Original Research Article

Genomic Characterization of Bacteria and Fungi Associated with Palm wine (*Raphia Raphia* and *Elaeis guineensis)* in Port Harcourt Rivers State Nigeria

Abstract

Nigerian Palm wine has several nutritional, medical, religious and social uses which have been reported to have increasingly enhanced the demand for this natural product. However, the sap undergoes spontaneous fermentation, which promotes the proliferation of microorganism due to its nutritional content. The study was therefore aimed at investigating the pattern of microbial contamination of palm wine of different origin. In the present study, the genomic characterization of bacterial and fungal isolates associated with palm wine (*Raphia Raphia* and *Elaeis guineensis)* in Port Harcourt, Rivers State, Nigeria, was carried out using standard methods. The bacterial and fungal species were isolated on standard microbiological media, followed by the Sanger method, for their genomic identities. The result revealed that the palm wine harboured several species of bacteria of different genera which included *Bacillus pumilus, Bacillus altitudinis, Bacillus circulans and Bacillus* amyloliquef. The molecular characterization also revealed the fungal isolates included *Pichia* species. Results of enumeration of the microbial population revealed that palm wine from oil *Elaeis guineensis* had the highest values of total heterotrophic bacteria and total heterotrophic fungi (2.67 x 10^4 cfu/ml and 1.64 x 10^3 cfu/ml, respectively) compared to *Raphia raphia* (raphia palm) with values of 1.58 x 10^4 cfu/ml and 1.60 x 10^3 cfu/ml, respectively, for the different samples. The presence of these microbial populations in the drink is of public health importance.

Keywords: Bacteria, *Elaeis guineensis*, fungi, Genomic, *Raphia hookeri*, palm wine, microbiological.

1. INTRODUCTION

Palm wine is a collective name for an assortment of alcoholic beverages prepared from fermented palm sap. Agu *et al.* (2010) defined palm wine as a generic name for a group of alcoholic beverages obtained by fermentation from the saps of palm trees. It is consumed throughout the tropics and appears as a whitish liquid produced by natural fermentation of the sap of *Elaeis guineensis* and *Raphia hooker*i. Palm wine was recently reported to be the phloem exudates from the palm tree (Ojimelukwe, 2002). It is probably the most popular naturally fermented alcoholic beverage in West Africa, and in some parts of Nigeria, its production has developed into small scale industries. Palm sap is a sweet, clear colourless and nearly neutral juice containing 10-12% of sugar mainly sucrose. It is usually a whitish effervescent liquid (Agu *et al.,* 2009). These properties are derived from the metabolic activity of numerous microorganisms found in the wine (Mmegwa, 2007).

The quality of the wine as indicated by the taste is highly variable and depends among other factors, on the variety of palm, which the sap is obtained (Okafor, 2006). Within the same palm-type, the method of tapping induces microbial changes that apparently affects the composition of the sap and hence the quality of the wine. Upon fermentation by the natural microbial flora, the sugar level decreases rapidly as it is converted to alcohol and other products (Obire, 2005), whereas, the sap becomes milky-white due to the increased microbial suspension resulting from the prolific growth of the fermenting organism. Palm wine is characterized by an effervescence of gas resulting from the fermentation of the sucrose content by the fermenting organisms. Previous studies on the microbiology of *E. guineensis* and *R. hookeri* have incriminated several bacterial and yeast flora to be involved in the fermentation process (Ejiofor, 2004; Nester *et al*., 2004). These organisms have also been reported to originate from several sources, which include tapping equipment, containers, the environment, etc. Generally, both brands of palm wine have several nutritional, medical, religious and social uses which have been reported elsewhere (Iheonu, 2000), to have increasingly enhanced the demand for this natural product. The microflora of palm wine is believed to originate from the endogenous flora of the palm tree, the tapping implements such as the tapping knives, funnels and calabash (Karamoko *et al.,* 2012), the visiting fruit fly, Drosophila melanogaster and other insects attracted by the sweet aroma of the palm wine (Amoa-Awua *et al.*, 2007). Many microorganisms can be present during the palm wine production; however, they cannot be detecting because a good identification technique is not apply, and there are only a limited number of advanced identification studies. The microbial population identification in palm wine has been performed by the application of traditional methods and some molecular techniques (Amoa-Awua *et al.* 2007).

Conventional identification methods are commonly called traditional methods, their disadvantage is that only easily culturable microorganism can be detected, and members of microbial communities that need elective enrichment are not identified (Stringini *et al.,* 2009). Moreover, this phenotypic identification is time consuming and there is possible inaccuracy in the results (Martín-Platero *et al.,* 2009). However, others techniques applied to isolated microorganisms have been employed in the studies of the palm wine microbiology, those are based on polymerase chain reaction (PCR) amplification and analysis of restriction of the complex Internal Transcribed Spacer (ITS) regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) useful in measuring close fungus phylogenetic relationships (López *et al.*, 2006). Recent advances in microbial ecology and distribution render it possible to combine molecular and culture-dependent approaches in order to describe bacterial diversity (Brito *et al.*, 2012). For few decade microbiologists have relied on the use of 16S rRNA gene sequencing for identification, classification and estimation of bacterial diversity/dynamics in samples through PCR and DNA sequencing (Chikere *et al.*, 2012). PCR is an extremely sensitive technique that allows the amplification of millions of copies of a portion of a desired gene, entire gene or gene clusters with high fidelity within three (3) to four (4) hours with the help of a DNA polymerase enzyme and specific primers (Chikere, 2013), the amplified genome is therefore sequenced and compared with genomes in the data NCB base for the identification of the organisms to species level.

The presence of microorganisms in palm wine and its products play a major role in their shelf life. A previous report had indicated the preservative potential of *Zingiber officinale*, *Thaumatococcus daniellii* and *Vernonia amygdalina* in *Raphia hoiokeri* and *Elaeis guineensis* palm wine by possible suppression of microbial activities in the product (Ugboma *et al.*, 2021). Thus this present study was therefore aimed at isolating, enumerating and characterizing the bacterial and fungal species associated with palm wine.

2. Materials and Methods

2.1. Study Area/ Sample Collection

The study was carried out in Choba, Obio-Akpor Local Government Area in the metropolis of Port Harcourt, one of the major centres of economic activities in Nigeria, and one of the major cities of the Niger Delta, located in Rivers State.

Freshly taped palm wine from *Raphia raphia* (raphia palm tree) and *Elaeis guineensis* (oil palm tree) were collected at the point of tapping from traditional palm wine tappers in Choba, Rivers State, Nigeria. The samples were collected and transported to the laboratory in coolers equipped with ice packs within one hour of tapping in 1.5 litres sterile flasks for treatments and analyses. This procedure is intended to keep the samples at about 2 - 4°C (Ibekwe *et al*. 2006).

2.2 Isolation and Enumeration of Microbial Population

Serial dilution

A serial dilution is a series of sequential dilutions used to reduce a dense culture of cells to a more usable concentration or countable colonies usually 30 to 300 colonies. Each dilution will reduce the concentration of bacteria by a specific amount. Thus, prior to inoculation of the samples to the respective culture medium used in this study, serial dilutions were carried out using standard method as adopted by Ogbonna *et al.,* (2019). One millilitre each of the palm wine samples were separately added to 9 ml of normal saline (diluents). After thorough shaking, further 10-fold (v/v) serial dilutions were made by transferring 1 ml of the original solution to freshly prepared normal saline diluents to a range of 10^{-4} dilutions (Prescott *et al.*, 2005).

Inoculation, Incubation and Enumeration

Total Heterotrophic Bacteria (THB)

Total Heterotrophic Bacteria was enumerated as described by Prescott *et al* (2005). Bacterial Colonies that appeared on the nutrient agar plates which were inoculated in duplicate with an aliquot of 0.1ml from 10^{-4} dilutions were counted and the mean expressed as cfu/ml for the samples (Ogbonna *et al.,* 2019). While discrete colonies on the nutrient agar plates that were inoculated with an aliquot of 0.1ml from the direct samples were sub cultured on freshly prepared nutrient agar plate in order to isolate pure cultures. The colony forming unit per milliliter was calculated using the formula below;

$$\text{CFU/ml} = \frac{\text{number of colonies}}{\text{Dilution x volume plated}}$$

Total Heterotrophic Fungal Counts (THF)

This was determined using the Sabouraud Dextrose Agar (SDA) amended with Tetracycline to suppress bacterial growth (Okerentugba and Ezereonye 2003). The spread plate technique as described by Prescott *et al* (2005) was adopted. An aliquot 0.1ml from 10^{-2} dilution of the serially diluted samples was inoculated onto pre-dried SDA agar plates in duplicates. The inocula were then spread evenly on the surface of the media using a flamed bent spreader. The plates were then incubated at room temperature (25 ^oC) for 5 days after which the colonies that developed were counted and the mean total Fungi counts were recorded accordingly.

2.3 Purification of Isolates

After incubation, pure isolates were obtained by picking (with sterile inoculating loop) distinct culturally and morphologically different colonies from the various plates. These were subjected to streaking on sterile nutrient agar in plates until pure distinct colonies were formed.

2.4 Identification of Isolates by Conventional and Genomic Studies

2.4.1 Conventional method

The discrete bacteria isolated from the samples were characterized based on their cultural morphological which includes colour, texture, shape, size, elevation, etc of the isolate while, biochemical characteristics which include test include; Gram' reaction, motility, catalyse, oxidase, spore formation, indole production, methyl red, citrate utilization, voges proskauer test and sugar fermentation of the discrete bacterial isolates were compared with the recommendation by Cowan and Steel (1994), for the conventional identification of the bacterial isolates.

The fungal isolates were identified based on morphological and microscopic characteristics such as colony growth pattern, conidial morphology, and pigmentation. The isolates were further subjected to microscopy. This was done by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the aerial mycelia from the representative fungi cultures was removed and placed in a drop of lacto phenol. The mycelium was well spread on the slide with the needle. A cover slip was gently placed with little pressure to eliminate air bubbles. The slide was then mounted and viewed under the light microscope with ×10 and ×40 objective lenses. The morphological characteristics and appearance of the fungal isolates seen were identified in accordance with standard scheme for identification of fungi as adopted by Okerentugba and Ezereonye (2003).

2.4.2 Genomic Identification

Bacterial DNA extraction (Boiling method)

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95^0C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^oC for other downstream reactions.

16S rRNA Amplification

The 16s rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; anealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 20 minutes and visualized on a blue light transilluminator.

Fungal DNA extraction

Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of the suspected isolates was suspended in 200 microlitre of isotonic buffer into a ZR BashingBead Lysis tubes, 750 microlitre of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processedat maximum speed for 5 minutes. The ZR bashingbead lysis tube were centriguged at 10,000x*g* for 1 minute.

Four hundred (400) microlitres of suprnatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrufuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) microlitres of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 microlitre, 800 microlitre was then transferred to a Zymo-Spin IIC colum in a collection tube and centrifuded at 10,000x*g* for 1 minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microlitre of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 1 minute followed by the addition of 500 microlitre of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute.

The Zymo-spin IIC colum was transferred to a clean 1.5 microlitre centrifuge tube, 100 microliter of DNA elution buffer was added to the colum matrix and centrifuged at 10,000x*g* microlitre for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other dowstream reaction.

Internal Transcribed Spacer (ITS) Amplification

The ITS region of the rRNA genes of the isolates were amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; anealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator.

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The sofware of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestral, the upper pedestral was brought down to contact the extracted DNA on the lower pedestral. The DNA concentration was measured by clicking on the “measure” button.

Sequencing

Sequencing was done using the Bi-dye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25µl Bigdye terminator v1.1/v3.1, 2.25 µl of 5 x Bigdye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditio were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Jukes and Cantor 1969). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

Statistical Analysis

Data obtained from experiments were subjected to statistical analysis. Analysis of variance was used to determine the variation among the treatments. Duncan’s Multiple Range Test was used to separate the means at 5% level (Steel and Torrie, 1980).

3.0 Results and Discussion

In this study, total heterotrophic bacteria and fungi were enumerated from two different fresh palm wine sources, such as *Elaeis guineensis (*oil palm tree) and *Raphia raphia* (*raphia palm tree*). Results obtained revealed palm wine from oil palm tree (*Elaeis guineensis*) recorded higher values of THB and THF (2.67 x 10^4 cfu/ml and 1.64 x 10^3 cfu/ml, respectively) compared to *Raphia raphia (raphia palm tree)* with values of 1.58 x 10^4 cfu/ml and 1.60 x 10^3 cfu/ml, respectively. The counts expressed in $\log_{10}$ CFU//ml are presented in (Figure 1). The higher microbial counts obtained from *Elaeis guineensis* (oil palm tree) may be attributed to the nature of palm sap of the oil palm tree which has the ability to encourage the growth of more microorganisms. Also other organoleptic properties such as taste of palm wine can influence the rate of microbial growth in the palm wine. Also other organoleptic properties such as taste of palm wine can influence the rate microbial growth in the palm wine.

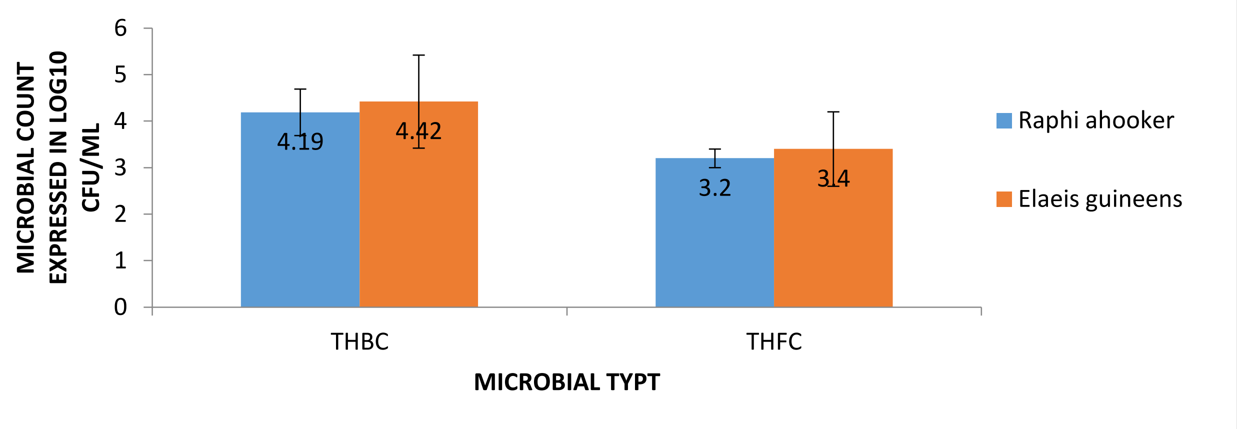

Figure 1: Results of the Mean microbial counts from the two Palm wine

Key: **THBC**= Total Heterotrophic Bacterial counts; **THFC** = Total Heterotrophic Fungal counts.

Data obtained from the study further showed that the palm wine samples harboured several strains of microorganism which may be as a result of the poor hygienic condition involved in the tapping, collection and distribution of the wine. However, Obi *et al.*, (2015) also reported similar high microbial counts in palm wine sample from Ikwuano Local Government Area of Abia State, Nigeria. Generally, both brands of palm wine have several nutritional, medical, religious and social uses which have been reported else where (Iheonu, 2000), to have increasingly enhanced the demand for these natural products. The sap of the palm trees, which is originally sweet (Naknean *et al.*, 2010; Santiago-Urbina *et al.*, 2013) serves as a rich substrate for the growth of various types of microorganisms. Soon after the sap is collected, and within an hour or two, the product becomes reasonably high in alcoholic content (up to 4%). If allowed to continue to ferment for more than a day, it starts turning into vinegar. The sap undergoes spontaneous fermentation, which promotes the proliferation of yeasts and bacteria for the conversion of the sweet substrate into several metabolites mainly ethanol, lactic acid and acetic acid (Amoa-Awua *et al.*, 2007; Stringini *et al.*, 2009; Ouoba *et al.*, 2012; Santiago-Urbina *et al.*, 2013).

Plate 1 showed the agarose gel image of amplified 16s rRNA of the isolates which is a unique gene for only bacteria species while Figure 4.2 showed the Evolutionary history of the isolates. It was inferred using the Neighbour-Joining method and bootstrap consensus tree inferred was from 500 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Plate 2 shows the amplied ITS gene of the fungi isolates while Figure 3 showed the phylogenic tree of the fungal isolates

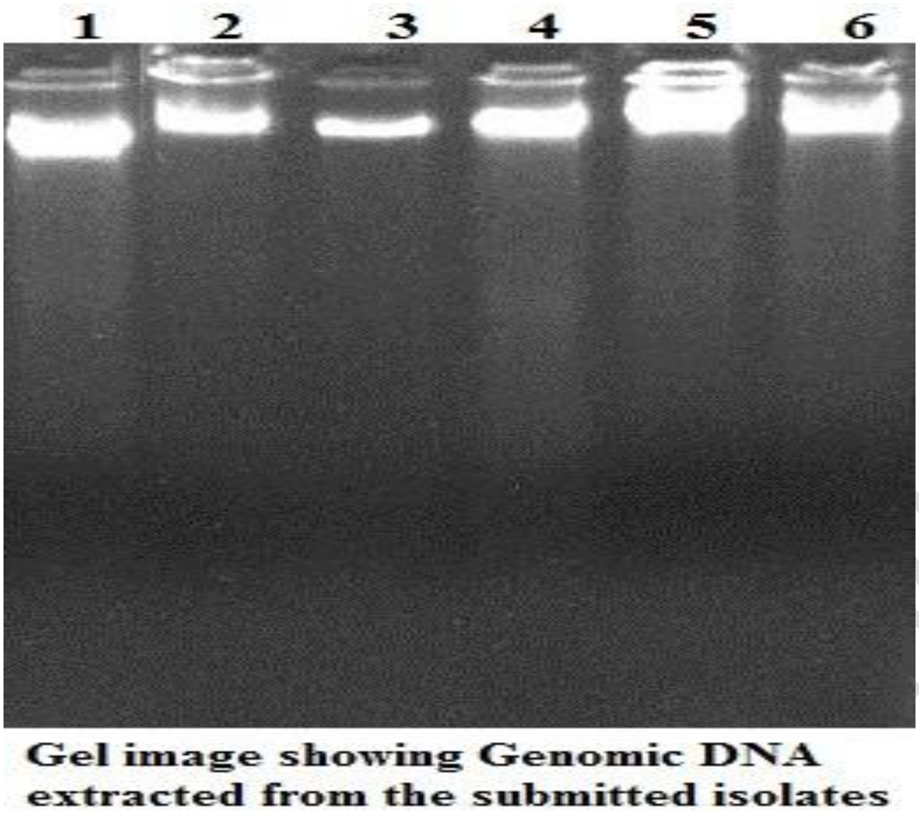

Plate 1: agarose gel image of amplified 16s rRNA of the isolates

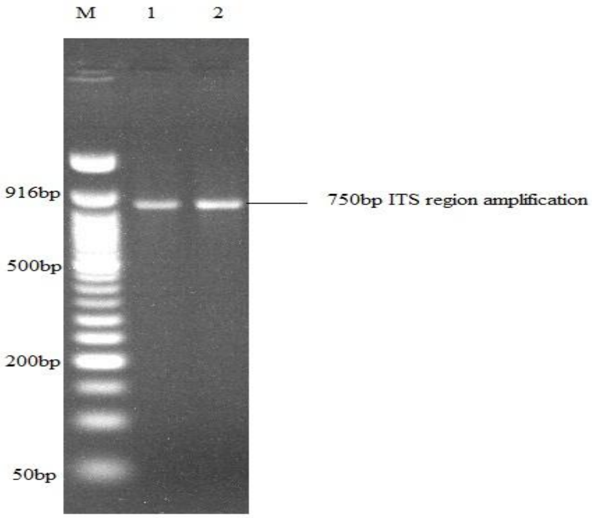

Plate 2: Gel image of ITS region amplification of the Fungi isolates. Lane M is 50bp Molecular weight ladder. Lane 1 and 2 are isolates 1 SDA and 2 SDA respectively.

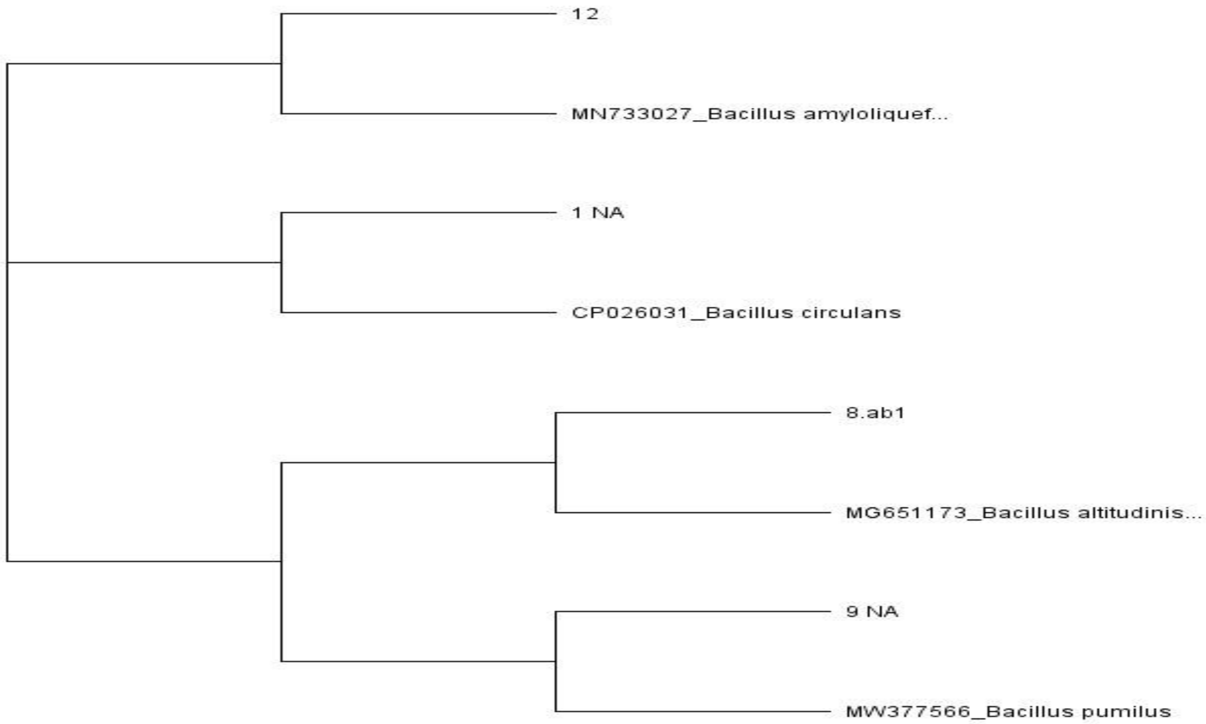

Figure 2: Phylogenic Tree Showing the Evolutionary Distance of the Bacterial Isolates

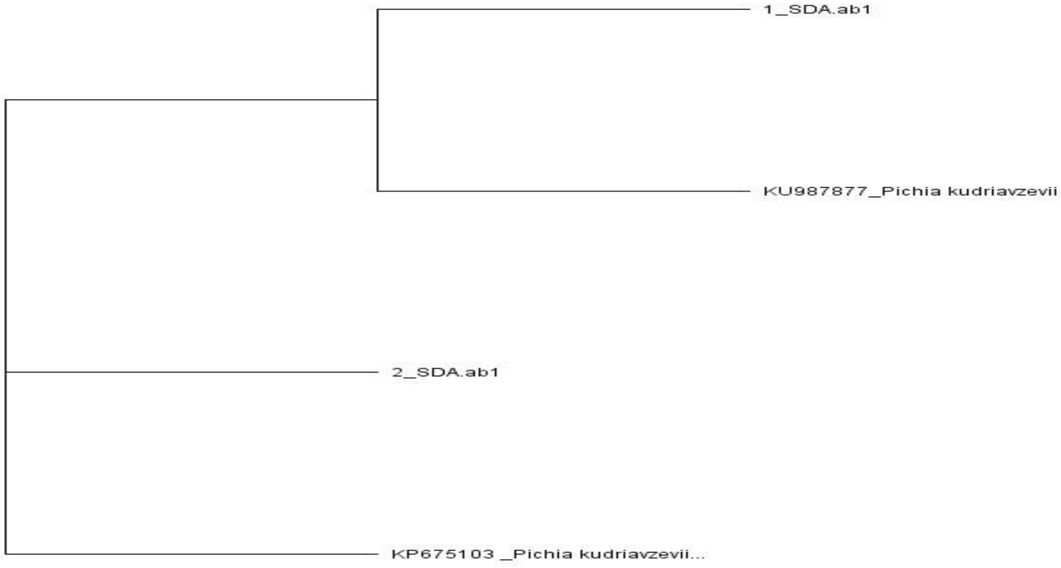

Figure 3: Phylogenic Tree Showing the Evolutionary Distance of the Fungal Isolates

Ranjard *et al*. (2000) reported that cultural techniques are no longer reliable in the identification of microorganisms due to anomalies and human errors, hence the use of molecular identification. Bacterial 16S rRNA sequences were aligned with BLAST algorithm of National Centre for Biotechnology Information (NCBI) database. Sequences aligned showed 100% similarity with those deposited in GenBank and the isolates were genotypically identified as *Bacillus pumilus, Bacillus altitudinis, Bacillus circulans and Bacillus* amyloliquef which were isolated and identified from the two brands of the palm wine. Also, the molecular characterization revealed the presence of *Pichia* species in the palm wine. Molecular techniques have been employed recently, to facilitate the reliable identification of microorganisms. Amplification of sequences that is specific for an organism can be done through polymerase chain reaction. The presence of these organisms in the wine is an indication of the poor hygienic state of the tappers, materials used and the method of tapping involved. This occurrence is of public health importance as the wine is consumed in almost every part of the country due to its nutritional, health and social significance. There is therefore the need for public awareness in the consumption of these wines to help promote the quality of these products as well avoiding the health risk that may be associated with the consumption of contaminated palm wine. Karamoko *et al.,* (2012) also reported the presence of yeast and other bacteria isolate from palm wine sample. However, according to Ogbulie (2007), the methods of palm wine tapping and collection of palm sap influence the microbial content of the sap.

4.0 Conclusion

Palm wine and its distillate are important solvent in herbal medicinal administration. Pregnant women consume it fresh for the sweetness and nutrition while nursing mothers drink it warm to enhance breast milk production. In addition, majority of people drink palm wine during social activities and other ceremonies. The presence of these organisms in the samples as identified in the present study is of public health importance, if not properly handled. Therefore, there is need to promote the quality of these products to enhance their nutritional and health benefits as widely consumed in this part of country.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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