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Bacterial diversity study on fermentation of *Parkia biglobosa* using 16s rRNA gene analysis

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ABSTRACT

The diversity and dynamics of the bacterial community during the fermentation of *Parkia biglobosa* to produce '*Iru*', a condiment, were studied, using 16S rRNA gene analysis. Fresh *P. biglobosa* seeds were processed using the traditional method at ambient temperature (30±2°C) for 72 h. The total bacterial community was obtained by vigorously rinsing the seeds in phosphate-buffered saline at 0 h and subsequently at 24, 48 and 72 h. Community DNA was extracted directly from the rinsed water and from nutrient culture of the rinse water. The 16S rRNA genes of bacteria present were amplified by polymerase chain reaction from the extracted DNA, using the primer pair 27F and 1492R. The amplicons were sequenced and aligned with reference fragments in the database of the ribosomal RNA project. The bacterial populations in fermented '*Iru*' increased from $1.2x10^1$ cfu/g at start of fermentation to 1.63×10^9 cfu/g at 72 h. The results obtained from the database were as follows: Bacilli–Bacillales represented by *Bacillus algicola*; *Bacillus cereus; Bacillus thuringiensis; Bacillus subtilis; Bacillus pumilus; Bacillus* sp.*; uncultured Staphylococcus* sp.*; Bacillus anthracis; Bacillus foraminis*; *Bacillus clausii; Lysinibacillus* spp.; Lactobacillales was represented by *Streptococcus sanguinis; Fusibacter* sp.*;* Clostridia–Clostridales was represented by *Butyrivibrio fibrisolvens*; Bacteroidia –Bacteriodales was represented by *Bacteroides nordii; Parabacteroides merdae*; Alpha proteobacter was represented by *Acetobacter pasteurianus*; gamma proteobacter represented by *Acinetobacter baumannii and Enterobacter aerogenes.* The dominance of *Bacillus* spp. in cultured bacterial community increased from 8 to 83%, while that of alpha proteobacteria increased from 2 to 15% and gamma proteobacteria decreased from 28 to 1%. As fermentation progressed *Bacillus* species decreased. Gamma proteobacteria increased to 17% at 72 h. The succession of the bacteria associated with the fermentation of *P. biglobosa* to '*Iru*' was shown to be co-dominated by *Bacillus* and the Proteobacteria spp. Molecular method adopted showed varieties of microorganisms that had hitherto not been identified by the conventional culture-dependent methods.

INTRODUCTION

Iru is an indigenous protein-rich soup condiment produced by fermenting the cotyledons of African locust bean (*Parkia biglobosa*). It is consumed mostly by the local rural dwellers as protein supplement in the diets in many West African countries. *Iru* is known as 'Soumbala' in Burkina Faso (Ouoba et al., 2003b) and 'Dawadawa' by the Hausa-speaking ethnic groups in West Africa (Odunfa and Adewuyi, 1985). *Iru* is not only consumed

as a soup flavouring food additive, but also serves as a cheap meat substitute amongst poor families. In the production of *Iru,* being a traditional art, the fermentation is initiated by chance inoculation of natural microflora and thus the product varies considerably in quality and shelf-life. Condiments are also known to contribute to the calorie and protein intake (Umoh and Oke, 1974; Simmons, 1976).

Fermentation is one of the oldest methods of food preservation known to man. In Africa, the art of fermentation is widespread including the processing of fruits and other carbohydrate sources to yield alcoholic and non-alcoholic beverages, as well as the production of sour tasting *ogi* – a fermented cereal product, which provides energy in breakfast and convalescent diets (Adewusi et al., 1991; 1992). Oil seeds such as African locust bean, melon seed, castor oil seed and soybean are also fermented to give condiments. In food systems, estimates of "true" microbial diversity are still difficult because the majority of the food-associated microflora cannot be cultivated on standard laboratory media (Giraffa and Neviani, 2001). DNA technology has triggered research advances in almost all fields of biology. The new techniques opened up the study of the molecular details of eukaryotic gene structure and function. Today, hundreds of useful products are produced by genetic engineering, the manipulation of genetic material for practical purposes. It has become a routine to combine genes from different sources – often different species, in test tubes, and then transfer this recombinant DNA into living cells where it can be replicated and expressed. *Escherichia coli* is often used as a host because it is easy to grow and its biochemistry is well understood (Giraffa and Neviani, 2001). Molecular biology -based identification methods for biodiversity of food microorganisms can greatly enhance the specificity, sensitivity and speed of microbial detection. These molecular tools can be used to provide molecular characterization and predict phylogenetic relationship between microorganisms in fermented food (Simpson et al., 2002). The use of 16S rRNA gene for identification of microbial diversity stems from its stability and conservativeness through the evolutionary period. Genetic-based culture independent techniques have suggested the existence of a vast undiscovered microbial diversity.

Numerous studies have been carried out on the production of fermented condiments – *Iru*, from African locust bean (Eka, 1980; Odunfa, 1986), and soybean 'Dawadawa' (Omafuvbe et al., 2000, 2002). Succession of microorganisms involved during the fermentation of African locust bean seeds have been studied by many researchers (Odunfa and Oyewole, 1986; Ogbadu and Okagbue, 1988, Steinkraus, 1995; Ouoba et al., 2003a; Oguntoyinbo et al., 2007) and it has been reported that the major microorganism present is *Bacillus subtilis.* Microorganisms involved in the fermentation of *P. biglobosa* have been extensively studied using the culture–dependent approach, biochemical and morpho-logical features. So far, no investigation has been carried out on the bacterial diversity of fermenting *Iru* using 16S rRNA gene analysis.

The objective of this study was to evaluate the bacteria community of fermenting African locust bean seeds '*Iru'* using 16S rRNA gene analysis in order to confirm/dispute results of previous studies that employed the conventional (biochemical and culture-dependent) methods for the identification and characterization of microorganisms during the fermentation.

MATERIALS AND METHODS

The African locust bean seed (*P. biglobosa*) used for this research was purchased from Lafenwa (local market) in Abeokuta, packed in sterile polythene bags (Ziploc) and transported to Biological Sciences Laboratory, Florida Atlantic University, Davie campus, Florida, U.S.A. All chemicals and enzymes were supplied by the Biotechnology Centre of Federal University of Agriculture, Abeokuta and Biological Sciences Laboratory, Florida Atlantic University, Davie, Florida, U.S.A.

Traditional processing of African locust beans to *Iru*

African locust beans were processed to *Iru* following the traditional method (Figure 1). The fresh seeds (1 kg) were cleaned to remove extraneous materials such as shafts and stones, washed in tap water and poured into a large cooking pot. The seeds were then cooked in 2000 ml of tap water for about 6 h until over 50% of the seed coats were cracked. The coats with un-cracked seeds were pounded gently in a cyclical manner in a mortar to separate the seed coat from the cotyledons. The cotyledons were washed with several changes of tap water and then sieved to remove the non-cotyledon materials. The cotyledons were parboiled for between 45 min and 1 h. The colour of the cotyledons changed from whitish yellow to light brown at this stage. A sieve was used to remove the cotyledons and the water was allowed to drain before pouring into a basket lined with jute sack and covered tightly immediately to prevent the heat from escaping. The basket was wrapped with several layers of thick cloth and the seeds were fermented at 37°C in a dark, warm cupboard for three days. At the end of fermentation, the colour of the cotyledon changed to ash white. Sodium chloride (salt)

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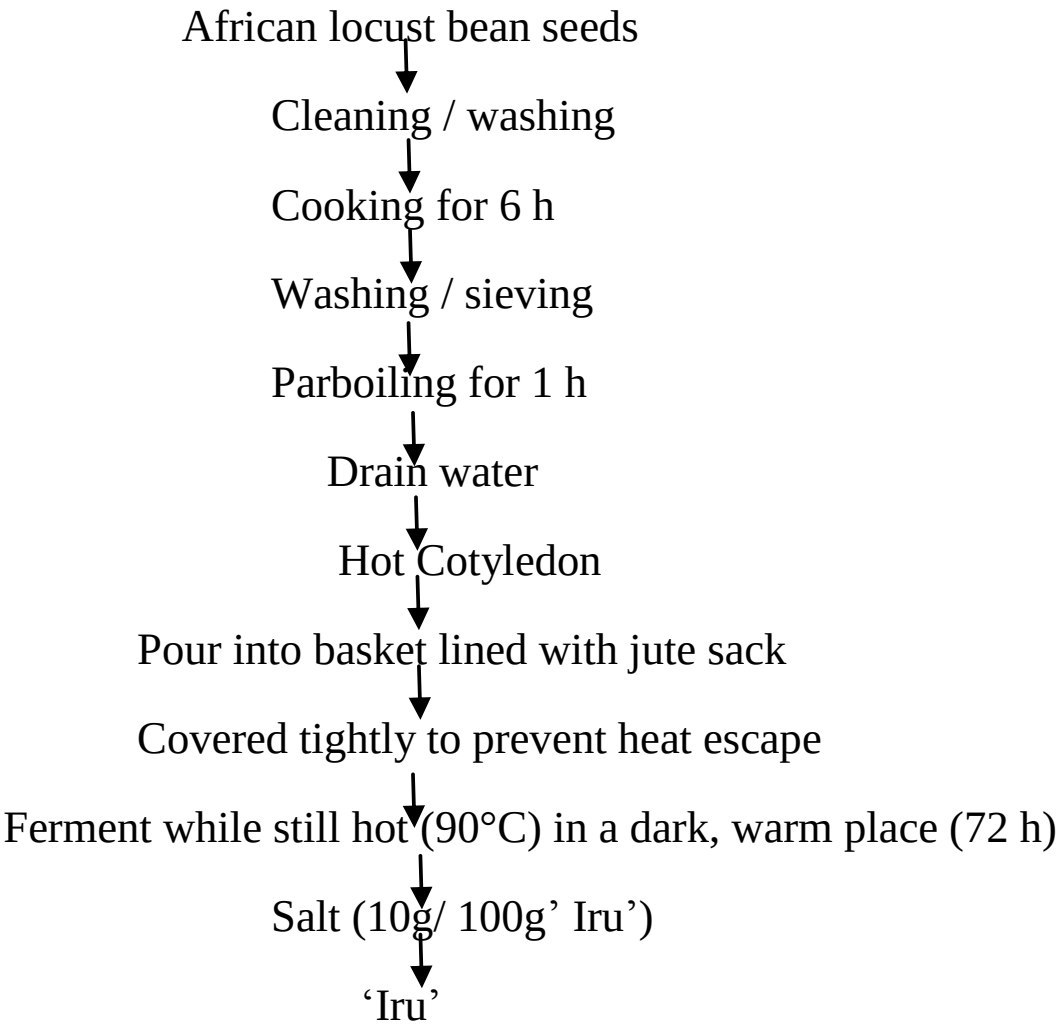

Figure 1. Traditional processing of fermented African locust beans to *Iru*.
Source: Odunfa and Oyewole (1986).

was added to enhance the taste.

Isolation of microorganisms and pH determination of fermenting *Iru*

The African locust beans were fermented for three days. At 0 h and at 24 h intervals, 1 g of the seeds was obtained with a sterile spatula from each wrap and buffer rinsed with Phosphate buffered saline (100 ml). On the rinsed water, a 10-fold serial dilution was made and dilutions were plated out on Tryptone Soya agar, (all obtained from Oxoid) and incubated overnight (24 h) at 37°C. The colonies were counted using the standard plate count method and stored at 4°C in the Biological Laboratory of Florida Atlantic University, Davie Campus, Florida, U. S. A. until required for DNA extraction.

The pH of the raw, cooked and fermenting African locust beans 'Iru' were measured by mixing 1 g of each sample with 9 ml of distilled water and determining the pH with a Jenco Bench top pH meter.

Cultured bacterial community DNA extraction of fresh, fermenting African locust beans seeds

The Qiagen DNeasy Tissue protocol (Qiagen, Valencia, California) was used for the DNA extraction. The community of all the colonies on each agar medium was taken by rinsing each agar surface with 1000 µl of 1 × Phosphate buffered saline. The rinse saline was transferred into a clean micro centrifuge tube and centrifuged for 10 min at 7500 rpm. The supernatant was discarded. Pellet in the tubes were re-suspended in 180

µl Buffer ATL, Proteinase K (20 µl) was added, mixed by vortexing and incubated at 55°C for minimum of 1 h, until the cells were completely lysed. After incubation, the tubes were vortexed for 15 s, and Buffer AL (200 µl) was added to the samples, mixed thoroughly by vortexing and incubated at 70°C for 10 min. It is important that the sample and Buffer AL were mixed immediately and thoroughly to yield a homogeneous solution. An aliquot of 200 µl of 95% (v/v) ethanol was added to the sample and mixed thoroughly by vortexing. The mixture was pipetted into the DNeasy spin column placed in 2 ml collection tube and centrifuged at 8000 rpm for 1 min, flow through was discarded. The spin column was placed in a new 2 ml collection tube and 500 µl buffer AW1 was added and centrifuged for 1 min at 8000 rpm, flow through was discarded. The spin column was placed in a new 2 ml collection tube and 500 µl buffer AW2 was added and centrifuged for 3 min at full speed (8000 rpm) to dry the DNeasy membrane, flow through and collection tubes were discarded. This centrifugation ensured that no residual ethanol was carried over during the elution stage. The DNeasy spin column was placed in a clean 1.5 or 2 ml micro-centrifuge tube and 200 µl buffer AE (or nano pure water) was pipetted directly into the DNeasy membrane, incubated at room temperature (37°C) for 1 min and centrifuged for 1 min at 8000 rpm to elute. Elution with 100 µl increased the final DNA concentration in the eluate, but decreased the overall DNA yield. The extracted DNA in the tubes was kept in the fridge till further analyses.

Agarose gel electrophoresis

The success of the DNA extraction was investigated by subjecting the DNA to agarose gel electrophoresis as described by Neelam (2004) and visualizing with ethidium bromide under UV light.

Procedure

Agarose powder (1 g) was added to 100 ml of 1 × TBE buffer in a 250 ml conical flask. The flask was placed in a microwave (100°C) for 1 min to dissolve the agarose and then cooled to about 50°C (or to a temperature safe enough for the flask to be hand-held). Ethidium bromide (EtBr) (30 µl of 2.5 mg/ml) solution was added and the cooled agarose gel was poured into the gel holder with the comb properly placed in the case. The gel was allowed to set at room temperature. Gel-loading buffer (2 µl) was added to each DNA extract and then mixed with a vortexer. The new mixture (10 µl) was loaded into a well of the gel using a micropipette. Marker DNA (1 kb) (exaACTGene, Fisher BioReagents) (5 µl) containing DNA fragments of known sizes were loaded alongside the samples. The order in which they were loaded was recorded. The electrodes were connected to the electric power source and the DNA run through the gelat 100 V for 1 h. The gel was viewed under UV light on a trans-illuminator and recorded by photographing.

Amplification by polymerase chain reaction (PCR)

A polymerase chain reaction (PCR) amplification of extracted community DNA was carried out using Eppendorf Master Gradient Thermo Cycler (Eppendorf, New York). Universal bacterial primer 27F (5'-AGAGTTGATCATGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3') (Martin-Laurent et al., 2001) were used to amplify the 16S rRNA genes. These nucleotides correspond to bases 9 to 27 and 1510 to 1491 of *E. coli* 16S rRNA gene sequence (Brosius et al., 1978). The PCR conditions were: Initialization at 96°C for 6 min, then 30 cycles of denaturation at 94°C for 1 min; annealing at 44°C for 30 s; extension at 72°C for 2 min final extension at 72°C for 4 min. To validate PCR results, each PCR reaction batch was always carried out with positive and negative controls. Positive control had all PCR reagents and *E. coli* DNA with known (ATTC 25922) genomic DNA as template. Negative control had all reagents except DNA template. Nano pure water was used as negative control. The PCR was carried out in triplicates for each sample. All extracted community genomic DNA was amplified with 27F and 1492R primers (Bioscience, Ramona, California) using Qiagen PCR Master Mix (Qiagen Inc, Valencia, CA). All PCR products were verified by electrophoresis on 1% agarose gel made with 1 × TBE (Tris boric-EDTA) buffer stained with ethidium bromide. The 9 µl of each DNA sample plus 1 µl loading dye were loaded into each well and with 5 µl of exaACTGene, Fisher BioReagents DNA marker (1 kb) at 100 volts for 30 min to verify the presence of DNA.

Cloning of 16S rRNA amplified gene

The amplified 1500 bp PCR product was cloned by pCR Blunt TOPO vector cloning kit (Invitrogen, - California). Two replicates of each sample were cloned. The amplified PCR product was ligated with Blunt TOPO cloning vector as per manufacturer's instructions (Figure 2). The ligation time was increased to 1 h thereby giving sufficient time for vector to ligate with PCR product. Ligated product (2 µl) was added to 50 µl *E. coli* TOPO 10 chemically competent cells. Incubation time on ice was increased to 30 min to increase the time for attachment of the vector to the host cells. Cells at 0°C were heat shocked at 42°C for 30 s and returned to ice, then, 250 µl of S.O.C. medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM

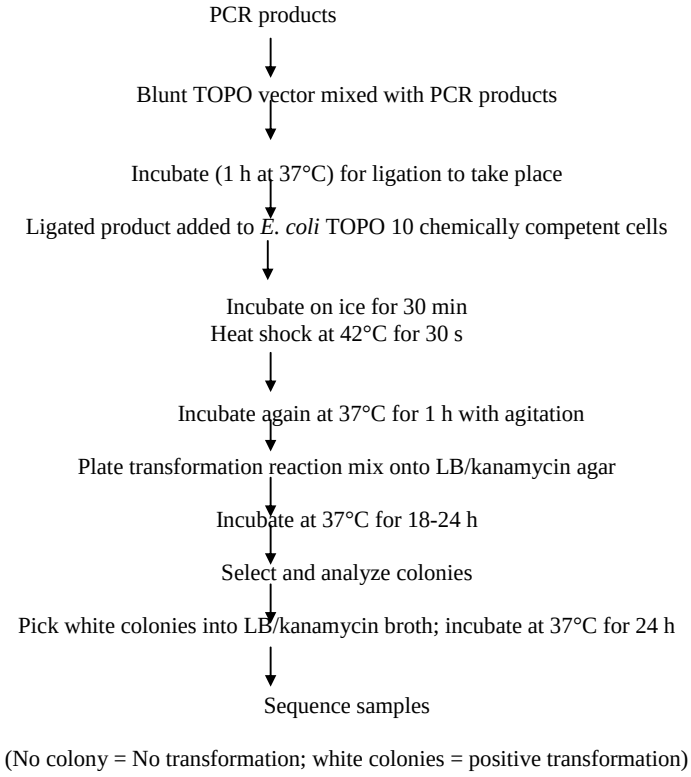

Figure 2. Blunt end TOPO vector DNA cloning flow chart for bacterial community in fermenting *Iru*.

$MgSO_4$, 20 mM glucose) was added and incubated at 37°C for 1 h with agitation on a rocking platform. An aliquot of 300 µl was then plated on LB plates containing 50 µg/ml kanamycin. The plated cells were incubated for 18-24 h at 37°C. Non-transformed cells did not grow on the medium due to presence of kanamycin and due to activation in cells containing self-ligated-vectors, of toxic *ccd B* gene present in the vector (Bernard and Couturier, 1992; Bernard et al., 1993, 1994). Colonies which grew on the plate were assumed to be transformed.

Preservation of clones

From the transformants, ten colonies were randomly picked from the agar plates and each single colony was inoculated and grown in 1 ml Luria-Bertani broth/ kanamycin overnight at 37°C in 1.5 ml Eppendorf tube. To the grown clones, sterile 10% glycerol was added, mixed properly and preserved at -80°C for further study.

Sequencing

The transformants in LB/Kanamycin were sent for sequencing at Macrogen Inc., South Korea. The samples were sequenced with the manufacturer's primers (M13 Reverse and M13 Forward). Templates from samples were prepared by Rolling Cycle Amplification (RCA) Technology. Sequencing was performed by dye terminator chemistry, products were separated by capillary electrophoresis with ABI 3730 XL automated DNA Sequencer. All the sequences were submitted to Sequence Match Program and Classifier tool of Ribosomal Database version (RDP 9.0 Version) to find close relatives and taxonomical classification based on Bayesian rRNA classifier.

RESULTS

Fermenting pH of African locust beans and bacterial count

The fermenting pH and bacterial count of fresh African locust beans, *Iru* are shown in Figure 3 and Table 1. The bacterial colonies increased with increase in fermentation days and the fermentation was shown to be an alkaline fermentation.

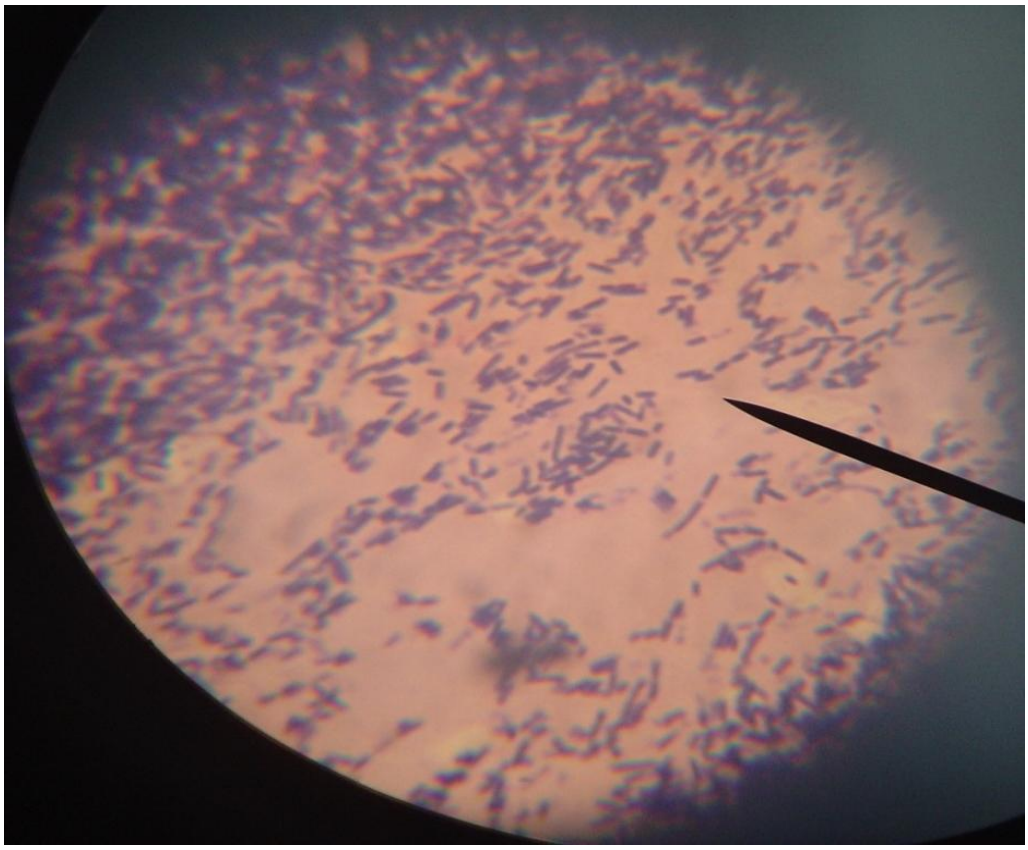

Figure 3. Rod-like *Bacillus* spp. when viewed under the microscope at 48 h fermentation of African locust beans.

Table 1: The pH and bacterial count of fermenting African locust bean seeds 'Iru' at different stages of fermentation.

Fermentation stage	pH	Bacterial count (cfu/g)
Raw seed	5.91±0.09	4.0×10^3
0 h	6.41±0.11	1.2×10^1
24 h	6.61±0.03	3.59×10^4
48 h	7.68±0.09	1.28×10^5
72 h	8.44±0.08	1.63×10^9

Means ± standard deviation.

Sequencing

Sequencing data was retrieved as FASTA and individual chromatogram format. Species which had less than 90% sequence identity with known representatives in the database were classified as unknown species and sequences which had more than 90% sequence identity were classified as known species.

Identification of bacteria from fermented African locust beans, '*Iru*' using their 16S rRNA gene sequences

From the fermented African locust beans, *Iru* cultured bacterial clone library, 70 clones were detected and sequenced. Identities and relative abundance of organisms whose 16S rRNA gene fragments had

matching sequences in the database (>90%) are identified. Analyzed sequences suggested the presence of lineages of the bacteria domain as follows: Fresh African locust beans appeared to contain organisms from one major lineage of bacteria domain -the Proteobacter. *A. pasteurianus* from the Alpha sub-division of Proteobacteria and *A. baumannii* and *Pectobacterium atrocepticum* from Gamma sub-division of Proteobacteria matched the queried fragments.

Boiled African locust beans contained organisms in two major lineages of bacteria as follows: From Bacilli–Bacillales sub-division were *B. algicola* and *B. clausii,* and from Alpha sub-division of Proteobacteria was *A. pasteurianus.* From the gamma sub-division of Proteobacteria there was a close match for *Pseudomonas acephalitica.*

At 24 h fermentation, analyzed sequences indicated the presence of two major lineages of bacteria domain as follows: Bacilli–Bacillales sub-division (*B. subtilis*; *B. algicola; B. cereus; B. anthracis* and *B. thuringiensis*) and Proteobacteria from Alpha sub-division (*A. pasteurianus*).

At 48 h fermentation, analyzed sequences suggested the presence of two major lineages of bacteria domain as follows: Bacilli–Bacillales sub-division (*B. cereus; B. algicola; B. clausii; B. thuringiensis* and *B. subtilis*) and Proteobacteria from gamma sub-division (*A. baumannii*).

At 72 h fermentation, analyzed sequences indicated the presence of two major lineages of bacteria domain as follows: Bacilli–Bacillales sub-division (*B. cereus; B. algicola; B. clausii* and *Lysinibacillus sphaericus*) and Proteobacteria from gamma sub-division (*A. baumannii*).

DISCUSSION

The amplification and sequencing of the 16S rRNA genes of microorganisms involved in fermentation of African locust beans to produce '*Iru*' has proven to be an effective tool for the identification of the microbial community associated with their fermentation. The presence of *A. pasteurianus* after boiling indicates that it has been residing in the raw African locust bean seeds, the bacterium dominated the microbial flora in the raw and boiled seeds because it is a plant bacterium and also that it is thermostable. The ability of some of these strains to form endospores which can germinate after boiling, may explain their presence during this stage of fermentation.

At 24 h fermentation, the cultured bacterial community was dominated by *Bacillus* group (90% relative abundance). The predominance of *Bacillus* species has been demonstrated in African locust bean seed fermentation and other fermenting legume proteins (Odunfa and Oyewole, 1986; Achi, 1992; Barimalaa et al., 1994; Barber et al., 1998; Omafuvbe et al., 2002). What is different in this study is the predominance of *B. cereus* group rather than the *B. subtilis* reported to be dominated by these workers. Odunfa (1985) stated that it is evident that production of fermented condiments is initially mediated by a diverse microbial flora, which eventually becomes Gram-positive flora (a reflection of many African fermented foods). The indication through this study is that fermentation was initiated by *B. cereus.* The presence of *B. cereus* (70%) at 24 h fermentation (cultured) clearly points toward a potential health risk, these strains have the potential to produce enterotoxins linked with the diarrhoeal syndrome (Oguntoyinbo et al., 2007). Ouoba et al. (2008) found that *B. cereus* strains isolated from 'Soumbala' and 'Bikalga' similarly produced enterotoxin. There appeared to be a decreasing trend in the percentage abundance of *Bacillus* group after 48 h in the cultured samples. Between 24 h and 48 h of fermentation, *B. cereus* group (*B. cereus, B. thuringiensis, B. clausii*) dominated the fermentation. *B. clausii* was present throughout the fermentation stages. *B. clausii* can tolerant extreme environmental conditions and also produce the proteolytic enzyme, subtilisin, like *B. sublitis* (Kageyama et al., 2007). Its poor growth on culture media in this study, as suggested by its low percentage abundance in cultured samples but high abundance in uncultured samples, suggests that this may be why its importance was missed by previous workers. The succession of the organisms associated with the fermentation of African locust beans to *Iru* was shown by this study to be dominated by *B. cereus* group, especially *B. clausii.* The use of molecular methods to investigate the bacterial community of fermenting African locust beans in this study has revealed other organisms that had not hitherto been reported by previous researchers who employed the biochemical and culture based methods, especially in the identification of *B. clausii* and the proteobacteria species. The application of molecular based techniques in the field of microbiology is certainly allowing a better understanding of the ecology of food fermentations. These findings revealed a high dominance and frequency of the *B. cereus* group (*B. cereus, B. thuringiensis* and *B. clausii)*, as compared to *B. subtilis*, *B. pumilus* and *Bacillus licheformis* which had been consistently found by other workers (Odunfa and Oyewole, 1986; Ogbadu and Okagbue, 1988, Steinkraus, 1995; Ouoba et al., 2003a; Oguntoyinbo et al., 2007) to be dominant in African locust beans fermentation. These results indicate that these *Bacillus* spp. may be common members of the fermenting African locust bean seeds microbiota, since at least almost all of the samples analyzed harbored these bacteria.

The *B. cereus* groups are supposed to be enterotoxin producers but undoubtedly, traditionally fermented condiments have not been incriminated in food poisoning (Oguntoyinbo et al., 2007). *B. cereus* produces three types of enterotoxins; of which two are involved in food poisoning. One of the two is heat stable while the other is

heat labile. The heat stable one is responsible for the diarrhoeal syndrome associated with *B. cereus* food poisoning. It is possible that the effect of the heat stable toxins is not felt because of the small quantities in which the product is normally used as a condiment. This may be why consumers of *Iru* have observed that a high concentration of the product in food leads to abdominal discomfort with cramps, gas and diarrhoea. This used to be ascribed to the oligosacharride often found in legumes and which give similar symptoms. It may be that it is a combination of the toxin and the oligosaccharides that is responsible for the observed symptoms.

Conclusion

Identification of organisms present during African locust beans fermentation to *Iru* using Molecular methods revealed one major group of bacteria - the *Bacillus* group. Majority of the strains belonged to the *B. cereus* group, contrary to previous studies that singled out *B. subtilis* as the dominant microorganism in the fermentation of African locust beans to *Iru.* Results of the present study suggested *B. clausii* to be the dominant in the fermentation process. The use of molecular methods in the study of bacterial diversity of fermenting African locust beans has revealed many organisms that had not hitherto been reported by previous researchers that use biochemical methods. It is necessary to carry out further studies to confirm these findings. The study also shows the superiority of the non-culture dependent method in establishing the true bacterial diversity of a food system.

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