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

Palm wine has been severally reported for its nutritional, medical, religious and social uses in Nigeria. The ever increasing importance of this product has resulted in an increased demand and consumption of the product. The aim of this study therefore, was to investigate 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)* was carried out using classic microbiological procedures. The bacterial and fungal species were isolated on standard microbiological media, followed by the Sanger method for their genomic identities. The data obtained indicated the presence of several species of bacteria such as *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 the association of palm wine from oil *Elaeis guineensis* with higher counts of total heterotrophic bacteria (THB) and total heterotrophic fungi, THF (2.67 x 10^4 cfu/ml and 1.64 x 10^3 cfu/ml, respectively) as against palm wine from *Raphia raphia* (raphia palm) with values of 1.58 x 10^4 cfu/ml and 1.60 x 10^3 cfu/ml, for THB and THF, respectively. The microbiological quality of the palm wine samples reported in this study has accentuated some industrial and health importance of the product.

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

1. INTRODUCTION

Beverages from natural sources has been given much attention for both traditional and religious reasons. Palm wine has been classified as a natural alcoholic beverage due to its alcohol content following fermentation. Agu *et al.* (2010) had classified this product, palm-wine among beverages obtained from palm trees that become alcoholic drinks, after a fermentation process. The product is whitish liquid taped from *Elaeis guineensis* and *Raphia hooker*i, and is well known and used mostly in the tropical regions. Palm-wine was recently reported to be the phloem exudates from the palm tree (Ojimelukwe, 2002. 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).

Different factors, including the source or variety of the palm tree influence the quality of the palm-wine products (Okafor, 2006).Variations in the quality, indicated mostly by taste and appearance have been observed within the same palm wine type, and have been reported to be influenced by the tapping technique, which in part causes changes in microbial community structure, that seemingly affect the constituent and conditions of the sap. Fermentation of palm wine by indigenous microbial flora, causes a rapid decrease in the sugar level due to its conversion, causing it to become alcoholic in nature (Obire, 2005).

Studies by earlier researchers (Ejiofor, 2004; Nester *et al.*, 2004) had implicated different microbial flora to be associated with the fermentation process of palm-wine. These microbial groups have been reported to be originating from sources such as the tapping devices and methods, as well as the surrounding environment, etc. Palm-wine from both *Elaeis guineensis* and *Raphia hooker*i sources has reported to have different nutritional, religious, medical and social uses which has resulted in an increased demand for palm-wine products (Iheonu, 2000). 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 flavour of the palm-wine (Amoa-Awua *et al.*, 2007). Application of good and advanced techniques has limited the identification of diverse microbial groups in this product. The application of both conventional and nucleic acid based approaches have however aided some researchers in the identification of microorganisms associated with palm-wine (Amoa-Awua *et al.* 2007).

Conventional methods also known as traditional methods for the identification of microorganisms are limited, since only a proportion of microbial cells, mostly those amenable to culture, can be isolated based on cultural techniques, while the fastidious organisms with special growth requirements are not isolated with ease (Stringini *et al.*, 2009). Also culture based characterization is usually rigorous with various degrees of uncertainty in the classification and identification of the species (Martín-Platero *et al.*, 2009). This notwithstanding, an array of nucleic acid based techniques have been employed in recent studies to characterize the microorganisms associated with palm wine. These techniques include the use of polymerase chain reaction (PCR) dependent methods and methods that target special regions to determine the evolutionary relatedness of the microbial species (López *et al.*, 2006). The combination of molecular and cultural methods provides veritable approaches to describe bacterial diversity (Brito *et al*., 2012). For few decade researchers have adopted the sequencing and analysis of the 16S rRNA gene, using PCR and DNA sequencing techniques, for the characterization and identification of microorganisms (Chikere *et al*., 2012). With the PCR-based methods, specific regions of the entire gene, clusters of genes or a target region can be amplified, and studied. The technique is a highly sensitive approach that allows for the extension of the chain length of a gene fragment within a short period of time, usually, three to four hours, with the aid of a special enzyme, DNA polymerase and specific target-primers (Chikere, 2013). The resulting amplified gene fragments are sequenced using a sequencer and checked for corresponding or similar sequences using the basic local alignment search tools (BLAST). This therefore enables 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, Port Harcourt, Rivers State, Nigeria. 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 palm wine samples were collected and conveyed to the laboratory in coolers containing 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

2.2.1 Serial dilution

The samples were subjected to a stepwise decimal dilution process that enables the reduction of the microbial number to a countable level as adopted by Ogbonna *et al.,* (2019). This was achieved by introducing 1ml of the palm wine sample into a test tube containing 9ml of normal saline. This sequential dilution process was done serially up to 10^{-4} dilutions was obtained and used for the enumeration of microbial population in the sample (Prescott *et al.,* 2005).

2.2.2 Estimation of microbial population

Enumeration of Total Heterotrophic Bacteria (THB)

The population of Total Heterotrophic Bacteria in the sample wa**s** carried out as described by Prescott *et al* (2005). This was achieved by transferring 0.1ml of the serially diluted sample (10^{-4} dilutions) unto freshly prepared nutrient agar plates, in duplicates and incubated at 37^{o}C for 24hrs. The resulting colonies, after 24 hours incubation were counted and expressed as cfu/ml for the samples (Ogbonna *et al.*, 2019). The total colonial population was determined by the formula given below;

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

Estimation Total Heterotrophic Fungal (THF)

The estimation of the Total Heterotrophic fungi was carried out as described by previous researchers (Okerentugba and Ezereonye 2003). This was done using using the Sabouraud Dextrose Agar (SDA), using the spread plate method. The inhibition of bacterial growth was achieved by amending the media, SDA, with Tetracycline as described by Prescott *et al* (2005). A 0.1ml volume from 10^{-2} dilution of the serially diluted sample was inoculated and spread over the surface of the solid fungal growth media. The plates were then incubated at room temperature (25 ^{o}C) for 7 -5 days after which the colonies that developed were counted and the mean total Fungi counts were recorded accordingly.

2.3 Production of pure cultures

The isolation of pure colonies of the isolates was done using an inoculating wire to transfer colonies from the plates with microbial growth unto freshly prepared nutrient agar plates, using a back and forth pattern to provide different zones of colonial growth in a manner that the organisms are caused to grow distinctively as discrete colonies. After incubation, pure isolates were obtained by choosing (using a sterile inoculating loop) unique culturally and morphologically different colonies from the several plates.

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 cultural identification of the fungal isolates done by checking their morphological as well as microscopic characteristics, which included colonial growth pattern on the media, the morphology of the conidia, and presence cum type of pigmentation. The microscopy of the fungal isolates was done using lacto phenol cotton blue to stain the fungal cell on a clean grease-free slide. "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 isolates were further characterized and identified in accordance with standard scheme for identification of fungi" as adopted by Okerentugba and Ezereonye (2003).

2.4.2 Genomic Identification

Extraction of Bacterial DNA by boiling method

The extraction of the bacterial DNA was done by spinning five milliliters of the bacteria previously cultured overnight in Luria Bertani (LB). This was done at a using a centrifuge at 14000rpm for 3 min. This was followed by re-suspending the cells in 500ul of normal saline and later heated at 95^{0}C for 20 min. Bacterial suspension, after heating was cooled on ice and spun for a period of 3 min at 14000rpm. The resultant supernatant containing the DNA was later transferred to a 1.5ml micro centrifuge tube and stored at -20^{o}C.

16S rRNA Amplification

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

Fungal DNA extraction

Fungal DNA extraction was carried out using a ZR fungal DNA mini prep extraction kit by Inqaba South Africa. Pure cultures of the suspected isolates were suspended in a 200 microlitre volume of isotonic buffer and transferred into a ZR Bashing Bead Lysis tubes, with 750 microlitre of lysis solution added to the tube. The ZR bashing bead lysis tube were centrifuged at 10,000x*g* for 1 minute.

After the centrifugation, Four hundred (400) microlitres of the supernatant was later transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) microliters of the fungal DNA binding buffer was added to the filtrate, making a final volume of 1600 microliter in a Zymo-Spin IIC column. This was followed by centrifuging (at 10,000x*g* for 1 minute) 800 microlitre volume of the final volume, with the flow through discarded from the collection tube. Two hundred (200) microlitre of the DNA Pre-Wash 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 microliter of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute.

The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube, 100 microliter of DNA elution buffer was added to the column 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 downstream reaction.

Internal Transcribed Spacer (ITS) Amplification

The implication of the 16s rRNA region of the fungal isolates' genes was done using the primers, 27F ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3', on an ABI 9700 Applied Biosystems thermal cycler. This involved a final volume of 25 microlitres for 35 cycles. The PCR mix had: the primers at a concentration of 0.4M, the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), as well as the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. After the PCR process, the product was set on agarose gel (1%) at 120V for 20 minutes and visualized with the aid of a blue light transilluminator.

DNA quantification

After the DNA extraction, Nanodrop 1000 spectrophotometer was used to quantify the extracted genomic DNA as previously described (Sampson and Yom, 2021). The involved initializing the equipment with 2 µl of sterile distilled water and blanked using normal saline, and loading the lower pedestal with two microliter of the extracted DNA, while the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was then determined by clicking on the “measure” button.

Sequencing

The determination of the order of nucleic acid in the fungal DNA genome was done by Inqaba Biotechnological, Pretoria South Africa, using the Bi-dye Terminator kit on a 3510 ABI sequencer. The sequencing process involved a final volume of 10ul, in the presence of 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 condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

Phylogenetic Analysis

“The evolutionary relatedness of the isolates was evaluated using the bioinformatics algorithm Trace edit, by down loading similar sequences 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

“Statistical analysis was carried out using Analysis of variance, to check for differences in the mean of the two samples. 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.

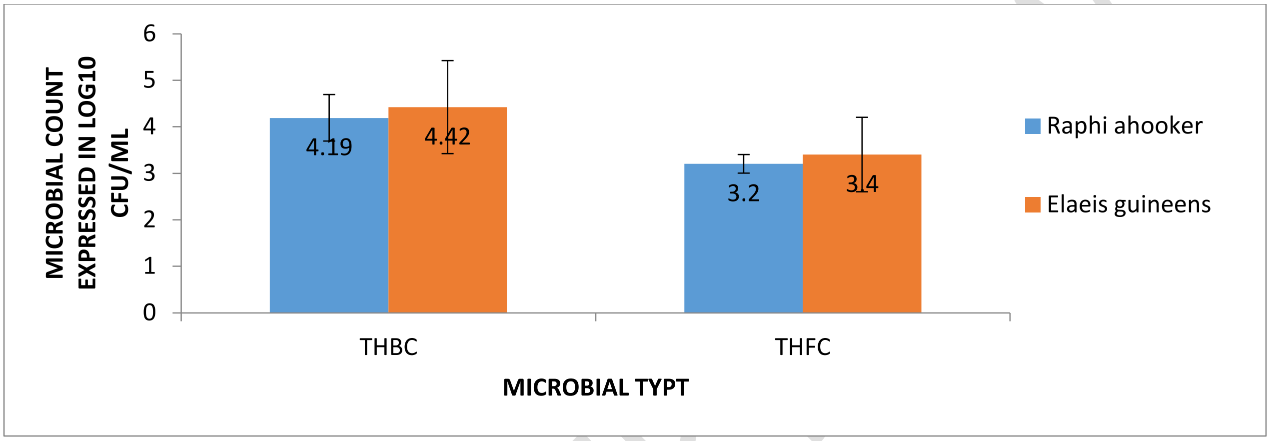

Figure 1: Results of the Mean microbial counts from the two Palm wine

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

Data obtained from the study further showed that the samples from the two different palm wine types contained different species of microorganism which might be due to poor hygienic condition during the tapping, collection and distribution of the palm wine. This finding is however, in line with that of Obi *et al.*, (2015). They also reported similar high microbial counts in palm wine sample from Ikwuano Local Government Area of Abia State, Nigeria. Generally, the two brands of palm wine are well known reported for their nutritional, medical, religious and social uses (Iheonu, 2000). This has made the demand for these natural products to be on the increase. “The sap of the palm trees, which is originally sweet (Naknean *et al.*, 2010; Santiago-Urbina *et al.*, 2013) provides a rich substrate for the proliferation 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 2 showed the Evolutionary history of the isolates. The evolutionary distances were calculated using the Maximum Composite Likelihood method and are expressed in terms of the number of base substitutions per site. Plate 2 shows the amplified ITS gene of the fungi isolates while Figure 3 showed the phylogenic tree of the fungal isolates.

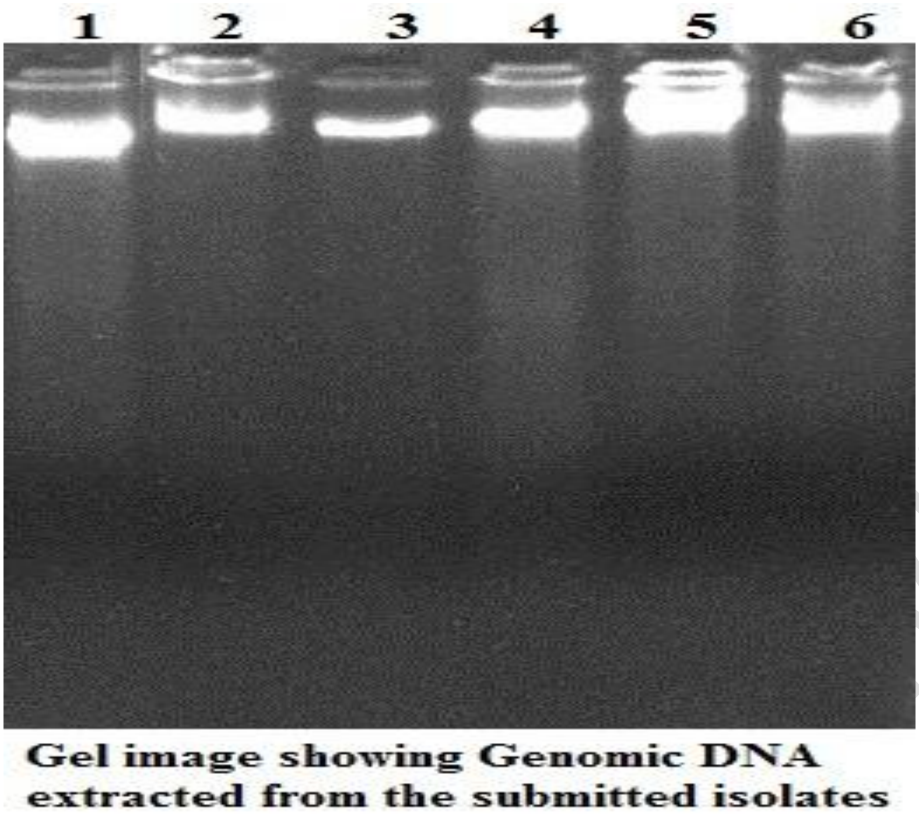

Plate 1: agarose gel image of amplified 16s rRNA of the isolates

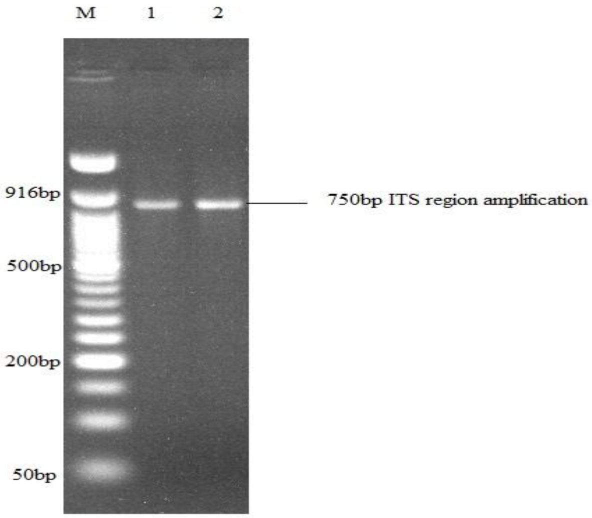

Plate 2: Gel image of ITS region amplification of the Fungi isolates.
Lane M is 50bp Molecular weight ladder. Lane 1 and 2 are isolates
1 SDA and 2 SDA respectively.

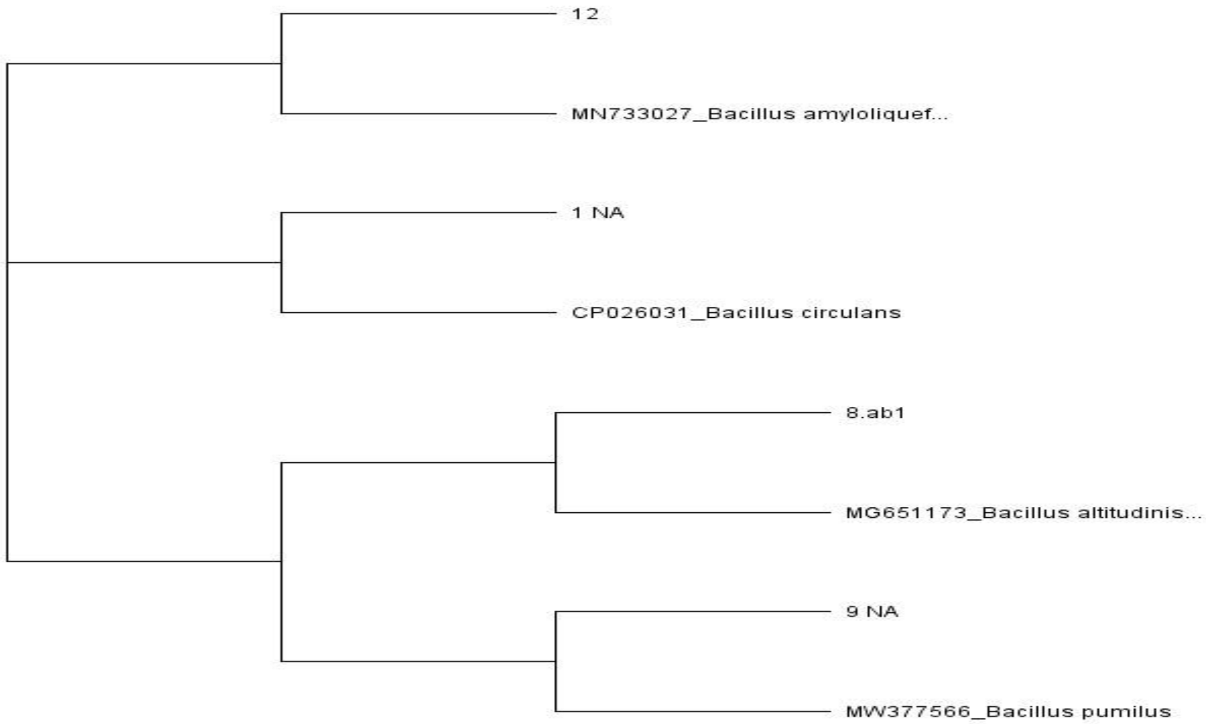

Figure 2: Phylogenic Tree Showing the Evolutionary Distance of the Bacterial Isolates

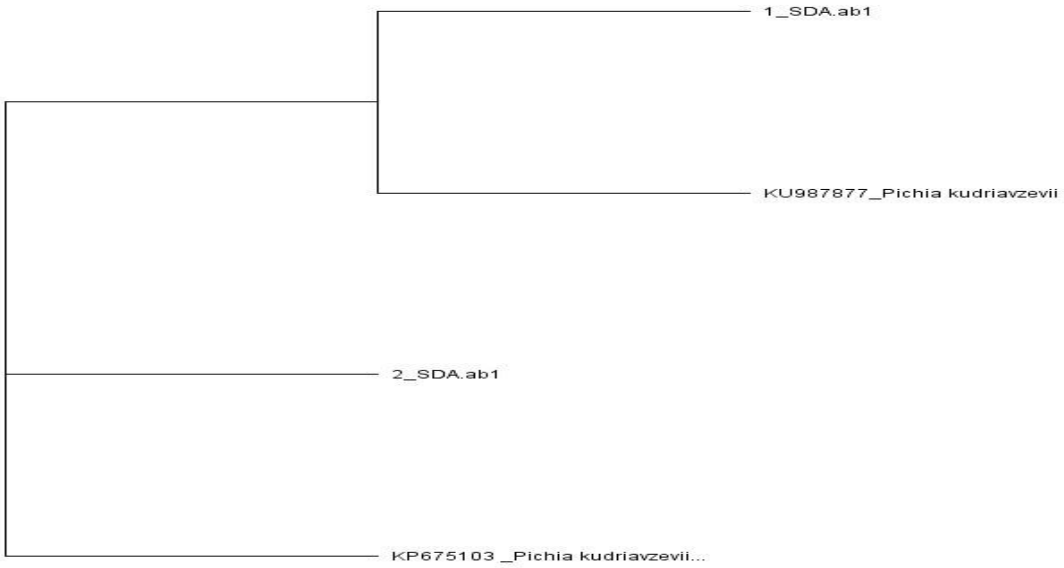

Figure 3: Phylogenic Tree Showing the Evolutionary Distance of the Fungal Isolates

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.

This present study has employed a culture-dependent molecular technique in characterizing the microbial population associated with palm wine contamination. This provides a more reliable result compared to the traditional or conventional phenotypic identification. This has therefore shown the quality of pal wine consumed in this area of study. These microbial groups are frequently associated soil and other related unhygienic milieu. Their presence in the pal wine study could therefore be attributed to poor hygienic state of the tappers, materials used and the method of tapping involved. Karamoko *et al.,* (2012) also reported “the presence of yeast and other bacteria in palm wine samples”. However, according to Ogbulie (2007), “the methods of palm wine tapping and collection of palm sap influence the microbial content of the sap”.

While these microorganisms presents some public health concerns, they can also be explored for other biotechnological applications. Locals involved in the production of this products should therefore be enlightened on the need for sanitary practices in the handling of this product.

4.0 Conclusion

From the study it was observed that palm wine harbored microbial population that varied based on the type of the palm wine product. The study further revealed that palm wine from *Elaeis guineensis (*oil palm tree) had more bacterial load than *Raphia raphia* (*raphia palm tree*).

This product is consumed by many including pregnant women for different reasons ranging including medicinal and social purposes. This study has indicated the microbiological implications of consuming this product in its crude form as it serves as substrate for the proliferation of different microbial populations, including yeast. Exposure of this product to conditions capable of controlling the growth of disease causing microbial population will increase the demand and acceptability of this product.

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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