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Molecular Characterization of Bacteria from Cereal Based Pap Enriched with Soyabean in Abakaliki, Ebonyi State.

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ABSTRACT

The Molecular Characterization of Bacteria from Cereal Based Pap Enriched with Soyabean in Abakaliki, Ebonyi State was analyzed. This study was done in Abakaliki Metropolis of Ebonyi State, Nigeria. Total bacterial DNA from freshly prepared bacterial cultures was extracted using the Bacterial DNA MiniPrep™ kit (Zymo Research) at IITA Ibadan. The results showed that the 16SrRNA was amplified at bout 1500 bp using a 1000 bp DNA ladder. After aligning the sequences of the gene 16S rRNA, a region of 1500bp was used for phylogenetic analysis. The 16S rRNA sequences of the following strains representing the identified *Escherichia coli*, *Pseudomonas* specie and *Lactobacillus* species were identified. Based on molecular characterization the result obtained showed the species and strains of bacteria isolated from the akamu. In conclusion, Molecular characterization of bacterial isolates revealed that 16s rRNA sequences of the strain of *E. coli, Pseudomonas* species and *Lactobacillus* species isolated from the analyzed samples were identified
Keywords: Molecular, Characterization, Bacteria, Cereal, Pap and Soyabean.

INTRODUCTION

A cereal is any grass yielding starchy seeds which are suitable for human consumption [1]. Cereals have been major staple foods for the world population since the beginning of the modern civilization [2,3,4]. They are produced worldwide and mainly include rice, wheat, maize, sorghum, millet, rye, barley, triticale and oat. They are a good source of energy providing about 350 kcal per 100 grams of whole grains [5,6,7] and contribute to about half of the food protein available in the world, mainly in developing countries [8,9,10]. Cereals fulfil an important role in the diet as a source of starch and dietary fibre that make up to 70 to 77 % of the whole grain [11] while proteins account for only 6 to 15 % [12,13,14]. As whole grain, cereals can potentially contribute to vitamins [15] and minerals intake [16]. One of the popular indigenous cereal-based fermented foods in Nigeria is *akamu* or *ogi*, a kind of pap, which is a fermented cereal porridge made from maize (*Zea mays*), sorghum (*Sorghum vulgare*) or millet (*Pennisetum typoideum*) [17]. Ogi can be simply described as a kind of diet that does not require chewing. The cereal-based ogi is very smooth in texture and has a sour taste reminiscent of that of yoghurt. Typically, Ogi has a distinct aroma and fine texture. The colour of the Ogi is mainly depending on the type of feedstock used for the processing. It could either be consumed as porridge (pap) or as a gel-like product (*agidi*) in some West African countries [18,19]. It is an essential weaning food for infants as well as a dietary staple for adults in West Africa [8]. Generally, traditional cereal foods play a vital role in the diet of the people of tropical Africa particularly in cereal producing regions [10]. However, cereal proteins are low in several essential amino acids potentially causing protein malnutrition. Ogi or Akamu has been reported as being poor in protein and essential nutrients because during processing, the protein are lost in the steeping and wash water. Similarly, Ogi has been implicated in the cause of protein-energy malnutrition in children during the weaning period [13].

Aim and Objective of the Study

This study is aimed at Molecular Characterization of Bacteria from Cereal Based Paps Enriched with Soyabean in Abakaliki, Ebonyi State.

MATERIALS AND METHODS

Materials

The following materials were used for this study.

Bacteriological Media

The media used include: Nutrient Agar (Lab M Ltd, U.K.), tryptone soy agar, MacConkey agar, Plate Count agar (Micromaster, U.K.).

Equipment / Instruments

The major equipment and instruments that were used include: autoclave (Searchtech, England), incubator (Newlife, England), HY-4A cycling vibrator (PEC Medical, USA), microscope (Olympus Optical CO. Ltd, U.K.), refrigerator (Haier thermocool), weighing balance (Ohaus USA),/Bacterial DNA MiniPrep™ Kit (Zymo Research, USA) and Milling Machine (Crowen).

Reagents and Chemicals

The following laboratory grade analytical reagents and chemicals were used: Lugols iodine, Kovac's reagent, Griess-Ilovsay reagent, crystal violet, safranine, oxidase reagent, Methyl red reagent, normal saline, acetone – alcohol decolorizer, immersion oil, hydrogen peroxide, and bleach.

Methods

Study Area

The study area of this research was Abakaliki Metropolis of Ebonyi State, Nigeria. Ebonyi State is located in the south-eastern part of Nigeria which lies approximately within longitude 7 ^{0} 30 ^{1} and 7 ^{0}E and, latitude 5 ^{0} 40 ^{1} and 6 ^{0} 45 ^{1} N. It has a population of 149,683, and a land mass of about 5,935 square kilometers. Ebonyi State is bounded to the north by Benue State, to the south by Abia State and to the east by Enugu State and west by Cross River State respectively. Abakaliki, the State capital, has tropical climate with an average relative humidity of 75 % and may reach 80 % during rainy season. The vegetation characteristics are predominantly rainforest with atmospheric temperature of 30 ^{0}C. Two seasons are distinguishable in Ebonyi State: a dry season (November to March) and a wet season (April and October). Abakaliki residents utilizes various cereal-based food products for weaning purposes and general nutritionalpurposes.

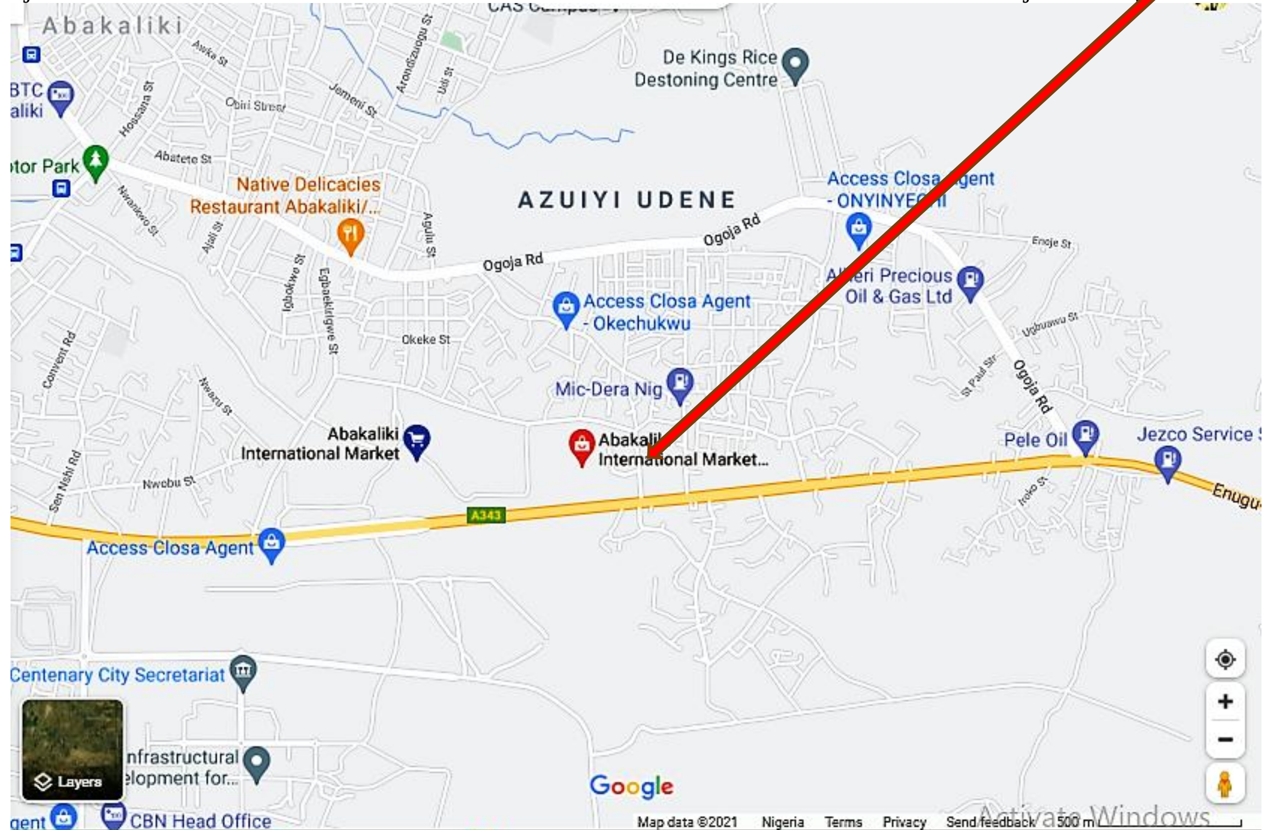


Figure 1: Map of Ebonyi State, showing the Study Area (Google Maps: 10/07/2021, 11:39pm).

Sample Collection

Exactly 2 kg each of white and yellow maize, white and red sorghum grains and 1 kg of soybean seed were randomly bought from traders at the Abakaliki international market Ebonyi State, Nigeria (i.e. the 5 samples were bought from 5 different shops). The samples were collected in polythene bags from the market and transported to the Applied Microbiology Laboratory of Ebonyi State University, Abakaliki for analysis. The cereal grains were authenticated by a taxonomist in the Department of Crop science, Faculty of Agriculture and Resource Management as *Zea mays, Sorghum bicolor* and *Glycine max* respectively.

Preparation of Cereal-Based Akamu

500g of each cereal grains were washed in distilled water to remove dust particles. The washed grains were steep in 1 Litre autoclaved distilled water for 48 h in covered cleaned plastic containers. Water was decanted and the fermenting grains were re-washed twice to reduce fermenting odour and then wet-milled with a blender. Wet milling of the different cereals grains was followed by wet-sieving to remove bran, hulls and germs. The waste was restrained on the muslin cloth and later dried as poultry feed, while the filtrate was fermented for another 24 h to yield ogi which is either white, cream or chocolate coloured depending on the pigment of the cereal grain.

Molecular Studies

Characterization of the Isolates

Total bacterial DNA from freshly prepared bacterial cultures was extracted using the Bacterial DNA MiniPrep™ kit (Zymo Research) according to [9] at IITA Ibadan. The process was carried out according to the manufacturer's protocol. Beta-mercaptoethanol was added to the Bacterial DNA Binding buffer to a final dilution of 0.5 % (v/v) *i.e.*, 500 µl per 100 ml. A 100 mg of bacterial cells that have been re-suspended in 200 µl of isotonic buffer was added to a ZR BashingBead™ Lysis Tube (0.5 mm). 750 µl Lysis solution was added to the tube and secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for ≥ 5 min. The tubes were centrifuged in a microcentrifuge at 10,000 x *g* for 1 minute. 400 µl of the supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection Tube and centrifuged at 7,000 x *g* for 1 minute. 1,200 µl of Bacterial DNA binding buffer was added to the filtrate in the collection tube. 800 µl of the mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10,000 x *g* for 1 min. The flow was discarded through from the collection tube. 200 µl of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000 x *g* for 1 minute. 500 µl of bacterial DNA Wash Buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10,000 x *g* for 1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl (35 µl minimum) DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000 x *g* for 30 seconds to elute the DNA. After this an Ultra-pure DNA was now ready for use. Extracted DNA was stored in 220 uC beforeanalysis.

Assessing the Quality and Purity of the Extracted DNA

DNA quality and purity was assessed using 1 % agarose gel for electrophoresis. All the isolated DNA samples were found intact and devoid of any form of shearing or degradation effects [9].

Polymerase Chain Reaction (PCR) Analysis using 16S rRNA Primer

The PCR preparation cocktail consisted of 10 µl of 5 x GoTaqcolourless reaction, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each of the 16S rRNA gene forward primer (16S rRNA: GTGCCAGCAGCCGCGCTAA) and reverse primer (16S rRNA: AGACCCGGGAACGTATTCAC) and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8 µl DNA template. PCR master mixture used in DNA amplification assay is presented in Table 1. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94 °C for 30 s, 30 s annealing of primer at 56 °C and 72 °C for 1 minute 30 seconds and a final termination at 72°C for 10 minwas maintained at 4 °C as its soaking temperature as described by [7] with little modification.

PCR Master Mixture

The PCR master mixture used in this study is presented in Table 1 below.

Table 1: PCR Mastermix

S/No	Reagent	1x(uL)
1	5 x GoTaq	10.00
2	25 mM $MgCl_2$	3.00
3	dNTPs (10 mM)	1.00
4	Forward primer	1.00
5	Reverse primer	1.00
6	10 pmol	1.00
7	DNA Taq (1000 U)	42.00
8	Ultrapure Water	8.00
9	**Total volume**	**67.00**

Primers for PCR Analysis

The primer that was used is 16S rRNA is listed in Table 2.

Table 2: Primers used for PCR Analysis

S/No	Primer Name	Primer Sequence Direction	Melting Temp. T_m (°C)	Amplicon Size (bp)
1	16S rRNA forward	GTGCCAGCAGCCGCGCTAA	56	1500
	16S rRNA reverse	AGACCCGGGAACGTATTCAC	56	1500

Assessment of Gel Integrity

The integrity of the DNA and PCR amplification was checked on 1 % and 1.5 % agarose gel respectively. The buffer (1 XTBE buffer) was prepared and subsequently used to prepare agarose gel. The suspension was boiled in a microwave for 5 min. The molten agarose was allowed to cool to 60 ˚C and stained with 3 µl of 0.50 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 min to form the wells. The 1 XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10 X blue gel loading dye was added to 4 µl of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products shall be estimated by comparison with the mobility of a hyper ladder 1 that was ran alongside experimental samples in thegel.

Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3 M and 240 µl of 95 % ethanol was added to each about 40 µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mixed thoroughly by vortexing and kept at -20 ˚C for 30 min. Centrifugation was carried out for 10 min at 13000 g and 4 ˚C followed by the removal of supernatant (tubes were

inverted on trash immediately) after which the pellet was washed by adding 150 µl of 70 % ethanol, it was mixed and centrifuged for 15 min at 7500 grams and 4 °C. All supernatant again was removed and the tubes were inverted on tissue paper and allowed to dry in the fume hood at room temperature for 10 min. It was re-suspended with 20 µl of sterile distilled water and kept in -20 °C prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel ran on a voltage of 110 V for about 1 h as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from Thermoscientific.

DNA Sequencing and Analysis

The amplified fragments were sequenced using a Genetic Analyzer 3130 xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator version 3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 was used for all genetic analysis. The samples were loaded on the machine and data in the form of adenine (A), cytosine (C), thyamine (T), and guanine (G) was released. The amplified 16S rRNA gene sequence was compared with the sequences in National Centre for Biotechnology Information (NCBI) sequence database using Basic Local Alignment Search Tool (BLAST) search program in accordance with the procedures of [10] with little modification.

Construction of Phylogenetic Tree

The phylogenetic/evolutionary history was inferred using the UPGMA method [12]. The optimal tree was determined. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. All positions with less than 95 % site coverage was eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases was allowed at any position. Evolutionary analyses were conducted in MEGA 5 [8].

Statistical Analysis

The raw data obtained in the course of the study was presented as mean ± standard deviation in tables and bar charts while relevant data was interpreted using simple descriptive statistics such as minimum, maximum, and one way analysis of variance (ANOVA) with the aid of IBM Statistical Package for Social Sciences (SPSS) version 22 and Microsoft Excel 2013 software. $P < 0.05$ was considered to be statisticallysignificant.

RESULTS

Molecular Analysis of Isolates

Plate 1 shows the genomic DNA template used in the PCR analysis of the isolated bacteria. The 16SrRNA was amplified at bout 1500 bp using a 1000 bp DNA ladder (Plate 2).After aligning the sequences of the gene 16S rRNA, a region of 1500bp was used for phylogenetic analysis. The 16S rRNA sequences of the following strains representing the identified *Escherichia coli*, *Pseudomonas* specie and *Lactobacillus*specie were identified. The ancestry of the tree was divided into two major groups. Group A was further divided into two sub-groups. Division 1 consists of isolates 4 & 5, isolate 10 and isolates 9 which are related. Isolate 4 & 5 are closely related to each other so as to isolates 6 & 7. Division 2 consist of isolate 1 & 3 which are closely related to each other. Group B consists of isolates 2 & 8 which are closely related to each other but more diverse from bacteria in Group A. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All

ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1473 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

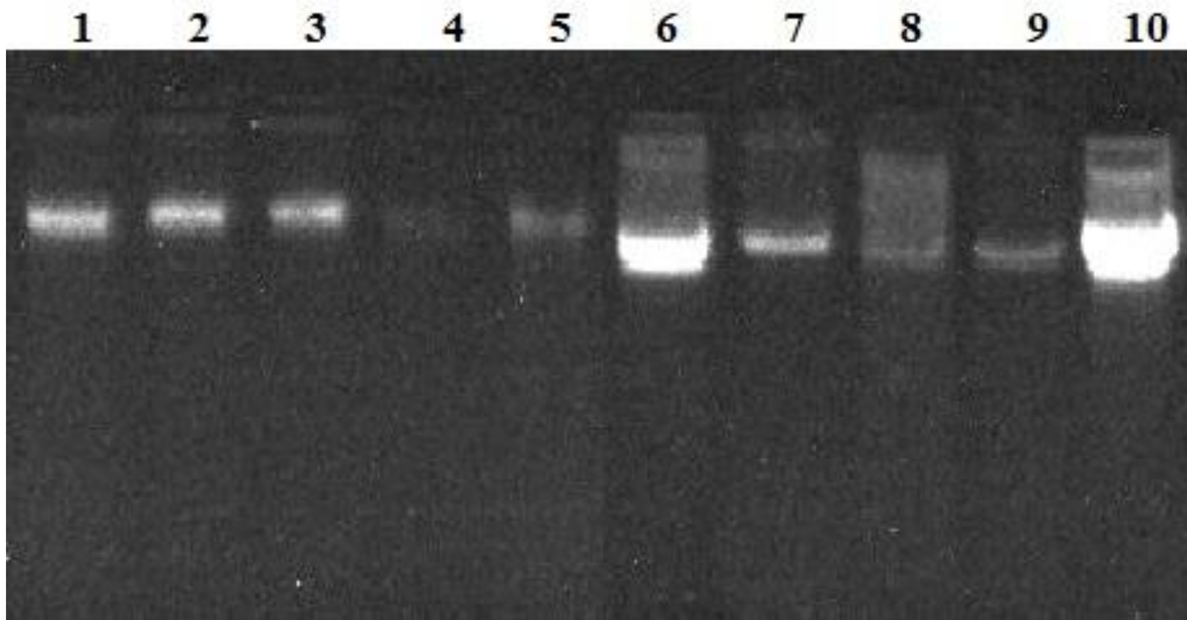


Plate 1: Gel electrophoresis image of the DNA template

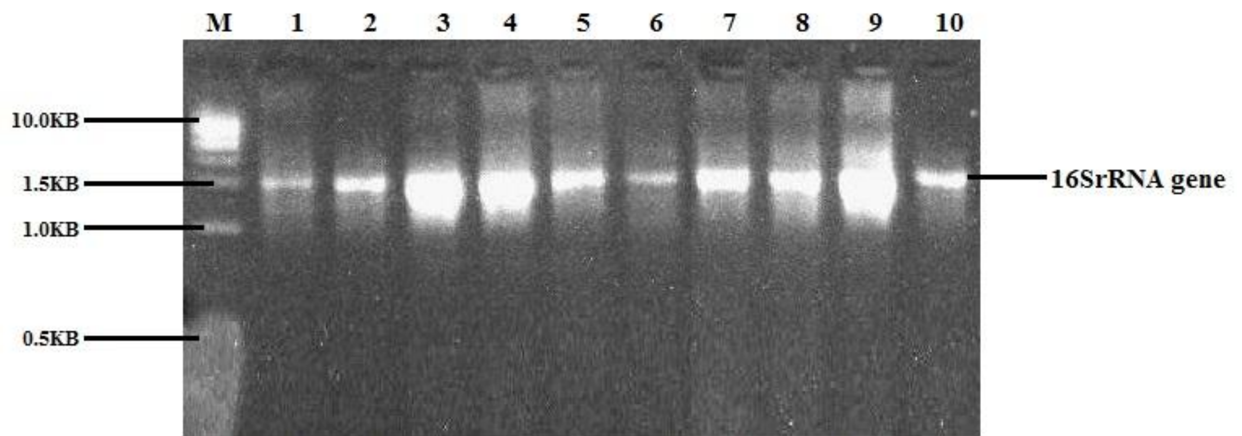
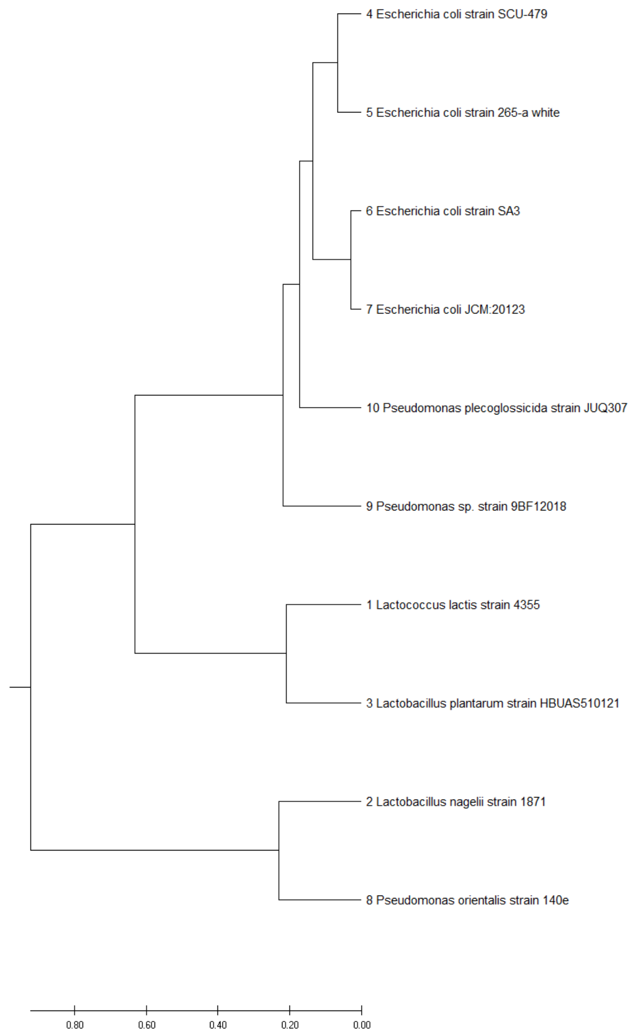


Plate 2: Gel electrophoresis image showing 16SrRNA gene amplification at about 1.5kb. (M is 1 kb DNA ladder NEB)



The ancestry of the tree above is divided into two major groups.
Group A is further divided into two sub-groups.

Division 1 consit of Isolate 4 & 5, Isolate 6 & 7, Isolate 10 and Isolate 9 which are related. Isolate 4 & 5 are closely related to each other so as Isolate 6 & 7.

Division 2 consist of Isolate 1 & 3 which are closely related to each other.

Group B consist of Isolate 2 & 8 which are closely related to each other but more diverse from organisms in Group A.

Figure 2: The Phylogenetic Tree.

DISCUSSION

Molecular analysis of isolates

Based on molecular characterization, the result obtained showed the species and strains of bacteria isolated. These groups of microorganisms have been reported to exist broadly in vast habitats including plants, within dairy and a wide range of fermented products of commercial interest globally as reported by [7]. Molecular characterization of bacterial isolates revealed that 16s rRNA sequences of the strain of *E. coli, Pseudomonas* species and *Lactobacillus* species isolated from the analyzed samples were identified. Based on molecular characterization, the result obtained highlighted *Lactobacillus* specie as the highest occurring organisms. This result is in agreement with the finding of [9] who reported that these group of microorganisms exist broadly in vast habitats including plants, within dairy and a wide range of fermented products of commercial interest globally. The ancestry of the tree showed three divisions within which isolate 4 and 5 are more related, isolate 6 and 7 are more related, isolate 1 and 3 are more related, while isolate 2 and 8 are equally closely related. This result is in agreement with the finding of [9] who reported a paired close relationship among isolates across the phylogenetic tree. Research has shown that the lactic acid bacteria *L. plantarum* is by and large one of the most predominant species of LAB with high occurrence rate isolated from plant sources through fermentation. These isolates are tested as probiotic candidates for their probiotic potential and applied in most food fermentation [6] *L. plantarum* was isolated from akamu a natural cereal food.

CONCLUSION

Food is essential for the survival of man and other living organisms. Nigeria has several staple food including plantain, cassava, maize, yam, etc. Majority of these food items are processed prior to consumption. Some also undergo fermentation processing via spontaneous process or with microorganisms found in the environment. Akamu is a typical fermented food largely produced from the fermentation of cereal grain (Maize/Sorghum) in Nigeria. Akamu is a major diet for the elderly and a weaning food in infants. During the fermentation of maize, several microbes are found in the medium including bacteria (of the Enterobacteriaceae family, lactic acid bacteria, obligate and facultative bacteria. Water, temperature of environment, storage conditions could be the major factor contributing to contamination and aflatoxin build-up of akamu as shown from the results of this research.

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