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Genetic diversity of *Lactobacillus plantarum* strains from some indigenous fermented foods in Nigeria

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ABSTRACT

Lactobacillus plantarum has been found to be commonly associated with Nigerian indigenous fermented foods. The intraspecies differentiation of *L. plantarum* using different molecular techniques is essential for the selection of functional strains. In the present study, 48 *L. plantarum* isolates from some Nigerian indigenous fermented foods; (*gari, fufu* and *ogi*) were phenotypically characterized. The intraspecies diversity of 17 selected *L. plantarum* strains with good acidification rates, hydrogen peroxide production and variation in carbohydrate fermentation patterns were carried out using molecular techniques, 16S-23 rDNA intergenic transcribed spacer and restriction fragment length polymorphism (ITS-PCR and ITS-RFLP), randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE). The ITS-RFLP-*Hae*III, RAPD-OPA5, OPA20 and PFGE-*Sfi*1 analysis showed genetic diversity among the strains of *L. plantarum* isolated from the different fermented foods, and it can be established that these molecular tools are useful for differentiation of *L. plantarum* strains. The molecular techniques used in this study may be considered useful tools for characterization of isolates and for in-depth examination of the strain diversity as the various strains isolated in this study can be used as adjunct and/or starter cultures in food fermentation processes.

1. Introduction

Lactic acid bacteria (LAB) are beneficial microorganisms that are commonly isolated from various food products such as; cabbage, cucumber, cereal and cassava. They have been widely reported to be involved in the fermentation of many African indigenous foods (Blandino, Al-seeri, Pandiella, Canteroa, & Webb, 2003; Holzapfel, 2002; Tamang et al., 2012), beside performing essential roles during the fermentation processes (Mills, 2004). These bacteria have equally been found to have several useful applications in the production of fermented foods and beverages which are widely consumed and are important for the livelihood of many people around the world (LeBlanc et al., 2013; Tamang et al., 2012). Africa has a long history of the production of lactic acid fermented foods which have pronounced influence on people's health, nutrition and socio economy status especially in the developing countries. In Nigeria, LAB are mainly responsible for the fermentation of indigenous foods such as: *ogi* (fermented cereal gruel from maize and sorghum), *fufu* and *gari* (fermented cassava mash and flakes from cassava tuber), *wara* (locally fermented cheese from cow's milk), *iru* and *ogiri* (fermented condiment from *Parkia biglobosa* plant and melon seed). Their occurrence in fermented foods is of interest not only for their role in fermentation but also in promoting positive health impacts such as the use as probiotics which are strain specific. It has equally been established that the selection of suitable bacteria for controlled fermentation, industrial application, probiotics, or as potential starter culture for enhancement process are strain dependent (Adesulu-Dahunsi, Sanni, & Jeyaram, 2017). Therefore, the correct identification of these strains is a fundamental step before examining their actual functionalities (Leroy & De Vuyst, 2004; FAO/WHO, 2006).

Several researchers have isolated and characterized *L. plantarum* for extensive studies. *L. plantarum* is a highly diverse and versatile specie which is commonly used in various food and health applications (Bringel, Curk, & Hubert, 1996; Kostinek et al., 2005). Among the LAB that are involved during the fermentation of foods, *L. plantarum* has an outstanding effect on the flavor and texture of such foods. They display specific metabolic and technological

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properties, and also play important roles during fermentative activities by providing desirable properties which may be used for the production of various probiotic functional foods. *L. plantarum* are widely used with other LAB for the production of some fermented foods with quality end products and good organoleptic properties (Salvucci, LeBlanc, & Perez, 2016; Tsafrakidou et al., 2016). Therefore, reliable identification of *L. plantarum* at the subspecies and strain level is of great interest.

The molecular based methods using polymerase chain reaction (PCR) and sequencing of the 16SrRNA gene have been developed and are widely used for LAB identification and allow differentiation between strains of the same species. PCR based fingerprinting methods have been reported by many researchers as useful and reliable tools for proper identification of these bacteria to species and strain level (Devi, Deka, & Jeyaram, 2015; Fusco, Quero, Stea, Morea, & Visconti, 2011; Santos et al., 2005). Also, the determination of the intra-species strain diversity is essential in examining the functionality of the strains (Leroy & De Vuyst, 2004). Several studies have explored the diversity within *L. plantarum* using different PCR-based methods such as RAPD-PCR (Bringel, Quenee, & Tailliez, 2001; Elegado, Guerra, Macayan, Mendoza, & Lizara, 2004), PFGE (Sànchez, Sesena, & Palop, 2004) multilocus sequence typing (MLST) (De las Rivas, Marcobal, & Munoz, 2006; Tanganurat, Quinquis, Leelawatcharamas, & Bolotin, 2009) and sequencing of 16S rDNA (Tannock, Tilsala-Timisjarvi, Rodtong, Munro, & Alatossava, 1999). The randomly amplified polymorphic DNA (RAPD-PCR) technique uses short random primers which bind under low stringency to complementary sequences. Pulsed-field gel electrophoresis is one of the most powerful molecular techniques for characterizing to strain level, and it involves digestion of the genomic DNA with rare-cutting restriction enzymes. The RAPD-PCR and PFGE techniques have been successfully used to differentiate *Lactobacillus* strains from various sources and different geographical origins (Chen et al., 2012; Tsafrakidou et al., 2016), but only a few studies have been carried out on strain level differentiation of indigenous LAB isolated from Nigerian fermented foods. This study therefore aims at characterizing *Lactobacillus plantarum* to strain level using ITS-PCR, ITS RFLP, RAPD and PFGE, to confirm subspecies identities and evaluating the intraspecies genetic diversity. This study is novel in that information on intraspecies genetic diversity of *L. plantarum* from Nigerian indigenous fermented foods using different molecular tools is limited and this report further serves as a precursor to future studies on the functional properties of the isolates for usage as probiotics and starter culture development.

2. Materials and methods

2.1. Collection of food samples

Thirty samples of freshly prepared *ogi, gari* and *fufu* were obtained from the local producers in two different Nigerian States (Oyo and Osun). The samples were collected in 500 ml capacity sterile sample containers and were transported immediately to the laboratory for microbiological analysis.

2.2. Isolation of LAB strains and phenotyping

At different fermentation times (12, 24 and 36 h), 10 g of each food samples was aseptically removed and diluted in 90 ml sterile physiological saline and homogenized using a stomacher for 2 min. The samples were diluted into 10-fold dilutions and were pour plated on de Man Rogosa and Sharpe (MRS) media with the addition of 0.1 g/l cycloheximide to the media to prevent yeast growth and the plates were incubated for 48 h at 37 °C under anaerobic conditions (Meroth, Walter, Hertel, Brandt, & Hammes, 2003). Colonies were randomly selected and repeatedly streaked on MRS agar plate until pure cultures were obtained. The isolates were preserved at −80° C with the addition of 20% (v/v) glycerol.

The isolates were phenotypically identified based on their cell morphology under phase contrast microscope (Leitz, Jena, Germany), Gram staining, catalase test, gas (CO_2) production from glucose, hydrogen peroxide production, arginine hydrolysis, growth at 15 °C and 45 °C, ability to grow at 6.5% NaCl, pH reduction in MRS broth and sugar fermentation patterns according to Dykes (1994), and the use of API 50CHL carbohydrates fermentation test strips on representative isolates.

2.3. Genotypic characterization

The L. *plantarum* reference strains used in the molecular analyses were obtained from the following cultures collection: *Lactobacillus plantarum* ATCC 8014 (American Type Culture Collection) and *Lactobacillus plantarum* SD1S6L2 (MRC culture collection centre, Institute of Bioresources and Sustainable Development, Imphal, India).

The genomic DNA extraction was performed using lysozyme-heat lysis method as described (Jeyaram et al., 2011). The DNA samples were quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Germany). The cell free lysate with absorbance ratio (A_{260}/A_{280}) of 1.8 and 2.2 were used as a template for PCR analysis. The genomic DNA lysate of the LAB isolates were amplified as previously described by Adesulu-Dahunsi et al. (2017) using universal forward and reverse primers; fD1 and rD1. The ribosomal DNA restriction digestion of the amplified 16S rRNA gene of about 1500 bp were performed with the restriction endonucleases *Hae*III (Sigma), *Hinf*I (Sigma) and *Rsa*I (Sigma) according to Jeyaram et al. (2010).

To confirm the identity of the selected isolates, the 16S rRNA gene of the five representative *L. plantarum* isolates having similar ARDRA profile with the reference strains was purified using NucleoSpin® Extract II gel extraction kit (Machery-Nagel, Germany) following manufacturer's instructions. The sequencing reactions were performed using ABI 3100 DNA sequencer (Applied Biosystems, USA) in both direction with universal primers used for amplification and an additional forward primer F515. The nucleotide database searches were performed in NCBI GenBank and Ribosomal Database Project (RDP) SEQmatch programmes for the identification of the closest known relatives of *L. plantarum*.

2.4. 16S-23S rRNA gene intergenic transcribed spacer (ITS) and restriction fragment length polymorphism (ITS-RFLP) PCR amplification

The16S-23S rRNA (ITS-PCR and ITS-RFLP-PCR) amplification was carried out following Jeyaram et al. (2010) with forward primer 16SF-R2 and reverse primer 23SR-R10 (Sigma). The amplified products were digested with *Hae*III (Promega). The reaction mixture contained; 5.0 μl of the ITS-PCR amplified product, 1.0 μl of *Hae*III (1× final concentration), 0.2 μl of 10 mg/ml BSA (Promega), 2 U of multicore buffer and 3.6 μl of sterile nuclease free water.

2.5. Randomly amplified polymorphic DNA (RAPD-PCR) analysis

The RAPD-PCR reactions of the selected isolates were performed with oligonucleotide primers OPA 5 and OPA 20. The amplified products were separated by electrophoresis on 1.8% agarose gel (Table 1). The reaction mixture contained; 2.5 μl of 10× PCR reaction buffer (Sigma Aldrich), 0.2 μl each deoxynucleotide triphosphate (dNTPs) (Promega) at final concentration of 200 μM, 2.5 mM

Table 1
Sequences of the PCR primers and conditions used in this study (Pulido et al., 2005; Weisburg, Bams, Peletiar & Lane, 1991).

Primers name	Primer Sequence (5′ - 3 ′)	Thermal cycling	References
fD1	AGAGTTTGATCCTGGCTCAG	94^0C for 10min } 1x	Weisburg et al., 1991
rD1	AAGGAGGTGATCCAGCCGCA	94^0C for 1min 65^0C for 1min } 35x	
f515	GTGCCAGCCGCCGCGGTAA	72^0C for 30sec } 1x	
		72^0C for 7min } 1x	
16SF-R2	CGCGGGATCCTTGTACACACCGCCCGTC	94^0C for 5min } 1x	Lechner et al., 1998
23SR-R10	GGCCGTCGACCCTTTCCCTCACGGTACTG	94^0C for 30sec 60^0C for 3min } 29x	
		72^0C for 1min } 1x	
		72^0C for 7min } 1x	
OPA5	AATCGGGCTG	94^0C for 5min } 1x	Pulido et al., 2005
OPA20	GTTGCGTCC	94^0C for 1min 35^0C for 1min } 34x	
		72^0C for 2min } 1x	
		72^0C for 10min } 1x	

x is number of PCR cycles

$MgCl_2$ (Promega), 0.1 μl of 0.1 mM primer, 0.3 μl of *Taq*DNA polymerase (5 U/ml) (Sigma Aldrich), and 2 μl of cell free lysate with 50 ng DNA and the volume of the PCR mixture was made up to 25 μl with nuclease free water.

2.6. Pulsed-field gel electrophoresis (PFGE) analysis

Eighteen hours old freshly prepared cultures with optical density (OD_{600}) of 1.5 were harvested by centrifugation at 8,000xg for 5min and were used for the preparation of the chromosomal plugs. The cells were washed gently for 5 times with 1 ml of TEN buffer (10 mM Tris Cl, pH 8.0; 100 mM EDTA, pH 8.0, 1M NaCl) and resuspended in 290 μl TEN buffer containing 10 μl mutanolysin (10 U/μl) (Sigma Aldrich, Banglore, India), incubated at 37° C for 30 min for pre-lysis treatment. The bacterial cell suspension was mixed with equal volume of 2.0% (w/v) low-melting agarose (LMP) (Promega, Madison, USA) in 0.125 M EDTA and equilibrated at 50° C, for 10 min, 1000 rpm in a thermomixer (Eppendorf, Hamburg, Germany). The cell suspension mixtures were immediately transferred into the plug molds (10 mm × 5 mm x 2 mm) and the plugs were left to solidify at 4° C for 20 min.

In situ cell lysis I was carried out by suspending the solidified plugs for each isolates into 3.8 ml 1× lysis buffer (6 mM Tris Cl, pH 8.0; 1 M NaCl, 100 mM EDTA, pH 8.0, 0.5% w/v sarcosyl and 0.2% w/v sodium deoxycholate), 200 μl of 10000 KU/ml lysozyme (2000 KU) (Sigma Aldrich) and incubated overnight (16–18 h)at 37° C, 160 rpm. *In situ* cell lysis II was performed by replacing the lysis solution with 3.9 ml ESP buffer (0.5 EDTA, pH 8.0, 1.0% (w/v) sarcosyl) and 100 μl of 20 mg/ml Proteinase K (Himedia, Mumbai, India) were added and incubated at 37° C overnight (16–18 h) at 160 rpm. After incubation, plugs which were clear and transparent when observed visually showed complete cell lysis, and were washed gently in 25 ml preheated (50° C) sterile demineralized water twice in an incubator shaker for 20 min, 65 rpm at 50° C, followed by five times washing in 25 ml preheated (50° C) TE10/0.1 buffer (10 mM Tris Cl, pH 8, 0.1 mM EDTA, pH 8), in an incubator shaker for 20 min, 65 rpm at 50° C for each wash. The plugs that were not used immediately were stored at 4° C in TE10/100 buffer (10 mM Tris Cl, pH 8, 100 mM EDTA, pH 8).

After pre-digestion, the plugs were repeatedly washed with sterile demineralized water and TE10/0.1 buffer (10 mM Tris Cl, pH 8, 0.1 mM EDTA, pH 8) as described above, chromosomal DNA plugs with the plug dimension 8–9 mm x 2.5 mm × 2 mm were equilibrated at room temperature for 30 min in 200 μl of 1× Multicore restriction buffer (Promega). For restriction enzyme digestion, 40 U of two different restriction enzymes were used; *Sfi*I and *Apa*I (Promega), the plugs were placed in tubes containing 200 μl reaction mixture of 20.0 μl of 1× restriction buffer, 0.1 mg/ml acetylated BSA, 40 U of the restriction enzyme, and incubated overnight (12–14hr) at 37° C in a water bath (Memmert). After incubation, the enzymes were inactivated by removing the digestion mixture and incubating the slices with 200 μl 0.5× TBE buffer (45 mM Tris borate, 1 mM EDTA, pH 8.0) for 30 min at room temperature. Plug slices of 8–9 mm x 1 mm × 2 mm wide were gently loaded into the wells of 1.2% PFGE agarose gel (Sigma Aldrich) and the wells were sealed by using 1% LMP agarose. After solidifying, PFGE was performed in an electrophoresis apparatus (Gene Navigator, Amersham Biosciences). The electrophoresis was carried out in 1.2% (w/v) PFGE agarose gel (Sigma Aldrich) and 0.5× TBE buffer at 9°Cand155 V. The electrophoresis was run for 26 h with a switch interval of 0.5–20.0 s for 24 h followed by 20.0–50.0 s for 2 h with lambda ladder PFG marker (48.5–727.5 kb) (New England Biolabs)

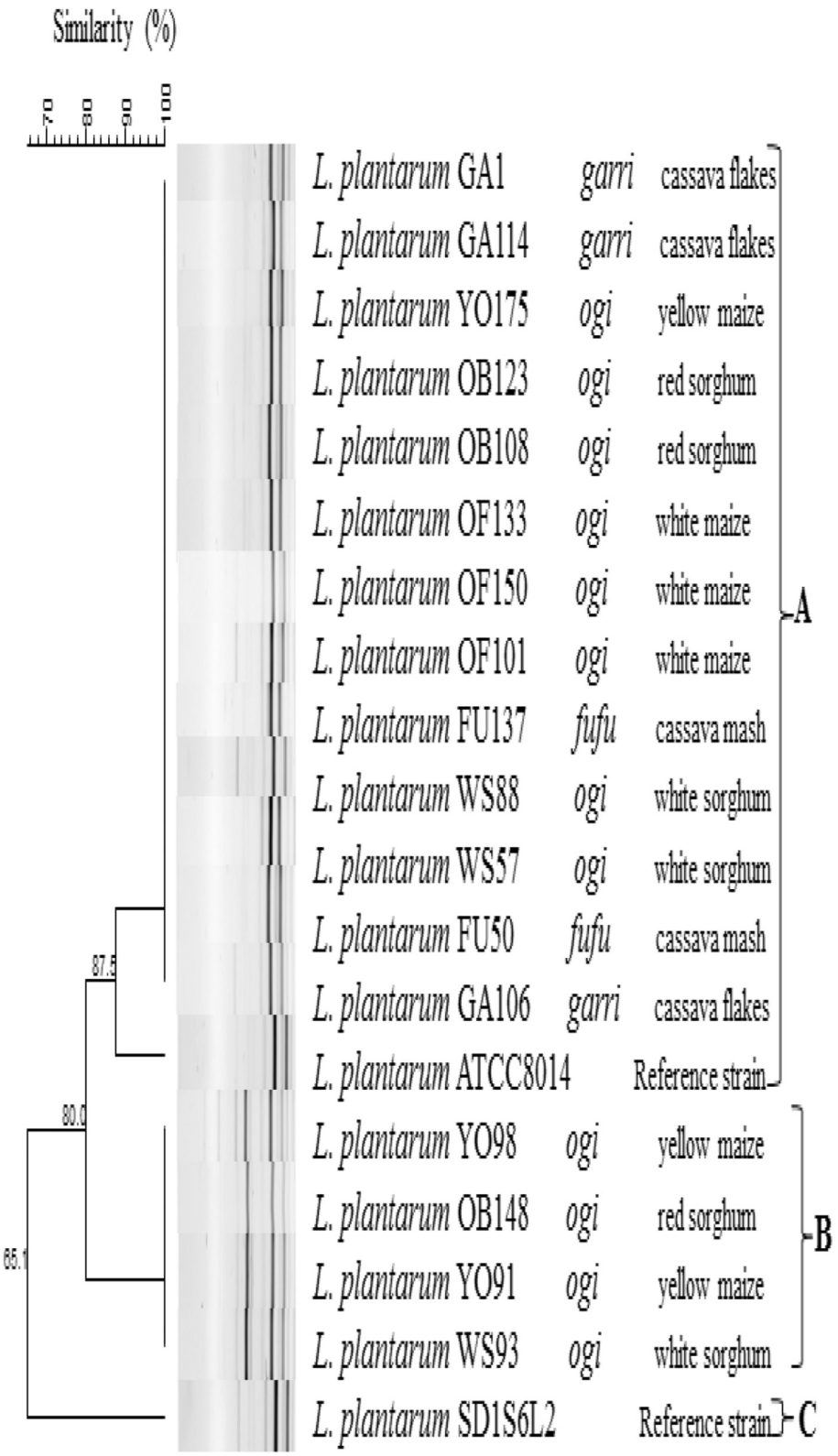

Fig. 1. Simplified dendrogram based on the UPGMA clustering of Jaccard coefficients (SJ) of ITS-RFLP profiles of selected *L. plantarum* strains isolated from some Nigerian traditional fermented foods with reference strains.

on the first and last lane of the gel. The gel was then stained in sybr gold for 30 min on a rocker at 15 rpm and were visualized in a gel documentation system (Gel doc EQ, Biorad, USA).

2.7. Phylogenetic analysis

The ITS-RFLP, RAPD and PFGE data were scored manually and

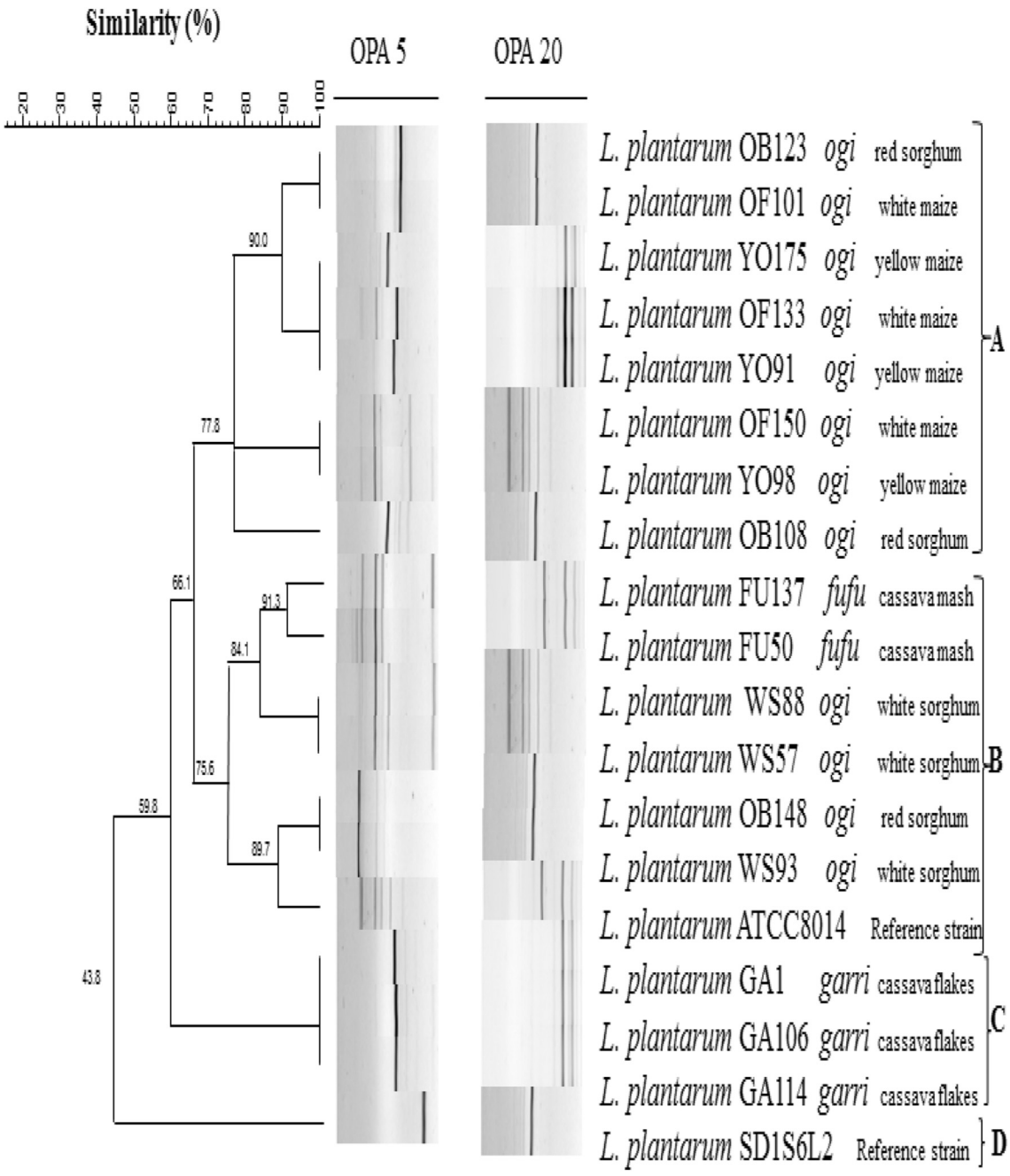

Fig. 2. Dendrogram obtained from RAPD-PCR fingerprinting pattern using primers OPA5 and OPA20 of selected *L. plantarum* strains isolated from some Nigerian traditional fermented foods with reference strains.

the generation of clustering analysis in a dendrogram were processed separately using NTSYSpc software version 2.20f based on the Jaccard similarity coefficient (S_J) and the un-weighted pair group method using arithmetic averages (UPGMA).

3. Results and discussion

Forty-eight (48) LAB species isolated from *gari, fufu* and *ogi* were presumptively identified as *L. plantarum* based on these phenotypic key characteristics; Gram positive, catalase-negative, hetero-fermentative rods and oxidase negative. The use of phenotypic methods in the identification and characterization of *L. plantarum* strains isolated from Nigerian indigenous fermented foods have been found to be insufficient over the years especially in the proper selection and inclusion in starter culture development. This is largely due to the significant roles played by these strains during fermentation processes. Researchers have reported the predominance of *L. plantarum* in Nigeria indigenous fermented foods (Banwo, Sanni, Tan, & Tian, 2012; Oguntoyinbo & Narbad, 2015). It has been reported that for controlled fermentation, and the use of the microorganisms as probiotics, or as potential starter culture for enhancement process, selection of suitable bacterial strains are strain dependent (Oguntoyinbo & Narbad, 2012). To reduce the number of isolates to be studied using molecular techniques, 17 isolates with good acidification rates, hydrogen peroxide production and variation in carbohydrate fermentation patterns were

selected.

The genomic diversity among the *L. plantarum* species was carried out using genotypic methods of characterization to confirm the identity of the selected strains and for strain characterization and differentiation. The amplified PCR products contained approximately 1500 bp, corresponding to the expected size of the 16S rRNA genes based on the nucleotide sequence data for the identification of LAB. The isolates were identified as *L. plantarum* on the basis of the ARDRA profile using *Hae*III restriction endonuclease (data not shown). The applicability of this method for rapid identification of *L. plantarum* species was previously confirmed (Adesulu-Dahunsi et al., 2017). The 16S rDNA gene sequencing has been recognized as a well-established standard technique for the identification of bacterial species (Lechner et al., 1998). The partial 16S rDNA gene sequences of selected isolates were identified as *L. plantarum* (*L. plantarum* GA106, *L. plantarum* OF101, *L. plantarum* FU137, *L. plantarum* YO175, *L. plantarum* OB123) and were submitted to the GenBank database with accession numbers: KU892392, KU892393, KU892394, KU892395, KU892396.

The strain level differentiation of *L. plantarum* was achieved by ITS-RFLP, RAPD and PFGE analysis. Application of these typing methods allows for identification to strain levels. There were no differences in the restriction patterns among the strains of *L. plantarum* with the amplification of the 16S-23S rDNA ITS-PCR, but the result of ITS-RFLP pattern digested with *Hae*III restriction enzyme showed diversity among the *L. plantarum* strains from the indigenous fermented foods with the reference strains, and were divided into 3 clusters (Fig. 1), cluster A consisted of 13 *L. plantarum* strains and having 100% similarity. *L. plantarum* ATCC 8014 showed 87.5% similarity with the 13 strains and 4 *L. plantarum* strains from *ogi* having 80.0% similarity were obtained for cluster B, *L. plantarum* SD1S6L2 showed 65.1% similarity with the other clusters. The use of restriction analysis ITS-RFLP-PCR with *Hae*III restriction enzyme digestion profile showed intraspecies variation among the strains of *L. plantarum*. This result is in agreement with Jeyaram et al. (2011) who reported a high intraspecies variation among the

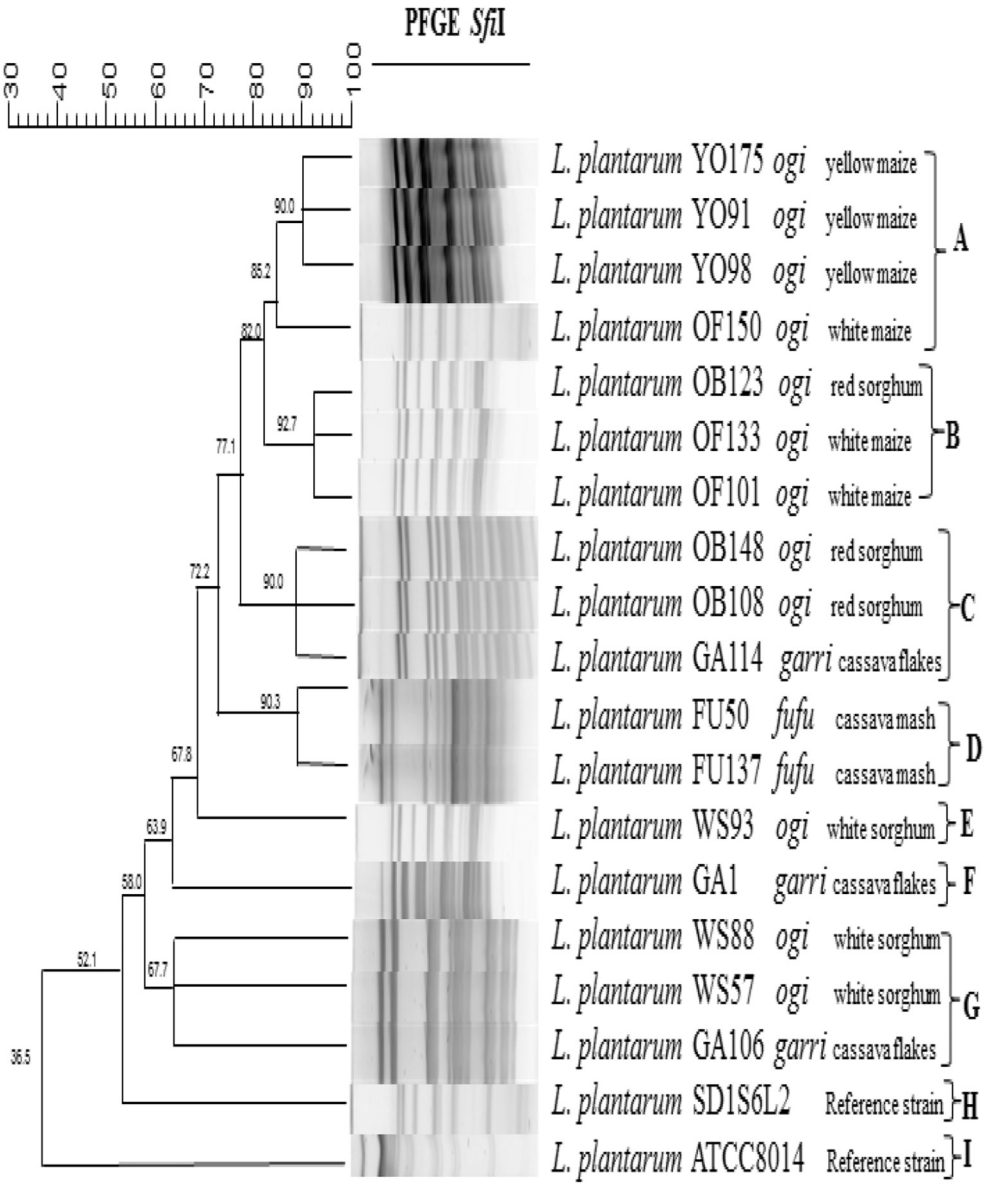

Fig. 3. Dendrogram generated after cluster analysis of PFGE patterns from *L. plantarum* strains after digestion with *Sfi*I. The dendrogram was constructed using the unweighted pair group methods using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficient.

Bacillus subtilis species using ITS-RFLP.

The use of RAPD-PCR method for *Lactobacillus* strain differentiation has been documented (Bouton, Guyot, Beuvier, Taillez, & Grappin, 2002; Cagno et al., 2010; Cocconcelli, Parisi, Senini, & Bottazzi, 1997). The RAPD pattern obtained with oligonucleotide primers OPA5 and OPA20 were combined in a dendrogram (Fig. 2). The result showed strain level differentiation among the *L. plantarum* strains while the strains and the reference strains were divided into 4 main clusters designated as A, B, C and D. Cluster A grouped 8 isolates from *ogi* produced from different varieties of maize and sorghum. Cluster B grouped 2 isolates from *fufu*, 4 from *ogi* and 1 reference strain *L. plantarum* ATCC8014. Cluster C consisted of 3 *L. plantarum* strain from *gari* and cluster D grouped only 1 reference strain *L. plantarum* SD1S6L2. The result is similar to the work of Zeljika et al. (2012), who reported differentiation among *Lactobacillus* strains that are involved during the fermentation of Croatian dry fermented sausages using RAPD-PCR technique. Similarly, Samarzija, Sikora, Redzepovic, Antunac, and Havranek (2001) affirmed that RAPD method is a useful and efficient method for the genetic diversity among LAB and also distinguished *Lactococcus lactis* subsp. *cremoris* at strain level. Lee et al. (2015) also reported the intra-species diversity among *L. sanfranciscensis* strains isolated from Korean sourdough using RAPD-PCR technique.

Pulsed-field gel electrophoresis (PFGE) has been widely used as a tool for the analysis of the genomic diversity of *L. plantarum* and also for identification and characterization of LAB from different food sources and geographical region to sub-species and strain level (Anna et al., 2008; Bouton et al., 2002; Sylvie, Annette, Stephane, & Michel, 1997). Two restriction enzymes *Apa*I and *Sfi*I were used to select the suitable enzyme for the digestion of the genomic DNA. *Sfi*I yielded unique PFGE patterns for the strains tested digesting 5–12 fragments (data not shown). The dendrogram developed using Dice similarity coefficient (Fig. 3) divided the isolates into 9 main clusters designated as A-I. Cluster A consisting of 4 *L. plantarum* strains, the strains displayed very similar chromosomal pattern, 2 subclusters were defined by coefficient of similarity above 85.2%. Cluster B and C contained 3 strains each with the similarity level of 82.0% and 77.1%. The remaining strains were grouped into minor clusters designated as D through I with less than 72.2% similarity level. Clustering of these *L. plantarum* strains revealed a high degree of strain specificity. Several researchers have observed that PFGE can discriminate more strains between LAB species than any other molecular typing methods (Roussel, Colmin, Simonet, & Decaris, 1993; Busse, Denner, & Lubitz, 1996; Marco & Ralf, 2002; Pepe et al., 2004). Analysis resulting from more than one molecular method is of great value for the *L. plantarum* typing, and a high degree of genetic polymorphism observed among *L. plantarum* species isolated from Nigerian indigenous fermented foods in this study, is in accordance with results from researchers who isolated LAB from foods.

4. Conclusion

Strain level typing of *L. plantarum* is essential for the selection of the best performing strains for diverse applications including biotechnological, industrial, pharmaceutical and environmental studies. Different genomic typing techniques were employed to determine phylogenetic relationship and diversity among *L. plantarum* species isolated from Nigerian indigenous fermented foods. From this study, it can be established that RAPD and PFGE are useful for differentiation of *L. plantarum* strains isolated from the different fermented food products. The isolates showed distinct profile which confirmed huge diversity among the *L. plantarum* strains from Nigerian indigenous fermented foods. In conclusion, polyphasic genomics techniques employed were useful in strain identification, differentiation and broad understanding of the diversity of *L. plantarum* strains isolated from different indigenous fermented foods in Nigeria, this study provides a basis for selection of adjunct and starter cultures for industrial food fermentation processes and also production of foods with desirable functional properties.

Conflict of interest

Authors declares that there are no conflicts of interest whatsoever.

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