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Molecular Characterization of Bacteria Associated with the Spontaneous Fermentation of Bambara Nut in "Ogirri"

*B.A. Omogbai and A.A. Aghahowa

[1]Department of Microbiology, University of Benin, Faculty of Life Sciences, University of Benin, Benin City
[1]Medical Department, National Biotechnology Development Agency, Abuja, Nigeria

*Corresponding author E-mail: barryomogbai@yahoo.com

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ABSTRACT: Fermentation is one of the oldest methods of food preservation known to man. Oil seeds and legumes have been fermented to give condiments. Molecular characterization of the bacteria associated with the spontaneous fermentation of Bambara nut (*Vigna subterranea*) seeds into "Ogirri" was carried out. *Vigna subterranea* seeds were subjected to pre-treatments of soaking and boiling and spontaneously fermented in 3 setups for a period of 96 h at 28±2 ^{0}C to yield ogirri product. The isolates were identified using cultural and morphological methods, and the identities of the bacterial isolates were confirmed using molecular characterization techniques. The total microbial count showed an increase from 2.0 x 10^3 cfu/g at 0 h to 9.50 x 10^4 cfu/g at 96 h for the bacterial isolates while the fungal isolate increased from 1.30 x 10^4 cfu/g at 24 h to 6.30 x 10^4 cfu/g at 96 h. The identities of four out of five bacterial isolates were successfully confirmed as *Bacillus cereus* strain BTD4-2, *Bacillus cereus* strain GBPS9, *Phyllobacterium leguminum*strain ORS1419 and *Lysinibacillus fusiformis* strain LMG 18474. The confirmation of the identities of the isolates from the spontaneous fermentation process showed that the product was predominantly fermented by species of the genera *Bacillus*. These organisms play a key role in the flavour development and influence the chemical composition of the fermented food through substrate modification.

Keywords: Genotypic, Bambara nut, Processing, Ogirri, Bacteria

Introduction

Bambara groundnut is a pulse which is related to cowpeas. It is botanically known as *Voandzeia subterranea* (L.) thesaurus, synonyms of *Vigna subterranea*. The crop belongs to the kingdom Plantae of the family *Fabeacea* and sub family of *Faboidea* and it is a legume species of African origin (Borget, 1992) with a subterranean fruit set which is widespread south of the Sahara (Ocran *et al*., 1998). It is cultivated by small holders over much of semi arid Africa and it serves as an important source of protein in the diets of a large percentage of the population in Africa, particularly to poorer people who cannot afford expensive animal protein (Linnemann, 1991).

The bambara groundnut has various names, each language and dialect has its own variation. The nuts are known as 'jugo' beans (South Africa), 'ntoyo ciBemba' or 'Katoyo' (Republic of Zambia), Congo groundnut (Congo), Nzama (Malawi), Nyimo beans (Zimbabwe) (Okonkwo and Okpara, 2010). Nigeria is one of the main producers of the crop and it is locally called 'Okpa' (Ibo), 'Epariro' (Yoruba), 'Gurjiya' or 'Kwaruru' (Hausa). It originated in the Sahelian region of present day West Africa, from the Bambara tribe near Timbuktu who now live in central Mali, hence its name Bambara groundnut (Nwanna *et al*., 2005).

The crop is indigenous to West Africa where it has a long history of cultivation although there is now limited production in parts of Asia and South Central America (Gibbon and Pain, 1985). Bambara groundnut is a hardy

plant, particularly well suited to the growing conditions found in the Savanna regions with a Sudanese and Sudano-Guinean climate. The crop is found wild in West Africa. It has been cultivated throughout tropical Africa for many centuries. It was taken at an early date to Madagascar, probably by Arabs. It reached Brazil and Surinam early in the seventeenth century and was later taken to the Philippines and Indonesia (Purseglove, 1992).

In West Africa, Bambara groundnut was for a long time at par with, or slightly ahead of cowpea (*Vigna unguiculata*) in terms of production, market availability and utilization. In Ghana, over 40,000 cans (various sizes) of Bambara groundnut were produced annually throughout the 1960's and early 1970's. The canned product was very popular throughout West Africa and competed favourably with Heinz baked beans. The status of the nut however began to decline from 1970's with the introduction of high yielding varieties of groundnut and pest control methods for cowpea (Doku, 1996).

Bambara groundnut is probably the most drought-resistant of the grain legumes and may be found growing successfully where annual rainfall is below 500 mm and optimum between 900-1000 mm per year (Ocran *et al*., 1998). The plant can be grown under dry climatic conditions where the rainfall during the rainy season would be adequate to enable them complete their vegetative cycle (Borget, 1992).

The protein of Bambara groundnut is of good quality and has surplus lysine which complements cereals in the diet (Ocran *et al*., 1998). The composition of the seeds from the point of view for human nutrition is very well balanced, as they contain 20% soluble carbohydrate and 8% fat. It is high in protein but unlike ordinary groundnuts, contains very little oil (Tweneboah, 2000).

Bambara groundnut has been ranked as the third most important grain legume, after groundnut (*Arachis hypogeae*) and cowpea (*Vigna unguiculata*) in Africa but due to its low status it is seen as a snack or food supplement but not a lucrative cash crop (Linnemann, 1992). Additionally, it is usually given less value and less priority in land allocation because it is grown by women. Between 10% - 40% of the harvest is sold and the rest is consumed by the rural farmers themselves. Bambara groundnut seeds vary in size, shape and colour of the seed coat. They may be round or elliptical in shape with cream, brown, red, mottled or black eyed with seed weight ranging between 280 and 320 g (Ojimelukwe, 1992). The crop has been widely cultivated in tropical region since the 17th century. In addition to Sub- Saharan Africa, it is now found in many parts of South America, Asia and Oceania. It can produce high yield levels with low input and it is an ideal crop for farmers. It was found that about 98% of farmers in Swaziland regard Bambara groundnut as a profitable crop (Sesay *et al*., 1999; Begemann *et al*., 2002).

Bambara groundnut is a promising commodity which needs more publicity both as a crop and as a food. The annual production is about 330,000 tons of which Africa produces half, with Nigeria being the major producing country. The yields are low because production and improvement of Bambara groundnut has been neglected for many years by researchers, even though the crop is important for small scale farmers due to its considerable commercial potential. Though grown extensively in Nigeria, it is still one of the lesser utilized and unexploited legume (Enwere, 1998).

Fermented foods are processed through the activities of microorganisms but in which the weight of the microorganisms in the food is actually small. The influence of microbial activity on the nature of food, especially in terms of flavour and other organoleptic properties is profound (Okafor, 2007). Some indigenous fermented foods in Nigeria include ogi', 'ogirri', 'dawadawa' and 'iru' (Steinkraus, 1995).

'Ogirri' is a flavouring made from fermented oil seeds such as sesame, melon and castor oil seeds (Oluwabukola *et al*., 2012). Ojinaka *et al*. (2013) evaluated the changes of enzyme activities during the fermentation of castor oil seed into ogirri and found the presence of amylase, proteinase and lipases. Ogirri serves as a cheap soup condiment particularly among the poor and rural dwellers (Odibo *et al*., 1990). Previous studies (David and Aderibigbe, 2010) have shown the microbiology of ogirri samples and identified organisms such as *Bacillus, Leuconostoc, Streptococcus, Pediococcus* and *Lactobacillus* based on physiological, cultural, morphological characteristics of the isolates. The condiments: iru and ogirri-isi were studied for microbiological components by Ibeabuchi *et al.* (2014). Reports from these authors indicated the presence of *Bacillus, Enterobacter faecalis, Staphylococcus aureus, Saccharomyces* spp as notable fermenter of locust bean iru and castor oil bean seed. David *et al.* (2016) evaluated the probiotic properties of *Bacillus* spp isolated from fermented locust bean (iru) and found them to be resistant to first line antibiotics. The presumptive results of the microbiological evaluation of ogirri by Peter-Ikechukwu *et al*. (2016) showed that the predominant bacteria were *Bacillus* spp, *Enterococcus and Corynebacteria* species.

Molecular approaches are now employed to characterize the nucleic acids of microorganisms isolated from samples (Li *et al.,* 2009). Genotyping is the process of determining the genetic constitution of an organism by examining the DNA sequence. Various genetic methods have been developed for genotyping bacteria since the late 1980s. These methods have become frequently used in bacterial identification due to their high resolution. The identified genetic profile of any bacteria by a specific genotyping method can be as unique as fingerprint (Li *et al*., 2009). The aim of

this study was to carry out the molecular characterization of bacterial isolates from the spontaneous fermentation process of Bambara groundnut into ogirri.

Materials and methods

Sample collection: Cream coloured seeds of *Vigna subterranea* (Bambara groundnut) were purchased from New Benin market, Benin City (6° 20' 21.0660" N, 5° 37' 2.8092" E). The healthy seeds were selected, stored inside a clean nylon bag and refrigerated at 4±1 °C until needed for use.

Preparation of sample: 120 g of *Vigna subterranea* seeds was weighed using a weighing balance (Model Search Tech KLS 10001, Germany). A stainless steel vessel was weighed empty and weighed again on filling with the Bambara nut. The nuts were shared into 3 portions of 40 g each, rinsed, and then soaked in 500 ml of distilled water each for 18 h (cold soaking) as described by Fadahunsi and Olubunmi, (2009). The water was decanted and the nuts were transferred into a hot plate pan containing 400 ml distilled water. The nuts were boiled using a hot plate (Model, Stuart SB160, Germany) for 90 min until soft. The boiled nuts were sieved, allowed to cool for 15 min and then transferred to a sterile mortar and gently mashed to a pulp.

Spontaneous fermentation: The mashed nuts was rolled into 3 balls and each wrapped in banana leaves which were prewashed in running tap water, and sterilized with 70 % ethanol. The setups were labeled SF_1, SF_2 and SF_3 denoting fermentation set up 1 - 3. This was placed in an air-tight container and covered with a few sheets of banana leaves. The setups were then kept on a surface sterilized laboratory bench to ferment naturally at room temperature (28±2 °C) for 96 h.

Isolation of microorganisms: Sampling was carried out at 24 h interval. 1 g of the fermenting Bambara nut mash was aseptically transferred into a test tube containing 9 ml of sterile water and shaken vigorously to dislodge the associated microorganisms, making a stock suspension. The 10^{-1} suspension was subsequently serially diluted using ten-fold serial dilution up to 10^{-10}. 1 ml aliquot of various dilutions were added to sterile petri dishes (duplicate for each dilution) to which approximately 15 mL of sterile cool molten (45 °C) media was added. Enumeration of bacteria and fungi were carried out using nutrient agar supplemented with nystatin and potato dextrose agar supplemented 0.6 mL chloramphenicol. The plates were swirled to mix and the agar was allowed to solidify. Upon solidification, the plates were incubated in an inverted position. Nutrient agar plates were incubated for 24 h while the potato dextrose agar plates were incubated at room temperature (28±2 °C) for 72 h to 96 h.

Enumeration of microorganisms: Discrete colonies on the nutrient agar and potato dextrose agar were selected and counted. The mean colony count on the nutrient agar and potato dextrose plates of each given dilution was used to estimate the total viable count for the samples in colony forming units per gram (cfu/g) (Holt *et al*., 2000).

Sub-culturing of bacterial isolates: A single isolated colony of the bacteria was picked up with the help of sterilized wire loop and was streaked on fresh nutrient agar medium. The nutrient agar plates were incubated at 28±2 ºC for 24 h. The isolated and purified bacterial strains were stored under refrigeration in slants.

Characterization and Identification of bacterial Isolates: Cultural characteristics of the bacterial isolates were observed on nutrient agar plates. The cultural characteristics include; colony size, shape, surface appearance, opacity, texture, elevation and pigmentation. These were determined by visual observation. Morphological and biochemical tests for the bacterial isolates were carried out according to the method of Cheesbrough (2000); Holt *et al.,* (2000).

Characterization/Identification of fungal isolates: A drop of lactophenol blue stain was dropped on a clean grease-free sterilized glass slide and a sterile inoculating wire loop was used to pick the mycelium unto the glass slide from the mold culture. The mycelium was spread evenly on the slide. Teasing was carried out to separate the mycelium in order to get a homogenous mixture and the mixture was then covered with cover slips gently and then allowed to stay for some seconds before observing under the microscope using the ×40 objective. The microscopic examination of actively growing mold was on the basis of structures bearing spores, presence or absence of septa on hyphae (Barnett and Hunter, 1998).

Molecular techniques: Five bacterial isolates were selected for molecular analysis (Polymerase chain reaction and DNA sequencing) which was carried out at the Nigerian Institute of Medical Research (NIMR), Lagos, Nigeria.

(a) *Chromosomal DNA extraction*: DNA extraction was carried out directly from the samples by boiling as follows: 1.5 mL of the organisms in broth was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the pellets were washed twice with sterile distilled water. This was followed by addition of 200 µl of sterile water to the pellets, the pellets were vortexed to homogenize and boiled in a dry bath at 100 °C for 10 min. The boiling mixture was vortexed and centrifuged at 12,000rpm for 5minutes. The supernatant

containing the DNA was transferred to another tube and stored at -20 °C. The concentration and purity of the extracted DNA was estimated using a Nanodrop spectrophotometer (ND 1000 Thermoscientific, USA)

(b) *PCR amplification of bacterial 16srRNA PCR gene*: The PCR amplification of the 16S rRNA gene was carried out using the primer set 27F-5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. The reaction was carried out in a 25µl reaction mixture containing 1XPCR buffer (Solis Biodyne), 1.5mM magnesium chloride (Solis Biodyne), 0.2mM of each dNTP (Solis Biodyne), 2 U Taq DNA polymerase (Solis Biodyne), 20 pMol of each primer and sterile water was used to make up the reaction mixture. PCR was carried out in an Eppendorf Nexus thermal cycler with the following cycling parameters; an initial denaturation step at 95 °C for 5 min, followed by 30 consecutive cycles of denaturation at 95 °C for 1min, annealing at 55 °C for 1min and 72 °C for 2 min. After this, a final extension at 72 °C for 10 min was carried out.

After the PCR reaction, PCR products were separated on a 1.5 agarose gel. 100 bp DNA Ladder (Solis Biodyne) was used as DNA molecular weight marker. Electrophoresis was done at 80V for 90 min. DNA bands were visualized by ethidium bromide staining using a UV trans-illuminator (model UVP M-10E , Fisher Scientific, USA). An amplicon size of 1,500bp signifies amplification of the 16S rRNA gene.

(c) *DNA sequencing*: The DNA sequence of each isolate was determined using Sanger sequencing at GATC Biotech, Germany. The nucleotide sequence for each isolate was identified by blasting on the National Center for Biotechnology Information (NCBI) website.

Results

The total viable microbial count obtained during the spontaneous fermentation revealed that the bacterial count increased from 2.0 x 10^3 cfu/g at 0 h to 9.50 x 10^4 cfu/g at 96 h while the fungal count increased from 1.30 x 10^4 cfu/g at 24 h to 6.30 x 10^4 cfu/g at 96 h (Fig. 1).

During the spontaneous fermentation of Bambara nut seeds, five (5) different bacteria and three (3) fungi were isolated. The five bacteria isolates were tentatively identified as species belonging to the genera *Bacillus*, *Staphylococcus*, *Lactobacillus* and *Corynebacterium* (Table1). The fungal isolates were presumptively identified as species of the genera *Rhizopus*, *Aspergillus* and *Penicillium* (Table 2).

The PCR amplification of the 16S rRNA gene of each bacterial isolate revealed that four of the isolates showed amplification for the Universal primer set after the PCR products were separated using agarose gel electrophoresis (Fig.2). The DNA sequencing of each isolate was carried out and the identities of four of the organisms were successfully confirmed as *Bacillus cereus* strain BTD4-2, *Bacillus cereus* strain GBPS9*, Phyllobacterium leguminum*strain ORS1419 and *Lysinibacillus fusiformis* strain LMG 18474 showing homologies of 83 %, 78 %, 89 % and 78 % respectively (Table 3).

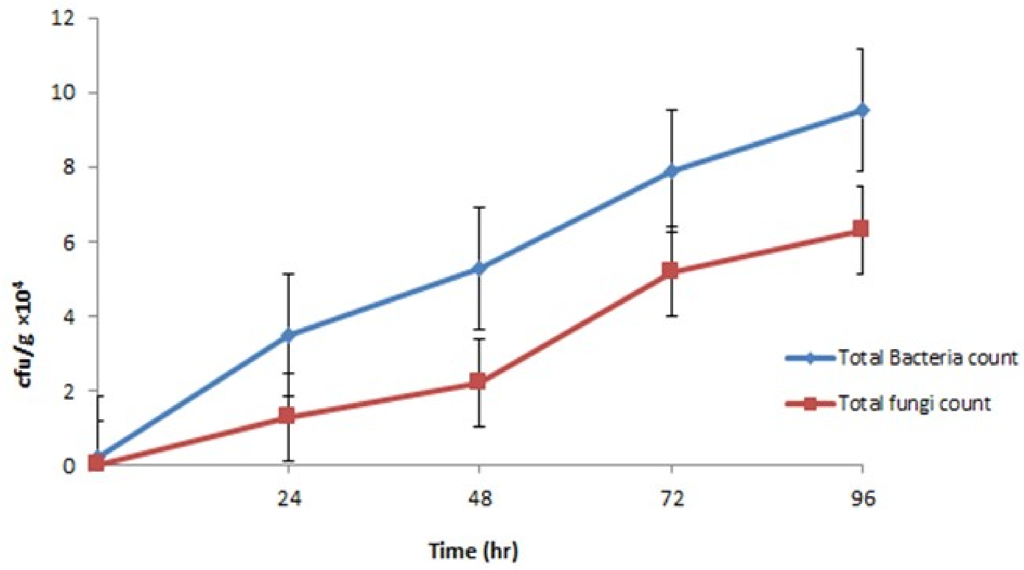

Fig. 1: Total viable microbial count during the spontaneous fermentation of bambara nut seeds.

Table 1: Characterization of Bacteria isolates from the fermented bambara nut seeds

	Isolates				
Cultural Characterization	**A**	**B**	**C**	**D**	**E**
Shape	Circular	Circular	Rhizoid	Irregular	Irregular
Colour	Milky	Milky	Milky	Milky	Yellow
Elevation	Flat	Raised	Raised	Flat	Raised
Margin nature	Entire	Crenate	Pointed	Lobate	Lobate
Wetness/dryness	Wet	Dry	Dry	Wet	Wet
Transparency	Translucent	Translucent	Opaque	Translucent	Opaque
Morphological Characterization					
Gram staining	+	+	+	+	+
Shape	Branched rod	Rod	Rod	Cocci	Bipolar Thick Rod
Arrangement	Chains	Pair	Single	Clusters	Pair
Biochemical Test					
Catalase	+	+	-	+	+
Oxidase	-	+	-	-	-
Indole	+	-	+	+	+
Spore forming	+	-	-	-	+
Citrate	+	+	+	+	+
Motility	+	+	+	+	+
Vogues Proskeur	+	-	+	+	+
Acid fast	-	-	-	-	-
Strict Anaerobe	-	-	-	-	-
Coagulase	-	-	-	+	-
Sugar Test/Starch Hydrolysis					
Lactose	+	+	+	+	+
Glucose	+	-	+	-	-
Starch	-	+	+	-	+
Mannitol	+	-	+	-	+
Arabinose/Acid	+	-	-	+	-
Isolates	*Bacillus* sp. (1)	*Corynebacterium* sp.	*Lactobacillus* sp.	*Staphylococcus* sp.	*Bacillus* sp.(2)

Table 2: Characterization of fungal isolates from the fermented Bambara nut seeds

	Isolate		
Cultural characteristics	**F_1**	**F_2**	**F_3**
Growth Form	Brown woolly with profuse growth	Black velvety with profuse growth	Greenish velvety with profuse growth
Colour of Reverse plate	Dirty Brown	Black	Greenish
Microscopy:			
Hyphae	Septate	Septate and broad	Septate
Conidiophore & Conidia	Conidiophores occur singly and in groups, multicellular crescent shaped macroconidia	Distinct conidiophores terminated by a swollen vesicle bearing flask-shaped phiallides	Conidiphore developed into branched phalide bearing chains of conidia, brush-like appearance
Isolate	*Rhizopus* sp.	*Aspergillus niger*.	*Penicillium* sp.

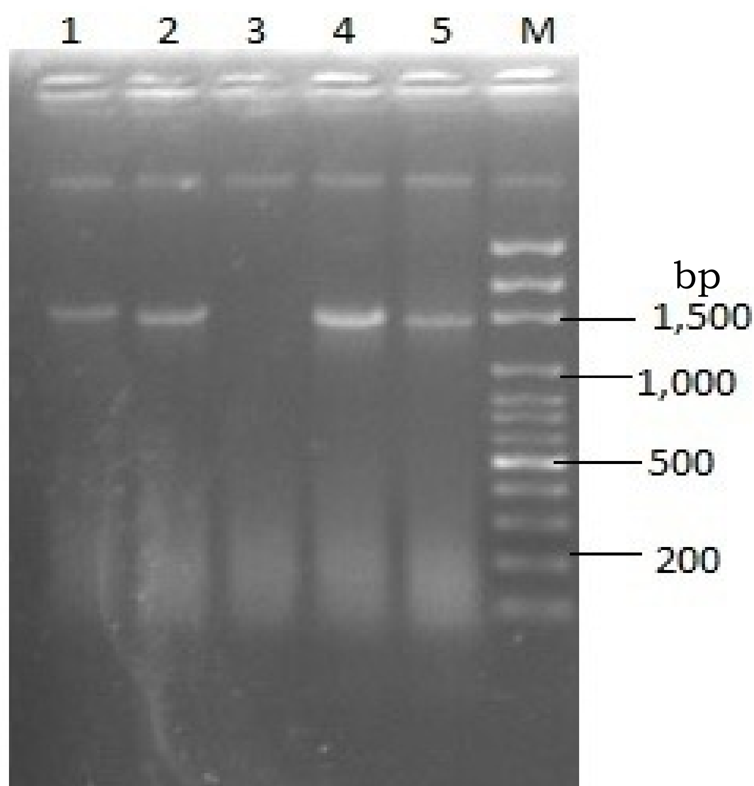

Fig.2: PCR amplification of bacterial 16srRNA PCR gene of the isolates
Key: 1 = Isolate A, 2 = Isolate B, 3 = Isolate C, 4 = Isolate D, 5 = Isolate E, M = DNA molecular weight maker where, an amplicon size of 1,500bp signifies amplification of the 16srRNA gene.

Table 3: Nucleotide Sequence of Genes of each Isolate and their homology

Isolate	Sequence	Organism	% Homology
A	NNNNNNNNNNNNNNNNNNNNTGCAGTCGNNNNAGCACTTANNN NNTTNCTTTNTGAAGATAGCCGNANNGNGTGANTACANGGGNN NTGCTGCGNANNGGAGTGNNATANCTCCNNGAAACCGNTATAN TACCGGATAACATTTTGAACCGCATGGTTCNNANTTGAAAGGCG GCTTCGGGGTGTGNCTTATGGATGAACCCGTGNTGGATTGNNTA GGTGGTTAGAAAGCGGCTCACCAAGGCAAGGNTGCGTAGCCNA CCNGACACGNTGGNCNNGACACTGGCCCCAGAACCCCCCCCCG AANCTCCGACGGGAGNNNTTGNAAAGGNGTTTTTACCCCTTTNN TCCCAAAATNGTNACGGGCGAGGNGNGGGCTTCATTTTTGTCTC CCATTTGTGGCANCCCTTAAANTNGGGNAAAAAAATTGGTAGGG AAAATAAAGGGGTTNTTTNNGGGCCCCCCCCGNAAANNNANNN	*Bacillus cereus* strain BTD4-2	83%
B	NNNNNNNNNNNNNNNNNNCTGCAGTCGNNNNANCACGNNNATTT NCNNTTTTGNCGATTGCCGCNCGTGANNNANTACANGNGNTNTG TTNTGNAGTGGAGTCTGANACCTCCAGAAAACCNNNNNNNNAC CGANTANCATTTTGTACCGCATGGNTCNTATGGAANGGCGGNNT CGGTNNNNNNGNATGGNTGAATCCGNNNNGGTTANNTAGNNTN NNANGANNGGCTCACNNNNGNTNGATTNATAGGCNNCNAGNNA GGTGNNNNNNAACTGNNCTGANNTNNNNGNNGGNNGGNNGGN NNAGTNGACGGGGNTNAACTNTNNNTGCCNTGCGNGNNGNCAG GNGNCATTGNTTTNNTATTAGNTNGTNAGGACNNTNTGGNNGTT TANATTNTNGNNGATAANGTGGATATTATNNNTTGCNNNNNTNN TNCANATTNGNNTNAAN	*Bacillus cereus* strain GBPS9	78%
C	NNNNNNNNNNNNNNTANNNNGNNGTCGAGCGCTCGCNCNGGGA GCGGCGGACGGGTGANTAACGCGTGGGAATCTACCCNTCNCTAC CGAATAACCCAGGGAAACTTGAACTAATACCGTATACGACCGA AAGGTGAAAGATTTATCGGTGATGGATGANCCCGCGTTGGATTN CTANTTGGTGGGGTAAAGGCCTACCAAGGCGACTATCCATAGCT GGNCTGANAGGATGATCAGCCACACGGGGACTGAAACACGGCC GTGACTCCTACNGNGGCACCANTGCGGAATATTNTACAATGNNN TNNTTGCCTGATCNAACAATGCCGCGTTNNNGATGANTNGTTNT ATGGT	*Phyllobacterium leguminum* strain ORS 1419	89%
D	NNNN	Not determined	
E	NNNNNNNNNNNNNNNNNNNNNNNNTNNNNNTGCAGTCGANCGG CACACNGNCTCTTGCCCCTTTGCCATTGGCGACGGACAGATNAT GNNACGTAACNTGCCTNTANATGGGGAGNNTACCTCCNGNAAA CCGGATATCATACCCACTNNTCTCTTTTGCTCCANGGTGAAAGA CTGCCCNNNNTCATTCNACTGACCC	*Lysinibacillus fusiformis* strain LMG 18474	78%

Discussion

The increase in microbial load during Bambara nut fermentation could have been influenced by the nutritional composition of the seeds. The seeds have been reported to make a complete food as it contains sufficient quantities of good quality protein, carbohydrate and fat which served as nutrition to the growing microbes (Owusu-Domfeh *et al*., 1970; Minka and Bruneteau, 2000). Fadahunsi and Olubummi (2009) reported progressive increase in the viable counts during the fermentation of Bambara nut seeds into 'iru' where the total viable microbial count recorded increased from 3.20 x 10^5 cfu/g at 0 h to 9.20 x 10^7 cfu/g at 96 h. In this study, the five bacterial isolates were identified as *Bacillus cereus* BTD4-1*, Bacillus cereus* GBPS9, *Phyllobacterium leguminum* ORS1419 and *Lysinibacillus fusiformis* LMG 18474. However, the identification of *Staphylococcus* sp. was not confirmed. The organisms confirmed are mesophillic in nature and have been reportedly found in farming soil as well as the rhizosphere of legumes. They can resist temperatures between 17 °C to 40 °C and pH 5 to 9.5 hence they could withstand the temperature (31.1 to 28.5 °C) pH (6.80 to 5.30) condition of the fermentation process (Vilain *et al*., 2006).

The predominance of *Bacillus* sp in traditional fermented protein condiments can be linked to different factors such as long cooking duration during the pre-treatment process, which select for heat resistant microorganisms especially spore-forming bacteria (Ouoba *et al*., 2008). In addition, *Bacillus* sp have been reported as producers of amylase, galactanase, glucosidase and fructofuranosidase which are involved in the degradation of carbohydrate to sugars. The sugar molecules are broken down into smaller organic end products such as acetic, formic, lactic, butyric, succinic and propionic acids. This was observed in the breakdown of the carbohydrate content of the nuts in this study, which led to a decrease in pH from 6.80 to 5.30. (Omafvbe *et al*., 2000; Kiers *et al* 2000; Sarkar *et al*., 1997). They have also been reported in the degradation of proteins during fermentation, with significant release of amino acids, part of which are a source of energy for microorganisms (Omafuvbe *et al*., 2000). Other studies, based on phenotypic characterization reported the presence of *Bacillus subtillis*, *Bacillus lichenformis* and *Bacillus pumilis* in the fermentation of bambara nut into 'iru' as well as other fermentation works on ogirri (Fadalunsi and Olubummi, 2009; David and Aderibigbe, 2010). The difference in results of the previous studies can be attributed to various factors such as the origin of the samples and the methods for identification. The previous studies were based only on traditional phenotypic tests which have been reported to be insufficient or unreliable for accurate identification of bacteria (Towner and Cockayne 1993). In this study, phenotypic tests were combined with genotypic methods for improved identification of isolates. It was observed that the rod shaped isolates tentatively identified (using phenotypic test) as species of the genus *Corynebacterium* and *Lactobacillus* which have previously been implicated in food fermentation (Njoku and Okemadu, 1988) were however confirmed as *Bacillus cereus* and *Phyllobacterium leguminum*.

The fermentation of traditional protein condiments is spontaneous by nature and may be carried out under unhygienic conditions therefore the presence of pathogenic and spoilage strains of *Bacillus* cannot be totally ruled out in some fermentation batches. As a soil bacterium, *Bacillus cereus* can spread easily to many types of foods (Vilian *et al*., 2006). The organism utilizes fermentation to generate energy and has been reported to metabolize a variety of compounds including carbohydrates, proteins, peptides and amino acids for growth and energy (Mols *et al*., 2007).

The occurrence of *Bacillus cereus* in African fermented foods is common (Ouba *et al*., 2004; 2008; Enujiugha 2009; Parkouda *et al.,* 2010; Thorsen *et al.,* 2011; Ahaotu *et al.,* 2012) but reported information on predominance of the species in food is rare. The limited publications on the subject include the study of Okanlawon *et al*. (2010) where occurrence of *Bacillus cereus* in a higher proportion than other *Bacillus* spp was recorded in 'ogirri' and 'iru' (six out of twelve isolates). Thorsen *et al*. (2011) also reported a high occurrence of *Bacillus cereus* (up to 68 %) of the isolates during Gergoush fermentation, a traditional Sudanese bread snack. Ahaotu *et al*. (2012) identified 49 bacterial isolates from 'ugba' produced in different areas of South-Eastern Nigeria by phenotyping and sequencing of 16srRNA gyrB and rpoB genes. They were identified as predominantly *Bacillus cereus sensu* lato and a combination of *Lysinibacillus xylanilyticus* and four other species and *Bacillus*. The occurrence of *Bacillus cereus* in a food is of considerable concern for human health. *Bacillus cereus*is recognized as a food born pathogen capable of causing food poisoning accompanied with diarrheal and or vomiting depending on the type of toxin produced.

The presence of *Lysinibacillus fusiformis* and *Phyllobacterium leguminum* in African fermented foods as noticed in this study seem not to have been previously reported. Interestingly, *Phyllobacterium* sp. is reported to have probiotic properties (Flores-Felix *et al*., 2015), while *Bacillus fusiformis* was isolated for the first time from cocoa fermentation using 16srRNA gene partial sequencing (Ouattara *et al*., 2014).

Aspergillus niger was observed to be the most predominant among the fungal isolates. The isolation of *Aspergillus niger* had also been previously reported by Difo *et al*. (2013) in their study of simultaneous production of phytase and tannase by *A. niger* isolated from three varieties of Bambara nut seeds, as well as Jeff-Agbola (2007) in his study of microorganism associated with natural fermentation of African yam bean seeds for the production of 'ogirri'.

Conclusion

This study has revealed the usefulness of genotypic molecular identification technique as key in identifying organisms present in the fermentative production of ogirri using bambara nut seeds. Organisms such as *Phyllobacterium leguminum* strain ORS1419 *and Lysinibacillus fusiformis* strain LMG18474 have been reported for the first time in Bambara nut fermentation studies.

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References

Ahaotu I, Anyogu A, Njoku OH, Odu NN, Sutherland JP, Ouba LII: Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba. Int J Food Microbiol 162: 95-104. 2012.

Barnett HL, Hunter BB: *Descriptions and Illustrations of Genera. Illustrated Genera of Imperfect Fungi*. The American Phytopathology Society Press, St Paul Minnesota 218p. 1998.

Begemann FI, Mukema I, Obel-Lawson J: Promotion of bambara ground nut (*Vigna subterranean*). Latest development of bambara ground nut research. Proceedings of the Second International Bambara groundnut Network (BAMNET). Accra, Ghana, 122p. 2002.

Borget M*: Food legumes.Technical Centre for Agricultural and Rural Cooperation, Wageningen*. The Netherlands. 111p. 1992.

David OM, Aderibigbe EY: Microbiology and proximate composition of "Ogiri", A pastry produced from different melon seeds. NY Sci J 3(4): 18- 27.2010.

David OM, Olagunju JL, Adebayo AA, Oluwaniyi TT, Olajide MO: Probiotic properties and antibiotic resistanc pattern of *Bacillus* spp isolated from two types of locust bean (iru). Br Biotechnol J. 10(4) :1-12. 2016.

Doku EV: Problems and prospects for the improvement of bambara groundnut: *Proceedings of the International Bambara Groundnut Symposium,* July 23-25, University of Nottingham UK, pp. 17 -27. 1996.

Enujiugha VN: Nutrient changes during the fermentation of African oil bean (*Pentaclethra macrophylla Berth*) seed. Pak J Nutr 2(5): 320- 323. 2003.

Enwere NJ: Foods of Plants Origin. .Proceeding and utilization with recipes and Technology profiles. Afro-Orbis Publishers, Nsukka, Nigeria. 301p. 2008.

Fadahunsi IF: The effect of soaking, boiling and fermentation with *Rhizopus oligosporous* on the water soluble vitamin content of bambara groundnut. Pak J Nutr 8(6): 835-840. 2009.

Fadahunsi IF, Olubunmi PD: Microbiological and enzymatic studies during the development of an "Iru" (a local Nigerian indigenous fermented condiment) like condiment from Bambara nut (*Voandzeia subterranean* (L) Thours). Malays J Microbiol 6(2):123-126. 2009.

Flores-Felix JD, Silva LR, Rivera LP, Marcos-Garcia M, Garcia-Fraile P Martinez-Molina E: Plants probiotics as a tool to produce highly functional Fruits: The case of *Phylobacterium* and Vitamin C in Strawberries. PLoS One 10(4): 1281-1287. 2015

Gibbon D, Pain A: *Crops of the Drier Regions of the Tropics*. Longman Publishing Company, Singapore. 155p. 1985.

Holt JG, Kreig NR, Sneath PHA, Staley JT Williams ST: Bergey's Manual of Determinative Bacteriology. (9th Ed.) Lippincott, Williams and Wilkins, Philadelphia. 679p. 2000.

Ibeabuchi JC, Olawuni IA, Iheagwara MC, Ojukwu M, Ofoedu CE: Microbiological evaluation of 'Iru' and 'Ogiri-Isi' used as food condiments. IOSR J Environ Sci Toxicol Food Technol 8(8): 45-50. 2014.

Jeff- Agboola A: Microorganisms associated with natural fermentation of African yam bean seeds for the production of 'otiru'. Res J Microbiol 2: 816-823. 2007.

Kiers JL, Van-Laekan AEA, Rombouts FM Nout MJR: *In vitro* digestibility of *Bacillus* fermented soya bean. Int J Food Microbiol 60: 163-169. 2000.

Linemann AR: Cultivation of bambara groundnut in northern Nigeria. Trop Crops Commun.15**:** 1-14. 1988.

Linnemann AR: *Bambara groundnut (Vigna subterranean)* literature: a revised and update bibliography. Tropical Crops Communication.Wageningen Agricultural University, Netherlands.124p. 1992.

Linnemann AR: Preliminary observation on photoperiodic regulation of phonological development in bambara groundnut (*Vigna subterranean* (L) verdc). Field Crops Res 26:295-304.1991.

Minka SR, Bruneteau M: Partial Chemical composition of bambara pea (*Vigna subterranean* (L) verde). Food Chem 68: 273-276. 2000.

Mols M, De Been M, Zwieteruig M, Moezelaar R, Abee T: Metabolic Capacity of *Bacillus cereus* strains ATCC 14579 and ATCC 10987 interlinked with comparative genomics. Environ Microbiol 60: 2023 – 2030. 2007.

Njoku HO, Okemadu CP: Biochemical changes during the natural fermentation of the African oil bean for the production of *ugba.* J Sci Food Agric 49: 457-465. 1989.

Nwanna LC, Enujiugha VN, Oseni AO, Nwanda EE: Possible effects of fungal fermentation on bambara groundnuts (*Vigna subterranean* (L) Verde) as a feedstuff resource. J Food Technol 3(4): 572-575. 2005.

Ocran VK, Delimini LL, Asuboah RA, Asiedu EA: *Seed management. Manual for Ghana*, MOFA, Accra, Ghana. 67p. 1998.

Odibo FSC, Nwabunia F, Osuigwe DI: Biochemical changes during fermentation of *Telfairia occidentalis* seeds for Ogiri production. World J Microbial Biotech 6:525-427. 1990.

Ojimelukwe PC, Ayernor GS: Oligosaccharide composite and functional properties of flour and starch isolates from cultivars of Bambara groundnut seeds. J Food Sci Technol 29**:** 319-321. 1992.

Ojinaka MC, Ojimelukwe PC: An assessment of the microbial and amino acid contents of 'ogiri' produced by fermenting oil bean seeds of *Ricinus communis.* Am J Food Nutr 3(3): 155-161. 2013.

Okafor N: *Modern Industrial Microbiology and Biotechnology*. Science Publishers. Enfield, New Hampshire, U.S.A. 551p. 2007.

Okanlawon BM, Ogunbanwo ST, Okunlola AO: Growth of Bacillus cereus isolated from some traditional condiments under different regimens. Afr J Biotech 8**:** 2129–2135. 2010.

Okonkwo SI, Opara FM: The analysis of Bambara groundnut (*Voandzeia subterranean* (L) Thouars) for sustainability in Africa. Res J Appl Sci 5(6): 394-396. 2010.

Oluwabukola O, Asagbra A, Faderin M: Comparative soluble nutrient value of Ogiri obtained from dehulled and undehulled boiled melon seeds. Food Sci Quality Manag 4**:** 10-16. 2012.

Omafuvbe BO, Oyedapo OO: Observed biochemical changes during natural fermentation of African oil bean (pentaclethramacrophylla) seeds. Nig J Nutri Sci 21**:** 19-23. 2000.

Ouattara HG, Reverchon S, Niamke SL, Naser W: Molecular identification and pecitate lyase production by *Bacillus* strains involved in cocoa fermentation. Food Microbiol 28(1):1-8. 2014.

Ouoba LII, Diawara B, Amoa-Awua WK, Traore´ AS, Lange Møller P: Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for fermentation of African locust bean (Parkiabiglobosa) to produce Soumbala. Intl J Food Microbiol 90: 197–205. 2004.

Ouoba LII, Parkouda C, Diawara B, Scotti C, Varnam AH: Identification of *Bacillus* spp. from Bikalga, fermented seeds of Hibiscus sabdariffa: phenotypic and genotypic characterisation. J Appl Microbiol 104: 122–131. 2008.

Owusu-Domfeh K, Christensen DA, Owen BD. Nutritive value of some Ghanaian feed stuffs. Can J Anim Sci 50**:** 11-618. 1970.

Parkouda C, Thorsen L, Compaoré CS, Nielsen DS, Tano-Debrah K, Jensen JS, Diawara B, Jakobsen M: Microorganisms associated with Maari, a Baobab seed fermented product. Int J Food Microbiol 142: 292–301. 2010.

Peter-Ikechuwu AI, Kabuo NO, Alagbaoso SO, Njoku NE, Eluchie CN, Momoh WO: Effect of wrapping materials on physico-chemical and microbiological qualities of fermented melon seed (*Citrullus colocynthis* L) used as condiment. Am J Food Sci Technol 4(1): 14-19. 2016.

Purseglove JW: *Tropical Crops* (Dicotyledons).Longman House Burnt Mill, Harlow, Essex, England. 305p. 1992.

Sarkar PK, Jones L, Craven GS, Somerset SM, Palmer C: Oligosacharide profiles of soybeans during kinema production. Lett Appl Microbiol 24: 337-339. 1997.

Sesay A, Kunene IS, Earnshaw DM: Farmers Knowledge and Cultivation of Bambara groundnut (*Vigna subterranean*) in Swaziland. J Agric Sci Technol 3(1): 27- 37. 1999.

Steinkraus KH: *Handbook of Indigenous Fermented Foods* (2nd Ed).Marcel Dekker, Inc., New York. 792p. 1995.

Thorsen L, Azokpota P, Munk-Hansen B, HvillumRønsbo M, Fog Nielsen K, Hounhouigan DJ, Jakobsen M: Formation of cereulide and enterotoxins by *Bacillus cereus* in fermented African locust beans. Food Microbiol 28: 1441–1447. 2011.

Towner KJ, Cockayne A: *Molecular Methods for Microbial Identification and Typing*, 1st edn. Chapman and Hall, London. 193p. 1993.

Tweneboah CK: *Modern Agriculture in the Tropics, Food Crops*. Co-wood Publishers. 237p. 2000.

Vilain S, Luo Y, Hildreth M, Brozel V: Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. Appl Environ Biol 72(7): 4970-4977. 2006.