Nigerian Food Journal

Official Journal of Nigerian Institute of Food Science and Techonology www.nifst.org

NIFOJ Vol. 31 No. 1, pages 83 – 90, 2013

Evaluation of Bacterial Diversity in Palm Wine by 16S rDNA Analysis of Community DNA

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ABSTRACT

Bacterial diversity and fermentation dynamics in palm wine, a traditional alcoholic fermented beverage, collected from upright palm trees from Idiaba community, Abeokuta, Ogun State, Nigeria were evaluated by DNA based method using the 16S rDNA of the microbial community to verify and complement previous reports, improve our understanding and document yet unreported, uncultured microbial diversity associated with palm wine. The 16S rRNA gene fragments were amplified from microbial community and genomic DNA of isolates, by Polymerase Chain Reaction (PCR) using universal primers; and sequenced. The partial sequences were identified by comparison with sequences deposited in the non-redundant nucleotide database of National Center for Biotechnology Information (NCBI). This analysis revealed that 32 community clones were identified as *Lactobacillus sp*, *Lactobacillus casei strain zhang*, *Lactobacillus plantarum*, *Leuconostoc mesenteriodes ssp dextranicum*, *Leuconcostoc lactis*, *Pediococcus parvulus strain Bpe-299, Acetobacter pomorum*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Acinetobacter calcoaceticus*, *Enterobacterium bacterium*, *Acidovorax sp*, *Comamonas sp*, *Bacillus subtilis*, *Staphylococcus piscifermentans* and uncultured bacteria clone D1-78. The results showed that bacterial diversity in the palm wine sample is dominated by *Lactobacillus* and *Leuconostoc* species as reported by previous workers and uncultured bacteria clone D1-78 (1 clone) was detected for the first time in palm wine.

Keywords: Palm wine, 16S rRNA gene, community DNA, PCR, lactic acid bacteria.

Introduction

Palm wine is a traditional African alcoholic fermented beverage produced from sap of trees from the family Palmea (Uzochukwu *et al.*, 1994a).

It has been proposed that the fermentation process starts immediately after the sap has been collected; naturally occurring microorganisms even with the collecting container, ferment the available carbohydrate. However, the process is accelerated by increase in temperature. Fermentation time varies from a few hours to a few days, depending on the time of the day and climatic conditions when the sap is collected.

Palm wine production consists of three stages of fermentation: acid, alcoholic and acetic, making this beverage an interesting environment where micro-organisms or genes with potential biotechnology application could be isolated.

Studies on the microbiology of palm wine have focused on the isolation and identification of microorganisms present in the fresh sap and in the fermented palm wine using traditional culture and characterization methods. Microorganisms frequently identified in palm wine samples comprise

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several yeast and bacterial species including homo and hetero-fermentative lactic acid bacteria, the alcohol producing bacteria *Zymomonas mobilis* (Okafor, 1975a) and the dextran-producing bacteria *Leuconostoc mesenteriodes* and *Leuconostoc dextranicum* (Uzochukwu *et al.*, 1997).

Culture-based methods for evaluation of microbial diversity have shown that phenotypic identification of fermentative micro flora is strenuous, time-consuming and often problematic due to the ambiguous biochemical or physiological traits, leading sometimes to incomplete or incorrect characterization. Further still, the difficulty in recovering known micro organisms which are viable but enter a non-cultivable state (Giraffa and Neviani, 2001) also contributes in hampering the efficiency of culture-dependent golden reference method in microbial characterization. Moreover, when biodiversity is studied by conventional approaches, such as cultivation of microbes on media, the results are quite biased since majority of food associated micro flora cannot be cultivated on standard laboratory media (Giraffa and Neviani, 2001); thus allowing only a rough measure of true microbial genera to be isolated. Therefore, alternative and complementary non-culture dependent approaches have been developed and evaluated to study microbial communities. PCR-based-molecular techniques such as 16S rRNA gene amplification and sequence analysis are being widely used, and have been demonstrated to be powerful tools in investigating the biological diversity present in environmental samples (Felske *et al.*, 1996). The application of this approach in studying microbial food ecology is not an exception. The community structure in traditional Nigerian fermented foods has not been well studied using this approach.

In this study, we analyzed for the first time 16S rRNA clone libraries generated from the total DNA of the bacterial community present in palm wine samples. The palm wine was pooled from different Eleasis gunineasis trees collected from Idiaba location in Abeokuta, Nigeria.

The aim of this study was to evaluate the bacterial diversity in Nigerian palm wine with a view to confirming, complementing and comparing data reported by previous workers who employed conventional culture-dependent methods.

Materials and Methods

Palm wine origin and sampling

Fresh palm sap samples were collected at Idiaba, Ogun state, Nigeria from different Eleasis gunineasis trees. The samples were collected in sterile collection bottles and transported on ice packs to the laboratory.

Experimental design

Analysis on the pooled sample from the different trees was carried out at the pH range of 7 and 4 for the first day. After the sample had attained the pH of 4, subsequent analyses were conducted every 24 h for three (3) days.

Bacterial DNA extraction

A 50 ml palm wine sample obtained by mixing all collected from different trees was used as the substrate for direct community bacterial DNA extraction. Extraction of bacterial DNA from the community bacteria was carried using DNeasy DNA extraction kit from Qiagen, USA. The integrity and quantity of extracted DNA were evaluated in 1.2% agarose gel electrophoresis and by using UV-7504 spectrophotometer respectively.

Bacteria isolation

Palm wine sample was homogenized for 2 min, appropriately diluted in sterile distilled water saline, pour plated onto Nutrient agar and were incubated at 37°C for 24 – 48 h. Distinct colonies were sub-cultured twice and pure cultures were stored in Nutrient agar slants and stored at -20°C.

Pure bacterial culture isolates grown overnight at 37°C in 3 ml of Luria Bertani (LB) broth were harvested and their DNA extracted and qualified using the same procedure adopted for the direct community DNA extraction.

PCR amplification

16S rRNA genes of both community bacteria and culture isolates from palm wine obtained at 24 h

intervals were amplified by PCR using the pair of 16S rRNA gene universal primers. Forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and Reverse primer (5′-ACGGCTACCTTGTTTACGACTT-3′) from integrated DNA Technology (IDT) Inc. USA, which are targeted to bacterial 16S rRNA gene were used. The forward and reverse 16S rRNA gene universal primers generate a 1.5 kb fragment.

The PCR reaction was performed in 0.5 ml micro-centrifuge tubes (Eppendorf, UK) with 25 ml of reaction mixture: 12.5 ml "Go Taq Green" master mix (2.5 units Taq DNA polymerase, 1X Qiagen PCR buffer and 200 μM of each dNTP), 0.5 μl forward primer (10 μM), 0.3 μl reverse primer (10 μM) and 1.5 μl DNA template, and made up to 25 μl with 10.2 μl of nuclease-free sterile distilled water (Norgen, Canada).

The PCR protocol consisted of an initial denaturation step of 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 44°C for 30 sec and elongation at 72°C for 2 min, final holding at 73 °C for 4 min. PCR reaction was performed in a 20 well block thermocycler (TECHGENE, UK).

A 5ul sample of reaction mixture was analyzed by 1.5% agarose gel electrophoresis in TAE 1X buffer. The gel was stained with 30 μl of ethidium bromide and the bands were visualized under UV illumination (Figure 1).

Cloning of 16S rRNA gene fragment

Amplified 16S rRNA gene fragment from both bacterial communities and isolates from palm wine samples from each analysis interval were separately ligated into PCR-TRAP vector (PCR-TRAP cloning Kit, GenHunter, USA). Ten microliters of ligation reaction were used to transform GH competent Escherichia coli cells included in the PCR-TRAP cloning kit. Transformation of *E. coli* cells were carried out according to the manufacturer's protocol. Transformed *E. coli* cells were plated on LB/Tet/X-gal plates. Positive clones carrying ≈1000 bp of 16S rRNA gene fragment were able to grow due to the inactivation of the repressor gene and thus the expression of the TetR gene on transcription.

To confirm the presence of the 1.5-kb PCR products ligated to the plasmid (PCR-TRAP Vector), two procedures were carried out (i) plasmid minipreps obtained with colony lysis buffer procedure. (ii) PCR amplification reactions with a slight modification (PCR parameter was as follows for 30 cycles: denaturation at 94°C for 30sec, annealing at 52°C for 40 sec; extension at 72°C for 1min, final extension step at 72°C for 5 min and final incubation at 4°C), using colony lysates as the template material for each positive clone. Amplification products were visualized on agarose gel electrophoresis as described above, against a 100 base pair molecular marker.

16S rRNA sequencing

Double-stranded plasmid templates were prepared from the positive clones with 16S rRNA gene inserts and partially sequenced. Nucleotide sequences were determined by analysis of fluorescently labelled DNA products generated by AmpliTaq DNA Polymerase on an AB 373a Stretch (Shot-gun) DNA sequencer. Primers: 518F and 800R were used in all sequencing reactions (Macrogen Inc., North Korea).

Identification of 16S rRNA gene sequences

The obtained sequences of 16S rRNA genes from the palm wine microbial community and isolates were aligned by submitting them to the non-redundant nucleotide database at Genbank using the BLAST program (http://www.ncbi.nlm.nih.gov) in order to determine the identities of the bacteria.

Results and Discussion

Microbial community of palm wine has been studied using traditional plating techniques (Amoa-Awua *et al.*, 2006; Okafor, 1975a and Uzochukuwu *et al.*, 1994a). The use of an experimental approach that does not depend on microbial cultivation was explored in this work to re-analyze the bacterial diversity in palm wine fermentation. Till date, no study has been carried out on the identification

of bacteria community associated with palm wine fermentation using molecular techniques. The findings of this study, however, are similar to the report by Escalante *et al.*, (2004) on fermenting Mexican pulque beverage; where *Lactobacillus* species had 80.97% dominance of all the organisms detected by molecular methods. The amplification of the 16S rRNA gene subunit has proven to be an effective tool for the characterization of the microbial community associated with palm wine fermentation.

The succession of organisms associated with palm wine fermentation was shown (Table 1) to be dominated by LAB. *Pediococcus parvulus* and *Staphylococcus piscifermentans* had the highest dominance of 33.33% respectively on the 1st day of the analysis when the pH of the sample was about 6.9, while still at the same pH, clones identified as *Acidovorax sp* and uncultured bacteria clone D1-78 were found to have 16.66% dominance. These organisms were replaced mainly by LAB as the pH of sample decreased to 4.0. The change in pH value of the sample from pH of 6.9 to 4 terminated the dominance of these organisms due to the acidification of the sample.

Table 1: Identity and relative abundance (%) of organisms identified from the 16S rRNA clone library of palm wine bacteria community DNA, based on analysis in the non-redundant nucleotide database from National Center for Biotechnology Information (NCBI).

Clone Identity	pH7	Day 1 pH4	Day 2	Day 3	Day 4
Lactobacillus sp		1 (20)	2 (33.33)	2 (22.22)	
Lactobacillus casei strain Zhang		2 (40)	1 (16.66)		
Lactobacillus plantarum			1 (16.66)	1 (11.11)	
Leuconostoc mesenteriodes ssp Dextranicum		1 (20)	2 (33.33)	2 (22.22)	1 (14.28)
Leuconostoc lactis				1 (11.11)	1 (14.28)
Pediococcus parvulus strain Bpe-299	2 (33.33)				
Acetobacter pomorum				1 (11.11)	2 (28.57)
Acetobacter pasteurianus					2 (28.57)
Gluconobacter oxydans				1 (11.11)	
Acinetobacter calcoaceticus		1 (20)			
Acidovorax sp	1 (16.66)				
Comamonas sp					1 (14.28)
Bacillus subtilis				1 (11.11)	
Staphylococcus piscifermentans	2 (33.33)				
Uncultured bacteria clone D1-78	1 (16.66)				

At the pH of 4, *Lactobacillus casei* strain zhang was found to be most dominant organism (40%) while *Lactobacillus sp*, *Acinetobacter calcoaceticus*, *Leuconostoc mesenteriodes sp*, *dextranicum* had 20% dominance each. This finding was earlier reported by Uzochukwu *et al.*, (2000). As the pH decreased on the 2nd day, percentage dominance of *Lactobacillus casei* strain zhang decreased to 16.66% while *Lactobacillus sp* and *Leuconostoc mesenteriodes sp dextranicum* increased to 33.33%. *Lactobacillus plantarum* was found in the palm sample with the dominance of 16.66%.

Leuconostoc mesenteriodes is a LAB previously reported in palm wine and it has been identified as the major microorganism responsible for the development of viscosity, one of the distinctive characteristics of

palm wine (Uzochukwu *et al*., 1994b). Leuconostoc mesenteriodes were detected throughout the period of fermentation of palm wine sample. Another ESP producing bacteria, *Lactobacillus sp* was detected in the sample. These families of microorganisms have been reported as a LAB, producer of the capsular polysaccharide Kefiran. The percentage abundance of *Lactobacillus sp*, *Leuconostoc mesenteriodes sp dextranicum*, *Lactobacillus plantarum* and *Lactobacillus casei* strain zhang decreased to 22.22%, 22.22%, 11.11% and 0% respectively as the pH of the sample dropped to 3.42 on 3rd day, while a new organism, leuconostoc lactis emerged with the relative abundance of 11.11%. Clones identified as *Gluconobacter oxydans* (11.11%), *Acetobacter pomorum* (11.11%) and *Acetobacter pasteurianus* (11.11%) were also detected on the 3rd day of analysis of the sample. As expected, acetic acid bacteria were detected on 4th day of palm wine fermentation. These organisms had the highest dominance of 28.57% on the 4th day of the palm wine fermentation and confirmed that palm wine spoilage is mainly caused by the presence of acetic acid bacteria in the wine. The abundance of *Leuconostoc mesenteriodes sp. dextranicum* in the palm wine further reduced to 14.28% while that of *Leuconostoc lactis* increased from 11.11% to 14.28% on day of the fermentation.

Table 2: Distribution of organisms identified in the 16S rRNA clone library of palm wine bacteria community DNA into lineages of the Bacteria domain and their accession number

Clone Identity	Number (%) of clones detected in 16SrRNA library	Accession Number (NCBI)	Clones sequenced
Bacillus-Lactobacillus subdivision			
Lactobacillus sp	5	DQ 682969.1	P3G, P2F, P14
Lactobacillus casei strain Zhang	3	EF 536364.1	P3H, P2C, P14A, P14D
Lactobacillus plantarum	2	FJ3 84624.1	P3F, P2E
Leuconostoc mesenteriodes ssp Dextranicum	6	AB 326298.1	P4E, P3C, P3E, P2B, P2G, P14B
Leuconostoc lactis	2	FJ2 15667.1	P4A, P3A
Pediococcus parvulus strain Bpe-299	2	EU 331259.1	P17C, P17D
Proteobacter – alpha subdivision			
Acetobacter pomorum	3	AJ 419835.1	P4G
Acetobacter pasteurianus	2	EU 096228.1	P4B, P4C
Gluconobacter oxydans	1	AB 436557.1	P4F
Proteobacter – gamma subdivision			
Acinetobacter calcoaceticus	1	FJ 263918.1	P2A, P2D
Proteobacter – beta subdivision			
Acidovorax sp	1	AF 508114.1	P17F
Comamonas sp	1	EF 426453.1	P4D
Bacillus – Bacillales subdivision			
Bacillus subtilis	1	FJ 197160.1	P3I
Staphylococcus piscifermentans	2	Y 15753.1	P17A, 917B
Uncultured bacteria clone D1-78	1	DQ 113756.1	P17E

By the analysis of the PCR amplified 16S rRNA gene sequence, it was discovered that lactic acid bacteria (LAB) species were the most abundant organisms in the palm wine sample (Table 2). Bacteria from the *genera Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Acetobacter* have been identified earlier in previous studies of palm wine fermentation using traditional cultivating technique (Okafor, 1975, Bassier, 1968, and Uzochukwu *et al.*, 1994, a and b) and the presence of the micro organism in fermenting palm wine sample were confirmed by 16S rRNA gene molecular analysis in this study. Other organisms identified in this current study such as *Acidovorax sp*, *Acinetobacter calcoaceticus* and uncultured bacteria clone strain D1-78, were not detected earlier in palm wine samples using culturing approach possibly due to the use of selective media and conditions that were not optimal for the growth of these organisms. *Staphylococcus pisciferm*entans were identified during the early stages of fermentation at pH of 6.9 and are likely to be incidental contaminants from human handling, as it failed to grow during fermentation. This conforms to the report of Okafor, (1975) that of probable presence of *Staphylococcus sp* in palm wine sample is as a result of chance contamination. This organism disappeared as the fermentation continued. This may be due to the large pH decrease from 6.9 to 4.0 occurring during the first day of fermentation and continued acidification of the drink down to pH 3.39 at the end of the day.

Table 3: Distribution of organisms identified in the 16S rRNA clone library of palm wine bacteria isolates DNA based on the outputs results from analysis in the non-redundant nucleotide database form NCBI

Clone Identity	pH7	Day 1 pH4	Day 2	Day 3	Day 4
Uncultured *Lactobacillus sp*	1 (50)				
Lactobacillus plantarum	1 (50)				
Lactobacillus sp		1 (50)	1 (50)		
Leuconostoc mesenteriodes ssp dextranicum	1 (50)	1 (50)			
Acetobacter sp				1 (100)	
Acetobacter ghanensis					1 (100)

The results of the analysis on palm wine showed that only eight bacteria isolates were obtained during the fermentation periods when culture-dependent method was employed and were identified as uncultured *Lactobacillus sp, Lactobacillus plantarum*, *Leuconostoc mesenteriodes sp. dextranicum*, *Lactobacillus sp*, *Acetobacter sp* and *Acetobacter ghanensis* (Table 3). Whereas, a total of thirty two 16S rRNA clones were obtained and identified from the same palm wine during the fermentation periods when culture-independent method involving the analysis of community 16S rRNA gene from palm wine was used.

Conclusion

The application of culture-independent methods in analyzing the microbial profile of palm wine have shown by way of comparison with the culture-dependent method to be more promising and effective in studying microbial diversity in palm wine. The method has shown the presence of numerous other microorganisms in palm wine as against the result from culture-dependent method. The results obtained from traditional plating and 16S rRNA gene approach have shown that palm wine fermentation was characterized by strong LAB activity with 62.50% of the total 16S rRNA clones. Prominent organisms like *Lactobacillus plantarum*,

Leuconostoc mesenteriodes sp. dextranicum, *Leuconostoc lactis*, *Lactobacillus casei strain zhang*, *Lactobacillus sp* and *Pediococcus parvulus* have been identified in this present work as the dominant lactic acid bacteria responsible for the sour taste of palm wine tapped from the live upright palm tree.

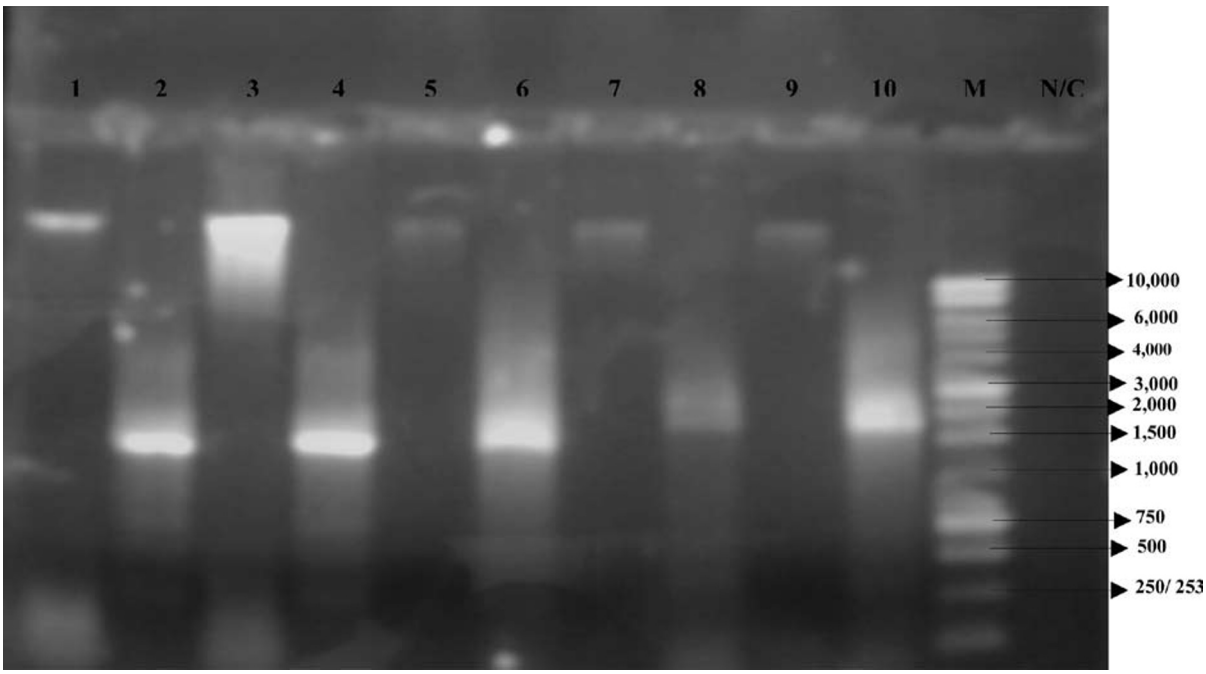

Fig. 1: Agarose gel electrophoresis of bacteria genomic community DNA prepared from palm wine with the corresponding 16S rRNA gene PCR products

Lanes 1 and 2 are community genomic DNA (CGD) and its amplicons respectively for the 1st day at pH of 7; Lanes 3 and 4 are CGD and amplicons for the 1st day at pH of 4; lanes 5 and 6 are CGD and amplicons for the 2nd day, lanes 7 and 8 are CGD and amplicons for the 3rd day, lanes 9 and 10 are CGD and amplicons for the 4th day, lane M is a 1kb DNA marker and lane N/C is a negative control (no DNA).

Acknowledgements

The author would like to thank the Biotechnology Centre, Federal University of Agriculture, (FUNAAB) Abeokuta, Nigeria for the provision of the enabling laboratory for this study.

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