Diversity and functionality of *Bacillus* species associated with alkaline fermentation of bambara groundnut (*Vigna subterranean* L. *Verdc*) into dawadawa-type African condiment

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Running title: *Bacillus* species associated with alkaline fermentation of bambara groundnut into *dawadawa*-type condiments

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ABSTRACT

The aim of this study was to investigate the diversity of *Bacillus* species in dawadawa; aiding potential starter cultures selection for alkaline fermentation of bambara groundnut into dawadawa-type condiments based on their genotypic and volatile compound profiles. *Bacillus* species (n = 71) isolated from spontaneously fermented dawadawa were identified using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) as *B. cereus* (35%), *B. licheniformis* (30%), *B. pumilus* (21%), *B. subtilis* (10%) and *B. amyloliquefaciens* (4%). Further molecular typing was performed using GTG_5 rep-PCR typing, 16S rRNA and *gyr*A gene sequencing. The *gyr*A gene sequence analysis exhibited the highest species discriminatory power with *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* subsp. *plantarum*, *B. pumilus* and *B. licheniformis* as the distinct clusters. Representative strains from each cluster were then used as starter cultures for the production of dawadawa from bambara groundnut. Volatile compounds analysis using headspace solid phase microextraction (SPME) and comprehensive gas chromatography coupled to time of flight mass spectrometry (GC × GC–TOF MS) identified distinct chemical profiles produced by each of the four strains. Volatile compounds produced by *B. subtilis* subsp. *subtilis* (strain SFBA3) were categorized by dimethyl disulfide, methanethiol and nonanal while *B. amyloliquefaciens* subsp. *plantarum* (strain SFBA2) produced acetic acid and hexadecanoic acid. *B. cereus* (strain PALB7) produced 2,5-dimethyl pyrazine and 2-butanone which were not detected in the other condiments. Hexanal was the main compound produced by *B. licheniformis* (strain OALB7).

Keywords: Alkaline fermentation, *Bacillus* species, dawadawa condiments, *Vigna subterranean* (L.) *Verdc.*, Volatile compounds

INTRODUCTION

Dawadawa is an African condiment produced solely from the spontaneous alkaline fermentation of African locust beans (*Parkia biglobosa*) [9, 22] and occasionally from soybeans (*Glycine max*) to produce soy dawadawa or bambara groundnut (*Vigna subterranean* L. *Verdc*.) which is an underutilized African legume for dawadawa-type condiments production [6-8, 13, 29]. Dawadawa is an integral part of the African diet due to its distinct aroma and flavour enhancing properties when added during cooking of soups or stews which are imparted by volatiles compounds in the condiments [22, 29]. *Bacillus* species are known to dominate the alkaline fermentation of legumes in the production of various traditional foods and condiments in Asia and Africa. These fermented condiments include Indian kinema, Chinese doushi, Thai thuanao, Japanese natto and West African dawadawa/soumbala [1-3, 7, 31]. The major role of *Bacillus* species in such condiments involves hydrolyzing proteins to peptides and amino acids and releasing ammonia, thereby creating an alkaline pH which aids the inhibition of spoilage microorganisms [31]. *Bacillus* species isolated from dawadawa made from African locust bean include *B. amyloliquefaciens*, *B. atrophaeus*, *B. badius*, *B. cereus*, *B. firmus*, *B. fumus*, *B. licheniformis*, *B. megaterium*, *B. mojavensis*, *B. mycoides*, *B. pumilus*, *B. subtilis*, *B. sphaericus* and *B. thuringiensis* [2, 4, 26, 31]. The use of starter cultures does not find widespread application in the traditional fermentation of food in Africa.

In the past decade, several works were dedicated to the characterization of the *Bacillus* species of these fermented products [4, 23, 26-28]. However, *Bacillus* strains elucidating desirable biochemical changes in traditional dawadawa have not yet been accurately identified. Unlike the application of *Bacillus subtilis* var *natto* in the production of the Japanesse natto, the use of indigenous commercial starter cultures is not available for dawadawa production. Limitations of the phenotypic identification methods to accurately differentiate individual *Bacillus* species and complexity in the use of simply16S rDNA PCR characterization to fully distinguish closely related species within these *Bacillus* groups has imposed constraints on the successful identification of potential starter cultures for alkaline fermentation and production of African food condiments [10, 23, 39]. The use of polyphasic classification methods such as the repetitive sequence-based PCR (Rep-PCR), DNA sequencing of the 16S rRNA gene in combination with core protein-coding genes (*gyr*A gene for instance), to accompany conventional phenotypic tests are important tools useful to identify and differentiate genetic diversity between closely related *Bacillus* species [10, 31, 33]. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been highlighted as an effective method used to rapidly identify whole microbial cells growing on a solid medium [5, 30, 35]. The profiling of volatile

compounds production during controlled microbial alkaline fermentation of legumes is one of the steps which could give information for the future selection of starter cultures [3]. Pyrazines, aldehydes, ketones, esters, alcohols, acids, alkanes, alkenes, benzenes, phenols, sulphurs and furans groups chemical groups identified in dawadawa produced from other legumes (African locust bean and soybeans) but no report from bambara groundnut [3, 4].

Therefore, the main objective of this study was to classify the genetic diversity of *Bacillus* species involved in alkaline fermentation of bambara groundnut using polyphasic identification methods and evaluating volatile compounds that would aid the selection of potential starter cultures for the production of dawadawa-type condiment.

MATERIALS AND METHODS

Sources of traditional *dawadawa*

Dawadawa condiment in form of sun-dried balls that was produced from African locust bean were purchased during January – March from open-markets in Ibadan, Lagos and Akure in Nigeria and also from Accra, Ghana. Three samples each were collected from these different locations and stored at air tight cooler boxes before analysis.

Microbiological and biochemical analyses

Ten grams (10 g) each condiment were suspended in 90 ml of 0.1% peptone buffer water (Merck, Darmstadt, Germany) and homogenized in a stomacher (Lab Blender, Model 400, Art Medical Instrument (Pty) Ltd, Johannesburg, South Africa). For spore-forming bacteria, 10 mL of the primary dilution was heated at 80 °C for 10 min, then 50µL of each dilution was incubated on Luria Bertani agar (LB) (Merck) at 37 °C for 48 h. A total of 71 spore-forming isolates obtained were stored in LB broth (Merck) cryopreserved at − 70 °C for further analysis. The pH of the dawadawa condiments were also determined using a pH meter (Model pH 211; Hanna Instruments, Woonsocket, RI, USA).

Phenotypic characterizations of spore forming isolates were examined for cell morphology, motility and presence of endospores by microscopy. Gram stain determination was performed using the KOH method, a portion of bacteria colony were mixed with a drop of 3% KOH on a glass slide for 1 min, colonies with stringy mixture when lifted with a loop are gram negative. Catalase production, hydrolysis of starch, growth at different pH values, temperatures and NaCl concentrations, Voges Proskauer and methyl red test, nitrate reduction, citrate

utilisation, propionate utilisation, and fermentation of sugars were determined according to prescribed methods [18]. Proteolytic activity was determined on plate count skim milk agar with 0.1% skim milk powder at 10 °C for 7 days. Presumptive aerobic spore-forming bacteria colonies were purified and further identified using PCR analysis and MALDI-TOF MS.

MALDI-TOF confirmation of the presumptive spore-forming bacteria isolates

Purified bacterial cultures isolated from the selective media were transferred, in duplicate, directly to the MALDI TOF steel polished target plate (Bruker, Bremen, Germany) and overlaid with the α-cyano-4-hydroxy-cinnamic acid matrix (Bruker) [30]. The target plate was subsequently analyzed using microflex LT MALDI TOF (Bruker) in conjunction with Biotyper automation software and library (Bruker). A score-oriented dendrogram was generated based on crosswise minimum spanning tree (MSP) matching.

Genotypic characterization

Bacillus isolates were grown overnight in LB broth at 37 °C. DNA was extracted by resuspending bacterial pellets in 0.2 ml lysis buffer [10 $mmol^{-1}$ Tris–HCl (pH 8.0), 1 $mmol^{-1}$ EDTA, 1% Triton X-100] (Sigma-Aldrich, Steinheim, Germany) and incubated for 30 min at 95°C with agitation. The tubes were then cooled to 4°C and centrifuged for 10 min at 12,000g; the supernatant was used directly for amplification [14].

$(GTG)_5$-Rep-PCR fingerprinting

The 20 µL reaction consisted of 10 µL, 2 x PCR Master Mix (Kapa Biosystems, Boston, MA, USA) containing KapaTaq DNA polymerase (0.05 U/µl, 1.25 U per 25 µl), reaction buffer with Mg^{2+} and 0.4 mM of each dNTP with loading dye, 0.8 µM $(GTG)_5$ primer [38], 2 µL DNA template and 4% dimethylsulfoxide (Sigma-Aldrich, St Louis, USA). Amplification was carried out as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 3 min; and a final elongation step at 72 °C for 10 min.

16S rRNA and gyrA gene amplification and sequencing

A total of 24 isolates were selected based on the Rep-PCR clustering for sequencing of the 16S rRNA gene. Partial 16S rRNA gene amplification using forward primer fD1 (5´-AGA GTT TGATCC TGG CTC AG-3´) and reverse primer rD1 (5´-AAG GAG GTG ATC CAG CCG CA-3´) [40]. The PCR was carried with initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 2 min; 42 °C for 30 s and 72 °C for 4 min; and a final

elongation step at 72 °C for 10 min. The PCR products were confirmed by electrophoresis and visualized under UV light with a Gel Doc system (Bio-Rad, Hercules, CA, USA) Grouping of the rep-PCR fingerprints was performed using GELCOMPAR II version 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium) based on the Dice similarity coefficient and the UPGMA algorithm to obtain a dendogram.

Phylogenetic analysis

The amplified PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), 16S rRNA sequencing reactions were conducted using the ABI BigDye version 3.0 sequencing kit (Applied Biosystems, Waltham, MA, USA) following the manufacturer's suggested protocol. Reaction products were purified via ethanol precipitation and run on an ABI 3730 genetic analyzer (Applied Biosystems). The obtained sequences were analysed and subjected to BLAST for comparing sequence homologies in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). Representative isolates tentatively identified as *B. subtilis* group members (based on 16S rRNA gene sequencing) were selected for sequencing of the *gyr*A genes as described by Chun and Bae [10]. The resultant partial 16S rRNA and *gyr*A gene sequences were assembled and aligned online using MAFFT version 7 multiple sequence alignment program (http://mafft.cbrc.jp/alignment/software/). The was used to Evolutionary distances calculated using Molecular Evolutionary Genetics Analysis (MEGA) v 5.2 program [37] and trees inferred based on neighbour-joining (NJ) and Jukes & Cantor-model [17, 34].

Production of dawadawa from bambara groundnut using starter cultures

Fermentation of bambara groundnut

The fermentation and production of dawadawa-type condiments from bambara groundnut was carried out as previously described by Barimalaa et al [6]. Briefly, 40 g of seeds were steeped in 200 ml distilled water at 24°C for 24 h, after which the seeds were dehulled manually using a mortar and pestle. Cotyledons recovered were boiled in distilled water at 100°C for 15 min. The cooked cotyledons were then drained with a sieve (1 mm pore size). Fifty (50) grams of the cooked cotyledons were placed in sterile0mm x 70mm zip lock perforated polythene bags (Apak Packaging, Johannesburg, South Africa). The *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. cereus* PALB7 and *B. licheniformis* OALB2 strains were then used to inoculate the bambara groundnut to obtain final inoculum level of 4 X 10^4 cfu/g. Fermentation was carried out at 30 °C for 120 h. A control fermentation was conducted without any inoculation. After fermentation, 10 g of fermenting bambara groundnut was aseptically placed in stomacher bag and 90 ml of

sterile distilled water was added and homogenized in a stomacher, 1 ml of this 10^{-1} dilution was removed and further diluted for in a ten-fold dilution series.

Determination of volatile compounds produced by *Bacillus* species

Chemical standards

Five grams of dawadawa were spiked with 6.25 µg 1,8-cineole (eucalyptol) analytical reference standard (Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa). The headspace sampling of the spiked sample was extracted as described below and analysed with GCxGC-TOFMS. For linear retention index determination *n*-alkanes (C_8–C_{28}) were used (Merck, Pretoria, South Africa).

Gas chromatography - time of flight mass spectrometry (GC x GC-TOFMS)

Headspace sampling were done with solid phase microextraction (SPME) device fitted with a 2-50/30µm DVB/Carboxen/PDMS StableFlex fibre (Supelco, Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa). The fibre was exposed to the headspace above the sample for 20 min. After extraction the SPME device was removed from the vial and desorbed in the injection port of a GC x GC-TOFMS. Compound separation was done using a LECO Pegasus 4D GC x GC-TOFMS with an Agilent 7890 GC (LECO Africa (Pty) Ltd., Kempton Park, South Africa). The system included a secondary oven and a dual stage modulator. Nitrogen gas was used for the hot jets and nitrogen gas cooled with liquid nitrogen was used for the cold jets. The carrier gas, helium, was of ultra-high purity grade (Afrox, Gauteng, South Africa) and was set at a flow rate of 1.4 ml min^{-1} in the constant flow mode. The capillary column set consisted of an apolar Rxi-5SilMS 30 m x 0.25 mm ID x 0.25 µm df (Restek, Bellefonte, PA, USA) as the primary column and a high temperature midpolar Rxi-17Sil MS 0.97 m x 0.25 mm ID x 0.25 µm df (Restek, Bellefonte, PA, USA) as the secondary column. Identification of compounds in the samples was done by comparison of mass spectra to that of the National Institute of Standards and Technology (NIST14) library and by experimental linear retention indices (RI_{exp}). Compounds reported had a spectral match quality of ≥ 80%. Semi-quantification of the compounds was performed by using the internal standard method of quantification [21].

RESULTS AND DISCUSSION

Isolation and phenotypic identification of spore-forming bacteria

The total aerobic counts ranged from 9.04 ± 0.14 log_{10} CFU/g to 9.58 ± 0.19 log_{10} CFU/g. Sporeformers counts ranged from 8.0 ± 0.1 log_{10} CFU/g to 8.3 ± 0.2 log_{10} CFU/g (Table 1). All spore-forming isolates from

spontaneous fermentation of African locust bean and bambara groundnut dawadawa were characterized as Gram positive, catalase positive, endospore forming and rod shaped based on the phenotypic characteristics. The predominant *Bacillus* isolate was *B. cereus*, followed by *B. pumilus*, *B. licheniformis* and *B. subtilis* in decreasing order. The *B. amyloliquefaciens* was identified only in the bambara groundnut *dawadawa*-type condiment. The *B. cereus* has the highest number of isolates, however; the combined population of *B. pumilus*, *B. licheniformis* and *B. subtilis* which are members of the *B. subtilis* group outnumbered the *B. cereus* population. The presence of *B. amyloliquefaciens* in the bambara groundnut condiment alone may be attributed to the composition of the substrate which has a higher carbohydrate content (63.5%) compared to African locust bean (49%) [9, 15]. Studies on African food condiments such as *soumbala*, *sonru*/*Iru*, *bikalga* and *mbuja* have reported the occurrence of *Bacillus* species, with *B. subtilis* group (*B. subtilis*, *B. licheniformis*, *B. pumilus* and *B. amyloliquefaciens*) often dominate the fermentation process [4, 20, 26-28]. The incidence of *B. cereus* highlighted its importance as part of the microflora of alkaline fermentation as with other food condiments based on alkaline fermented seed.

Table 1 The average count of *Bacillus* species associated with spontaneous fermentation of African locust bean and bambara groundnut in the production of dawadawa (n = 3)

Samples		AFL_{Accra}	AFL_{Ibadan}	AFL_{Lagos}	AFL_{Akrure}	BGN
pH		7.9	8.2	8.5	8	7.9
Log_{10} cfu/g	Total Aerobic count	9.06 ± 0.13	9.23 ± 0.07	9.04 ± 0.14	9.58 ± 0.19	9.07 ± 0.09
	Sporeformers	8.29 ± 0.04	8.33 ± 0.16	8.04 ± 0.12	8.38 ± 0.11	8.03 ± 0.17

AFL – African locust bean

BGN – Bambara groundnut

Isolates identified by MALDI-TOF Mass Spectrometry

MALDI TOF MS analysis identified all the aerobic spore forming bacteria isolates as *Bacillus* species. Overall, two distinct cluster groups were identified namely *B. cereus* and *B. subtilis* based on 85% distance level and higher (Fig. 1). African locust bean dawadawa condiments had a higher incidence of *B. cereus* than bambara groundnut *dawadawa*. The mass spectra fingerprinting deduced revealed a clear interspecies divergence in the *B. subtilis* groups; however, *B. cereus* species was the only specie identified in its group. Of the 71 *Bacillus* isolates from both dawadawa-types, *B. cereus* accounted for 25 isolates while *B. subtilis* group accounted for 46

isolates which comprised members; *B. lichenformis* (9 isolates), *B. pumilus* (12 isolates), *B. amyloliquefaciens* (12 isolates) and *B. subtilis* (13 isolates), respectively. MALDI TOF MS an emerging proteomics based rapid identification system, identified greater divergence with more species identified in the *B. subtilis* group than the phenotypic test. However, the only limitation was that of identifying those closely related *Bacillus* on subspecies level. Similar discrepancies in identifying closely related species were reported by other authors [30, 41]; and this has been attributed to the lack of adequate databases for strain comparison or inadequate method optimization for the MALDI-TOF MS. Classification using MALDI-TOF MS analysis for several closely related bacteria can be achieved by using additional sample preparation steps [5, 35].

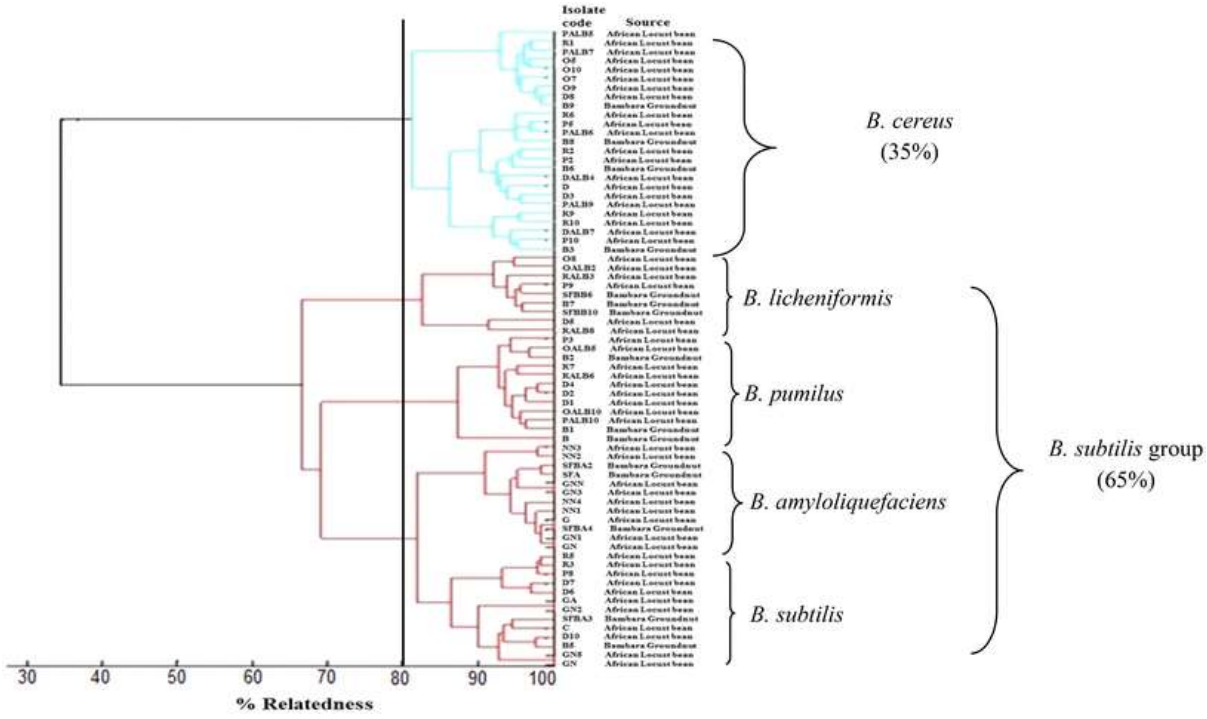

Fig. 1 Score-oriented dendrogram of MALDI-TOF mass spectrometry profiles showing genetic relationships between *Bacillus* species isolated from dawadawa African condiments produced from spontaneous alkaline fermentation of African locust bean or bambara groundnut. The vertical line represents clusters of isolates that showed 80% strain similarity which was taken as the threshold for closely related isolates

Genotypic identification

GTG_5 rep-PCR and 16S rDNA sequence identity

The isolated *Bacillus* species were grouped by GTG_5 rep-PCR fingerprinting analysis into five clusters (Fig. 2). The clusters confirmed intra-species diversity within the *B. subtilis* group. Representative isolates from the individual clusters were picked for 16S rRNA gene sequencing and identities of isolates were confirmed by blasting it against the available databases. The isolates belonging to GTG_5 rep-PCR cluster 1A (Fig. 2) were

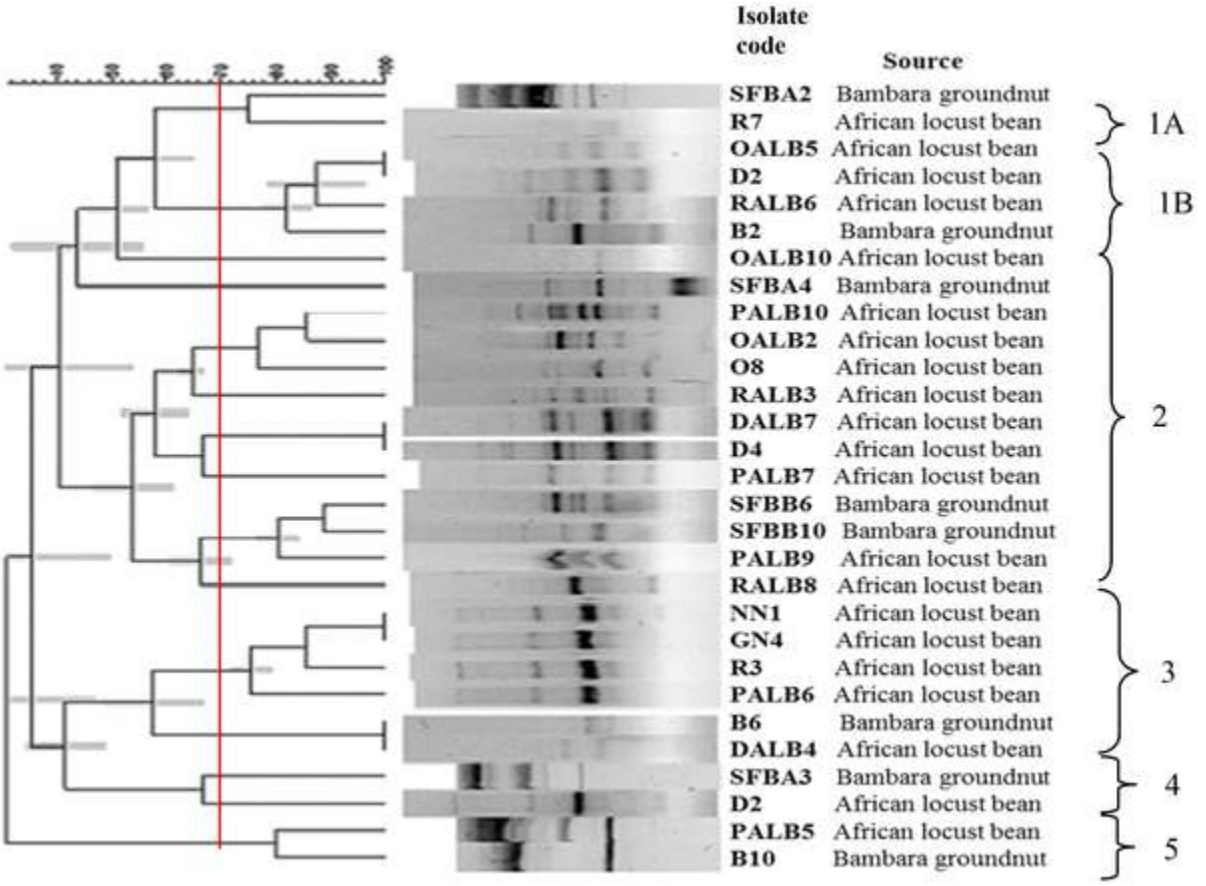

Fig. 2 Dendrogram based on Dice coefficient of similarity with the Unweighted Pair Group Method with Arithmetic averages (UPGMA) of GTG_5 rep-PCR fingerprint patterns of species in the *Bacillus subtilis* group sampled from spontaneous alkaline fermentation of African locust bean and bambara groundnut. Presumptive *Bacillus* species isolates in cluster 1A are *B. amyloliquefaciens*, 1B are *B. pumilus*, 2 are *B. lichenformis*, 3 and 4 are *B. subtilis* and 5 are *B. cereus*

found to belong to the *B. amyloliquefaciens* by 16S rRNA gene sequencing (99.8–100% similarity ratio to EzTaxon deposited sequences) while groups 1B belong to *B. pumilus* by 16S rRNA gene sequencing (97.8–100% similarity ratio). Cluster 2, the largest, which comprised of 12 isolates were confirmed with 16S rRNA gene sequencing (85–100% similarity ratio) as *B. licheniformis*. Cluster 3 and 4 represents the *B. subtilis* which by 16S rRNA gene sequencing had 99.8–100% similarity ratio. Cluster 5 is the *B. cereus* by 16S rRNA gene sequencing with 97.8–100% similarity ratio. The phylogenetic tree based on the 16S rDNA sequences showed 5 groups (Fig. 3). Isolate SFBA 3 clustered with the *B. subtilis* subsp *subtilis* strain 168 and *B. subtilis* subsp *natto* strain MBS04-6, the latter a commercial strain for *natto* fermentation. Isolate SFBA 2 clustered with *B. amyloliquefaciens* strains. Five strains each segregated into the *B. licheniformis* and *B. pumilus* strains respectively. A total of six strains segregated with the *B. cereus* strain which formed a distinct cluster on the phylogenetic tree (Fig 3); however, a number of the isolates were previously identified as *B. pumilus* with

phenotypic tests and proteomics (Fig. 2). GTG_5 rep-PCR have been shown to be a useful technique in the subtyping of *Bacillus* species [12, 16, 32]. However, protein coding genes such as *gyr*A used in this study exhibited much higher genetic variation for the classification of closely related taxa within the *B. subtilis* group. The large degree of variation in the individual group fingerprints suggests substantial intra-species genetic diversity may exist and highlights the very high resolution of 16S rDNA and *gyr*A gene sequencing.

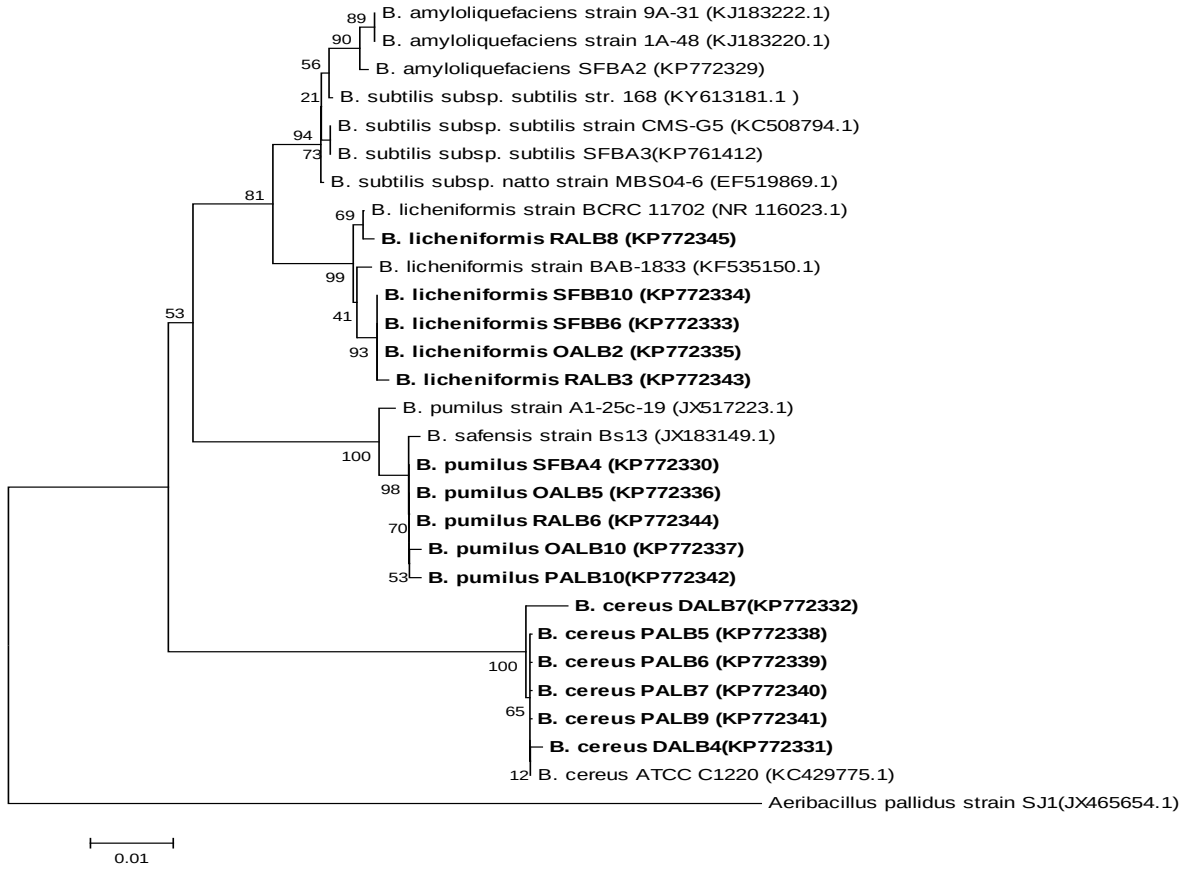

Fig. 3 Phylogenetic relationship of *Bacillus* strains from the spontaneous alkaline fermentation of African locust bean and bambara groundnut inferred from the alignment of the 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; > 50% were considered significant. Because the ML tree was very similar to the NJ tree, only the latter is shown here. Tree was rooted with *Aeribacilus pallidus* strain SJ1. *Bacillus* species isolated in the study represented in bold

GyrA sequence identity

Further classification using *gyr*A genes sequencing with a total of 15 isolates selected, a representation of each group identified by 16S rRNA sequence, were used to differentiate closely related species of the *B. subtilis* group (99–100% similarity ratio to GenBank sequences). The *gyr*A gene differentiated isolates SFBA 3 and SFBA 4 as *B. subtilis* subsp. *subtilis* and *B. amyloliquefaciens* subsp. *plantarum* respectively which could not be

identified with the phenotypic, proteomics and 16S rDNA sequence methods. The *B. licheniformis* strains identity were all confirmed with *gyr*A sequence, however, there were no positive *gyr*A sequences for *B. pumilus* isolate in this study (Fig 4). The *B. subtilis* subsp. *natto* type strain MS04-6 which is a commercial starter culture for Japanese natto alkaline fermentation shared 90.2% *gyr*A sequence similarity with the *B. subtilis* subsp. *subtilis* strain SFBA3 which was isolated from the spontaneous fermentation of bambara groundnut. This suggests the potential of this strain as a commercial starter culture for dawadawa production. *B. amyloliquefaciens* subsp. *plantarum* identified from the spontaneous fermentation of bambara groundnut has rarely been reported in alkaline fermentation. Previous authors only recorded the isolation of *B. lichenformis* as the sole organism responsible for fermentation of bambara groundnut [1, 6, 13]. However, findings in this study revealed other *Bacillus* species present such as *B. subtilis* subsp. *subtilis* and *B. amyloliquefaciens* subsp. *plantarum*. This work not only establishes the genetic relationship between *Bacillus* species from alkaline fermentation of African locust bean and bambara groundnut but with the *Bacillus* strain from the Japanesse *natto*.

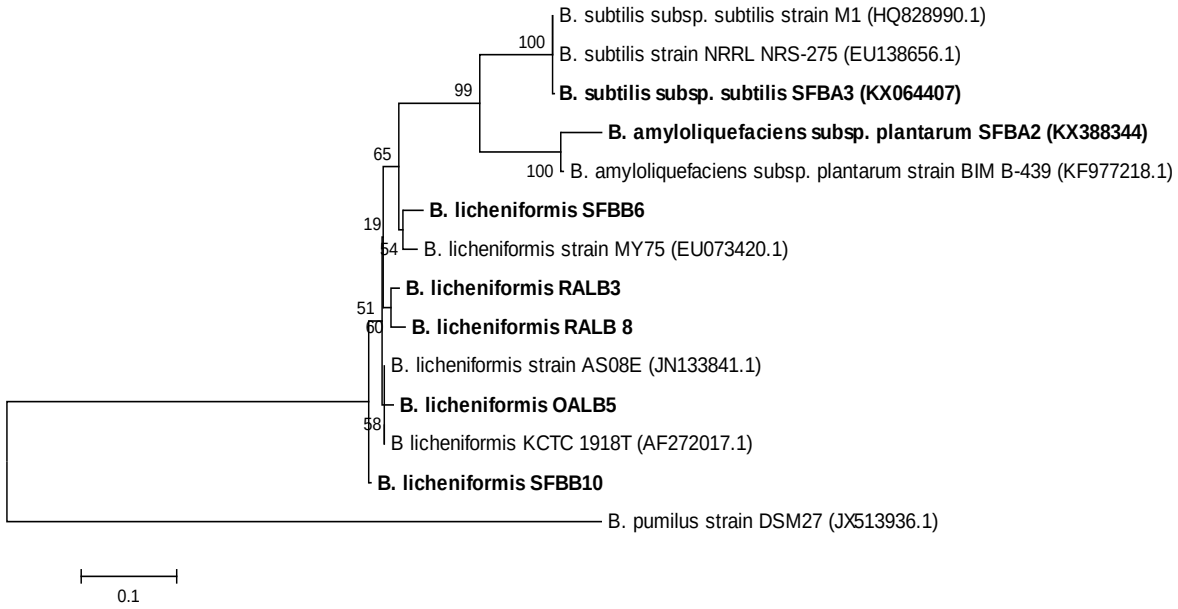

Fig. 4 Phylogenetic relationship of *Bacillus* strains from the spontaneous alkaline fermentation of African locust bean and bambara groundnut inferred from the alignment of the *gyr*A gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; > 50% were considered significant. Because the ML tree was very similar to the NJ tree, only the latter is shown here. Tree was rooted with *B pumilus* strain DSM27. *Bacillus* species isolated in the study represented in bold

Microbial growth on bambara groundnut

The study identified two major groups of *Bacillus* species complex; the *B. subtilis* group (*B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*) and *B. cereus* group identified from the spontaneous fermentation of African locust bean and bambara groundnut. Two strains *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2 were strains isolated from spontaneous fermentation of bambara groundnut while *B. licheniformis* OALB2 and *B. cereus* PALB7 strains were isolated from African locust bean to evaluate their potential as starter cultures for the controlled fermentation of bambara groundnut into dawadawa-type condiment.

The microbial cultivation of *Bacillus* species on bambara groundnut showed a maximum specific growth rate of 0.13, 0.14, 0.13 and 0.10 h^{-1} for *B. cereus* PALB7, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. subtilis* subsp. *subtilis* SFBA3 and *B. licheniformis* respectively. Both *B. cereus* PALB7 and control had a rapid growth throughout the fermentation as indicated by an increase in spore-forming bacteria counts with a concomitant pH increase from 6.90 to 8.20 for *B. cereus* PALB7 at 48 h. The *B. amyloliquefaciens* subsp. *plantarum* SFBA2 and *B. licheniformis* OALB2 strains had the highest growth at 72 h while *B. subtilis* subsp. *subtilis* SFBA3 at 96 h. The highest alkaline pH recorded for all of the *Bacillus* species was pH 8.5 at 96 h. The controlled fermentation with *Bacillus* strains starter culture highlighted *B. cereus* PALB7 to have the highest growth and alkaline pH at the short time of fermentation of 48 h. The *B. cereus* PALB7 was chosen as potential starter culture because *B. cereus* is a common natural microflora of various spontaneously fermented African locust bean [22, 24, 25]. *B. cereus* had been used as starter culture for African locust bean fermentation with satisfactory product [25]. Though *B. cereus* are known to cause mild food poisoning due to the production of up to three enterotoxins and one emetic toxin [36]. However, it should be noted that not all strains of *B. cereus* carry enterotoxin genes and are used as probiotics in feed and food [11].

Volatile compounds production

Aldehydes and acids are generally the major groups of compound formed by all the *Bacillus* strains (Table 2). *B. licheniformis* OALB7 produced the highest number (58) and level (15 030 ng/g) of compounds. The profile of OALB7 was dominated by aldehydes, in particular hexanal at 4 710 ng/g. Hexanal is generally responsible for a green odour [42]. Esters was produced by *B. licheniformis* OALB7 with decanoic acid methyl ester described as having a fermented odour, wine-like, fruity, floral, oily. Esters have been known to constitute a major volatile compound in various African fermented condiments [7, 19]. The esters are presumably the consequence of

chemical reactions between microbial acidic and alcoholic metabolites [19]. A trace amount (2 ng/g) of 2,5-dimethyl pyrazine was present, while sulfur and phenol compounds were not detected (Table 2).

Table 2 Volatile compounds in the headspace of dawadawa-type condiment produced from *Bacillus* fermented Bambara groundnut using GC x GC-TOFMS

Compounds	**Relative concentration ng/g**				
Aldehydes	**OALB2**	**PALB7**	**SFBA2**	**SFBA3***	**RI_{exp}**[a]
Acetaldehyde	ND[b]	300	ND	50	379
Propanal, 2-methyl-	40	ND	ND	ND	386
Butanal	40	ND	ND	ND	618
Hexanal	4710	50	ND	ND	791
Heptanal	480	ND	ND	ND	886
2-Heptenal, (E)-	140	ND	ND	ND	891
Benzaldehyde	250	570	230	90	953
Octanal	360	ND	ND	ND	1014
Benzeneacetaldehyde	240	ND	90	ND	1062
2-Octenal, (E)-	310	ND	ND	ND	1062
Nonanal	950	150	140	260	1108
2-Nonenal, (E)-	60	ND	ND	ND	1162
Decanal	90	ND	ND	ND	1204
2,4-Nonadienal, (E,E)-	30	ND	ND	ND	1217
4-Oxononanal	310	ND	ND	ND	1244
2-Decenal, (Z)-	220	ND	ND	ND	1263
Undecanal	70	ND	ND	ND	1309
2,4-Decadienal, (E,E)-	680	ND	ND	ND	1320
2-Undecenal	200	ND	ND	ND	1364
Dodecanal	110	20	ND	ND	1406
Pentadecanal-	ND	ND	ND	20	1713
Hexadecanal	30	40	20	ND	1713
Total ng/g	**9 310**	**1 140**	**470**	**420**	
Acids					
Acetic acid	280	70	3460	ND	737
Acetic acid, methoxy-	350	50	ND	ND	738
Acetic acid, methoxy-, anhydride	100	ND	ND	ND	740
Propanoic acid, 2-methyl-	ND	170	310	ND	791
Butanoic acid, 3-methyl-	ND	1270	860	ND	828
Phosphonic acid, (p-hydroxyphenyl)-	50	ND	ND	ND	895
5-Oxotetrahydrofuran-2-carboxylic acid	30	ND	ND	ND	1360
Undecanoic acid	ND	ND	30	ND	1795
Phthalic acid, cyclohexyl isohexyl ester	10	ND	4	ND	1847
n-Hexadecanoic acid	990	240	2160	90	1955
9,12-Octadecadienoic acid (Z,Z)-	590	130	1360	80	2128
Octadecanoic acid	ND	ND	140	ND	2157

Cyclopentaneundecanoic acid	60	ND	ND	ND	2257
Oleic Acid	50	ND	710	ND	2484
17-Octadecynoic acid	ND	60	ND	90	2648
Total ng/g	**2 510**	**2 000**	**9 040**	**250**	
Ketones					
2-Butanone	ND	1380	ND	ND	398
1-Octen-3-one	110	ND	ND	ND	915
2,3-Octanedione	260	ND	ND	ND	924
2(3H)-Furanone, 5-ethyldihydro-	10	ND	ND	ND	1028
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	20	ND	ND	ND	1445
2-Decanone	70	40	20	ND	1495
Benzophenone	ND	10	ND	10	1631
2-Dodecanone	ND	40	100	70	1897
ç Dodecalactone	ND	ND	30	40	2099
Total ng/g	**470**	**1 470**	**150**	**110**	
Esters					
Hexadecanoic acid, methyl ester	ND	ND	ND	10	1921
Decanoic acid, methyl ester	20	ND	ND	ND	1921
Benzoic acid, 2-hydroxy-, phenylmethyl ester	2	ND	ND	ND	1874
11,14-Eicosadienoic acid, methyl ester	110	ND	ND	ND	2162
Total ng/g	**130**	**ND**	**ND**	**10**	
Pyrazine					
Pyrazine, 2,5-dimethyl-	ND	30	ND	ND	861
Total ng/g	**2**	**30**	**ND**	**ND**	
Alcohols					
Ethanol	ND	710	ND	ND	369
1-Butanol, 2-methyl-	570	ND	ND	ND	530
Cyclobutanol, 2-ethyl-	ND	110	ND	ND	642
1-Octen-3-ol	120	ND	ND	ND	919
1-Hexanol, 2-ethyl-	40	ND	ND	ND	993
Ethanol, 2-phenoxy-	50	50	ND	ND	1223
trans-2-Undecen-1-ol	ND	ND	ND	30	1409
n-Tridecan-1-ol	ND	10	ND	ND	1490
2-Tridecen-1-ol, (E)-	40	ND	ND	20	1510
10-Undecyn-1-ol	ND	110	ND	ND	1660
8-Dodecenol	ND	ND	ND	10	1667
(Z)6,(Z)9-Pentadecadien-1-ol	ND	ND	10	ND	1863
1-Dodecanol	ND	20	ND	ND	1879

Total ng/g	**820**	**1 010**	**10**	**60**	
Nitrogen-containing compounds					
dl-Alanine	ND	ND	ND	60	719
1,2-Propanediamine	ND	290	ND	ND	721
Alanine	ND	ND	460	ND	726
Hydroxyurea	ND	ND	ND	90	731
2-Ethynyl pyridine	10	ND	ND	ND	894
1H-Pyrrole-2,5-dione	60	ND	ND	ND	1010
2,5-Pyrrolidinedione	10	ND	ND	ND	1154
Pyridine, 1-acetyl-1,2,3,4-tetrahydro-	10	ND	ND	ND	1192
Indole	20	ND	ND	ND	1297
Morpholine, 4-octadecyl-	ND	ND	30	ND	1884
Total ng/g	**110**	**290**	**490**	**150**	
Sulfur compounds					
Methanethiol	ND	ND	3	110	734
Propanesulfonylacetonitrile	ND	ND	ND	210	736
Disulfide, dimethyl	ND	ND	610	770	776
Dimethyl trisulfide	ND	ND	70	20	885
Benzoyl isothiocyanate	ND	30	ND	ND	1653
1,3-Benzenediol, monobenzoate	ND	10	ND	ND	1776
Benzene, [(methylsulfinyl)methyl]-	ND	1	ND	ND	1874
Total ng/g	**ND**	**40**	**680**	**1 110**	
Alkanes					
Octane, 3,5-dimethyl-	30	ND	ND	ND	1002
2,3,5-Trioxabicyclo[2.1.0]pentane, 1,4-bis(phenylmethyl)-	ND	ND	60	ND	1056
Octane, 1,1'-oxybis-	ND	110	50	60	1660
Undecane	10	ND	ND	ND	1699
Total ng/g	**40**	**110**	**110**	**60**	
Phenols					
Phenol	ND	ND	530	70	1000
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	ND	40	50	50	1619
9,12-Octadecadien-1-ol, (Z,Z)-	ND	ND	40	ND	2055
Phenol, 4,4'-(1-methylethylidene)bis-	ND	ND	80	30	2171
Total ng/g	**ND**	**40**	**700**	**150**	
Alkenes					
3-Tetradecene, (E)-	ND	ND	10	ND	1490

1,E-11,Z-13-Octadecatriene	ND	ND	60	ND	1660
1,6,11-Dodecatriene, (Z)-	ND	ND	ND	50	1660
1,11-Dodecadiene	ND	30	20	ND	1667
1,12-Tridecadiene	ND	ND	30	ND	1866
1-Nonadecene	ND	ND	70	ND	1889
5-Octadecene, (E)-	ND	ND	10	ND	1953
1-Docosene	290	ND	ND	ND	2314
Total ng/g	**290**	**30**	**190**	**50**	
Furans					
Furan, 2-pentyl-	1090	70	20	30	896
2-n-Octylfuran	20	ND	ND	ND	1293
Total ng/g	**1 110**	**70**	**20**	**30**	
Total ng/g (all classes)	**15 030**	**6 572**	**11 952**	**2 398**	
Number of compounds produced	**58**	**34**	**36**	**27**	

[a]RI_{exp}:Experimental Retention Index on a Rxi-5SilMS x Rxi-17SilMS column system. [b]ND: not detected. *SFBA3, *B. subtilis* subsp. *subtilis*; PALB7, *B. cereus*; OALB2, *B. licheniformis* and SFBA2, *B. amyloliquefaciens* subsp. *plantarum*.

B. amyloliquefaciens subsp. *plantarum* SFBA2 produced the second highest number (36) and level (11 952 ng/g) of compounds (Table 2). SFBA2 was characterised by organic acids, notably acetic acid (3 460 ng/g) and *n*-hexadecanoic acid (2 160 ng/g). The level of phenols was 700 ng/g, dominated mainly by phenol (530 ng/g). Dimethyl trisulfide and dimethyl disulfide were present at levels 70 ng/g and 610 ng/g, respectively. Dimethyl trisulfde and dimethyl disulfde showed meaty and vegetal aromas, respectively. Concentrations of aldehydes (470 ng/g) and ketones (150 ng/g) were the second lowest, while pyrazines and esters were not detected for this product. *B. cereus* PALB7 produced 34 compounds which was the second lowest number of compounds for the four *dawadawa*-type condiments. *B. cereus* PALB7 produced the second lowest level of total compounds at 6 572 ng/g (Table 2). *B. subtilis* subsp. *subtilis* SFBA3 produced the lowest number (27) and quantitatively the lowest level (2 398 ng/g) of compounds (Table 2). This product was characterised by the high level of sulphur compounds (1 110 ng/g) with methanethiol and dimethyl disulfide were detected. No pyrazines were detected. *B. cereus* PALB7 was characterised by ketones, alcohols and 2,5-dimethyl pyrazine. Esters were not detected for *B. cereus* PALB7. Of the four *dawadawa*-type condiments, 2,5-dimethyl pyrazine was produced only *B. cereus* PALB7 (30 ng/g). 2-Butanone (methyl ethyl ketone) was the major ketone (1 380 ng/g) present in *B. cereus* PALB7 and was not detected in the other three samples (Table 2). The major volatile compound groups

reported for traditional dawadawa made from African locust bean were pyrazines, aldehydes, alkenes, ketones, alcohols, esters and benzene derivatives. In contrast, aldehydes, acids and ketones dominated dawadawa-type condiments from bambara groundnuts. Pyrazines have been found in highest concentrations in dawadawa from African locust bean [3]. Only *B. cereus* PALB7 produced 2, 5-dimethyl-pyrazine in relation to the traditional condiments.

CONCLUSIONS

The present work identified the mixed-culture of *Bacillus* species involved in spontaneous fermentation of African locust bean and bambara groundnut with members of the *B. subtilis* group being the main species. The polyphasic approach for identifying these *Bacillus* species was successful in classifying closely related members of the *B. subtilis* group. Regarding the results obtained from the present work, the investigated *B. subtilis* subsp. *subtilis* SFBA3, *B. amyloliquefaciens* subsp. *plantarum* SFBA2, *B. cereus* PALB7 and *B. licheniformis* OALB2 were highlighted as potential starter cultures for the alkaline fermentation of bambara groundnut into *dawadawa*. However, evaluation of the organoleptic quality and consumer acceptability of the condiments produced are required to ascertain the preferred *Bacillus* strain.

Conflict of Interest

The authors declare that they have no conflict of interest.

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