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COMPARATIVE IDENTIFICATION APPROACH FOR GUM-PRODUCING LACTOBACILLI IN PALM WINE

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ABSTRACT

Introduction: Species of lactobacilli are responsible for the consistency and soluble white colouration of palm wine through the production of gum. Phenotypic and physiological characteristics as basis for identification of lactobacilli is often with low level of discrimination.
Objection: This study aimed at comparative identification approach of gum-producing lactobacilli using amplified ribosomal DNA restriction analysis (ARDRA) and API kit 50 CHL.
Methodology: Four hundred colonies of lactobacilli isolated from seventy-two fresh palm wine samples and two hundred were used for genomic DNA extraction, 16S rDNA analysis and digestion of amplicons with two endonucleases: *Hae* III and *Bash* 12361.
Results: Twenty ARDRA pattern group were generated and representatives of each were randomly chosen for sequence and API kit analysis. The size of the amplified products (~1500 bp), digested amplicons (100 - 1000 bp) and the ARDRA profiles; A-T. ARDRA screening revealed unique patterns among the two hundred isolates, with the same pattern for some of the isolates. The 16S rDNA gene sequence analysis showed that the isolates were divide into 20 groups of three genera: *Lactobacillus* (65%), *Leuconostoc* (30%), and *Lactococcus* (5%) whereas API kit identified two genera; *Lactobacillus* (75%) and *Leuconostoc* (25%). Gene sequence similarity of all the representative isolate for 16S rDNA (99 - 100%) while API kit (74.8 – 99.9%) with 55% overall similarity in bacterial identified. ARDRA showed higher similarities in identification and discrimination of bacterial species or between closely related groups isolated from palm wine compared to API kit.
Conclusion: ARDRA is a good tool for lactobacilli identification based on the results from this study.

Keywords: Lactobacilli, ARDRA bacteria identification, endonucleases, genomic DNA

INTRODUCTION

Lactic acid bacteria are known over the years for their wide application in food, pharmaceutical and chemical industries. Microorganisms, including lactic acid bacteria (LAB), have been reported to produce polysaccharides that are potentially useful as thickeners, stabilizers, emulsifiers and bodying agents (**Chawla *et al.*, 2009**; **Badel *et al.*, 2011**; **Adamu-Governor *et al.*, 2020**a; **2020b**). Exopolysaccharides are long-chain polysaccharides containing branched, repeating units of sugars or sugar derivatives. Also, exopolysaccharides have reproducible physicochemical properties and are non-toxic with immunostimulatory, anti-tumor and antioxidant activity (**Hosono *et al.*, 1997**; **Chabot *et al.*, 2001**).

The accurate and definitive identification of microorganisms is one of the cornerstones of microbiology (**Janda and Abbott, 2002**). The identification of microorganisms (especially bacteria) up till about two centuries ago relied on biochemical profiling in addition to morphology, serology and colonial appearance. However, sole dependence on these techniques has been reported to be sometimes misleading and significantly limiting as regards proper bacteria identification to strain level (**Houpikian and Raoult, 2002**; **Janda and Abbott, 2002**; **Spratt, 2004**; **Singh *et al.*, 2009**; **Rhoads *et al.*, 2012**).

Sugar fermentation and gas production are biochemical methods commonly used for identifying bacteria. However, these techniques are limited in terms of time and expense, as well as in the ambiguity of test results (**Lick, 2003**; **Callon *et al.*, 2004**). The used of biochemical tests and sugar utilization pattern in the identification of lactic acid bacteria have been documented (**Chantaraporn and Somboon, 2007**; **Patil *et al.*, 2010**). **Gever *et al.* (2001)** argued that the usage of biochemical and other conventional methods is often not adequate to discriminate closely related lactic acid bacteria species. Detection and evaluation of genetic variability in LAB at the strain level is very important and the use of reliable and accurate methods will be required. Further, genus rank differentiation of LAB has largely relied on catalase test, Gram staining and determination of carbohydrate utilization using tube or API 50 CHL kit. Studies have also shown that API 50CHL have been used in tentative identification of *Lactobacillus* and *Leuconostoc* species in palm wine (**Amoa-Awua *et al.*, 2007**; **Ziadi *et al.*, 2011**; **Adamu-Governor *et al.*, 2018**). However, data overlapping occasionally occurs and this underscores the limitations of API 50CHL identification method (**Suhartatik *et al.*, 2014**).

Amplified ribosomal DNA restriction analysis (ARDRA) has been used for the identification of Comamonadaceae (**Vaneechoutte *et al.* 1992**); the identification of *Lactobacilli* isolated from dairy products (**Giraffa *et al.*, 1998**; **Delly *et al.*, 2002**), from faecal and vaginal samples (**Ventura *et al.*, 2000**); and for differentiation of *O. oeni* from other wine LAB (**Sato *et al.*, 2000**). ARDRA only limitation is that when used alone, it can only produce a fingerprint. It does not allow for the detection or identification of specific phylogenetic groups within a community profile (**Spiegelman *et al.*, 2005**).

With advancements in molecular biology, nucleic acid-based methods of microbial identification have been developed, and reported to provide a higher degree of reliability than the classical phenotypic methods (**Spratt, 2004**; **Amor *et al.*, 2007**), making it possible to detect even the smallest of variations within microbial species and even within individual strains. However, according to **Janda and Abbott (2002**), "all systems used to identify bacteria, whether phenotypic or genotypic, have limitations, because no single test methodology will provide results that are 100% accurate". However, there is a general advocacy for a polyphasic approach that combines both phenotypic (e.g., biochemical testing, cellular fatty acid analysis, and numerical analysis), and genotypic methods (e.g., DNA-DNA hybridization, analysis of GC content, and 16S rDNA gene sequencing); as one method, will complement the other (**Vandamme *et al.*, 1996**; **Rossello-Mora, 2001**; **Houpikian and Raoult, 2002**; **Janda and Abbot, 2002**; **Spratt, 2004**; **Croci *et al.*, 2007**).

LABs are food grade organisms, generally recognized as safe (GRAS). A number of LAB have been reported to be responsible for the consistency and soluble white colouration of palm wine through their production of gums, largely dextrans and levans, in the fermentation of the beverage (**Uzochukwu *et al.*, 1991**; **Uzochukwu *et al.*, 1994b**; **Uzochukwu *et al.*, 1994**; **Uzochukwu *et al.*, 1999**; **Uzochukwu *et al.*, 2002**). From these studies, *Leuconostoc* and a number of *Lactobacillus* spp. have been identified as gum producers in palm wine. However, the identification of these microorganisms was based on cultural, morphological and biochemical characteristics which are subject to several limitations (**Janda and Abott, 2002**). Earlier, **Ehrmann *et al.*, (2009**) isolated a novel *Leuconostoc* species from palm wine sample which was named *Leuconostoc palmae* using 16S rRNA gene analysis. **Okolie *et al* (2013)** evaluated bacterial diversity in palm wine using 16S rRNA analysis of community DNA and reported that 62.50% of the total 16S rRNA clones were lactic acid bacteria and are responsible for palm wine fermentation. This study facilitated the screening and identification of two hundred

(200) LAB isolates obtained from palm wine using comparative approach of ARDRA and API 50 CHL kit.

MATERIALS AND METHODS

Collection of samples

Fresh palm wine samples were collected from six states in the South West of Nigeria, namely; Ogun (Aiyepe and Ogere, Mowe and Ibafo, Odogbolu), Oyo (Oloshoko, Alabat and Ajibade), Lagos (Badagry, Imota and Keleb, Epe), Osun (Ife, Ikire and Modakiki), Ondo (Oniparaga, Ikoya, Omotosho and Okitipupa) and Ekiti (Ijesa Isu-Ekiti, Iluomoba and Fatunla), according to the method described by Adamu-Governor *et al*. (2018).

Gum-producing bacteria Isolation

Isolation of gum-producing bacteria from palm wine was done according to the method described by **Adamu-Governor *et al.* (2018**). Distinct colonies were obtained after several subculture and pure cultures were inoculated in 6% sucrose agar slants and stored at 4°C. Four hundred gum producing bacteria was used for this study.

Extraction of DNA

Gram staining and catalase activity were used to screen isolated bacteria prior to molecular Identification. Overnight cultures of the selected mucoid isolates on Tryptone Soy Broth (TSB) were used for genomic DNA extraction by using the ZR Fungal/Bacterial DNA kit (Zymo Research, California, USA) according to the instructions of the manufacturer.

Partial amplification of 16S rRNA gene and sequencing

The amplification of partial 165 rRNA gene from genomic DNA was done using 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) bacterial primers. PCR amplification was carried out in PC 200 Thermocycler (Germany), in 25µl reactions containing 12.5µl of 2× PCR coloured Master Mix (Inqaba biotech, SA), 2.0Ml of template DNA, 0.2Ml of broth forward and reverse primers and 10.1µL of nuclease free water in a tube added in that order. The PCR mixture was mixed thoroughly and span down before placing the PCR tube in the thermocycler. PCR conditions were carried out using an initial denaturation step at 94ºC for 5min, followed by 30 cycles of 30s of denaturation at 94ºc, 30s of hybridization at 55ºc and 1 min of elongation at 72ºc, followed by a final extension step at 72ºc for 10 min. Amplicons were verified by agarose gel electrophoresis.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Restriction endonuclease digestion of 16S rRNA PCR product was carried out according to the manufacturer's instructions with FastDigest restriction endonuclease (FastDigest) HaeIII - GG/CC and Bash12361 - CG/CG (Thermo Scientific), in 30 µl reactions containing 17 µl of nuclease free water, 2.0 µl of 10X FastDigest Green buffer, 10 µl PCR product and 1 µl FastDigest enzyme in a tube added in that order. The restriction endonuclease reaction mixture was mixed gently and spun down for a few seconds. The reaction mixture was then incubated at 37ºC for 5 min. The restriction patterns or ARDRA profiles were examined using 1.5% (w/v) agarose gels in 1X TAE buffer with a 100 bp DNA ladder (Inqaba biotech, SA). The visualization of the gel was done using a UV – transilluminator and the bands were photographed using gel documentation.

Construction of GelCompar II database

PCR- RFLP fingerprints of LAB were analyzed using GelCompar II version 6.5 (Applied Maths, Kortrijk, Belgium). Gel image processing, band position tolerance and optimization was carried out according to the method described by **Kopermsub and Yunchalard, (2010)**. The unweighted pair group method using arithmetic averages (UPGMA) was used to construct dendrograms from the similarity matrix using GelCompar II.

DNA sequencing and analysis

The 16s rRNA gene amplicons were sequenced using the same set of primers used for PCR at Inqaba biotech (South Africa) using the Big Dye Terminator v 3rd cycle sequence kit (Applied Biosystems, UK), purified sequencing PCR products were run on a 3130 Genetic analyze (Applied Biosystems/Hitachi, Japan). Sequences were then assembled and subsequently aligned with the sequences deposited in the National Centre for Biotech Information (NCBI) GenBank, using the Basic Local Alignment System Tool (BLAST) for their identity.

Analytical profile index analysis (API kit 50 CHL)

Twenty representative of gum producing bacterial isolates from twenty distinct finger print pattern generated by amplified ribosomal DNA restriction analysis (ARDRA) were used for tentative identification with API kit 50 CHL according to the method described by **Adamu-Governor *et al.* (2018).**

RESULTS

Preliminary identification of bacterial isolates

Gums producing bacterial isolates isolated from palm wine sample were all Gram positive, catalase and oxidase negative reactions

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

In this study, ARDRA facilitated the screening and identification of four hundred (400) LAB isolates obtained from palm wine. The isolates were categorized into twenty distinct groups on the basis of their finger prints generated by two restriction enzymes (*Bash* 12361 and *Hae* III) and representative of the twenty groups were sequenced, and the identity of LAB species identified. PCR – RFLP photograph of 16S rRNA products of gum producing bacteria DNAs digested with endonucleases electrophoresed on agarose is shown on plates 1 – 5. The amplicons size for 16S rRNA gene is about 1500 bp as shown in plates 1-2. The sizes of fragment generated by the two restriction enzymes ranged from 100 to about 1000 bp (plates 3-4). The twenty distinct finger print patterns generated by ARDRA were assigned alphabetic codes from A – T as shown in plate 5.

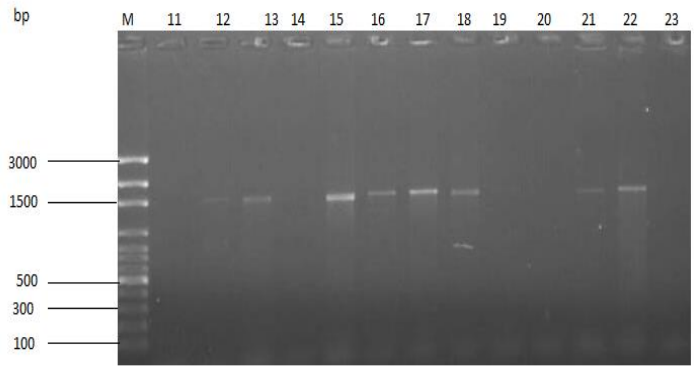

Plate 1 Gel electrophoresis profile of Amplified 16S rRNA gene fragments of gum producing lactic acid bacteria from palm wine. Lane M, 100 bp DNA Ladder; Lanes 11-23 are isolates 11-23.

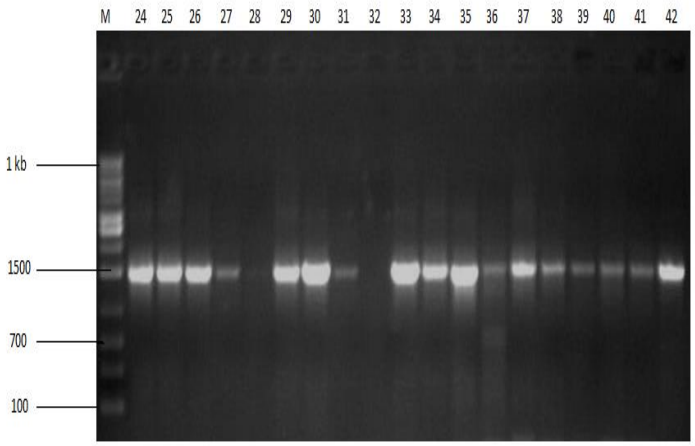

Plate 2 Gel electrophoresis profile of Amplified 16S rRNA gene fragments of gum producing lactic acid bacteria from palm wine. Lane M, 100 bp DNA Ladder; Lanes 24-42 are isolates 24-42.

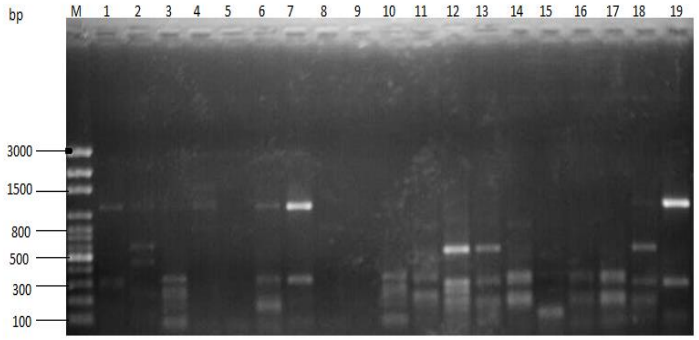

Plate 3 Gel electrophoresis profile of Amplified 16S rRNA gene fragments of gum producing lactic acid bacteria from palm wine digested with *Hae* III. Lane M, 100 bp DNA Ladder; Lanes 1-19 are isolates 1-19.

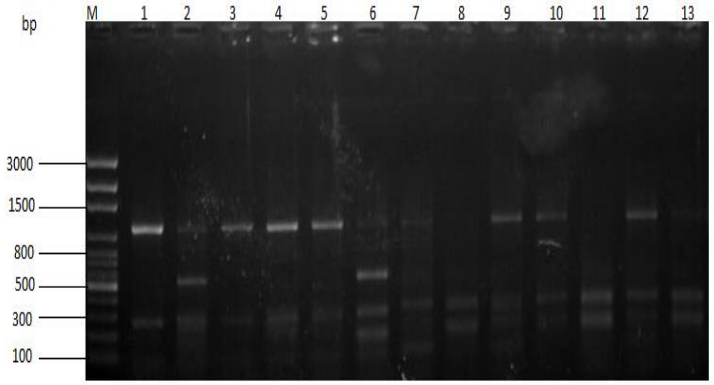

Plate 4 Gel electrophoresis profile of Amplified 16S rRNA gene fragments of gum producing lactic acid bacteria from palm wine digested with *Bash* 12361. Lane M, 100 bp DNA Ladder; Lanes 1-13 Plate 4: Gel electrophoresis profile of Amplified 16S rRNA gene fragments of gum producing lactic acid bacteria from palm wine digested with *Bash* 12361. Lane M, 100 bp DNA Ladder; Lanes 1-13 are isolates 1-13.

Sequencing and Identification

A representative of each ARDRA pattern was randomly chosen for sequencing and identification. The sequences fell into a major lineage of the bacteria order namely; *Bacillus- Lactobacillales.* Twenty bacterial isolates were detected in the 16S rRNA gene sequence analysis of bacteria isolates DNA as shown in table 1. These included *Leuconostoc* (6 species), *Lactobacillus* (13 species) and *Lactococcus* (1 species). *Leuconostoc* species (30%) and *Lactobacillus* species (65%) were the dominant species. Homology searches of the sequences revealed (with 98.2 – 100% homology) that profile A, *Leuconostoc lactis*; B, *Lactobacillus fermentum*; C, *Lactobacillus lactis*; D, *Lactococcus lactis ssp lactis*; E, *Lactobacillus rhamnosus*; F, *Leuconostoc carnosum;* G, *Lactobacillus plantarum*; H, *Leuconostoc mesenteroides*; I, *Lactobacillus acidophilus*; J, *Leuconostoc mesenteroides*; K, *Lactobacillus pentosus;* L, *Lactobacillus acidophilus*; M, *Lactobacillus brevis*; N, *Lactobacillus crispatus*; O, *Lactobacillus delbrueckii*; P, *Leuconostoc cremoris*; Q, *Lactobacillus plantarum*; R, *Leuconostoc citreum*; S, *Lactobacillus casei*; T, *Lactobacillus plantarum.*

Table 1 ARDRA profile and Identification of gum producing bacteria isolated from palm wine

PCR-RFLP pattern	Representative isolate	Closest relative	% Identity	GenBank identification no
A	IM_a 06	*Leuconostoc lactis*	100	AB023968
B	IBA_b 03	*Lactobacillus fermentum*	99	AF477498.1
C	IFE_a 05	*Lactobacillus lactis*	98.2	AY675257.1
D	MOD_a 01	*Lactococcus lactis* ssp *lactis*	100	AY920468
E	FAT_a 10	*Lactobacillus rhamnosus*	100	AB626049
F	IJE_a 05	*Leuconostoc carnosum*	100	AB022925
G	BAD_a 02	*Lactobacillus plantarum*	99	EU121672
H	AIY_a 13	*Leuconostoc mesenteroides*	99.08	CP000414
I	OMO_a 02	*Lactobacillus acidophilus*	100	FJ556999.1
J	MOW_a 05	*Leuconostoc mesenteroides*	99	AB023243
K	IM_a 02	*Lactobacillus pentosus*	100	AF375905.1
L	ILU_a 03	*Lactobacillus acidophilus*	99	FJ749655.1
M	IKI_b 04	*Lactobacillus brevis*	99.7	AB626062
N	IFE_c 10	*Lactobacillus crispatus*	99	AB008206.1
O	FAT_d 01	*Lactobacillus delbrueckii*	100	X52654.1
P	MOD_b 03	*Leuconostoc cremoris*	100	M23034.1
Q	IBA_b 06	*Lactobacillus plantarum*	99	EU121673
R	MOW_c 01	*Leuconostoc citreum*	100	NR_041727.1
S	MOW_c 08	*Lactobacillus casei*	99	GU299083.1
T	KEL_c 07	*Lactobacillus plantarum*	100	EU148598

Letter A to T correspond to the PCR-RFLP profile. IM_a 06, IMOTA; IBA_b 03, IBAFO; IFE_a 05, IFE; MOD_a 01, MODAKIKI; FAT_a 10, FATUNLA; IJE_a 05, IJESA ISU-EKITI; BAD_a 03, BADAGARY; AYI_a 13, AIYEPE; OMO_a 02, OMOTOSHO; MOW_a 05, MOWE; ILU_a 03, ILUOMOBA; IKI_b 04, IKIRE; KEL_b 07, KELEB. subscript a, palm wine tapper one; b, palm wine tapper two; c, palm wine tapper three; d, palm wine tapper four.

As expected, the two enzymes distinguished the *Lactobacillus sp*., *Leuconostoc sp*. and *Lactococcus sp*. from each other. In all of the cases, the PCR-RFLP profiles were reproducible and except for variation in the restriction profiles of strains of *Leuconostoc mesenteroides* (H and J), *Lactobacillus acidophilus* (I and L) *and Lactobacillus plantarum* (G, Q and T) belonging to the same genera were observed. Comparison of representative isolates from each ARDRA profile identified by API 50CHL and 16S rRNA gene sequence analysis is presented in Table 2. Eight representative isolates (D, E, F, H, L, P, R, S) examined gave discrepant identifications by biochemical testing and 16S rRNA PCR-RFLP pattern. Isolates D, E, F, L and S were biochemically characterized as *Lactobacillus delbrueckii ssp delbrueckii, Lactobacillus acidophilus, Lactobacillus delbrueckii ssp lactis, Lactobacillus* coprophilus and *Lactobacillus rhamnosus,* and PCR-RFLP analysis of 16S rRNA gene showed a pattern typical of *Lactococcus lactis ssp lactis, Lactobacillus rhamnosus, Leuconostoc carnosum, Lactobacillus acidophilus, Lactobacillus casei.* Similarly, isolates H, P and R were biochemically identified as *Leuconostoc lactis, Leuconostoc mesenteroides ssp. Mesenteroides/dextranicum* and *Leuconostoc mesenteroides ssp* while PCR-RFLP analysis of 16S rRNA gene identified *Leuconostoc mesenteroides, Leuconostoc cremoris, Leuconostoc citreum.*

Table 2 Comparison of identification methods of gum producing bacteria from palm wine

PCR-RFLP pattern	Representative isolate	API50CH	% Identity	16S rRNA	% Identity
A	IM_a 06	*Leuconostoc lactis*	85.2	*Leuc lactis*	1 100
B	IBA_b 03	*Lactobacillus fermentum*	98	*L. fermentum*	99
C	IFE_a 05	*Lactobacillus delbr.* ssp *lactis*	84	*L. lactis*	98.2
D	MOD_a 01	*L. delbr.* ssp *delbrueckii*	87.0	*Lactococcus lactis ssp lactis*	100
E	FAT_a 10	*Lactobacillus acidophilus*	86.3	*L. rhamnosus*	100
F	IJE_a 05	*Lactobacillus delbr.* ssp *lactis*	84.0	*Leuc carnosum*	100
G	BAD_a 02	*Lactobacillus plantarum*	99.5	*L plantarum*	99
H	AIY_a 13	*Leuconostoc lactis*	92.8	*Leuc mesent.*	99.08
I	OMO_a 02	*Lactobacillus acidophilus*	92.9	*L acidophilus*	100
J	MOW_a 05	*Leuconostoc mesenteroides subsp. Mesenteroides*	93.5	*Leuc mesent.*	99
K	IM_a 02	*Lactobacillus pentosus*	99.7	*L pentosus*	100
L	ILUa 03	*Lactobacillus coprophilus*	88.1	*L acidophilus*	99
M	IKI_b 04	*Lactobacillus brevis*	99.9	*L brevis*	99.7
N	IFE_c 10	*Lactobacillus crispatus*	81.4	*L crispatus*	99
O	FAT_d 01	*L delbr. Ssp delbrueckii*	84.8	*L delbrueckii*	100
P	MOD_b 03	*Leuconostoc mesent ssp. Mesenteroides/dextranicum*	82.5	*Leuc cremoris*	100
Q	IBA_b 06	*Lactobacillus plantarum*	95	*L plantarum*	99
R	MOW_c 01	*Leuc mesenteroides ssp. Mesenteroides/dextranicum*	85.1	*Leuc citreum*	100
S	MOW_c 08	*Lactobacillus rhamnosus*	88.1	*L casei*	99
T	KEL_c 07	*Lactobacillus plantarum*	90.9	*L plantarum*	100

Letter A to T correspond to the PCR-RFLP profile. IM_a 06, IMOTA; IBA_b 03, IBAFO; IFE_a 05, IFE; MOD_a 01, MODAKIKI; FAT_a 10, FATUNLA; IJE_a 05, IJESA ISU-EKITI; BAD_a 03, BADAGARY; AYI_a 13, AIYEPE; OMO_a 02, OMOTOSHO; MOW_a 05, MOWE; ILU_a 03, ILUOMOBA; IKI_b 04, IKIRE; KEL_b 07, KELEB. Leuc; *Leuconostoc*, L; *Lactobacillus*, mesent; *mesenteroides*, ssp; subspecies.

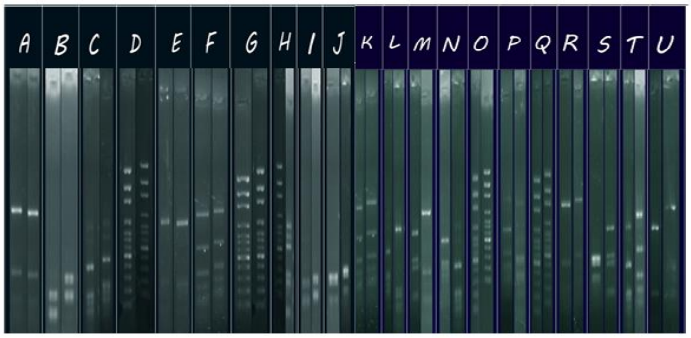

Plate 5 Amplified 16S rRNA restriction analysis (ARDRA) profiles generated after the restriction digestion of the PCR product with *Hae* III and *Bash* 12361; M, 100 bp marker. A, Leuconostoc lactis; B, *Lactobacillus fermentum*; C, *Lactobacillus lactis*; D, *Lactococcus lactis ssp lactis*; E, *Lactobacillus rhamnosus*; F, *Leuconostoc carnosum;* G, *Lactobacillus plantarum*; H, *Leuconostoc mesenteroides*; I, *Lactobacillus acidophilus*; J, *Leuconostoc mesenteroides*; K, *Lactobacillus pentosus;* L, *Lactobacillus acidophilus*; M, *Lactobacillus brevis*; N, *Lactobacillus crispatus*; O, *Lactobacillus delbrueckii*; P, *Leuconostoc cremoris*; Q, *Lactobacillus plantarum*; R, *Leuconostoc citreum*; S, *Lactobacillus casei*; T, *Lactobacillus plantarum*.

Further, comparison of the data obtained with API 50CHL and 16S rRNA gene marker identification showed discrepancy in intra-species identity as shown in Table 2. Isolates MOD_a01 and IJE_a 05 were identified with API kit as *Lactobacillus delbrueckii sub-species delbrueckii* (77%) and *Lactobacillus delbrueckii sub-species lactis* (84%) similarities but were identified at genus level with 16S rRNA as *Lactococcus lactis sub-species lactis* and *Leuconostoc carnosum* (100%) respectively. Similarly, discrepancies were observed at strains level with isolates FAT_a 10, AIY_a 13, ILUa 03, MOD_b 03, MOW_c 01 and MOW_c 08 which were identified as *Lactobacillus acidophilus* (76.3%); *Leuconostoc lactis* (92.8%); *Lactobacillus coprophilus* (78.1%); *Leuconostoc mesenteroides sub-species Mesenteroides/dextranicum* (82.5%); *Leuconostoc mesenteroides sub-species Mesenteroides/dextranicum* (85.1%) and *Lactobacillus rhamnosus* (78.1%) similarities and were also later identified as *Lactobacillus rhamnosus* (100); *Leuconostoc mesenteroides* (99.08%); *Lactobacillus acidophilus* (99%); *Leuconostoc cremoris* (100); *Leuconostoc citreum* (100%) and as *Lactobacillus casei* (99%) with 16S rRNA gene bank.

Cluster/Phylogenetic analysis

Phylogenetic tree based on the PCR-RFLP fingerprint data of the endonuclease digestion of 16S rRNA gene of bacteria isolated from fresh palm wine is shown in Figure 1-4. Cluster analysis calculated all pairwise similarity values of the two endonucleases fingerprint data with a similarity coefficient. The derived similarity matrix is converted into dendrogram with a cluster alogarithm. Similarity coefficient is dice with optimization of 0.50% and a band tolerance of 0.50%. The tree is drawn to scale, with branch lengths in the same units as those of the

evolutionary distances used to infer the phylogenetic tree. The analysis involved all the fingerprint data generated by the two endonucleases on the amplified 16S rRNA gene. All position containing gaps and missing data in the fingerprint data were eliminated. As expected, bacteria from the same species were located in the same cluster. Similarly, bacteria with the same fingerprint but from different locations were located in the same cluster. *Lactobacillus fermentum* is more closely related to *Lactobacillus plantarum* and *Lactobacillus brevis* than to *Leuconostoc lactis, Leuconostoc cremoris* and *Leuconostoc mesenteroides*.

DISCUSSION

Four hundred bacteria isolates identified from the different palm wine samples were Gram positive, catalase and oxidase negative reactions, thus, considered as presumptive LAB. All bacteria isolated from palm wine samples fit the classification of lactic acid bacteria as Gram positive, catalase negative and oxidase negative (**Salminen and Von-wright, 1993**; **Manel *et al*., 2011; Adamu-Governor *et al*., 2018**). The bacteria isolates were clustered into two major broad groups on the basis of their macro-morphology and micro-morphology. One hundred representative bacteria isolates were randomly chosen per group for 16S rRNA gene analysis. This is in agreement with **Manel *et al.* (2011**) who reported that ten strains of lactic acid bacteria isolated from palm sap were chosen according to the differences in cell morphology. Earlier studies had reported that thirteen strains of exopolysaccharides-producing lactic acid bacteria isolated from fermented milk samples in Burkina Faso were selected for identification according to their cultural and cellular morphology (**Savadogo *et al*., 2004**). Similarly, **Adebayo-tayo and Onilude, (2008)** reported that one hundred and thirteen lactic acid bacteria isolated from seven fermented foods were initially differentiated on the basis of their cultural and cellular morphology before they were subjected to various physiological and biochemical tests.

The 16S rRNA genes PCR has been used as a tool for bacterial identification because it contains conserved regions coexisting with variable sequences as specific targets for molecular identification, and 16S rRNA gene is present in all bacteria (**Jandal and Sharon, 2007**; **Frederick, 2015)**. Amplification of variable sequences of the rRNA gene is made possible by PCR primers targeting the conserved regions of rRNA and co-migration of amplified DNA fragments from all bacterial isolates indicated their identical size. The PCR products contained approximately 1500 bp and corresponded to the expected size of the 16S rRNA genes based on the nucleotide sequence data for lactic acid bacteria. This results is in agreement with **Jandal and Sharon (2007)** who reported the 16S rRNA sequence is about 1,500 base pairs long and universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540 bp or at the whole sequence -1,550 bp regions. The 16S rRNA gene is universal in bacteria and has been used for phylogenetic and taxonomic studies as it is highly conserved between different species of bacteria and archeea (**Tortoli, 2003**; **Clarridge, 2004**).

Several studies have shown the merits of ARDRA for rapid grouping and identification of isolates of LAB (**Sato *et al*., 2000**; **Vaneechoutte and Heyndrickx, 2001**; **Chen *et al*., 2005**; **2006**) and bacterial species (**Alfa, 2012**). As expected, the two enzymes distinguished the bacterial isolates by generating 20 distinct profiles (A – T). Further, ARDRA profiles were reproducible and no variation in the restriction profiles of strains belonging to the same genera was observed. Usefulness of the RFLP analysis of 16S rRNA gene has been proved by **Yu *et al*. (2009)** who characterized 171 strains of lactic acid bacteria from home-made fermented milk. Previously, **Jang *et al*. (2003)** and **Kim *et al*. (2003)** reported the used RFLP analysis of 16S rRNA gene for the identification of *Leuconostoc* species isolated from kimchi. Similarly, RFLP analysis with *Hind* III and *AcyI* enzymes for differentiating *Lactococcus* strains that produced extracellular exopolysaccharides has been proposed (**Deveau and Moineau, 2003**). **Otlewska *et al*. (2010)** differentiated between *Lactococcus* and *leuconostoc* species based on RFLP analysis of 16S rRNA. using four enzymes; *EcoRI, BamHI, Hind*III and *Taq*I.

The fragments size generated in this study are similar to sizes generated in previous studies (**Kopermsub and Yunchalard, 2010**; **Otlewska *et al*., 2010**). Further, the differences observed in the fragment sizes generated by the two restriction enzymes in this study could be attributed to variations in gels and electrophoresis conditions. This result is in agreement with **Otlewska *et al*., (2010)** who reported that the difference in the fragment sizes generated by four restriction enzymes when differentiating between *Lactococcus* and *Leuconostoc* species were presumably due to variations in gels, buffers, ethidium bromide concentration, and electrophoresis conditions.

Comparing the identified results of LAB obtained in API tests and RFLP analysis of 16S rRNA gene showed that only 55% of the examined species/strains were correctly identified by the API 50 CHL system. **Otlewska *et al*. (2010)** reported 64% correct identity of examined strains when the API 50 CHL results were compared with RFLP analysis of 16S rRNA gene in differentiation between *Lactococcus* and *Leuconostoc* species. Differences between sequencing and phenotypic tests have already been observed previously, not just for LAB but also for many other bacteria (**Aymerich *et al*., 2003**; **Velasco *et al*., 2004**; **Gomes *et al*., 2008**; **Alfa, 2012**). In this study, disparities were observed with the results from carbohydrate utilization, estimated by API 50 CHL system and RFLP analysis of 16S rRNA gene.

Bacterial sugar fermentation and enzymatic activities, has been commonly used characteristics for the identification of lactic acid bacteria through API 50CHL system (**Dickson *et al*., 2005**; **Manel *et al*., 2011**; **Adamu-Governor *et al*., 2018**). However, the unreliability of API 50CHL to differentiate phylogenetically closely related lactobacilli has been reported (**Amarela *et al*., 2009**). This has been attributed to variation in some properties of LAB due to changes in growth and environmental conditions, and spontaneous mutations (**Deveau and Moineau, 2003**). Consequently, the identification of LAB only by API 50CHL or biochemical tests can be misleading (**Amarela *et al*., 2009**; **Moraes *et al*., 2013**). This may result in the non-reproducibility of the tests or difficulties in interpretation and therefore limits the use of traditional methods. Results of this study agrees with the advocates of molecular methods of microbial identification argue that classical biochemical methods as sole means of microbial identification are unreliable and misleading. Therefore, the application of molecular methods is more accurate than that of the conventional phenotypic methods (**Riebel and Washington, 1990**; **Alfa, 2012**).

The used of GelCompar II to verify the identity of isolates by finding the closest match and/or characterize isolates based on band pattern and percentage similarity number has been documented (**Chan *et al*., 2003**; **Kopermsub. and Yunchalard, 2010**). Similarly, for definitive confirmation of pattern identity requires visual comparison of unknown with the surrounding patterns to determine indistinguishable or closely related pattern when two or more strains are run side-by-side on the same gel (**Chan *et al*., 2003**).

In this study, identification of a representative gum producing lactic acid bacteria from the 20 distinct profiles generated by amplified ribosomal DNA restriction analysis (ARDRA) and API 50CHL showed that palm wine is largely dominated by *Lactobacillus* and *Leuconostoc* genera (**Amoa-Awua *et al*., 2007**, **Manel *et al*., 2011**; **Adamu-Governor *et al*., 2018**). This result further confirms the identification of the same bacteria isolates by API 50 CHL system. Studies have shown that polyphasic approach should be employed on confirmatory basis in the identification of bacteria (**Janda and Abbot, 2002**; **Otlewska *et al*., 2010**; **Kopermsub and Yunchalard, 2010**; **Afaf, 2012**).

CONCLUSION

Judging by the data obtained from this study, amplified ribosomal DNA restriction analysis (ARDRA) screened and grouped the two hundred gum-producing bacterial into twenty distinct group based on the finger print generated. Also, API 50 CHL system confirm the identity bacteria isolates earlier identified by 16S rRNA gene analysis which are largely *Lactobacillus* and *leuconostoc* species.

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Authors' inputs: The study was designed by LA and SU. LA developed the methodology and acquired the data. LA wrote the manuscript, TS, RA and SU corrected the manuscript, provided administrative support and aptly supervised the study. All authors read and approved the final manuscript.

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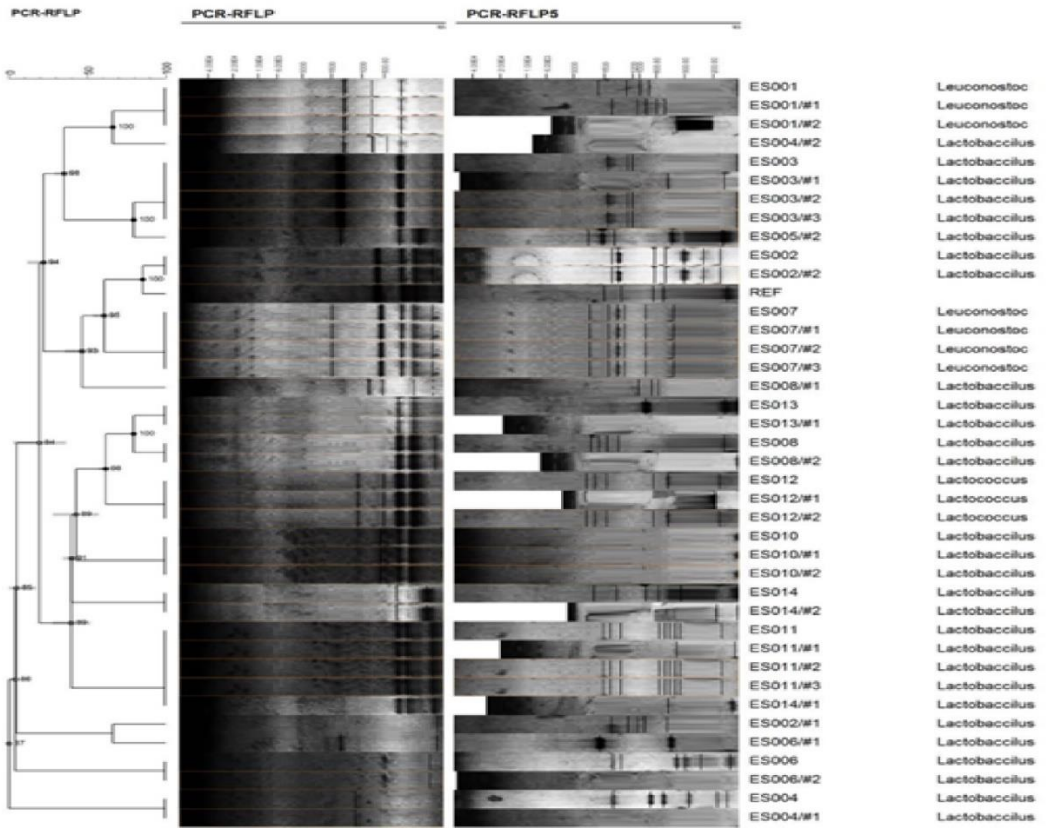

Figure 1 GelCompar II dendrogram showing similarity matrix of fingerprint data of amplified 16S rRNA gene of gum producing bacteria isolates 1-50 from palm wine. PCR-RFLP1, *Hae* III and PCR-RFLP5, *Bash* 12361.

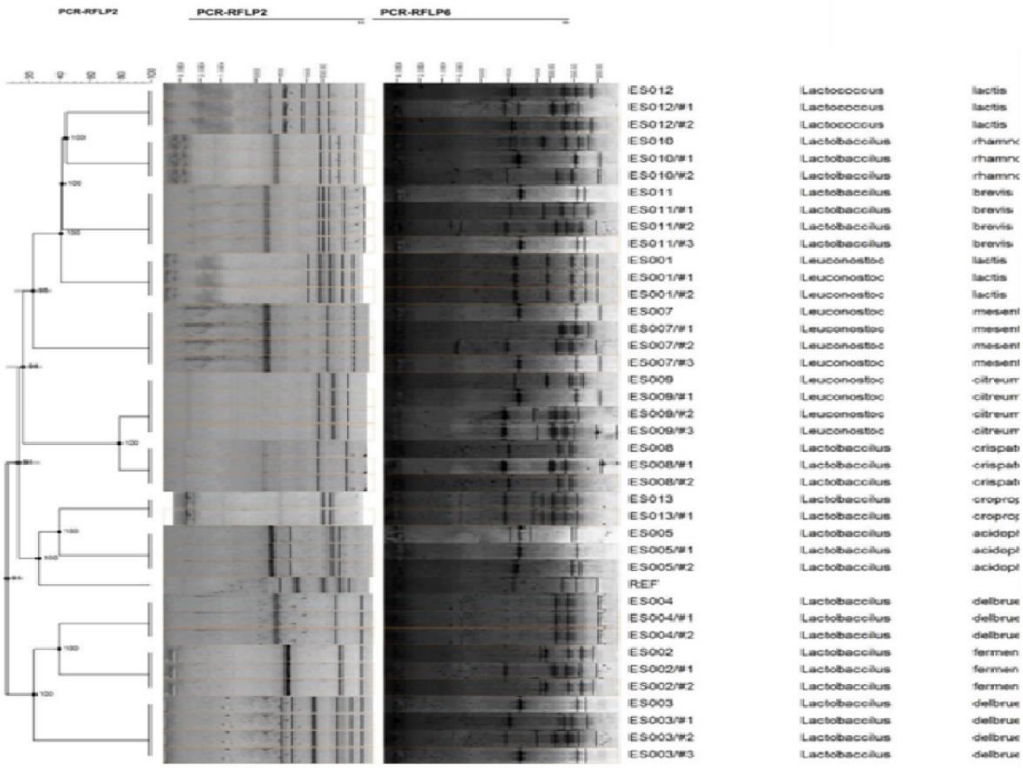

Figure 2 GelCompar II dendrogram showing similarity matrix of fingerprint data of amplified 16S rRNA gene of gum producing bacteria isolate 51-100 from palm wine. PCR-RFLP2, *Hae* III and PCR-RFLP6, *Bash* 12361.

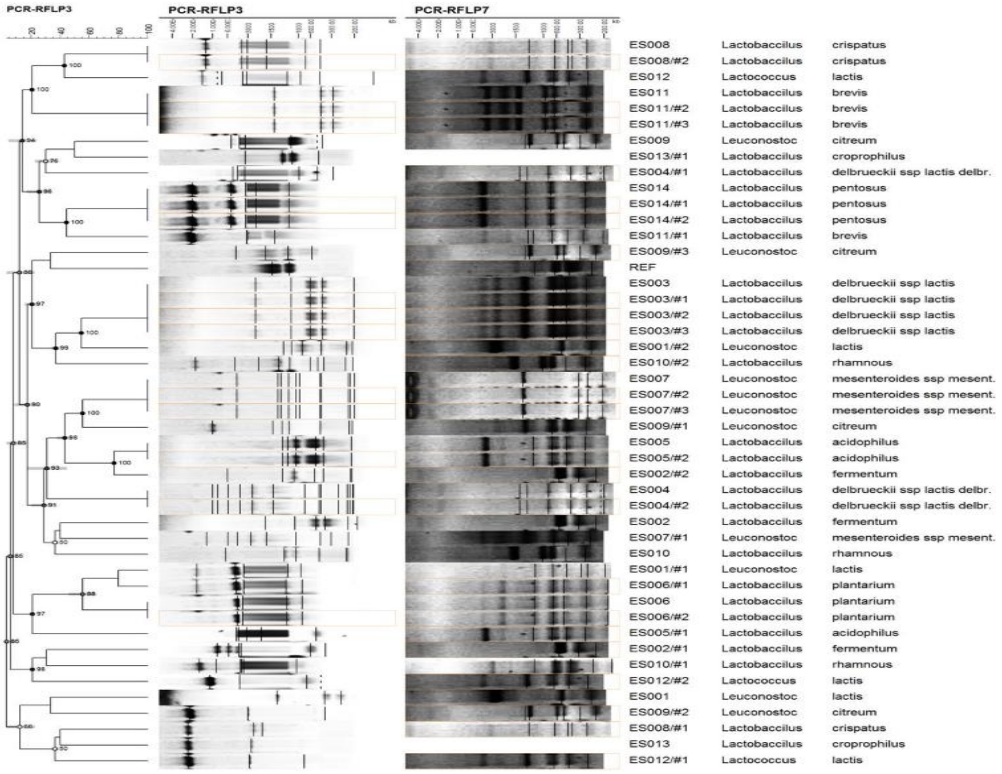

Figure 3 GelCompar II dendrogram showing similarity matrix of fingerprint data of amplified 16S rRNA gene of gum producing bacteria isolates 101-150 from palm wine. PCR-RFLP3, *Hae* III and PCR-RFLP7, *Bash* 12361.

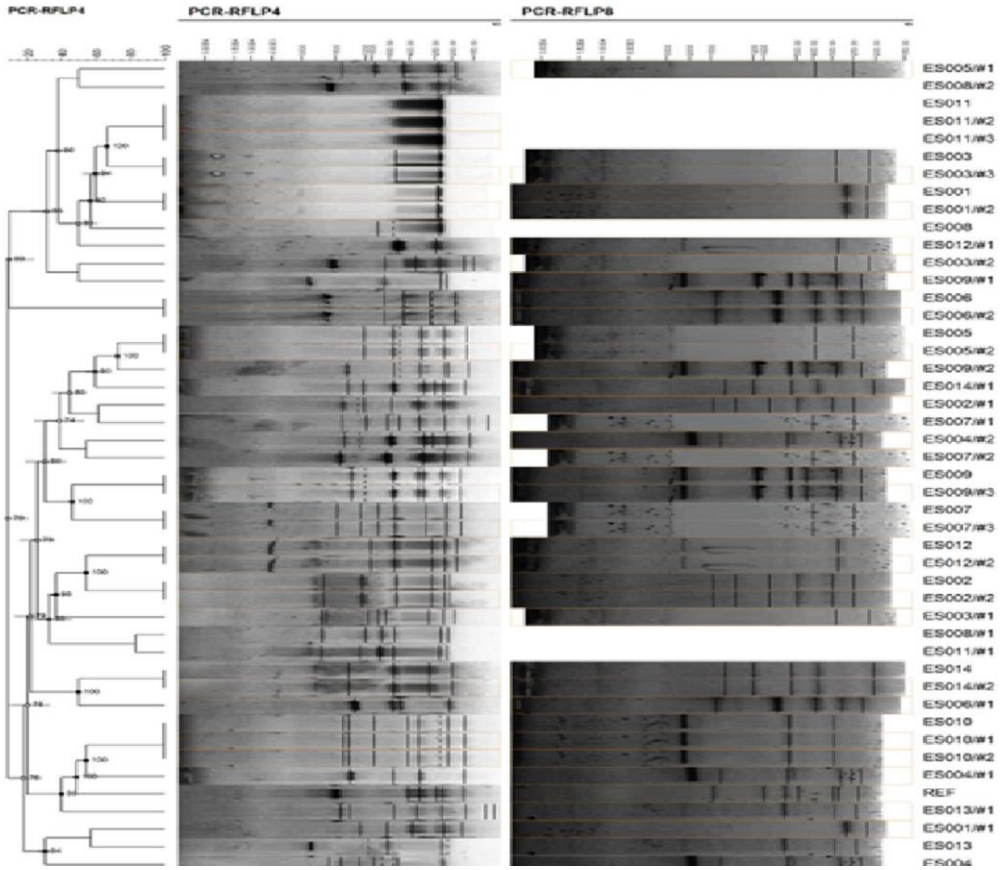

Figure 4 GelCompar II dendrogram showing similarity matrix of fingerprint data of amplified 16S rRNA gene of gum producing bacteria isolates 151-200 from palm wine. PCR-RFLP4, *Hae* III and PCR-RFLP8, *Bash* 12361.