Food Sci. Biotechnol. 25(4): 1081-1086 (2016)
DOI 10.1007/s10068-016-0174-8

Food Science
and
Biotechnology

Molecular analysis of bacterial community dynamics during the fermentation of *soy-daddawa* condiment

Obinna Ezeokoli[1,2,3], Arvind Gupta[1], Temitope Popoola[2], and Cornelius Bezuidenhout[1,*]

[1]*Unit of Environmental Sciences and Management, North-West University, Potchefstroom Campus, 2520, South Africa*
[2]*Department of Microbiology, Federal University of Agriculture, PMB 2240, Abeokuta, Nigeria*
[3]*Microbiology and Environmental Biotechnology Research Group, Agricultural Research Council-Institute for Soil, Climate and Water, Pretoria, 0001, South Africa*

Received November 27, 2015
Revised May 1, 2016
Accepted May 11, 2016
Published online August 31, 2016

*Corresponding Author
Tel: +27-0-824940221
Fax: +27-18-2992330
E-mail: carlos.bezuidenhout@nwu.ac.za

pISSN 1226-7708
eISSN 2092-6456

Abstract Bacterial community dynamics during *soy-daddawa* fermentation was investigated using culture-dependent and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) molecular methods. The total titratable acidity (TTA), pH, and bacterial counts (BCs) were monitored daily during a 72-h fermentation period. Bacteria were characterized based on 16S rRNA gene sequencing. TTA ranged from 0.08 to 0.26 mg lactic acid/g, whereas pH ranged from 7.01 to 8.19. BCs increased from 3.9 to 10.61 log CFU/g. Fifty-eight isolates were obtained by culture method and clustered into seven operational taxonomic units (OTUs) at 97% sequence similarity, whereas four OTUs were obtained from the PCR-DGGE method. Taxonomic identification revealed that bacteria belonged to the genera *Bacillus*, *Enterobacter*, *Enterococcus*, and *Staphylococcus* with *B. subtilis* being present throughout fermentation. Medically significant isolates, including *B. anthracis*, *Enterococcus casseliflavus*, and *Enterobacter hormaechei* were detected. These results emphasize the need for starter culture utilization and offer a platform for starter culture screening and selection.

Keywords: *Soy-daddawa*, fermented condiment, bacteria, 16S rRNA, PCR-DGGE

Introduction

Soy-daddawa is a fermented soybean (*Glycine max* (L.) Merr.) food condiment commonly used in the preparation of traditional dishes in Nigeria (1-3). As a condiment, it basically serves to improve the flavor of traditional diets (4) but can also contribute essential nutrients (5). The fermentation of soybean seeds for *soy-daddawa* production involves a number of biochemical changes, which are predisposing factors for bacterial population dynamics. Hence, investigation of the bacterial population dynamics of *soy-daddawa* is of interest to understand the bacterial consortia interactions involved in the fermentation of the product, and ultimately, for starter culture(s) screening, selection, and application.

To this end, a number of studies have investigated the microbial ecology of *soy-daddawa* (6) and the microbial dynamics during *soy-daddawa* fermentation (1,7). A few studies have also explored the use of starter cultures in the production of *soy-daddawa* condiment (8). However, these previous studies on the bacterial community dynamics and identification of starter cultures have been based on classical phenotypic methods, which could be misleading when relied upon independently (9,10). It suffices that a reliable identification and selection criteria for potential starter cultures be of utmost importance. Hence, a polyphasic approach, involving both phenotypic and genotypic methods for identification of potential starter cultures is essential. However, there is a paucity of genotypic-based studies involving the use of molecular tools to characterize bacterial communities during *soy-daddawa* fermentation.

Furthermore, molecular assessments of microbial communities are performed using a culture-dependent and culture-independent approach. The culture-independent approach, when used alongside the culture-dependent approach, helps to detect the presence of microbial species, which are largely uncultivable on currently available media formulations (11-13). The culture-independent technique PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is a relatively fast fingerprinting technique to profile microbial communities. PCR-DGGE has been successfully applied in estimating the bacterial structure of several fermented foods, including dairy products (11), soybean pastes (14), sausages (15), and beverages (16). However, there is a paucity of molecular based studies on the bacterial dynamics during *soy-daddawa* fermentation. This study aimed at applying both culture-dependent and PCR-DGGE techniques in assessing the bacterial dynamics during the spontaneous fermentation of *soy-daddawa*.

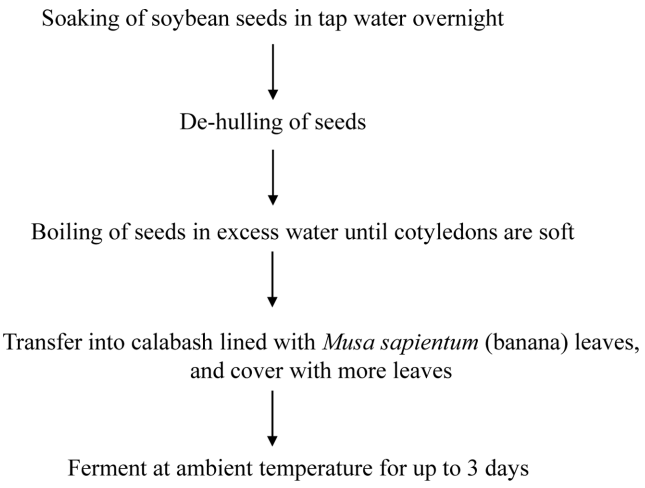

Fig. 1. Flow chart for *soy-daddawa* production. De-hulling of seeds was done manually by gently crushing of seeds between the palms. The duration of the boiling step was approximately 2 h. The prevailing ambient temperature during the 72 h fermentation period averaged 29±1°C.

Materials and Methods

Production of *soy-daddawa* Soybean (*Glycine max* (L.) Merr.) seeds used in this study were obtained from the Institute of Agricultural Research and Training (IART), Ibadan, Nigeria. Production of *soy-daddawa* was done by following the method described by Popoola and Akueshi (2) (Fig. 1) in three independent (replicates) small-scale bioreactors at prevalent ambient temperature. Sampling of the fermentation substrate was done aseptically at 24 h intervals over a 72 h period.

Isolation of bacteria during *soy-daddawa* fermentation Ten-fold serial dilutions of crushed *soy-daddawa* samples in maximum recovery diluent (0.85% NaCl, 1% peptone) were prepared and spread-plated on tryptone soy agar (TSA) (BioLab, Budapest, Hungary). Following incubation of plates at 35°C for 24-48 h, bacterial counts (BCs) were enumerated and expressed as colony forming units (CFUs). Distinct colonies morphotypes were picked, streaked, and successively sub-cultured on TSA to obtain pure cultures.

Determination of pH and titratable acidity For pH determination, samples were homogenized 1:10 in distilled water (7). pH was then determined using a precalibrated pH meter (Basic 20; Crison Instruments, Barcelona, Spain). Total titratable acidity (TTA) was determined by titrating a one-tenth dilution of the sample in distilled water against 0.1 N NaOH and using phenolphthalein solution (1%, w/v) as the indicator (17). One milliliter of 0.1 N NaOH was taken to be equivalent to 9.008×10^3 g lactic acid. TTA was expressed as milligram lactic acid per gram of sample.

DNA Extraction Two milliliters of an overnight growth of pure isolates in Luria and Bertani broth was centrifuged at 8,117 x *g* for 2 min (MiniSpin; Eppendorf Ag, Hamburg, Germany) to harvest cells. The NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) was used to extract DNA from cell pellets after cell wall lysis in lysozyme (10 mg/mL) by following the manufacturer's protocol. For culture-independent analysis, the method described by Kim *et al*. (14) was followed to dislodge bacterial cells from samples. In brief, 5 g of *soy-daddawa* sample was suspended in 15 mL cold phosphate buffered saline (pH 7.3) (Dulbecco A, Oxoid, England). Sample suspension was further homogenized by vortexing at a maximum (Vortex Genie G560E; Scientific Industries Inc., New York, NY, USA) and filtered through sterile muslin cloth. The filtrate was centrifuged at 18,625 x *g* for 5 min at 4°C (M-240R; BOECO, Hamburg, Germany) to pelletize cells. Total community DNA was then extracted using the ZR Soil microbe DNA kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instruction. Following extraction, DNA integrity and quality were ascertained on 1% (w/v) agarose gel, whereas concentration and purity were determined using a Nano Drop spectrophotometer (ND-1000; Nano Drop Technologies, Wilmington, DE, USA).

PCR amplification of bacterial isolates The partial 16S rRNA gene was amplified using the universal bacterial primers 27F (5'-AGAGTTT GATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). All PCR were performed in a thermal cycler C1000™ (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR reaction (25 μL volume) contained 0.2 μM of each forward and reverse primers, 12.5 μL of 2× Dream Taq PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA), DNA template (50 ng), and nuclease-free water. The amplification protocol was an initial 94°C for 3 min, 40 cycles of 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 5 min.

PCR-DGGE For the culture-independent PCR-DGGE method, a nested-PCR approach was adopted to attempt the detection of less dominant species populations, especially at the onset of fermentation (14). In the first PCR step, component and protocol were as described earlier for isolates. First PCR's amplicons were purified by a silicon dioxide matrix method (18) and used as template DNA in a subsequent second (nested) PCR targeting the V3-V5 region of the bacterial 16S rRNA gene using primer pairs 341F (5'-CCTACGGG AGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') (19). A 40 bp GC-rich clamp was attached to the 5'-end of the forward primer (19). The concentration of PCR components was the same as in the first PCR, except that 100 ng templates were now used. The PCR reactions were done in duplicates for each sample and involved an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min with a final extension step of 72°C for 5 min. All PCR amplicons were verified on 1.5% (w/v) agarose gel.

For DGGE, 30 μL of pooled amplicons from each sample PCR duplicates were directly loaded onto a 1 mm thick 8% (w/v) polyacrylamide gel of a denaturing gradient of 25-55% urea and

formamide (100% denaturant is 7 M urea and 40% (v/v) formamide) in 1× TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) running buffer. Equal mixtures of all PCR amplicons of community samples were loaded on separate lanes as a reference (for a later densitometric analysis of DGGE image). DGGE was run on the Dcode^{TM} Universal Mutation Detection system (Bio-Rad Laboratories) at 60°C, initially at 200 V for 15 min and afterwards at 100 V for 16 h.

Gel was stained in a 1.5 mg/μL ethidium bromide solution (in 1x TAE buffer) for 15 min, rinsed in sterile TE buffer, and photographed using the ChemiDoc^{TM} MP imaging system (Bio-Rad Laboratories). DGGE was performed twice to ascertain bands migrated to similar positions in the gel. Major bands were excised with sterile blades and incubated overnight at 4°C in nuclease-free water to elute DNA. DNA eluates (10 ng) served as the template for a "re-amplification PCR" involving same PCR components and cycling conditions stated earlier, except that no 40 bp GC-rich clamp was attached to the forward primer (341F).

Densitometric analysis of DGGE image The DGGE gel image was analyzed using Gene Tools software (v. 4.03.1.0) (Syngene, Cambridge, UK). Peaks (or bands) were first detected by the software and manually corrected for "true peaks" using a set of standard functions in the software. A similarity matrix of banding patterns was generated using relative band intensity and subjected to a hierarchical cluster analysis using the unweighted pair-group method using arithmetic averages (UPGMA) and expressed as a dendrogram using the R statistical software (20).

Sequencing PCR amplicons were purified using a silicon oxide method (18) and prepared for sequencing using the Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK). For sequencing PCR, forward primers 27F and 530F (5'-GTG CCAGCMGCCGCGG-3') were used for isolate amplicons, whereas primer 341F was used for excised DGGE bands. Following sequencing PCR, amplicons were purified using ZR DNA Sequencing clean-up kit (Zymo Research) and analyzed on a 3130 Genetic Analyser (Applied Biosystems/Hitachi, Tokyo, Japan). Sequence chromatograms were inspected and edited using FinchTV (v. 1.4.0, http://www.geospiza.com/Products/finchtv.shtml). The sequences of isolates obtained using primers 27F and 530F were assembled to form a contiguous sequence of >1200 bp using ChromaPro (v. 1.7.4, Technelysium Pty Ltd., South Brisbane, Australia).

Sequence-based taxonomic assignment and phylogenetic reconstruction For taxonomic assignments, sequences were first clustered into operational taxonomic units (OTUs) at 97% sequence similarity cut-off using Mothur software (21). OTU representative sequences were selected based on the minimum distance to the other sequences in the OTU cluster using the "get.oturep" algorithm in Mothur and aligned against type strains with validly published prokaryotic names through the EzTaxon server (http://www.ezbiocloud.net/eztaxon). For phylogenetic analyses, public sequences in the Genbank with close homology to sequences obtained in this study were selected and aligned (multiple sequence alignment) using Clustalx (v. 2.1) (22). The multiple sequence alignments were manually edited and corrected for gaps in DAMBE software (v. 5.0) (23). A neighbor-joining tree was then constructed in Mega (v. 6.0) (24) using the Tamura-Nei substitution model and a thousand bootstrap replications.

Nucleotide sequences obtained in this study have been deposited in the GenBank under the Accession numbers KT021481-KT021540 and KT122380-KT122386.

Results and Discussion

Bacterial counts, pH and TTA during *soy-daddawa* fermentation We monitored the bacterial count, pH, and total titratable acidity during *soy-daddawa* fermentation in a bid to determine that the fermentation was typical of *soy-daddawa* fermentation. The bacterial count increased during fermentation (Fig. 2A) as reported in previous studies (1,7). The greatest change in bacterial count occurred within the first 24 h of fermentation. During fermentation, bacteria utilize nutrients in the bean for growth by breaking down complex compounds in the fermenting matrix into simpler subunits through a number of biochemical pathways resulting in significant chemical changes (4). Overall, the pH and TTA of the fermenting *soy-daddawa* increased with fermentation time (Fig. 2B). The pH increased towards alkalinity from an initial value of 7.06 to a peak value of 8.19 (at 48 h). The increase in pH (towards alkalinity) during *soy-daddawa* fermentation is typical of fermented legume condiments (7,8,25). The release of ammonia gas during the proteolysis of protein in the fermented legumes is responsible for alkalinity (4,25). TTA increased from an initial value of 0.08-0.26 mg lactic acid/g of sample (Fig. 2B). Increase in the total titratable acidity (TTA), the amount of acids available for reaction with a base (acid-base reaction), is attributable to the organic acid formation from the enzymatic hydrolysis of carbohydrate component of the soybean seeds (4,26). The observed simultaneous increase in pH and TTA during *soy-daddawa* fermentation has been previously reported (1,7) and is attributable to the concomitant production of ammonia gas and organic acids during fermentation.

Bacterial isolate dynamics during *soy-daddawa* fermentation Seven OTUs were identified from 29 unique sequences obtained from cultured isolates (*n*=58 isolates) (Table 1). Majority (85.71%) of isolates belonged to the phylum *Firmicutes*, whereas the remaining (14.29%) belonged to the phylum *Proteobacteria*. Majority of isolates were of the genus *Bacillus* (Table 1). This observation indicates that *Bacillus* species are the dominant bacteria in *soy-daddawa* fermentation. Previous phenotypic-based studies have also reported the dominance of *Bacillus* (1,2,7), facilitating the use of species of the genus *Bacillus* in a number of controlled fermentation studies

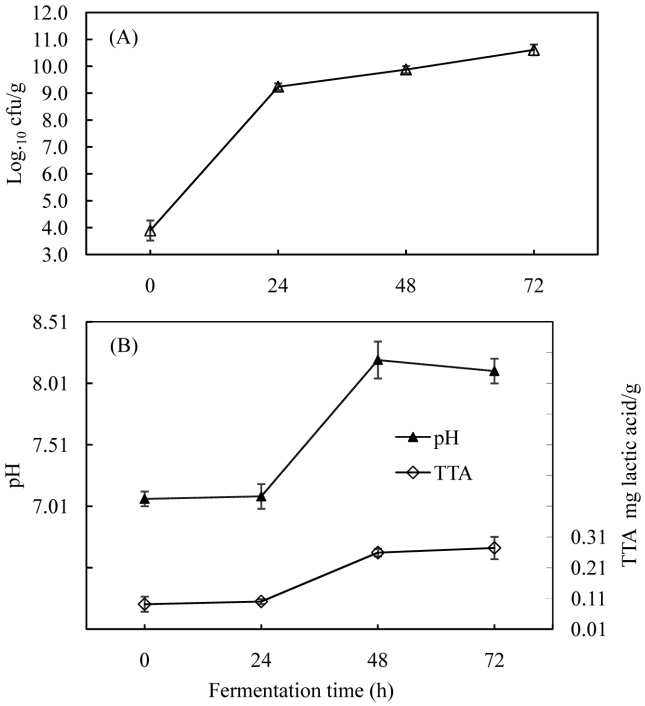

Fig. 2. Bacterial counts and chemical changes during *soy-daddawa* fermentation (A) Bacterial count. Values are means of counts from triplicate bioreactors. (B) pH and Titratable acidity changes during *soy-daddawa* fermentation. pH values are means of triplicate measurements, whereas TTA values are means of sextuplicate determinations.

(8,27,28). *Bacillus* species are known to secrete specific amylases, proteases, and lipases, which act on the fermenting bean substrate during *soy-daddawa* fermentation (4). Only *B. subtilis* was present at all sampling time points during fermentation (Table 1). The presence of *B. subtilis* in legume type fermentations is well documented (6,25,27) as well as the co-dominance of *Bacillus* and *Staphylococcus* species in *soy-daddawa*. However, relatively little is known of the safety and the role of other members of the genus such as *B. safensis* detected in the present study. The detection of organisms such as *B. anthracis,* enteric *Enterobacter*, and *Enterococcus* species as well as the skin commensal *Staphylococcus* species are pointers to the potential microbiological risks associated with spontaneously fermented foods (29-31). The rudimentary utensils and fermentation materials used in *soy-daddawa* processing are possible sources of these food contaminants (6,30).

PCR-DGGE bacterial community dynamics In the PCR-DGGE method, four OTUs were obtained from PCR-DGGE after analyzing a total of 28 band sequences (Fig. 3A). We observed multiple band positions (positions 1, 3, and 5) for OTU 3 (Fig. 3A and Table 1). According to Duarte *et al*. (32), heterogeneity in 16S rRNA genes among and within species could give rise to multiple banding patterns for the same species in DGGE.

The taxonomic identifications of the OTUs and bacteria dynamics are presented in Table 1. With the exception of *Enterobacter hormaechei*, all other bacteria species were present at all sampling times. The hierarchical cluster dendrogram of DGGE profiles in Fig. 3B revealed that the bacterial community diversity and population numbers changed with fermentation time, thereby corroborating bacterial counts obtained in the culture-dependent approach (Fig. 2A). The DGGE bacterial community at 48 h and 72 h were similar (Fig. 3B). This observation suggests that the bacterial population in the fermenting *soy-daddawa* had reached a climax community.

The presence of *B. tequilensis* and *B. subtilis* in either of the

Table 1. 16S rRNA gene-based identification of bacteria during *soy-daddawa* fermentation

OTU no. (OTU rep. accession number)	Homologue Representative (Abundance/Band number position)	% Similarity	Fermentation time (h)			
			0	24	48	72
Culture-dependent						
1 (KT021532)	**Bacillus subtilis* (29)	99.9	+	+	+	+
2 (KT021528)	$^{\alpha}$*Bacillus safensis* (3)	99.4	+	-	+	+
3 (KT021505)	$^{\beta}$*Bacillus anthracis* (1)	99.9	-	+	-	-
4 (KT021522)	*Enterobacter hormaechei* (15)	99.2	+	+	+	-
5 (KT021519)	*Enterococcus casseliflavus* (3)	99.8	-	-	+	+
6 (KT021535)	*Staphylococcus sciuri* (3)	99.8	-	-	-	+
7 (KT021502)	$^{\mu}$*Staphylococcus xylosus* (4)	99.8	+	+	-	-
Culture-independent						
1 (KT122380)	**Bacillus tequilensis* (2)	99.5	+	+	+	+
2 (KT122386)	*Bacillus methylotrophicus* (4)	99.6	+	+	+	+
3 (KT122384)	$^{\gamma}$*Bacillus siamensis* (1, 5, 3)	100.0	+	+	+	+
4 (KT122382)	*Enterobacter hormaechei* (6)	98.4	-	-	+	+

+, presence; -, absence; ***,** $^{\beta, \alpha, \gamma, \mu}$Taxonomic groups. A taxonomic group includes species/subspecies that are not distinguishable by 16S rRNA sequence; *Member of the *Bacillus subtilis* taxonomic group comprising *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, *B. subtilis* subsp. *inaquosorum*, *B. mojavensis*, *Brevibacterium halotolerans*, and *Bacillus tequilensis*; $^{\beta}$Member of the *Bacillus cereus* taxonomic group comprising *Bacillus anthracis*, *B. cereus*, *B. thuringiensis*, and *B. toyonensis*; $^{\alpha}$Member of the *Bacillus pumilus* taxonomic group comprising *B. pumilus* and *B. safensis;* $^{\gamma}$Member of the *Bacillus amyloliquefaciens* taxonomic group comprising *B. amyloliquefaciens* subsp. *amyloliquefaciens*, *B. amyloliquefaciens* subsp. *Plantarum*, and *B. siamensis*; $^{\mu}$Member of the *Staphylococcus saprophyticus* taxonomic group comprising *S. saprophyticus* subsp. *saprophyticus*, *S. saprophyticus* subsp. *bovis*, and *S. xylosus*.

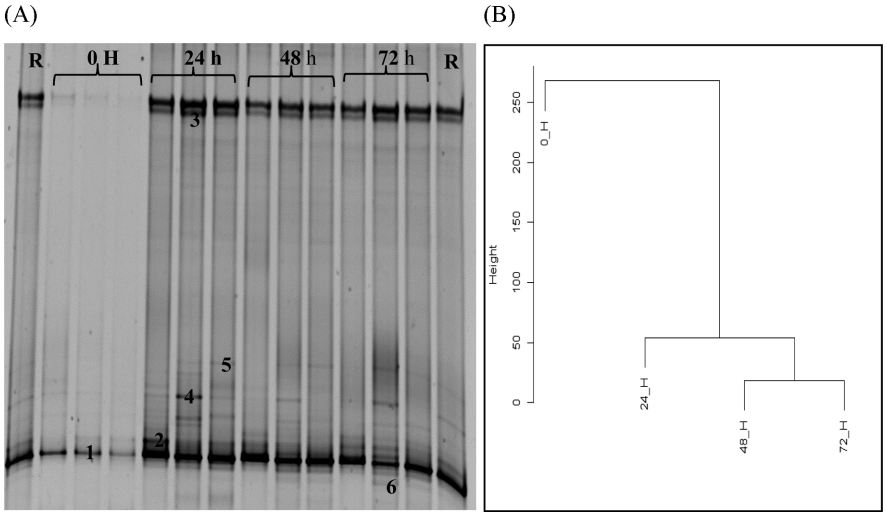

Fig. 3. PCR-DGGE profile and hierarchical cluster dendrogram of bacterial community during *soy-daddawa* fermentation. (A) Lanes designated as 0, 24, 48, and 72 h are fermentation-sampling times. R, reference lanes used in generating a similarity matrix for dendrogram construction. Numbered bands are major band positions excised and sequenced. (B) Hierarchical cluster dendrogram was constructed from the relative intensities band matrix of lanes in R software using unweighted pair-group method with arithmetic mean (UPGMA).

culture-dependent or culture-independent methods are likely similar observations (species), given that these species both belong to the same taxonomic group based on 16S rRNA gene homology (33). While the presence of *E. hormaechei* is of significant medical importance, the role (in *soy-daddawa* fermentation) and safety of *B. methylotrophicus* and *B. siamensis* are relatively unknown. These observations highlight the necessity of developing starter culture technologies for a controlled processing of *soy-daddawa* and other fermented foods (29-31,34).

The combination of both culture-dependent and culture-independent (PCR-DGGE) methods revealed bacterial genera such as *Bacillus* and *Staphylococcus*, which have been previously reported by culture-based phenotypic studies (1,2). However, we also report the presence of *B. methylotrophicus*, *B. safensis*, and *B. siamensis* in *soy-daddawa* microbial ecology for the first time. Further screening of the isolates will help elucidate roles and safety in *soy-daddawa* fermentations. The phylogenetic reconstruction of bacterial isolates and sequences obtained in this study confirmed taxonomic assignments (data not shown). *B. subtilis* and *B. tequilensis* obtained by culture-dependent and culture-independent analysis, respectively, belonged to the same clade (data not shown) thus suggesting close evolutionary relatedness.

In conclusion, our findings further suggest that in the event of applying starter cultures for *soy-daddawa* fermentation, should a mixed culture of "safe" *Bacillus* species (as an example) be selected for use, all the cultures can be included from the onset of fermentation rather than sequentially. The spontaneous fermentation of *soy-daddawa* involves a consortium of bacterial species. While we can speculate the specific contribution of some of the "safe microbiota" present in the fermented condiment, the risk posed by known human pathogens such as *B. anthracis* is certain. Our findings lend credence to the advocacy for a departure from the common spontaneous fermentation practices in the traditional processing of fermented foods in developing countries. This study further points toward screening and selection studies for potential and suitable starter cultures with the ultimate goal of optimizing a controlled *soy-daddawa* production process.

Acknowledgments The European Union (Grant Agreement 2011-204072) funded this study through the Intra-ACP Academic Mobility Scheme: Strengthening African Education through Academic Mobility (STREAM). Financial support from the funders to O. T. Ezeokoli (Stream Number STRE1101380) is hereby acknowledged. Post-Doctoral research fellowship awarded by the North-West University to A.K. Gupta is also acknowledged. We sincerely thank Charlotte Mienie and Abram Mahlatsi for their technical assistance.

Disclosure The authors declare no conflict of interest.

References

1. Omafuvbe B, Shonukan O, Abiose S. Microbiological and biochemical changes in the traditional fermentation of soybean for "soy-*daddawa*"-Nigerian food condiment. Food Microbiol. 17: 469-474 (2000)
2. Popoola TOS, Akueshi CO. Microorganisms associated with the fermentation of soybean for the production of soybean *daddawa* (a condiment). Nigerian Food J. 2&3: 194-196 (1985)
3. Popoola TOS, Kolapo AL, Afolabi OR. Biochemical deterioration of soybean *daddawa*-A condiment. J. Food Agric. Environ. 5: 67-70 (2007)
4. Achi OK. Traditional fermented protein condiments in Nigeria. Afr. J. Biotechnol. 4: 1612-1621 (2005)
5. Popoola TOS, Akueshi CO. Nutritional evaluation of *daddawa*, a local spice made from soybean (*Glycine max*). Mircen J. Appl. Microb. 2: 405-409 (1986)
6. Ezeokoli OT, Gupta AK, Mienie C, Popoola TOS, Bezuidenhout CC. PCR-denaturing gradient gel electrophoresis analysis of microbial community in *soy-daddawa*, a Nigerian fermented soybean (*Glycine max* (L.) Merr.)

condiment. Int. J. Food Microbiol. 220: 58-62 (2016)
7. Dakwa S, Sakyi-Dawson E, Diako C, Annan NT, Amoa-Awua WK. Effect of boiling and roasting on the fermentation of soybeans into *dawadawa* (*soy-dawadawa*). Int. J. Food Microbiol. 104: 69-82 (2005)
8. Omafuvbe BO, Abiose SH, Shonukan OO. Fermentation of soybean (*Glycine max*) for *soy-daddawa* production by starter cultures of *Bacillus*. Food Microbiol. 19: 561-566 (2002)
9. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. J. Clin. Microbiol. 45: 2761-2764 (2007)
10. Justé A, Thomma B, Lievens B. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiol. 25: 745-761 (2008)
11. Ercolini D, Moschetti G, Blaiotta G, Coppola S. The potential of a polyphasic PCR-DGGE Approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: Bias of culture-dependent and culture-independent analyses. Syst. Appl. Microbiol. 24: 610-617 (2001)
12. Takahashi M, Ohta T, Masaki K, Mizuno A, Goto-Yamamoto N. Evaluation of microbial diversity in sulfite-added and sulfite-free wine by culture-dependent and-independent methods. J. Biosci. Bioeng. 117: 569-575 (2014)
13. Hamdouche Y, Guehi T, Durand N, Kedjebo KBD, Monet D, Meile JC. Dynamics of microbial ecology during cocoa fermentation and drying: Towards the identification of molecular markers. Food Control 48: 117-122 (2015)
14. Kim T-W, Lee J-H, Kim S-E, Park M-H, Chang HC, Kim H-Y. Analysis of microbial communities in doenjang, a Korean fermented soybean paste, using nested PCR-denaturing gradient gel electrophoresis. Int. J. Food Microbiol. 131: 265-271 (2009)
15. Aquilanti L, Santarelli S, Silvestri G, Osimani A, Petruzzelli A, Clementi F. The microbial ecology of a typical Italian salami during its natural fermentation. Int. J. Food Microbiol. 120: 136-145 (2007)
16. Oguntoyinbo FA, Tourlomousis P, Gasson MJ, Narbad A. Analysis of bacterial communities of traditional fermented West African cereal foods using culture independent methods. Int. J. Food Microbiol. 145: 205-210 (2011)
17. Kimaryo VM, Massawe NA, Olasupo NA, Holzapfel WH. The use of starter culture for the fermentation of cassava for the production of "Kivunde," a traditional Tanzanian food product. Int. J. Food Microbiol. 56: 179-190 (2000)
18. Li J-F, Li L, Sheen J. Protocol: A rapid and economical procedure for purification of plasmid or plant DNA with diverse applications in plant biology. Plant Methods 6: 1-8 (2010)
19. Muyzer G, De Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microb. 59: 695-700 (1993)
20. R Core Team. R Foundation for Statistical Computing, Vienna, Austria (2013)
21. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microb. 75: 7537-7541 (2009)
22. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948 (2007)
23. Xia X. DAMBE5: A comprehensive software package for data analysis in molecuar biology and evolution. Mol. Biol. Evol. 30: 1720-1728 (2013)
24. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729 (2013)
25. Parkouda C, Nielsen DS, Azokpota P, Ivette Irène Ouoba L, Amoa-Awua WK, Thorsen L, Hounhouigan JD, Jensen JS, Tano-Debrah K, Diawara B, Jakobsen M. The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia. Crit. Rev. Microbiol. 35: 139-156 (2009)
26. Sauer M, Porro D, Mattanovich D, Branduandi P. Microbial production of organic acids: Expanding the markets. Trends Biotechnol. 25: 100-108 (2008)
27. Sarkar P, Cook P, Owens J. *Bacillus* fermentation of soybeans. World J. Microb. Biot. 9: 295-299 (1993)
28. Terlabie NN, Sakyi-Dawson E, Amoa-Awua WK. The comparative ability of four isolates of *Bacillus subtilis* to ferment soybeans into *dawadawa*. Int. J. Food Microbiol. 106: 145-152 (2006)
29. Adewumi GA, Oguntoyinbo FA, Keisam S, Romi W, Jeyaram K. Combination of culture-independent and culture-dependent molecular methods for the determination of bacterial community of *iru*, a fermented *Parkia biglobosa* seeds. Front. Microbiol. 3: 1-9 (2013)
30. Iwuoha CI, Eke OS. Nigerian indigenous fermented foods: Their traditional process operation, inherent problems, improvements and current status. Food Res. Int. 29: 527-540 (1996)
31. Achi OK. The potential for upgrading traditional fermented foods through biotechnology. Afr. J. Biotechnol. 4: 375-380 (2005)
32. Duarte S, Pascoal C, Cassio F. Denaturing Gradient Gel Electrophoresis (DGGE) in Microbial Ecology-Insights from Freshwaters. pp. 173-196. In: Gel Electrophoresis - Principles and Basics. Magdeldin S (ed). INTECH, Open Access (2012)
33. Wang L-T, Lee F-L, Tai C-J, Kasai H. Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA–DNA hybridization in the *Bacillus subtilis* group. Int. J. Syst. Evol. Micr. 57: 1846-1850 (2007)
34. Holzapfel WH. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int. J. Food Microbiol. 75: 197-212 (2002)