African Journal of Biotechnology Vol. 10(69), pp. 15607-15615, 7 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1770
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Microbiota of *Tayohounta*, a fermented baobab flavour food of Benin

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Accepted 12 October, 2011

The present work provides data on the microbial composition of *Tayohounta*, a product of natural fermentation of baobab seed kernels. Samples were collected from 3 different small scale producers from Benin at the end of the fermentation process. Microorganisms were enumerated and identified using phenotypic and molecular approaches. *Tayohounta* was also investigated using culture independent techniques, direct DNA extraction, polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) and cloning. Isolated microorganisms were tested for their functionality in baobab seed kernels fermentation. Total viable counts were around 9 log cfu/g representing mainly *Bacillus* spp., whereas lactic acid bacteria (LAB) (8 log cfu/g), yeasts and moulds represent a smaller part of the total flora in all *Tayohounta* samples. Sequencing of clones of polymerase chain reaction (PCR) products of bacterial DNA directly extracted from *Tayohounta* revealed large differences between the products made by different producers. In all products, *Bacillus licheniformis*, *B. pumilus*, *B. subtilis*, *B. thermoamylovorans* and *Lactobacillus fermentum* were present. Other microorganisms (*B. thuringiensis*, *Brevibacterium borstelensis*, *Enterococcus casseliflavus*, *E. durans*, *Lb. agilis*, *Pediococcus pentosaceus*, *Streptococcus equinus* and *Weissella confusa*) were present occasionally. In experimental pure culture fermentations, *B. subtilis* showed little effect on pH, but degraded protein and caused a typical pungent smell typical of *Tayohounta*.

Key words: *Adansonia digitata*, natural fermentation, *Bacillus* spp., DGGE, genotyping, functionality.

INTRODUCTION

In many African countries, the baobab (*Adansonia digitata* L.) is a widely used tree for multiple purposes. All parts of the tree (roots, bark, leaves, fruits and seeds) are used as ingredients in the preparation of traditional foods (Nordeide et al., 1996; De Caluwe, 2005) and some have therapeutic use (Codjia et al., 2001; De Caluwé et al., 2009). Up to 300 uses were recorded in Benin, Mali and Senegal and included medicinal, nutritional, spiritual and ethnoveterinary (Buchmann et al., 2010). In addition, the human/cultural perceptions of morphological variability, uses, preferences and the links between them were recorded; local people from Benin, Ghana, Burkina Faso and Senegal use 21 criteria to differentiate baobab individuals *in situ* and to link the desirable and undesirable characteristics of the edible parts of the tree (Assogbadjo et al., 2008). Outside Africa, baobab dried fruit pulp has been acknowledged as a novel food by the European Union (The Commission of the European Communities, 2008). The Food and Drug Administration of the US has also designated it as substances Generally Recognized as Safe in 2009 (GRAS Notice N°. GRN 000273) on request of PhytoTrade, and it is also reportedly been approved as a food ingredient in the US (Addy, 2009), which may boost the trade of this product from African countries and thus provide a valuable source of foreign exchange.

The most common baobab-derived foods in Africa are

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Abbreviations: **LAB**, Lactic acid bacteria; **TVC**, total viable count; **DGGE**, denaturing gradient gel electrophoresis, **PCR**, polymerase chain reaction.

juices from the pulp, powdered leaves used as an ingredient for sauces, and flavouring agents made from the seeds (Sidibe and Williams, 2002; Chadare et al., 2008). Up till now, data are available on the ethnobotanic, ecological and genetic aspects of the baobab tree, as well as on the chemical composition of baobab parts (Ajayi et al., 2003; Assogbadjo et al., 2005, 2006; Chadare et al., 2009; Nour et al., 1980; Osman, 2004). Research on traditional African foods can provide possibilities for valorization through improvement of traditional techniques and products leading to production of added value products for a larger market and a better price (Chadare, 2010; Sidibe and Williams, 2002). This has yielded valuable information (physico-chemical composition and microbiology of the foods) on some West-African traditional foods in general and fermented ones in particular, example, fermented cereal products such as *mawe*, *gowe*, *ogi* (Agati et al., 1998; Hounhouigan et al., 1993; Michodjehoun-Mestres et al., 2005), and fermented seed products such as *Afitin*, *Sonru* (Azokpota et al., 2006) and *Iru* (Azokpota et al., 2006; Sanni et al., 2000) made from African locust bean (*Parkia biglobosa*); *Otiru* (Jeff-Agboola, 2007) from African yam bean (*Sphenostylis sternocarpa*); *Ogiri* (Sanni et al., 2000) made from melon seeds (*Citrullus* spp.) or castor seeds (*Ricinus communis*); *Ugba* (Sanni et al., 2000) made from African oil bean seeds (*Pentaclethra macrophylla* Benth); *Dawadawa* (Dakwa et al., 2005) made from soya bean (*Glycine max*), and *Kpaye* (Omafuvbe et al., 1999) made from African mesquite seeds (*Prosopis africana*). Some *Bacillus* strains are showed to be functional (that is-contribute to desirable outcomes) of fermentation in *kinema* and *soumbala* for protein degradation, pH increase and development of desirable sticky consistency (Sarkar et al., 2002). Most of the fermented seed products are used as flavouring agents; therefore, the flavour is desirable and hence functional in those products. Volatile compounds resulting from the metabolic activities of *Bacillus* spp. contribute significantly to flavour during the fermentation of African locust beans to produce *soumbala* (Ouoba et al., 2005) or soya beans to produce *natto* or thai *thua-nao* (Leejeerajumnean et al., 2001). As such, many volatile compounds were found in Beninese *afitin*, *iru* and *sonru (Azokpota* et al., 2008). Such researches have, however, never been performed on any baobab products.

In Benin, thirty five baobab food products have been recorded, with several fermented products which are yet to be characterized (Chadare et al., 2008). *Tayohounta* is one of those indigenous fermented baobab foods from Benin, and its production process was only described recently (Chadare et al., 2010). It is a product belonging to the category of the alkaline fermented foods (Steinkraus, 1995) and it is used as a source of flavour when making soups. There is some diversity of *Tayohounta* from different producers, possibly because of the uncontrolled natural fermentation taking place in which high numbers of *Bacillus* spp. were observed. This would give it resemblance to other regional flavour products such as *afitin, iru and soumbala*. In order to better understand and characterize the common microflora and arrive at a basis for the future development of fermentation starter cultures for *Tayohounta*, the present analysis of the products of three individual small-scale producers was carried out.

MATERIALS AND METHODS

Sampling of *Tayohounta*

The *Tayohounta* production process was followed at 3 representative small-scale production sites in Boukoumbe, North-Benin. The production process was as described by (Chadare et al., 2010). All 3 processors were supplied from the same batch of baobab seed kernels which had been purchased at the local market of Boukoumbe. The seed kernels were roasted (5 to 10 min), cooked in water (about 30 min), transferred into a plastic container, covered with leaves (usually leaves of *Annona senegalensis*) and allowed to ferment for 2 days at 24 to 31°C (night-day ambient temperature). Cover leaves were in contact with the product. Processor 3 cooked for a longer time (about 75 min) and used different leaves (leaves of *Sarcocephalus latifolius*) cover from processors 1 and 2. Samples were collected just at the end of the fermentation period and refrigerated (± 4°C) prior to analysis.

Enumeration of the predominant groups of microorganisms

Sub-samples of 10 g were weighed aseptically and were made into 10^{-1} dilutions using peptone physiological saline (PPS) solution (8.5 g NaCl and 1 g neutralized bacteriological peptone (Oxoid, LP0034) in 1 L demineralised water) in sterile filter Stomacher bags. The samples were homogenized for 60 s with a Stomacher (Seward Laboratory Blender Stomacher 400) at normal speed and consecutive 10-fold dilutions were subsequently prepared with PPS.

Aerobic mesophilic bacteria were enumerated on plate count agar (PCA, Oxoid CM0325) (30°C, 2 days) and reported as total viable count (TVC). Lactic acid bacteria (LAB) were enumerated on de Man, Rogosa and Sharpe Agar (MRSA), 1.5% Technological Agar (Oxoid, LP0013) added to de Man, Rogosa and Sharpe broth (Merck, VM986641, mixed and sterilized) supplemented with natamycin (2 g Delvocid (50% natamycin, DSM) added to 1 L MRSA). After solidification the plates were stored in an air-tight jar under microaerophilic conditions (80% N_2, 10% CO_2 and 10% H_2 gas mixture leaving a final concentration of O_2 of 6%) and incubated at 30°C for two days. Presumptive LAB was confirmed by oxidase and catalase tests, and confirmed counts were reported as lactic acid bacteria (LAB). Enterobacteriaceae were enumerated on Violet Red Bile Glucose (VRBG) agar (Oxoid, CM0458), in microaerobic conditions (37°C, 24 h). Bacterial spores were enumerated on PCA (30°C, 2 days), after heating the 10^{-1} dilution at 80°C for 10 min to kill vegetative cells. Micrococci were counted on mannitol salt agar (MSA, Oxoid CM0085) (30°C, 2 days). Yeasts and moulds were enumerated on oxytetracycline glucose yeast extract agar (OGYEA, Oxoid CM0545 supplemented with oxytetracycline (25°C, 3 days). Yeast and mould colonies were counted separately (Nout, 1991).

Isolation, purification and confirmation

Distinct colonies were isolated (5 per group of microorganisms and per sample) from the counted plates to confirm the obtained counts

by microscopic observation and biochemical analysis, identification and experimental fermentations. Colonies were purified by streaking on adequate medium, incubated at the required temperature and further stored on slants kept at 4-7°C. Wet-mount preparations of the isolated strains were made and observed by microscope (Olympus, BX40). A preliminary grouping was based on morphological traits (cell shape and size).

DNA extraction and amplification from isolated bacteria

Confirmed isolates were grown in nutrient broth overnight in a shaking water bath at 30°C and 200 rpm, and DNA was extracted using the 'isolation genomic DNA for Gram positive and Gram negative bacteria' kit A1125 from Promega, Southampton, UK, according to the manufacturer's instructions. The DNA was stored at 4°C. The DNA isolated for bacterial isolates was used to amplify the 16S rDNA by polymerase chain reaction (PCR), using forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'- AAG GAG GTG ATC CAG CCG CA-3'(Oomes et al., 2007). PCR was performed with a total reaction volume of 50 µL containing 26.6 µL ddH_2O, 5 µL PCR buffer, 3 µL $MgCl_2$ (25 mM), 10 µL dNTP (2 mM), 1 µL of each primer (50 µM), 1 µL DNA template (20-50 ng/µL), and 0.4 µL Taq DNA polymerase (5 U/µL) (Fermentas, USA).

PCR was done using a GeneAmp PCR system 9700 (Applied Biosystems) with PCR conditions as follows: initial denaturation of double-stranded DNA for 5 min at 94°C; 35 cycles each consisting of denaturation for 30 s at 94°C, 20 s annealing at 56°C and 1 min extension at 72°C; and extension of incomplete products for 7 min at 72°C followed by cooling at 4°C. The PCR products were sent to GATC Biotech (http://www.gatc-biotech.com) in Germany for purification and sequencing.

DNA extraction from isolated yeasts and moulds

Cultures were grown in malt extract broth at 30°C for 1 day for yeasts and at 25°C for 2 days for moulds, and then centrifuged at 6000 rpm for 10 min at 4°C, followed by washing of pellets with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $NaH_2PO_4 \cdot 7\ H_2O$, 1.4 mM KH_2PO_4) and again centrifuged as aforementioned. After the second centrifugation, the pellets were resuspended in 0.5 ml PBS buffer and transferred into 2 ml Eppendorf tubes containing 0.5 g zirconia/silica beads (diameter 0.1 mm; Biospec products, Inc). Furthermore, 0.5 ml hexadecyltrimethylammonium bromide (CTAB) extraction buffer (5% CTAB in 0.35 M NaCl with 120 mM potassium phosphate buffer) and 0.5 ml phenol-chloroform-isoamyl alcohol (25:24:1) was added and tubes were beaten at maximum speed twice for 45 s using Mini beadbeater-8, Biospec, Bartlesville, USA. Afterwards the tubes were centrifuged at 13000 rpm for 5 min at 4°C using a microcentrifuge (Biofuge fresco, Heraeus) to separate the aqueous phase which contained nucleic acids. An equal volume of chloroform-isoamyl alcohol (24:1) was added to remove the phenol, followed by centrifugation at 13000 rpm for 5 min at 4°C.

To precipitate the total nucleic acids two volumes ice-cold (-20°C) isopropanol were added and stored at -80°C for 1 h. After centrifugation at 13000 rpm for 10 min at 4°C nucleic acids were washed using ice-cold 70% (v/v) ethanol and air dried. Nucleic acids were finally resuspended in 50 µL TE buffer (10mM Tris, 1mM EDTA, pH 7.4) and stored at -20°C.

Amplification and sequencing of fungal DNA

The DNA isolated from fungi was used to amplify the 26S rDNA using forward primer V9G (5′-TTACGTCCCTGCCCTTTGTA-3′) and reverse primer LS266 (5′-GCATTCCCAAACAACTCGACTC-3′) (van den Ende and de Hoog, 1999). PCR was performed with a total reaction volume of 50 µL containing 26.6 µL ddH_2O, 5 µL PCR buffer, 3 µL $MgCl_2$ (25 mM), 10 µL dNTP (2 mM), 2 µL of each primer (10 µM), 1 µL DNA template (20 to 50 ng/µL), and 0.4 µL Taq DNA polymerase (5 U/µL) (Fermentas, USA). PCR was done using GeneAmp PCR system 9700 (Applied Biosystems) with PCR conditions as follows: initial denaturation for 5 min at 95°C followed by 35 cycles comprising denaturation at 95°C for 60 s, annealing was at 52°C for 45 s, extension at 72°C for 60 s, and final 7 min extension at 72°C followed by cooling to 4°C. The PCR products were sent to GATC Biotech (http://www.gatc-biotech.com) in Germany for purification and sequencing.

Direct extraction of bacterial DNA from *Tayohounta samples*

Four grams of sample were suspended in 30 ml of PBS buffer (57.7 mM Na_2HPO_4, 42.3 mM NaH_2PO_4) and DNA was extracted according to Wang et al. (2008). From the DNA extracted directly from *Tayohounta*, the V6-V8 region of the 16S rDNA was amplified using the universal forward primer EUB-968-GC-for (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA GGG GAA CGC GAA GAA CCT TAC-3') and reverse primer EUBL1401-rev (5'-CGG TGT GTA CAA GAC CC-3) (Engelen et al., 1995) for DGGE analysis.

Denaturing gradient gel electrophoresis

PCR products were analyzed by DGGE as described by Wang et al. (2008); 4 to 10 µg DNA was applied and a range of 30 to 60% denaturant was used. The gel was run for 10 min at 200 V and 60°C, followed by 16 h at 85 V and 60°C. Then, the voltage was switched down to 10 V until the gel was removed from the buffer. Silver staining was done according to Sanguinetti et al. (1994). The dried gel was scanned on a GS 800 calibrated densitometer (BioRad). Bionumerics software (Applied Maths) was used to normalise the gel and analyse band positions. Band positions from the pure cultures were compared to the pattern given by mixtures of the pure cultures and of the original samples.

Cloning of PCR products

Identification of microbial strains was obtained by cloning 16s rDNA PCR fragments in *Escherichia coli* according to Röling et al. (2001).

DNA sequence analysis

Obtained sequences were viewed for errors using Chromas Lite v2.01 software (http://www.technelysium.com.au/) and if necessary corrected manually with the IUB/IUPAC nucleic acid code. Afterwards the forward and reverse sequence were assembled to form a contig using the program Seqman II (DNAStar Inc. USA, version 5.08) and exported to FASTA format. These files were used to obtain strain identities by using the nucleotide BLAST program from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The most probable match with a 16S rDNA sequence was selected based on the percentage of identification (%ID) and taxonomy report.

Laboratory-scale fermentation of Baobab seeds: Functional properties

A selection of microorganisms isolated from *Tayohounta* was

Table 1. Microbiota composition of Tayohounta made by three individual processors.

Parameter (Log cfu/g)	T 1*	T 2	T 3
Total viable aerobic count	9.0 ± 0.1^{a}	8.8 ± 0.1^{a}	8.7 ± 0.1^{a}
Bacterial spores	8.0 ± 0.0^{b}	8.5 ± 0.1^{a}	8.1 ± 0.0^{b}
Lactic acid bacteria	7.7 ± 0.0^{c}	8.5 ± 0.0^{a}	8.2 ± 0.0^{b}
Micrococci	7.2 ± 0.1^{a}	5.1 ± 0.1^{b}	5.3 ± 0.2^{b}
Enterobacteriaceae	6.0 ± 0.4^{a}	7.0 ± 0.0^{a}	$< 1^{b}$
Yeasts	7.2 ± 0.0^{a}	3.1 ± 0.1^{c}	5.9 ± 0.0^{b}
Moulds	2.9 ± 0.1^{a}	2.4 ± 0.1^{b}	$< 1^{c}$

*T1, T2, T3 = Tayohounta made by Processors 1, 2 and 3, respectively. For each group of microorganisms, samples with different letters are significantly different ($p < 0.05$). Means ± standard deviation.

inoculated on sterile baobab seed kernels and their effect was measured. A quantity of 1000 g dry seed kernels was soaked in 2 L of tap water overnight at 25°C. After pouring off the water, 50 g aliquots of the soaked kernels were transferred into 500 ml glass jars and sterilized by autoclaving at 121°C for 30 min. The seeds were inoculated with pure cultures, pre-grown for 48 h at 30°C in tubes containing 10 ml of nutrient broth (*Bacillus*), MRS broth (LAB) and malt extract broth (MEB; Oxoid CM57) (yeasts and moulds). One millilitre of a 10^{-1} dilution in PPS was used to inoculate each jar of kernels, and these were incubated at 30°C for 0 h (unfermented control) and 48 h.

Sample analyses

Microbial growth

Each sample of fermented kernels was analyzed as earlier described under “enumerations”.

Terminal amino nitrogen and pH

10 g sample was mixed with 90 g water and made into a suspension with a blender. This suspension (A) was used to measure pH and to perform the formol titration (Han et al., 1999) for terminal amino groups, which were expressed as mM/ g sample.

Volatile flavour

Of each fermented or non-fermented sample the flavour was examined by sniffing unfermented as well as fermented seeds. This was done by 3 untrained panellists.

RESULTS AND DISCUSSION

Predominant groups of microorganisms

Table 1 shows that the total viable aerobic count (TVC) in all samples was quite high around 9 log cfu/g. High numbers were expected because there was no process step performed on the samples which would decrease the number of microorganisms after fermentation. The groups of lactic acid bacteria (LAB) and bacterial spores with counts around 8 log cfu/g, represented a large part of the TVC, but considering the high number of spores it is probable that the remaining TVC consisted mainly of vegetative cells of *Bacillus* spp. Enterobacteriaceae counts were around 6 log cfu/g in T1 and T2 and below detection level in T3. Yeasts were present in considerable numbers in product T1 and T3, and less in product T2, while moulds represented only a minor part of the total flora.

Similarly, high numbers of aerobic mesophilic bacteria and *Bacillus* spp. (9 to 10 log cfu/g each) were also found in *afintin* and *sonru* made from African locust beans (*P. biglobosa*) while Enterobacteriaceae were not detected (Azokpota et al., 2006). This was also the case for *iru* (8.9 to 9.5 log cfu/g of TVC and 8.6 to 9.4 log cfu/g of spore-forming bacteria) made from African locust beans (*P. biglobosa*) (Azokpota et al., 2006; Sanni et al., 2000); *Ogiri* (8.5 to 9.2 log cfu/g of TVC and 8.4 to 9.0 log cfu/g of spore-forming bacteria) made from melon seeds (*Citrullus* spp.) or castor seeds (*R. communis*); and *Ugba* (7.6 to 8.8 log cfu/g of TVC and 7.6 to 8.7 log cfu/g of spore-forming bacteria) made from African oil bean seeds (*P. macrophylla* Benth) (Sanni et al., 2000). In *dawadawa* made from soya bean (*G. max*), TVC was up to 11 log cfu/g; LAB was 3 to 6 log cfu/g and the load of yeasts which had increased in the first 48 h of fermentation was very low at the end of fermentation (not detected on the 10^{-2} dilution plates) (Dakwa et al., 2005). Count of yeasts was also found to be low in *Dikouanyouri* (fermented baobab whole seeds) (Chadare et al., 2010) and in *Otiru* from African yam bean (*S. sternocarpa* Harms) (Jeff-Agboola, 2007). *Tayohounta* which has extended the list of African fermented seeds products is thus comparable to most plant seeds products for its high load of TVC and bacterial spores. Some discrepancies are however noticed especially with respect to the relatively high load of LAB also noticed in Maari, a fermented baobab whole seeds from Burkina Faso (Parkouda et al., 2010) and variable numbers of yeasts. Substrate depletion by the rapid growth and high numbers of bacteria, as well as formation of metabolites such as ammonia might retard the growth of yeasts and moulds present. As shown in a study on silage storage with various treatments, numbers of yeasts and moulds declined quickly in silage treated with ammonia (Kung et al., 2000). Reduced oxygen

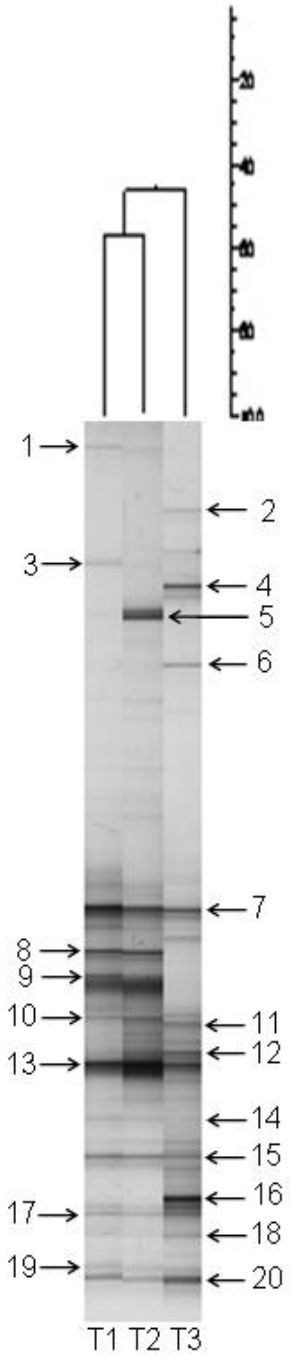

Figure 1. Annotated PCR-DGGE from direct extraction of *Tayohounta* made by 3 processors. T1, T2, T3 = *Tayohounta* from processor 1, 2 and 3. Phylogenetic tree from Bionumerics and neighbouring method. Lane 1: *Pediococcus pentosaceus*; lane 2: *Streptococcus* sp.; lane 3: *Streptococcus equinus;* lane 4: *Bacillus thuringiensis*; lane 5: *Streptococcus equinus*; lane 6: *Lactobacillus agilis*; lane 7: *Bacillus pumilus*; lane 8: *Weissella confusa*; lane 9: *Bacillus licheniformis*; lane 10: *Pediococcus pentosaceus*; lane 11: *Brevibacillus borstelensis*; lane 12: *Enterococcus casseliflavus*; lane 13: *Bacillus subtilis*; lane 14: *Enterococcus durans*; lane 15: *Bacillus subtilis*; lane 16: *Bacillus subtilis*; lane 17: *Lactobacillus fermentum*; lane 18: *Bacillus licheniformis*; lane 19: *Bacillus licheniformis*; lane 20: *Bacillus thermoamylovorans*.

tension encountered in solid substrate fermentation may reduce mould growth (Jeff-Agboola, 2007).

Since no starter is added after the kernels are boiled, likely origins of the microorganisms observed include kitchen utensils, cover leaves and other environmental sources (soil, water and air). Indeed, generally, the seeds are spontaneously fermented by microorganisms indigenous to the preparation site (Odunfa, 1981). It can also be expected that some spores were still viable after the long cooking procedure which correspond to moist heat treatment (Coleman and Setlow, 2009; Melly et al., 2002) or that the spores had formed clumps which increase their heat resistance (Furukawa et al., 2005). This long cooking process constitutes a selective step for heat resistant bacteria such as *Bacillus* sp. as the dominant microorganisms in such products (Ouoba et al., 2004). In addition, the cooking eliminates non spore-forming (pathogenic) bacteria rendering the plant seed fermented products somehow safe for human consumption (Parkouda et al., 2009).

Bacteria encountered in *Tayohounta*

Figure 1 shows a DGGE profile of the 3 products obtained after amplification of the V6-V8 region of the bacterial 16S rDNA obtained directly from the samples. Table 2 also shows the bacteria identified in *Tayohounta* based on sequencing 16S rDNA of isolated pure cultures. Both Figure 1 and Table 2 showed that although all 3 products contain a different microbiota, products T1 and T2 show more similarity than product T3.

Sequencing of clones of PCR products of bacterial DNA directly extracted from *Tayohounta* revealed that in all products, *B. licheniformis*, *B. pumilus*, *B. subtilis*, *B. thermoamylovorans* and *L. fermentum* were present. Other microorganisms were present occasionally; these were *B. thuringiensis* (T3), *B. borstelensis* (T3), *Enterococcus casseliflavus* (T2, T3), *E. durans* (T1, T2), *Lb. agilis* (T3), *P. pentosaceus* (T1, T2), *S. equinus* (T1, T2), and *W. confusa* (T1, T2). Sequencing of 16S rDNA based on isolated pure culture shows in addition to the aforementioned microorganisms, the presence of *Enterobacter cloacae* (T2), *Enterococcus faecium* (T2), *Enterococcus italicus* (T3), *Jeotgalicoccus halotolerans* (T3), *Klebsiella pneumoniae* (T1), and *Staphylococcus aureus* (T2). Moreover, previous research on similar products identified *B. subtilis* as dominating micro-organism within the *Bacillus* group, sometimes even responsible for 50% of the total *Bacillus spp.* count and often accompanied by *B. licheniformis, B. pumilus* and *B. cereus* (Azokpota et al., 2006; Dakwa et al., 2005; Omafuvbe et al., 1999).

Bacillus species were indeed reported as dominant in African fermented seeds such as *Ugba* produced by alkaline fermentation of African oil bean (*P. macrophylla*) (Isu and Ofuya, 2000); *Aisa* produced by alkaline

Table 2. Identification of bacteria in *Tayohounta* made by 3 processors, based on sequencing 16S rDNA of isolated pure cultures.

Group	Name	T1*	T2	T3
Spore-forming bacteria	*Bacillus subtilis*	+	+	+
	Bacillus licheniformis	-	-	+
	Bacillus pumilus	+	+	-
	Bacillus cereus	+	-	-
Enterobacteriaceae	*Enterobacter cloacae*	-	+	-
	Klebsiella pneumoniae	+	-	-
Lactic acid bacteria	*Lactobacillus fermentum*	-	-	+
	Pediococcus pentosaceus	+	+	-
	Weissella confusa	+	-	-
	Enterococcus italicus	-	-	+
	Enterococcus casseliflavus	-	+	--
	Enterococcus faecium	-	+	-
Micrococci	*Staphylococcus aureus*	-	+	-
	Jeotgalicoccus halotolerans	-	-	+

* T1, T2, T3 = Tayohounta made by Processors 1, 2 and 3, respectively; + indicate the presence of species; - indicate the absence of species.

fermentation of *Albizia saman* seeds (Ogunshe et al., 2006); *Bikalga*, a product of alkaline fermentation of *Hibiscus sabdariffa* L. seeds (Ouoba et al., 2008). It was also the case for Asian fermented foods product of soybean fermentation for example, *Kinema* (Sarkar et al., 2002); Thua-nao (Inatsu et al., 2006). Heat resistance of *B. subtilis* spores has been investigated by Fox and Eder (1969) and Warth (1978), who found that the decimal reduction time at 100°C (D_{100}) was in the order of magnitude of 10 min. Considering that the boiling step during the process can take up to half an hour, it is quite possible that certain *Bacillus* spores can survive the boiling procedure when present in high numbers. In addition, since there were differences in appearance and smell between the *Tayohounta* from the 3 different producers, it can be expected that in addition to *B. subtilis,* other microorganisms play a role during fermentation of the seed kernels as well. A different flora of the environment and leaves or on the skin of the producers and used utensils is suggested as probable causes for variability of microbiota (usually indigenous to production site as suggested by Odunfa (1981)) of *Tayohounta* from the different producers.

It will be interesting to investigate the microbial succession during the fermentation of baobab seeds kernels to obtain a more detailed understanding of the evolution of the microbiota; this may also help to assess the role of specific microorganisms for example flavour development of *Tayohounta*; in the preparation of *Maari,* a fermented whole baobab seed from Burkina Faso, fermentation was initiated by *B. subtilis* and *Staphylococcus sciuri*; no lactic acid bacteria were isolated at the beginning of the process; still, after 24h of fermentation *E. faecium* appeared in the fermenting seeds and remained until the end of the fermentation as the predominant LAB (Parkouda et al., 2010). A comparison of both processes and the microbial evolution during the fermentation can give some insight into the critical steps that affect microbiota development and related product quality.

Functionality

Visual inspection of the products prior to analysis revealed that T1 and T2 still had the structure of intact seed kernels with a dry or dull surface. On the other hand, in T3, the kernel structure had decomposed and the product had a moist appearance. Also the flavour of T3 was much more pungent than that of T1 and T2.

Table 3 presents the growth and impact of pure isolated strains from *Tayohounta*, on cooked and sterilized baobab seed kernels. All tested strains showed good growth within 48 h of incubation. Only a subset of isolates was tested for their effect on quality attributes of fermented kernels. *B. subtilis* showed only little effect on pH, but it degraded protein and caused a typical pungent smell reminding of *Tayohounta*. *Klebsiella* and *Enterobacter* spp. had no effect on pH or protein, but *Pediococcus* sp. caused a slight decrease in pH, slight degradation of protein, and minor change of smell. A strong and rather unpleasant smell was caused by *Aspergillus* sp. and two unidentified yeast isolates.

Table 3. Growth and impact of selected pure isolates from Tayohounta, during fermentation at 30ºC of cooked baobab seed kernels.

Species	Code	Log cfu g^{-1}		pH		Terminal Amino NH_4^+ (mmol g^{-1} dry matter)			Smell	
		t = 0 h	t = 48 h	t = 0 h	t = 48 h	t = 0 h	t = 48 h	Increase factor	t = 0 h	t = 48 h
Bacillus subtilis	*Aba*	6.3	8.8	5.8	5.9	0.26	0.62	2.4	Cooked pasta	Pungent, tayohounta-like
Bacillus subtilis	*Bba*	6.3	9.2	5.8	5.9	0.24	0.90	3.8	Cooked pasta	Pungent, but less strong than *Aba*
Klebsiella pneumoniae	*16bs*	6.3	8.2	5.8	5.9	0.24	0.24	1.0	Cooked pasta	Wet bread
Enterobacter cloacae	*17bs*	6.3	8.1	5.8	5.9	0.28	0.29	1.0	Cooked pasta	Wet bread
Pediococcus sp.	*3LAB*	6.3	7.5	5.8	5.7	0.22	0.40	1.8	Cooked pasta	Acidic beans
Pediococcus sp.	*4LAB*	6.3	7.6	5.8	5.8	0.26	0.36	1.4	Cooked pasta	Wet bread
Aspergillus sp.	*9ym*	4.3	7.3	5.8	6.1	0.24	0.52	2.2	Cooked pasta	Unpleasant, smoky nuts
Unidentified yeast	*10ym*	5.3	7.3	5.8	6.6	0.26	0.44	2.0	Cooked pasta	Unpleasant, less strong than *9ym*
Unidentified yeast	*Dym*	5.3	7.1	5.8	6.4	0.22	0.46	2.1	Cooked pasta	Unpleasant, rotting sour cream

Moreover, in the fermentation of soybeans by *Bacillus* strains for preparation of *Soumbala* and *Kinema*, a correlation was observed between increasing pH and level of terminal amino-N (Sarkar et al., 2002). In the present study, though *Bacillus* strains were able to degrade protein, increase of free amino-N was not significantly correlated with increasing pH in the fermentation of baobab kernels. The formation of volatiles by yeasts and moulds may find its origin in the degradation of lipids, resulting in free fatty acids and associated esters.

The present findings are still of an exploratory nature. More research on flavour production should include the use of objective analytical methods such as SPME-GC-MS (Plutowska and Wardencki, 2007). In addition, more microbial strains should be tested, either single or in combination to obtain a better understanding of their importance as fermentation starters. It is concluded that there is evidence of considerable microbial and quality diversity of *Tayohounta* made by different traditional processors even when using the same batch of seed kernels but different utensils and other ingredients such as plant leaf covers. Indeed, the type of leaves used for fermentation was reported to affect both the sensory and nutritional quality of *dawadawa* (Gernah et al., 2007). This needs further investigations. More also, similar to most fermented seed products (*afitin*, *iru*, *sonru*, *dawadawa*, etc.), the preponderance of *B. subtilis* and other *Bacillus* species in *Tayohounta* is evident, whatever the process/producer. A further study of the functionality of *Bacillus* species in *Tayohounta* quality is required prior to starter culture

development.

REFERENCES

Addy R (2009). Baobab fruit approved as food ingredient in US Food Navigator.com. http://www.foodnavigator.com/Publications/Food-Beverage-Nutrition/NutraIngredients-USA.com/Industry/Baobab-fruit-approved-as-food-ingredient-in-US.

Agati V, Guyot JP, Morlon-Guyot J, Talamond P, Hounhouigan DJ (1998). Isolation and characterization of new amylolytic strains of *Lactobacillus fermentum* from fermented maize doughs (mawe and ogi) from Benin. J. Appl. Microbiol. 85(3): 512-520.

Ajayi LA, Dawodu FA, Oderinde RA (2003). Fatty acid composition and metal content of *Adansonia digitata* seeds and seed oil. La Rivisita Italiana Delle Sosteanze Grasse. 80: 41-43.

Assogbadjo A, Glele Kakai R, Chadare F, Thomson L, Kyndt T, Sinsin B, Van Damme P (2008). Folk Classification, Perception, and Preferences of Baobab Products in West Africa: Consequences for Species Conservation and Improvement. Econ Bot. doi:10.1007/s12231-007-9003-6. 62(1): 74-84.

Assogbadjo AE, Kyndt T, Sinsin B, Gheysen G, Van Damme P (2006). Patterns of genetic and morphometric diversity in baobab (*Adansonia digitata*) populations across different climatic zones of Benin (west Africa). Ann. Bot. 97: 819-830.

Assogbadjo AE, Sinsin B, Codjia JTC, Van Damme P (2005). Ecological diversity and pulp, seeds and kernels production of the baobab (Adansonia digitata) in Benin. Belg. J. Bot. 138(1): 47-56.

Azokpota P, Hounhouigan DJ, Nago MC (2006). Microbiological and chemical changes during the fermentation of African locust bean (*Parkia biglobosa*) to produce *afitin*, *iru* and *sonru*, three traditional condiments produced in Benin. Int. J. Food Microbiol. 107: 304-309.

Azokpota P, Hounhouigan J, Annan N, Nago M, Jakobsen M (2008). Diversity of volatile compounds of *afitin*, *iru* and *sonru*, three fermented food condiments from Benin. . World J. Microbiol. Biotechnol. 24: 879-885.

Buchmann C, Prehsler S, Hartl A, Vogl CR (2010). The Importance of Baobab (*Adansonia digitata* L.) in Rural West African Subsistence. Suggestion of a Cautionary Approach to International Market Export of Baobab Fruits. Ecol. Food Nutr. 49(3): 145-172.

Chadare FJ (2010). Baobab foods from Benin: Composition, processing and quality. PhD thesis, Wageningen University, Wageningen, The Netherlands.

Chadare FJ, Gayet DP, Azokpota P, Nout MJR, Linnemann AR, Hounhouigan J, van Boekel MAJS (2010). Three undocumented fermented baobab foods from Benin: *Mutchayan*, *Dikouanyouri* and *Tayohounta*. Preparation, properties and consumption. Ecol. Food Nutr. 49(4): 1-19.

Chadare FJ, Hounhouigan JD, Linnemann AR, Nout MJR, van Boekel MAJS (2008). Indigenous knowledge and processing of *Adansonia digitata* L. food products in Benin. Ecol. Food Nutr. 47(4): 338-362.

Chadare FJ, Linnemann AR, Hounhouigan JD, Nout MJR, van Boekel MAJS (2009). Baobab food products: a review on their composition and nutritional value. Crit. Rev. Food Sci. 49: 254-274 .

Codjia JTC, Fonton-Kiki B, Assogbadjo AE, Ekue MRM (2001). Le baobab (Adansonia digitata) Une espèce à usage multiple au Bénin. Coco Multimédia Cotonou, Bénin.

Coleman WH, Setlow P (2009). Analysis of damage due to moist heat treatment of spores of *Bacillus subtilis*. J. Appl. Microbiol. 106(5): 1600-1607.

Dakwa S, Sakyi-Dawson E, Diako C, Annan NT, Amoa-Awua WK (2005). Effect of boiling and roasting on the fermentation of soybeans into dawadawa (soy-dawadawa). Int. J. Food Microbiol. 104(1): 69-82.

De Caluwe E (2005). Etnobotanie, morfologische variabiliteit en natuurlijke regeneratie van *Adansonia digitata* in Atacora (Benin). MSc. Thesis, University of Ghent, Belgium,

De Caluwé E, De Smedt S, Assogbadjo AE, Samson R, Sinsin B, Van Damme P (2009). Ethnic differences in use value and use patterns of baobab (*Adansonia digitata* L.) in northern Benin. Afr. J. Ecol. 47(3): 433-440

Engelen B, Heuer H, Felske A, Nübel U, Smalla K, Backhaus H (1995). Protocols for the TGGE. Abstracts for the Workshop on Application of DGGE *and* TGGE in Microb Ecol 1995. BBA for Agriculture and Forestry, Braunschweig.

Furukawa S, Narisawa N, Watanabe T, Kawarai T, Myozen K, Okazaki S, Ogihara H, Yamasaki M (2005). Formation of the spore clumps during heat treatment increases the heat resistance of bacterial spores. Int. J. Food Microbiol. 102(1): 107-111.

Gernah DI, Inyang CU, Ezeora NL (2007). Incubation and fermentation of African locust beans. *Parkia biglobosa*, in production of "*dawadawa*" J. Food Process Preserv. 31: 227-239.

Han BZ, Kiers JL, Nout MJR (1999). Solid-substrate fermentation of soybeans with *Rhizopus* spp.: comparison of discontinuous rotation with stationary bed fermentation. J. Biosci. Bioeng. 88(2): 205-209.

Hounhouigan DJ, Nout MJR, Nago CM, Houben JH, Rombouts FM (1993). Changes in the physico-chemical properties of maize during natural fermentation of mawe. J. Cereal Sci. 17(3): 291-300.

Inatsu Y, Nakamura N, Yuriko Y, Fushimi T, Watanasiritum L, Kawamoto S (2006). Characterization of *Bacillus subtilis* strains in *Thua nao*, a traditional fermented soybean food in Northern Thailand, Lett. Appl. Microbiol. 43: 237-242.

Isu NR, Ofuya CO (2000). Improvement of the traditional processing and fermentation of African oil bean *Pentaclethra macrophylla* Bentham, into a food snack '*ugba*'. Int. J. Food Microbiol. 59: 235-239.

Jeff-Agboola YA (2007). Microorganisms associated with natural fermentation of African yam bean (*Sphenostylis sternocarpa* Harms) seeds for the production of otiru. Res. J. Microb. 2(11): 816-823.

Kung L, Robinson JR, Ranjit NK, Chen JH, Golt CM, Pesek JD (2000). Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. J. Dairy Sci. 83(7): 1479-1486

Leejeerajumnean A, Duckham SC, Owens JD, Ames JM (2001). Volatile compounds in *Bacillus*-fermented soybeans. J. Sci. Food Agr. 81: 525-529.

Melly E, Genest PC, Gilmore ME, Little S, Popham DL, Driks A, Setlow P (2002). Analysis of the properties of spores of *Bacillus subtilis* prepared at different temperatures. J. Appl. Microbiol. 92(6): 1105-1115.

Michodjehoun-Mestres L, Hounhouigan DJ, Dossou J, Mestres C (2005). Physical, chemical and microbiological changes during natural fermentation of "gowe", a sprouted or non sprouted sorghum beverage from West-Africa. Afr. J. Biotechnol. 4(6): 487-496.

Nordeide MB, Hatloy A, Folling M, Lied E, Oshaug A (1996). Nutrient composition and nutritional importance of green leaves and wild food resources in an agricultural district, Koutiala, in Southern Mali. Int. J. Food Sci. Nutr. 47(6): 455-468.

Nour AA, Magboul BI, Kheiri NH (1980). Chemical Composition of baobab fruit (*Adansonia digitata*). Trop. Sci. 22(4): 383-388.

Nout M (1991). Ecology of accelerated natural lactic fermentation of sorghum-based infant food formulas. Int. J. Food Microbiol. 12: 217-224.

Odunfa SA (1981). Microorganisms associated with fermentation of African locust bean (*Parkia filicoidea*) during iru preparation. J. Plant Food. 3: 245-250

Ogunshe AAO, Ayodele AE, Okonko IO (2006). Microbial studies on *aisa*: a potential indigenous laboratory fermented food condiment from *Albizia saman* Jacq, F Mull Pakistan J. Nutr. 5: 51-58.

Omafuvbe BO, Abiose SH, Adaraloye OO (1999). The production of 'Kpaye' - a fermented condiment from *Prosopis africana* (Guill and Perr) Taub. seeds. Int. J. Food Microbiol. 51(2-3): 183-186

Oomes S, van Zuijlen A, Hehenkamp J, Witsenboer H, van der Vossem J, Brul S (2007). The characterisation of *Bacillus* spores occurring in the manufacturing of (low acid) canned products. Int. J. Food Microbiol. 120: 85-94.

Osman MA (2004). Chemical and nutrient analysis of baobab (*Adansonia digitata*) fruit and seed protein solubility. Plant Food Hum. Nutr. 59(1): 29-33.

Ouoba LII, Diawara B, Amoa-Awua WK, Traore AS, Moller PL (2004). Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for fermentation of African locust bean Parkia biglobosa, to produce soumbala. Int. J. Food Microbiol. 90: 197-205.

Ouoba LII, Diawara B, Annan NT, Poll L, Jakobsen M (2005). Volatile compounds of Soumbala, a fermented African locust bean (*Parkia biglobosa*) food condiment. J. Appl. Microbiol. 99(6): 1413-1421.

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

Parkouda C, Nielsen DS, Azokpota P, Ouoba LII, Amoa-Awua WK, Thorsen L, Hounhouigan JD, Jensen JS, Tano-Debrah K, Diawara B, Jakobsen M (2009). The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia. Crit. Rev. Microbiol. 35: 139-156.

Parkouda C, Thorsen L, Compaoré CS, Nielsen DS, Tano-Debrah K, Jensen JS, Diawara B, Jakobsen M (2010). Microorganisms associated with Maari, a baobab seed fermented product. Int. J. Food Microbiol. 142(3): 292-301.

Plutowska B, Wardencki W (2007). Aromagrams - Aromatic profiles in the appreciation of food quality. Food Chem. 101(2): 845-872.

Sanguinetti C, Neto E, Simpson A (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels BioTechniques, 17: 915-919.

Sanni AI, Ayernor GS, Sakyi-Dawson E, Sefa-Dedeh S (2000). Aerobic spore-forming bacteria and chemical composition of some Nigerian fermented soup condiments. Plant Food Hum. Nutr. 55(2): 111-118.

Sarkar PK, Hasenack B, Nout MJR (2002). Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala). Int. J. Food Microbiol. 77(3): 175-186.

Sidibe M, Williams JT (2002). Baobab: *Adansonia digitata* International center for underutilised crops, Southampton, UK.

Steinkraus KH (1995). Handbook of indigenous fermented foods 2nd edition, revised and expanded edn. Marcel Dekker, Inc., New York.

The Commission of the European Communities (2008). COMMISSION DECISION of 27 June 2008 authorising the placing on the market of Baobab dried fruit pulp as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council. Official J. Eur. Union, pp. 38-39.

van den Ende AHGG, de Hoog GS (1999). Variability and molecular diagnostics of the neurotropic species *Cladophialophora bantiana*. Stud. Mycol. 43: 151-162.