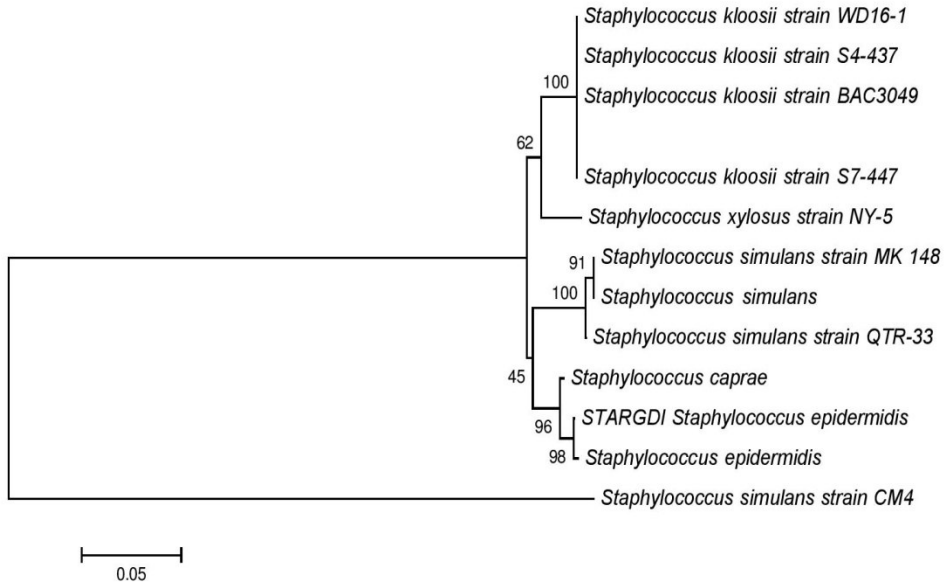

Fig. 1: Unrooted Neighbour - joining tree showing the phylogenetic relationships among CoNS Species based on a comparison of 500-bp 16S rRNA sequences.
The numbers are the estimated confidence levels expressed as percentages, for the the branching points when they are above 50% as determined by bootstrap analysis based on 1,000 replications. The scale bar evaluates the sequence divergence or evolutionary distance between sequences.
Key: Isolates KIL 2= *S. xylosus* NY-5, IRUJ 3= *S. epidermidis*, NOJ 4= *S. simulans* AYO26056.1, IRUJ 4= *S. simulans* MK 14-8, NOMA 6= *S. caprae*, KIM 5= *S. simulans* QTR-33, IRIL 5= STARGDI *S. epidermidis*, NOJ 4= *S. simulans* CM4, KUM 2= *S. kloosii* S4-437, KIL 4= *S. kloosii* S7-447, IRUL 3= *S. kloosii* WD-16-1, *S. kloosii* BAC 3040= outcast.

In this study, 79.3% of CoNS were incident in a total of 324 food samples of which 257 indicated the occurrence of CoNS. *Wara* (94.4%) and *kindirmo* (98%) had the highest occurrence of CoNS while *ogi* samples (33%) had the least occurrence (Table 1).

The occurrence of the CoNS species in the fermented food samples showed that *S. epidermidis* (30.5%)

was highest in *iru* samples; *S. kloosii* (28.6%), *S. simulans* (32%) and *S. xylosus* (33.3%) were predominant in *kindirmo* samples while *S. caprae* (38.5%) was highest in *nono* samples as shown in Table 2.

Table 1: Percentage Occurrence of CoNS in Food Samples.

Food Sample	Number of Sample Examined	Number of Positive Samples	Occurrence of CoNS in Sample (%)
Iru	54	49	90.7%
Ogi	54	18	33%
Nono	54	48	88.9%
Wara	54	51	94.4%
Kindirmo	54	55	98%
Kunu	54	36	66.7%
Total	324	257	79.3%

Table 2: Distribution of CoNS Species in Fermented Food Samples.

CoNS Species	Total	*Iru*	*Ogi*	*Nono*	*Kindirmo*	*Kunu*	*Wara*
S. epidermidis	128 (50%)	39 (31%)	22(17%)	10 (8%)	26 (10%)	21 (16%)	10(8%)
S. *kloosii*	28 (11%)	7 (25%)	0 (0%)	7 (25%)	8 (29%)	4 (14%)	2 (7%)
S.simulans	50 (20%)	10 (20%)	6 (12%)	11(22%)	16 (32%)	2 (4%)	5 (10%)
S.xylosus	36 (14%)	2 (6%)	0 (0%)	11(30.5%)	12 (33%)	0 (0%)	11(30.5%)
S.caprae	13 (5%)	0 (0%)	0 (0%)	5 (39%)	4 (30.5%)	0 (0%)	4 (30.5%)

Discussion

Coagulase-negative staphylococci have been previously dismissed as contaminants but recent studies have shown that they cause quite a number of diseases such as nosocomial infections, endocarditis, central nervous system shunt disease, neonatal infection and a few reported cases of food poisoning caused by their enterotoxins [17].

16S rRNA sequencing identified five species namely *S. epidermidis*, *S. kloosi*, *S. xylosus*, *S. simulans* and *S. caprae*. The polymerase chain reaction (PCR) method based on 16S rRNA gene for the detection and identification of pathogenic bacteria in food present a sensitive and rapid method. The use of 16S rRNA gene sequence to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker because of its presence in almost all bacteria often existing as a multigene family or operon, the function of the 16S rRNA gene has not changed over time and the 16S rRNA gene (1500bp) is large enough for informatics purposes and thus it has proven to be a reliable and golden molecular method for the identification of *Staphylococcus* species [18].

The result obtained in this study showed a high incidence of CoNS in *iru*, *nono*, *wara* and *kindirmo* samples. Their presence in indigenous milk products may have been directly introduced into the food by food handlers during handling or preparation since the organisms are commensals on the human skin or may be during milking from cow, since a lot of CoNS species have been isolated from cows suffering from mastitis (a disease condition in cows characterized by enlargement of the breast caused by *Staphylococcus* species thus the introduction of the organism into the milk while milking may have occurred. In the Northern part of Nigeria, consumption of locally processed raw milk is preferred to pasteurized milk because it is believed that locally processed raw milk and its by-products have nutritional advantages over the pasteurized one [8]. However, consumption of raw milk and its by-products is considered potentially hazardous and has been associated with several types of infections including brucellosis, tuberculosis, salmonellosis, yersiniosis, *Escherichia coli* O157 and staphylococcal enterotoxin poisoning [8]. Milk gotten from cows in Nigeria by the Fulanis and used for local cheese (*wara*) and yoghurt (*nono* and *kindirmo*) are not pasteurized, they are used directly and if the lactic acid bacteria does not grow equivalently as the pathogen present in the milk, it may not be able to eliminate them before they illicit their toxins into the food [8]. Sneezing or coughing represents other means through which these organisms may be disseminated in these foods [19]. In Nigeria, the occurrence of CoNS in traditional fermented foods have not been reported indepthly however, this study has established their occurrence in these foods.

Their occurrence may be from human handlers during preparation or as a result of exposure during display for sale. Their occurrence in Nigerian traditional fermented foods may be attributed to the poor hygiene of food producers caused by inadequate knowledge as to the implication of lack of hygienic practices during

food preparation in relation to public health.

Conclusion

This study has established the occurrence of CoNS in Nigerian fermented foods such as *kindirmo, nono, wara, iru, ogi and iru*. The occurrence of CoNS in food in the North Central Nigeria calls for public awareness as to the incidence of these organisms in food and their public health significance. The need for personal hygiene especially by food handlers and producers should be emphasized. Milk and milk products should be pasteurized before its use in food production.

Conflict of Interest

The authors declare no conflict of interests.

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