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Microbial Engineering of Flavour Compounds from African Oil Bean Seed (AOBS) (*Pentaclethra Macrophylla*)

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Abstract

The aim of this research was to evaluate the production of flavor compounds during the fermentation of *Pentaclethra macrophylla* seeds using mono and mixed cultures of *Bacillus subtilis* and *Lactobacillus fermentum* and to establish the quality of the fermented products. *Pentaclethra macrophylla* seeds were pre-processed and fermented with monocultures of *B. subtilis* and *L. fermentum* as well as a combined culture where *L. fermentum* was used as adjunct. Traditional fermentation was used as the control. When the pure microbial isolates were compared using the basic local alignment search tool (BLAST), the organisms showed 93.4% similarity to *Bacillus subtilis* DQ198162.1, and 100% similarity to *Lactobacillus fermentum* respectively. Naturally fermented *P. macrophylla* contained fungi, spoilage and pathogenic as well as beneficial microorganisms. The flavor compounds from each mono starter fermentation and combined starters were monitored for 96 hours. Fermentation with isolated *B. subtilis* yielded mainly fatty acids. Fermentation with isolated *Lactobacillus fermentum* yielded mainly aldehydes. Fermentation with mixed *B. subtilis* and *L. fermentum* for 72 hours yielded mainly esters and traditionally fermented samples yielded numerous flavor compounds (mainly esters and acids).

Keywords: *Pentaclethra Macrophylla* Seeds, *Bacillus Subtilis*, *Lactobacillus Fermentum*, Fermentation, Fatty Acids, Flavour Compounds

Introduction

Pentaclethra macrophylla (Benth) (African Oil bean) (AOB) is a leguminous tree found in the tropical lowlands of West Africa. It belongs to the family Leguminosae and sub-family Mimosoideae. It has flat greyish seeds, which turn to purplish brown on maturity [1]. Its common names are African Oil Bean, "Congo acacia (Congo), and Koumbala (Cameroon), In Nigeria it is called Apará (Yoruba), Ukana (Efik), Ugba or Ukpaka (Ibo) [2]. It is frequently found in forest areas. At maturity the pods dehisce and disperse the seeds. The unfermented seeds are not edible and contain a number of toxic constituents such as caffeoyl putrescence, and paucine; but pre-processing followed by fermentation renders them edible and improves their nutritive value [3]. Extracts from the *P. macrophylla* leaves, stem bark; seed and fruit pulp have anti-inflammatory and anthelmintic activities. They are used to treat gonorrhea, diarrhea, ocular hypertension, convulsions, and pain [4]. *P. macrophylla* bark, stem, and leaves possess health benefits which are largely confined to ethnobotanical uses due to lack of scientific data to substantiate the claims [5, 6,7]. According to the ripe fruits are applied externally to heal wounds [8]. The root bark is used as a laxative; an enema against dysentery, and a liniment against itching. Due to their anti-inflammatory properties, AOB seeds and bark are used in the manufacture of ointment for treating itches, insects' bites, wound and skin related problems. Fermented African oil bean seeds (AOBS) is one of several plants products commonly used in Nigeria as food and as a condiment. *P. macrophylla* seed improves

lipid metabolism and could protect against atherosclerosis. Fermentation of *P. macrophylla* seeds to produce “ugba” softens the cotyledon, improves its digestibility and enhances nutrient availability [9]. The colour of the fermented seeds vary from light to dark greenish brown depending on the packaging material. Different types of microorganisms such as *Bacillus*, *Streptococcus*, *Salmonella*, *Escherichia coli*, *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Micrococcus*, *Lactobacillus*, *Proteus* and *Saccharomyces cerevisiae*, *Penicillium*, *Aspergillus*, *Fusarium* and *Rhizopus* may be involved. The fermented seed in the diet demonstrated beneficial role in lipid, bilirubin and liver enzyme status [10]. The fermented African oil bean seed is used as a delicacy and in the preparation of traditional sauces which serve as an accompaniment to many dishes in the African setting. The packaging during the natural fermentation process determines the flavor of the fermented product. Several leaves (Cocoa leaves, banana leaves etc.) and polyethylene may be used for packaging the sliced seeds for fermentation.

Traditional fermentation of *P. macrophylla* seeds into Ugba (the fermented product) is associated with numerous challenges such as variable quality of the products, un-standardized processing conditions, poor packaging, varied sensory properties and safety issues [11]. Several research efforts have been made to standardize cultures for the controlled fermentation of African oil bean seed. *Bacillus* has been observed to play a key role, while *Lactobacillus fermentum* serves as adjunct starter, assisting in the development of acceptable flavours. Earlier research works have reported the changes in proteins and amino acid profiles during *Bacillus* fermentation of African oil bean seeds with *L. fermentum* as an adjunct starter. The present study was undertaken to investigate the biotechnological changes in the lipid and flavor profiles of fermented African oil bean seeds. The objectives of the present research is to identify the microorganisms which have shown promise for use as starter cultures in the fermentation of *P. macrophylla* seeds and to compare the changes in lipid and flavour of products fermented by them as mono and mixed cultures with that of naturally fermented AOB seeds.

Materials and Methods

Materials

The raw material used for this study, (African oil bean seeds), were purchased from Fiong Arang market in Akwa Ibom State, Nigeria. Pure cultures of *B. subtilis* and *L. fermentum* were obtained from traditionally fermented African Oil Bean and was molecularly characterized at International Institute for tropical Agriculture (IITA) Ibadan, Nigeria. The flavor compounds were analyzed at the GCMS laboratory, University of Nigeria, Nsukka.

Methods

African oil bean meal was obtained from traditionally fermenting African oil bean seeds and pure cultures were isolated at 24, 48, 72 and 96 hours fermentation periods.

Preparation of Traditionally Fermented African Oil Bean Seed (AOBS).

Two and half (2.5) kg of AOB seeds were sorted and cleaned. The seed samples were processed by modification of the method of Nwanagba. The seeds were boiled for 4 h and the hard coats were removed manually. The cotyledons were sliced longitudinally, washed and boiled again for 2 h. After draining, four (4) portions of two hundred grams (200 g) of African oil bean were put into different beakers designated C24 h, C48 h, C72 h and C96 h and fermented at 28-30°C for 96 h. At every 24 h intervals, ten (10) g sample was collected from the fermenting samples, dried and packaged in airtight containers for further analysis (see Figure 1)

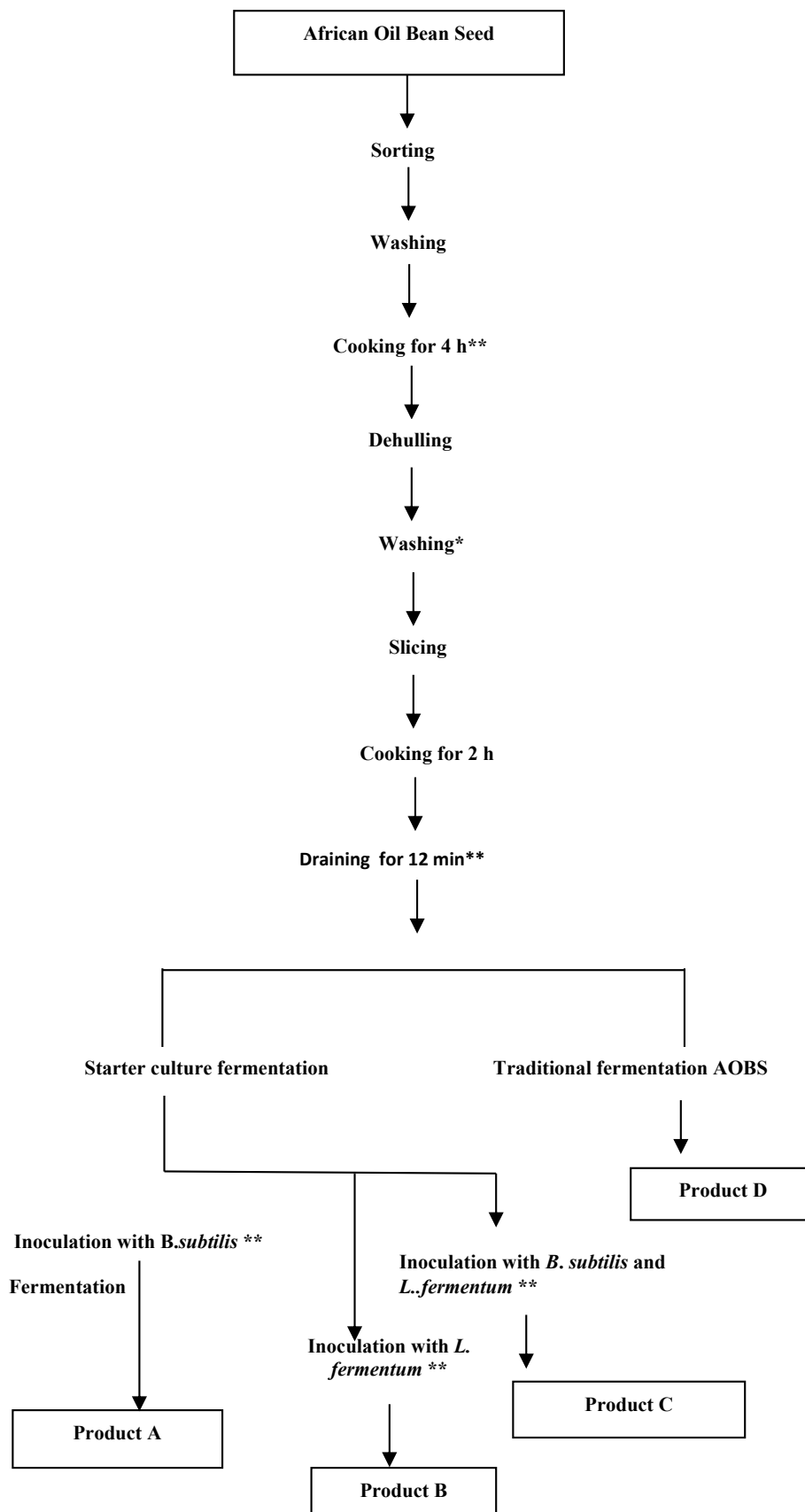


Figure 1.: Flowchart for the Processing of African oil bean Samples

(i) *--Hazard, (ii) **---Critical Control Point. (iii) Fermentation was for 24, 48, 72 and 96h Respectively. N/B: AOB = African Oil bean (Nwanagba et al., 2020) [15]. Product A= Fermented with B.subtilis alone; Product B = Fermented with L. Fermentum alone; Product C = Fermented with B.subtilis + L. Fermentum; Product D = Fermented traditionally.

Samples from Processed African Oil Bean Seed

Four samples were obtained from the different processing treatments as shown in Table 1

No	Sample code	Sample description
1	AOBS-1 (A).	Ugba produced from fermentation with B.subtilis only
2	AOBS-2(B).	Ugba produced from fermentation with L.fermentum only.
3	AOBS-3 (C)	Ugba produced from fermentation with B.subtilis and Lfermentum
4	AOBS-4 (D)	Ugba produced from traditional fermentation.
NB: AOBS= African Oil Bean Seeds		

Table 1: Samples from Major Processing Treatments of African Oil Bean Seeds

Determination of the Microbial Flora Responsible for the Traditional Fermentation of P. Macrophylla into "Ugba" Product

Isolates were prepared from traditionally fermented P. macrophylla samples using the method of [12]. Standard microbiological methods were used to determine the microbial flora responsible for the fermentation of P. macrophylla into "ugba". (Harrigan and McCance, 1990). Specific organisms were isolated on Nutrient agar and De Man Rogosa Sharpe agar (MRS). The colony characteristics of the microbial cultures were physically observed while the cell arrangement and the colony shape were observed with the help of a microscope.

Biochemical Characterization

Gram staining test, catalase test, spore test, gas production test; acid production; alcohol production, sugar utilization tests were carried out. The two major microbial cultures isolated were also cultured for growth on MRS agar at 15 °C, and 45 °C on Nutrient Agar at room temp (30±2 °C) [12].

Purification and Maintenance of Microbial Isolates

Discrete colonies of B. subtilis and L. fermentum from primary culture plates were sub-cultured repeatedly in freshly prepared Nutrient agar and MRS agar plates by streaking, and incubated for growth at optimum temperature. They were transferred and maintained in agar slants in the refrigerator for further use [12]. The two microbial isolates were identified using molecular techniques and used for the fermentation of African Oil Bean Seeds (AOBS).

Molecular Identification

Methodology for 16s rRNA PCR and Sequencing

The PCR cocktail mix consisted of 2.5ul of 10x PCR buffer, 1ul of 25mM MgCl₂, 1ul each of forward primer and reverse primer, 1ul of DMSO, 2ul of 2.5mM dNTPs, 0.1ul of 5u/ul Taq DNA polymerase, and 3ul of 10ng/ul DNA. The total reaction volume was made up to 25ul using 13.4ul Nuclease free water.

Primer Sequence for Bacterial Identification were

27F: AGAGTTTGATCMTGGCTCAG for B. subtilis and 1525R: AAGGAGGTGWTCCARCCGCA for L.fermentum

Initial denaturation at 94°C was for 5min, followed by 36 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and elongation at 72°C for 45sec. This was followed by a final elongation step at 72°C for 7 minutes and hold temperature at 10 °C. Amplified fragments were visualized on Ethidium bromide stained 1.5% agarose electrophoresis gels. The size of the amplicon was about 1500bp and the DNA ladder used was 1kbp ladder from NEB.

Cycle Sequencing

Cycle sequencing was done using 96 well plates and the products were purified using Ethanol/ EDTA precipitation method. Twenty-five (25) ng of the PCR product was used to perform cycle sequencing.

Preparing for 96 Well Reaction Plates

For each reaction the following reagents were added to a separate tube:- 8.0µL of Terminator Ready Reaction Mix; 3.2 pmol of Primer; 4 µL of ready reaction premix; 2 µL of BigDye sequencing buffer DNA template and deionized water. It was made up to a volume of 25 µL, mixed well and spun briefly.

Purifying Extension Products using ethanol/ EDTA Precipitation Method

Precipitating in 96-Well Reaction plates

The 25 µL 96-well reaction plates were removed from the thermal cycler and briefly spun. Five (5) µL of 125 mM of EDTA was added to each well, to reach the bottom of the well. Thereafter, 60 µL of 100% ethanol was added to each well. The plate was sealed with aluminum tape; inverted four times and incubated at room temperature for 15 min. The plate was spun at 1650 × g for 45 min using a Beckman Allegra 6A centrifuge set at 4 °C with a GH-3.8A rotor. The plate was inverted and spun up to 185 × g, before it was removed from the centrifuge. Sixty (60) µL of 70% ethanol was added to each well. The centrifuge was set at 4 °C and used to spin the plate at 1650 × g for 15 min. The plates were further inverted and spun up to 185 × g for 1 min, then removed. The plate was covered with aluminum foil and stored at 4 °C (Applied Biosystems- BigDye® Terminator v3.1 Cycle Sequencing Kit, 2010)

Sequencing of Bacterial 16s rRNA PCR Products for Homology Analysis

The PCR products were sequenced by the Applied Biosystems- BigDye® Terminator v3.1 Cycle Sequencing Kit, (2010). BLAST program was used to align the sequence with that from the GenBank database for homology analysis.

Electrophoresis of bacterial 16s rRNA PCR amplification products

After PCR amplification, 5 µl products were analyzed by 1% agarose gel electrophoresis at 110 V for 30 min along with a molecular weight standard DL 2000 Marker. The products were visualized on the gel imaging system, and images captured.

Activation of Starter and Adjunct Cultures for the Fermentation of African Oil Bean Seeds

A loopful of pure culture of *B. subtilis* was picked aseptically with a sterile loop, inoculated into 30 ml of 0.1% sterile peptone water in a test tube and incubated at 37 °C for 24 h. A quantity of 0.1 ml of it was aseptically transferred into a sterile solidified Tryptone Soya Agar (TSA) in petri dish. A sterile glass rod was used to spread the culture on solidified TSA plates to ensure even distribution. It was subjected to further incubation at 28±2 °C for 24 h. Due to the microaerophilic nature of *L. fermentum*, a loopful from a distinct colony was aseptically inoculated into 25 ml of MRS broth and incubated at 30 ± 1 °C for 48 h in an anaerobic jar. Thereafter, 0.1 ml each of *L. fermentum* culture suspension was aseptically transferred into different previously solidified MRS agar plates. A sterile glass rod was used to spread the culture for even distribution and incubated for 48 h in an anaerobic jar.

Preparation of McFarland standard

McFarland standard was prepared according to the method of Mahesh et al. (2025) and confirmed with the McFarland standard chart.

Preparation of Fermentation Culture Medium

A loopful of 16 h old culture of each single bacterium (*B. subtilis* & *L. fermentum*) from each plate was transferred into three different sterile transparent flasks, containing 30 ml each of normal saline. The addition of more organisms and dilution with normal saline stopped when the absorbance of the suspension compared favorably with that of McFarland standard. This was determined with a spectrophotometer at wavelength of 600 nm. The concentration (CFU/ml) of the starter used was determined from the McFarland standard chart by comparing the absorbance value equivalent (in millimeter) of the organism used for inoculation process.

Preparation of Starter Culture for Fermented African Oil Bean

Three (3) ml (9.0 x 10⁸ CFU/ml) each of *B. subtilis* was inoculated into 200 g each of African oil bean samples (AOBS) (designated A24 h, A48 h, A72 h and A96 h to represent different fermentation periods), Three (3) ml (9.0 x 10⁸ CFU/ml) each of *L. fermentum* inoculum was aseptically inoculated into 200 g each of AOBS designated B24 h, B48 h, B72 h and B96 h at pH of 6.3 and temperature of 36.5 °C and fermentation proceeded until 96 h. The 3rd sample was obtained by aseptically inoculating 3ml (9.0 x10⁸ CFU/ml) each of *B. subtilis* inoculum into 200 g each of the samples designated C24 h through C96 h for the initial fermentation period of 24 h followed by addition of 3ml (9.0 x 10⁸ CFU/ml) each of adjunct culture (*L. fermentum*) inoculum also into other samples designated C48 h, C72 h and C96 h at pH of 6.3 and temperature of 36.5°C. Fermentation proceeded until 96 h. The sample designated E0h (Cooked unfermented African oil bean sample) was dried immediately after cooking and drained for 12 min to prevent fermentation from taking place. For the fermenting samples, one lot was aseptically withdrawn at every 24h interval until the 96 h sample was taken, dried, packaged accordingly and kept in airtight containers for further analyses.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis Of Volatiles from Fermenting African Oil Bean Samples

Sample Preparation

An analytical grade N-hexane was used to extract oil containing fatty acid and flavour components from fermented AOBS sample, and the extract was used for GC-MS analysis to identify and quantify major volatile of fatty acids, esters, aldehyde, ketones and hydrocarbons.

Analysis

GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system comprising a AOC-20i auto-sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 x 0.25 mm ID x 1µm df, composed of 100 % dimethylpolydioxane); operating in electron impact mode at 70 eV; helium (99.999 %) was used as carrier gas at a constant flow of 1 mL/min and an injection volume of 0.5 µL was employed (split ratio of 10:1) injector temperature 250 °C; ion-source temperature of 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time was 36 min. Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) Abuja, having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained. The concentrations of the identified compounds were determined through area and height normalization.

The compounds detected in this work were characterized by MS data, together with the interpretation of the observed MS/MS spectra and compared with those found in the literature.

Data Analysis

GC-MS Data collected from the study were reported. Several public databases were consulted in the identification process, such as ChemSpider and PubChem [13,14].

Results and Discussions

Traditional Fermentation of Pentaclethra Macrophylla Seeds.

At least, ten microorganisms with different phenotypic characteristics (based on colony morphology, microscopy and biochemical tests) were found in the naturally fermenting *P. macrophylla* seeds. The probable organisms were *Staphylococcus* species, *Lactobacilli*, *Acetobacter*, *Bacillus*, *Micrococcus*, *Saccharomyces*, *Candida*, *Aspergillus* and *Penicillium* representing bacteria, molds and yeasts. *Bacillus subtilis* has been reported to play major roles in "ugba" fermentation [15]. *Micrococcus* sp., *Lactobacilli*, *Staphylococcus* sp., *Leuconostoc mesenteroides* *Proteus* and *Escherichia coli* were also found during natural fermentation of *P. macrophylla*. *Micrococcus* species increased with the keeping time showing that it is associated with the spoilage of ugba *Staphylococcus aureus* is associated with pathogenicity. *Lactobacillus* was the largest LAB genus and made significant contributions to the fermentation of *P. macrophylla* seeds. The numerous microorganisms associated with the traditional fermentation of pre-processed AOBS bean seeds indicate that during this spontaneous fermentation, beneficial, non-beneficial, spoilage and pathogenic microorganisms get involved. This emphasizes the need for controlled fermentation.

Molecular Characterization of Bacillus Subtilis and Lactobacillus Fermentum

Bacteria PCR amplification results Based on their size and electrical charge after electrophoresis, the PCR pattern depicted the gene expression of the microorganisms identified [16]. Molecular characterization confirmed the identity of *Bacillus subtilis* and *Lactobacillus fermentum* used as starter and adjunct for the conversion of sliced *P. macrophylla* seeds into the fermented product. reported that 95–99 % of the similarity of 16S rRNA gene sequence between two bacteria hints similarity in species while >99 % indicates the same bacteria [17]. Multiple sequence alignment showed conservation and homology of the isolated strains.

Chromatographic Patterns for Flavour Compounds during the Fermentation of P. Macrophylla using different Microorganisms

Raw and Cooked Samples

The chromatogram of flavour compounds of raw *P. macrophylla* raw sample showed 24 peaks, which increased to 37 peaks after cooking. Oils extracted from African oil bean seed contained several compounds and were made up of free fatty acids (FFA), triacylglycerols, glycolipids, diacylglycerols, phospholipids, and other minor components [18]. Cooked unfermented AOBS had comparatively lower content of oleic acid than raw ones but 7.9% content of hexanoic acid ester, cyclohexyl ester.

Flavour Compounds Associated with the Natural Fermentation of Pentaclethra Macrophylla

The GC-MS chromatogram of the traditionally fermented *Pentaclethra macrophylla* seeds is shown in Figure 2. There were about 10 clear observable peaks after fermentation for 96 hours.

The major fatty acids eluted in the GC-MS column were Linoleic acid, Oleic acid, trans-13-Octadecenoic acid , 9-Octadecenoic acid, (E), 9,12-Octadecadienoic acid (Z ,Z)-, cis-vaccenic acid, etc; (Linoleic acid and its derivatives). The flavour compounds were numerous in the traditionally fermented sample as a result of interplay of several microorganisms in the natural fermentation. Methyl, 10-trans, 12-cis-Octadecadienoate (24.978%); 6-Nonadecyltetra hydro-2H-pyran-2-one (35.62%); 9, 12-Octodecadialenal (34.89%) are some examples (see Figure 2).

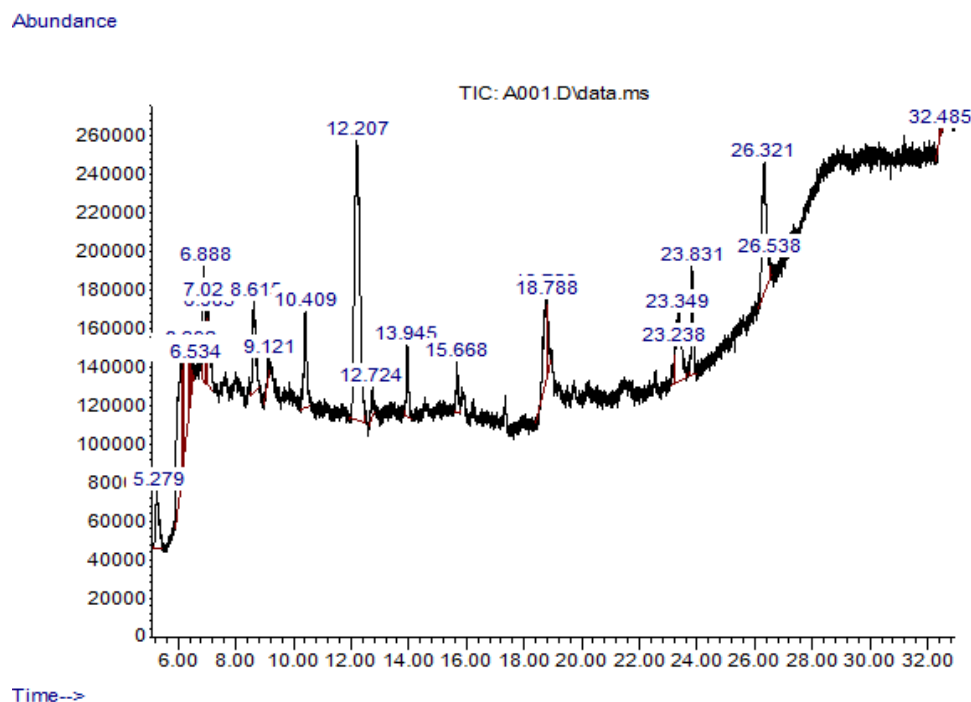


Figure 2: Chromatogram of Flavour Compounds from Traditionally Fermented *Pentaclethra Macrophylla* Seeds (24 hours) (AOBS D24)

Flavour Compounds from Fermenting *P. Macrophylla* seeds

Table 2 shows the flavor compounds from fermented *P. Macrophylla* Samples.

Organism	24 hours	48 hours	72 hours	96 hours
<i>Bacillus subtilis</i>	Fifteen peaks for flavor compounds, 7 were fatty acids. The others were esters, amides and alcohols	Six (6) compounds were observed. 3 were fatty acids.	Five (5) clear observable peaks. 1 fatty acid; alcohol; aldehyde; ester and hydrocarbon	Nine compounds. 5 fatty acids; 2 aldehydes; an ester and an alkane
<i>Lactobacillus fermentum</i>	Twenty six peaks. Fourteen (14) compounds. Two main types of fatty acids (oleic acid and cyclopentaneundecanoic acid). Others were aldehydes; and alkanes.	Twenty six peaks. Eight compounds were observed. Two types of fatty acids (linoleidic acid and octadecanoic acid). The other compounds were aldehydes.	Up to ten (10) compounds were observed. The fatty acids were oleic acid, octadecanoic acid; and cyclopentaneundecanoic acid. The other compounds were aldehydes and hydrocarbons.	Three compounds were observed-two aldehydes and one fatty acid (9,12, octadecadienoic acid (Z,Z)
<i>Bacillus subtilis</i> + <i>Lactobacillus fermentum</i>	The major fatty acid observed was oleic acid. Other compounds were esters and amides	Aldehydes, alcohol and alkenes were observed. There were many oleic acid fractions as well as cyclopentaneundecanoic acid	Esters, alcohols and amides were observed. The fatty acids observed were oleic acid; dodecanoic acid, n-hexadecanoic acid and cyclopentaneundecanoic acid	Esters, ethers, aldehydes and hydrocarbons were observed. The fatty acids present were mainly oleic acid and dodecanoic acid.
NB: Some of the peaks were found to be the same compound				

Table 2: Flavour Compounds Found in Fermenting African Oil bean Seeds using *Bacillus Subtilis* and *Lactobacillus Fermentum* as Mono and Combined Starter Cultures

Bacillus Subtilis Fermentation

The quantity of flavour compounds from *Bacillus subtilis* monoculture was highest on the first day and declined by the 3rd day. The compounds associated with 24 hour fermentation with *B. subtilis* were esters, amides and fatty acids. After 24 hours, substantial amounts of cis-13-Octadecenoic acid (96.6798 %) was observed. After 48 hours, aldehydes (1), fatty acids and their derivatives were observed (with large amounts of vaccenic acid-97.661%). After 72 hours, trans-13-octadecanoic acid constituted 92.2669% of the flavor compounds amidst esters, fatty acids and aldehydes. After 96 hours esters, alkane, aldehydes, fatty acid were present (with hexanoic acid constituting 94.3414% of the total concentration) as well as oleic acid. These compounds (except oleic acid) are derivatives of Linoleic acid. Cis-vaccenic acid is an omega-7 fatty acid with antibacterial activity [19]. Vaccenic acid lowers total LDL and triglyceride levels and raises

HDL [20]. 9,12-Octadecadienoic acid (Z,Z)-or alpha Linolenic acid possesses anti-inflammatory, hypocholesterolemic, cancer preventive activities and is beneficial for mental health, brain function and behaviour [21]. The predominant volatile which contributed to the flavour of AOBS A samples (Table 2) were the esters of unsaturated fatty acid although there were small quantities of aldehydes.

Flavour Compounds Produced by *Lactobacillus Fermentum*

Table 2 shows that the production of flavour compounds during *L. fermentum* starter fermentation was high for 2 days and declined by the 3rd day. After 24 hours, large amounts of aldehydes and fatty acids were observed: with cyclopentaneundecanoic acid constituting 35% and oleic acid constituting 17% of the flavor compounds, while the others were aldehydes. After 48 hours, aldehydes, fatty acids (with large amounts of octadecanoic acid) were found. After 72 hours, alkane; trans-octadecanoic acid (92.1309%) and cyclopentaneundecanoic acid (1.8542%) were found. After 96 hours, aldehydes, fatty acid (99.5923% octadecanoic acid Z Z) were observed. Oleic acid was 17.69% at 24 h and this changed to 9, 12-Octadecadienoic acid (Z, Z)-, a linoleic acid with 54.376% concentration. The Trans isomer of oleic acid (9-Octadecenoic acid (E)-) was observed at 48 h of fermentation with a concentration of 44.86%. It is an unsaturated fatty acid with code C18:1 trans-9. This compound is the major trans-fat found in hydrogenated vegetable oils. The result showed that the concentration of unsaturated fatty acid increases with fermentation time when fermented with *Lactobacillus fermentum*. (Table 2). *L. fermentum* monoculture fermentation led to the production of large amounts of aldehydes and trans fats.

Flavour Compounds from *Bacillus Subtilis* and *Lactobacillus Fermentum* Combined Culture Fermentation

The flavour compounds were highest on the second fermentation day for the mixed culture fermentation and declined on the 3rd day. Figure 3 shows the chromatogram of flavour compounds generated after 96 hours of fermentation by combined *B. subtilis* and *L. fermentum*. Peaks were much smaller when compared with that of flavour compounds from natural fermentation.

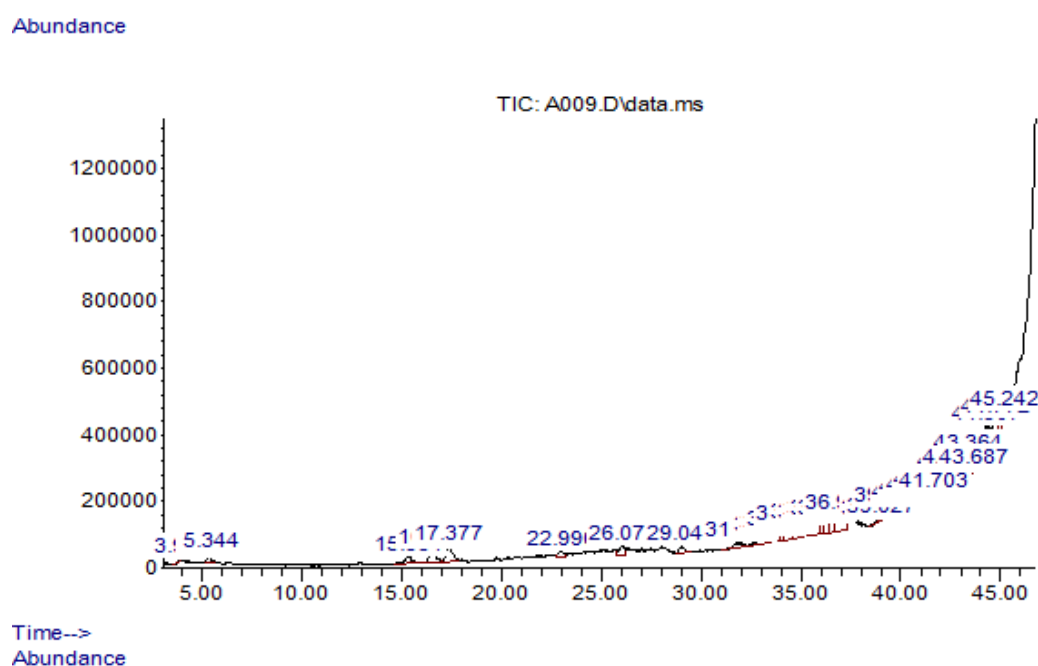


Figure 3: Chromatogram of Flavor Compounds Produced by Fermentation with Combined *B. Subtilis* and *L. Fermentum* Starter Cultures after 96 Hours

Oleic acid was the predominant unsaturated fatty acid observed, when the AOBS samples were fermented with a mixed culture of *Bacillus subtilis* and *L. fermentum*. Hydrocarbons, alcoholic acid, aldehydes, ketones and acetates were identified. Among them, Dodecanoic acid, 1-methylethyl ester had 26.314% (Table 2). This compound is also known as isopropyl dodecanoate, a fatty acid ester which is a metabolite found in the human saliva [22].

Also predominantly observed was 2, 4-Decadienal, (E, E)-(Table 2) an unsaturated aromatic aldehyde, also found in potato chips, roasted peanut and bread crumb. As an isolate, it has the deep fat flavor but at lower concentration, it has the odor of orange or grapefruit [23]. Trans-13- Octadecenoic acid, methyl ester (11.94%), a linoleic ester; pyrrole-2-carboxylic acid, 4-(1-chlorodec-1-enyl)-3,5-dimethyl ethyl esters (11.05%) and (S)-(1)-1,1-Bi-2-naphthol, O,O'-bis (trimethylsilyl)- (14.23%), were the other flavoring compounds observed when *B. subtilis* and *L. fermentum* were used to ferment the sample at 72h.

Methyl esters of fatty acids can be aromatic compounds produced in both single and mixed pure culture fermentations [23]. These compounds are produced by the methanolysis of acyl-CoA that is formed during fatty acid synthesis or

degradation. They contribute to quality sensory properties in fermented foods [23]. Esters can be products of reactions between microbial, acidic and alcoholic metabolites and are associated with nice flavour [24]. Using the mixed culture had a great impact on the flavor compounds of AOBS.

For fatty acids, oleic acid was found within the first 24 hour fermentation period. By the 48th hour, cyclopentaneundecanoic acid and hexadecanoic acid were also found in the fermenting substrate in addition to oleic acid. Dodecanoic acid was additionally found by the 72nd hour fermentation period, thereby further diversifying the fatty acid composition. By the 96th hour, oleic acid (82.98%) was found together with dodecanoic acid (17.22%). The fermentation process introduced variants of oleic acid by the 72 hour fermentation period (4 fatty acids) all of which were healthy fatty acids. Oleic acid improves the heart condition by reducing inflammation and lowering cholesterol levels. Dodecanoic acid is used to treat viral infections such as flu, the common cold; fever blisters, cold sores, and genital herpes caused by the herpes simplex virus (HSV) and HIV/AIDS. n-hexadecanoic acid has anti-inflammatory properties. Cyclopentane undecanoic acid has antioxidant and anti-bacterial properties. Thus fermentation diversifies the health benefits of *P. macrophylla*.

Flavor development during fermentation is achieved through biochemical reactions. Enzymes in the starter cultures generate metabolites that form the product. Proteases break down proteins into peptides and amino acids. Lipases break down fats into fatty acids and glycerol. Amylases break down starches into simple sugars. Galactosidases breakdown lactose into glucose and galactose [25].

AOBS fermented traditionally for 24h only had 1.8% oleic acid. Comparing this value to the values obtained at the same time when single and mixed cultures were used to ferment the samples, more of the fatty acids were eluted in the pure culture systems showing the positive impact of the starter cultures on the flavor compounds.

Udo and Ojimekwe, and several other researchers have made efforts to translate traditional fermentation of *P. macrophylla* into science using microbial biotechnology, exploiting the key role played by the *B. subtilis* [6]. Both *B. subtilis* and *L. fermentum* are known to possess probiotic properties [26]. Fermentation of oil seeds is accompanied by a change in the fatty acid profile. Lipases catalyze the hydrolysis of lipids to free fatty acids, Lipoxygenase produces conjugated hydroperoxy from the fatty acids. Hydroperoxide dehydrases and hydroperoxide lyases transform the products to carbonyl compounds [27].

Conclusions

The lipid fraction of African oil bean seed is rich in flavor compounds. Oleic acid and its derivatives showed predominance in monoculture fermentation with *Bacillus subtilis*. Aldehydes dominated monoculture fermentation with *Lactobacillus fermentum*. Esters dominated mixed fermentation by the two organisms. Fermentation with mono culture of *L. fermentum* is not recommended, but fermentation with a mixed culture of *B. subtilis*, using *L. fermentum* as adjunct produces a robust flavour with a lot of esters (known for their good flavours in fermented legumes). Acceptable Ugba can be produced from *P. macrophylla* seeds with *B. subtilis* and *L. fermentum* as starters. Fermentation for 72 h at 36.5 °C and pH 6.3 is appropriate for biochemical conversions of "ugba" using *B. subtilis* and *L. fermentum* as starter cultures. The mixed culture of *B. subtilis* (an alkaline fermenter) and *L. fermentum* (a probiotic lactobacillus) led to major biochemical transformations in this legume seed and enhanced flavor development.

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Conflict of Interest

The authors declare that there are no conflicts of interest associated in this research.

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