

Full Length Research Paper

Molecular identification of *Lactobacillus plantarum* isolated from fermenting cereals

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The identification of a microbial isolate to genus level only amounts to a partial characterization of the isolate, but this can tell us a lot about that organism. Knowing the species allows the laboratory access to the body of knowledge that exists on that species. Identification schemes using phenotypic characteristics such as colony and cell morphology, Gram reaction and other staining characteristics, nutritional and physiological requirements for growth and metabolic characteristics have been developed and improved over many decades to a point where laboratories are able to identify isolates to species level using simple conventional methods. This phenotypic method however have some limitations apart from being laborious and time consuming, some organisms may however be misidentified either at genus or species level. This work aims at looking directly at the genome of lactic acid bacteria (LAB) and from this identifies some species using its genotypic and phenotypic characteristics. These bacteria species were identified by sequencing specific sections of ribosomal DNA - the 16S rRNA gene, after amplification by PCR, and then comparing the results to sequences stored on a related database. The results from both conventional and molecular methods were then compared. Twenty (20) *Lactobacillus plantarum* were isolated from spontaneously fermented cereals made into "Ogi" and identified using classical methods. They were further characterized using molecular methods by polymerase chain reaction (PCR) amplification of 16S rDNA genes to confirm their identities. The genotypic characterization however showed that 85% of the organisms identified using conventional method as *L. plantarum* correlated, while 15% did not correlate; 2 were identified as *Lactobacillus pentosus* and one unidentified *Lactobacillus* sp. The method is a rapid and reliable way of producing a large number of copies of a specific DNA sequence for the identification of LAB. This method is however, able to solve the problem of poor identification that is usually associated with the identification of this fastidious organism that is regularly used as probiotics, starter culture and bio-preservatives in fermented foods that are consumed and in biotechnology because they are generally regarded as safe.

Key words: Molecular methods, conventional, *Lactobacillus plantarum* identification, fermented foods, species and genera level, rapid, reliable.

INTRODUCTION

Microorganisms have been isolated from different sources especially from different food samples and grown in pure cultures over the centuries. A major aspect of

microbiology and the work of food microbiologists and various microbiology laboratories is the ability to identify and characterize various isolates so that they can be

differentiated from one another. Different schemes that can be used to describe the characteristics and properties of microbial isolates are essential in every branch of microbiology. These schemes have been undergoing different forms of development and refinement over the years. The various methods are not static; but have been improved from time to time and proper identification is very essential when it has to do with foods that are consumed (Lucke, 2000; Olaoye and Onilude, 2009). The advent of molecular biology in the 1980s contributed a set of powerful new tools that have helped microbiologists to detect the smallest variations within microbial species and even within individual strains (Olaoye and Onilude, 2009). This is because different organisms have different genetic combination.

In fact, the technology has progressed far beyond the level needed by most routine laboratories, where identifying the species of any isolate is likely to be sufficient. Distinguishing between different strains of the same species (typing) is more likely to be of value in a research laboratory. Nevertheless, methods and equipment designed to help with both species identification and typing are commercially available for a range of applications (Lucke, 2000).

There are different molecular characterization techniques namely genotyping, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), ribotyping, repetitive sequence-based PCR (rep-PCR) and the use of 16S rDNA genes which relies on the relative stability of the 16S and 23S rRNA genes coding for ribosomal-RNA and so on (Ogier et al., 2002; Gomes et al., 2008; Paula et al., 2012).

Molecular characterization of microorganisms however has some distinct advantages over the known conventional methods. The molecular method of identification and characterization of microorganisms have been preferred over the classical ones which make use of the biochemical reactions and proteolytic activities of the organisms (Morgan et al., 2009). The classical and conventional method of identification is slow, laborious, time consuming and may not be 100% specific and accurate. It is also problematic and subjective due to ambiguous biochemical or physiological traits.

Bulut et al. (2005) reported that identification of lactic acid bacteria (LAB) by phenotypic methods such as sugar fermentation may be uncertain and complicated owing to the increase in species that vary with few characters. The commercially available system based on this technology is a valuable complementary tool to other routine identification technologies. However, identification based on the 16S rRNA gene is by no means infallible as the sequence stretch analysed is a reduced section of the full genome and the variability of this marker is low.

The development of molecular typing methods has offered the possibility of accelerating a great deal of bacterial identification which avoid so many biases that are related to the classical methods. The polymerase chain reaction (PCR) has however provided a method to detect DNA sequences with high speed and sensitivity. This technique is emerging as a new tool in identifying and selecting bacteria with specific and desirable functions (Bulut et al., 2005). A combination of different approaches in the identification of different organisms offer a solution to the use of the conventional method that makes use of the ability of LAB to produce acid from carbohydrate and other metabolic activities only (Morgan et al., 2009).

According to Merien et al. (2013), the nucleotide base sequences of *Lactobacillus* spp. 16S ribosomal DNA also provides accurate basis for phylogenetic identification of organisms that are slow growing, fastidious and are therefore poorly identified by conventional methods. These small ribosomal units exist universally among bacteria and include regions with species-specific variability which makes it possible to identify bacteria to species level.

The use of *Lactobacillus* sp. as probiotics in man has been found to enhance their immunity and increase their ability to fight and survive against food related pathogens. Also, nursing mothers prefer natural products with fewer artificial preservatives in foods that are used for weaning infants with natural fortification or supplements. They have also been found to be consumed in fermented foods that contain them for their health benefits (Adeyemo and Onilude, 2013).

Lactobacillus plantarum particularly has also been implicated in the reduction of raffinose- family of oligosaccharide content of soybeans used in the formulation of a weaning food blend by their ability to hydrolyse the raffinose to simple sugars and hence improve the weaning food (Adeyemo and Onilude, 2014). Fermentation with cultures containing LAB is able to produce healthy, safe, high quality and nutritious beneficial food products such as fermented milk, meat, vegetables, grains, cereals, legumes, meat, beverages, etc. These organisms produce lactic acid which has a way of preserving such fermented foods and also improve the flavour, texture and nutritional compounds of such foods through the metabolic activities of LAB during fermentation. Also, the metabolism and physiology of LAB is used in different biotechnological processes in industries to formulate LAB starters with useful metabolic activities and capabilities so as to ensure a wide range of quality fermented products with consistent characteristics (Adeyemo and Onilude, 2013).

Being used as probiotics and starter culture in many

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food industries and in fermentation technology, a prompt and rapid identification of *L. plantarum* is of utmost importance so as not to confuse this very important organism with other organisms of the same genus or species that are closely related. As a result of this, there is need for accurate identification of this organism, the importance of which cannot be over emphasized.

MATERIALS AND METHODS

Sample collection

Local varieties (LV) of sorghum (*Sorghum bicolor*) were obtained from a market and typed varieties (TV) from Institute of Agricultural Research & Training, Ibadan, Nigeria. They were all processed to *ogi* in the laboratory using the traditional method of Banigo and Muller (1972). *Ogi* was also obtained from traditional sellers within Ibadan (CO) and used for comparative studies. The samples were collected in clean polythene bags and transported to the laboratory.

Isolation of lactic acid bacteria

One gram each of the samples listed above was subjected to ten-fold serial dilutions using the method of Harrigan and MacCance (1976). Isolation of organisms was done with the pour plate method using molten MRS agar. After solidification, they were incubated anaerobically in an anaerobic jar at 30°C for 48-72 h. Pure cultures were selected and stored on slant overlaid with sterile glycerol.

Identification of the isolates

Morphological and macroscopic characteristics

For proper identification of the isolates, the cultural, morphological, biochemical and physiological characterization including microscopic and macroscopic examinations of the various isolates were carried out according to Sneath et al. (2009). Gram positive and catalase negative organisms were subjected to further biochemical tests.

Biochemical characteristics

Isolates were identified phenotypically on the basis of the following biochemical test after Gram's staining, catalase, oxidase, methyl red test, Voges Proskauer, nitrate reduction, starch, casein and gelatin hydrolysis, growth at different pH and temperature and NaCl ranges and the ability to produce CO₂ from glucose and production of acid from carbohydrates such as fructose, lactose, maltose, galactose, arabinose, mannose, xylose, dulcitol, inositol, mannitol, raffinose, trehalose, rhamnose, etc. (Sneath et al., 2009).

Genetic characterization of isolates

Extraction of genomic DNA of LAB isolates

DNA extraction from the LAB isolates was carried out using a modified GES (5M guanidine thiocyanate (Fisher scientific, England), 0.1 N EDTA (Sigma, England) and 0.5% N-lauroyl - sarcosine sodium salt (Sigma, England) (w/v) DNA extraction method (Pitcher et al., 1989). Aliquots of 1.5 ml of overnight cultures grown in appropriate broth were centrifuged (Biofuge,

Heraeus, Germany) in Eppendorf tubes at 13,000 g for 1 min. Pellets obtained were washed in 1 ml of ice cold lysis buffer (25 mM Tris-HCl (Sigma, England), 10 mM EDTA, 50 mM sucrose (BOH GPR 303997J), pH 8). The pellets were re-suspended in 100 µl of lysis buffer in addition to 50 mgml⁻¹ lysozyme (Sigma, England.) and incubated at 37°C for 30 min. 0.5 ml of the GES solution were added and mixed thoroughly. This was incubated at room temperature for 15 min. The lysate was then placed on ice for 2 min and 0.25 ml of 7.5 M ammonium acetate (Fisher scientific, England). Cooled ice was also added, vortexed and incubated on ice for 10 min. Aliquots (0.5 ml) of 24:1 chloroform : isoamylalcohol (Sigma, England) were added, vortexed and centrifuged for 10 min at 13,000 g. Aliquots of 800 µl of the upper phase were removed quantitatively and placed in a clean Eppendorf tube. Cold isopropanol (Fisher scientific, England) was added and mixed for 1 min. This was then centrifuged at 13,000 g for 5 min and the supernatant removed from the pellet. The pellet was washed three times in 500 µl of 70% ethanol and dried at 37°C for 15 min. Aliquots (50 µl) of TE buffer were added and 5 µl of the DNA were checked on 1% agarose (Biogene, Kimbolton, UK) gels in 200 ml 1X TAE buffer and the DNA samples were then stored at -20°C for future use.

Polymerase chain reaction (PCR) amplification of 16S rDNA gene

The method of Bulut et al. (2005) was used. Amplification of 16S rDNA gene - ITS region, was performed by using the following primer pairs. Forward (16S ITS For), 5'-AGAGTTTGTATCCTGGCCTCAG-3' and reverse (16S - ITS Rev), 5'-CAAGGCATCCACCGT - 3', 16S rDNA V3, forward 5'-CCTAGGGGAGGCAGCAG - 3' and 16S rDNA V3, reverse, 5'-ARRACCGCGTCTGC-3'. The forward 5'-CCTACGGGAGGCAGCAG-3' and reverse, 5'-ATTACCGCGTCTGCTGG-3', primers used occupied positions 341-358 and 518-534, respectively of the V3 region in the 16S ribosomal DNA of *Escherichia coli*. The primers specify about 200 bp of the PCR products (as could be seen on the gel after electrophoresis).

The V3 primer pair was used for ease of sequencing of the gene, using the variable region 3 (V3), for the genetic identification of the isolates.

Each of the polymerase chain reactions (PCR) was performed in a 50 µl reaction volume containing 50 µg genomic DNA as the template. 10 µl of 0.2 mM deoxynucleoside triphosphates, dNTPs (Promega UI20A - UI23A, Madison, WI, USA), 10 µl of 2.5 mM MgCl₂, 10 pmol each (0.1 µl volume) of the DNA primer in PCR buffer (Promega, UK), and 10 µl of 1.25 units Taq DNA polymerase (Promega, UK) and 18.9 µl distilled water. Amplification conditions were as follows: an initial denaturation step of 5 min at 94°C, 40 amplification cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min elongation at 72°C. Reactions were terminated with a final extension step for 10 min at 72°C. PCR amplification was performed in a Thermocycler (Techne- Progene, Cambridge, UK).

Gel electrophoresis of 16S rDNA PCR Products

Electrophoresis of the amplified 16s rDNA PCR products were performed on the Bio-Rad contour - clamped homogenous electric field (CHEF) DRII electrophoresis cell. This was done through 1.5% (w/v) agarose gel (Biogene, Germany) in 0.5 X TAE buffer at 84 V for 1.5-2 h. This was prepared by boiling 1.5 g of agarose powder in 100 ml of 0.5X TAE buffer. A 100 bp ladder (Promega, U.K) and 1 Kb DNA ladder (Promega, U.K) were used as molecular size markers.

Sequencing and analysis Of 16S rDNA gene

Purification of PCR 16S rDNA gene

75 µl of the PCR 16S rDNA amplified products (obtained above) were resolved in 1% agarose gels with the conditions earlier described. PCR products were resolved by gel electrophoresis, using an agarose gel (1.5%; Biogene) that was stained with of 0.5 µg/ml ethidium bromide, in 1xTAE buffer at 84 V for 1.5 - 2 h.

The DNA bands were then visualised using a UV transilluminator (Amersham Pharmacia Biotech, UK) with 313 nm emission and pictures were taken using Fuji Film Imaging system FT1-500 (Amersham Pharmacia Biotech, UK).

The resulting bands in agarose gel were carefully excised with sterile scalpels and then purified the Wizard PCR preps DNA purification kit (Promega, USA). The purified DNA was kept at 4°C until used.

Drying of the purified 16S rDNA genes

To a 50 µl of the purified DNA, 0.1 µl of sodium acetate buffer (3M, pH 5.0) and 2.0 µl of 100% ethanol were added. This was then incubated at -20°C for 1 h. It was brought out and left to stand at room temperature for 5 min, and then centrifuged at 13,000 g at 4°C for 45 min. The liquid was removed, leaving only the DNA in the Eppendorf tubes. The DNA was dried in an incubator at 37°C for 30 min.

Sequencing of 16S rDNA gene

The dry DNA samples (obtained using V3 primers) were sequenced using a computer analytical sequencer (MGW - Biotech, Germany) with the V3 and V5 primer Rev, acting as the basis according to manufacturer's instructions. The generated nucleotide sequences were subjected to analysis. Sequencing of the purified 16S rDNA DNA products was performed using the sequencing unit of the University of Nottingham; a 373 DNA sequence (Perkin-Elmer Applied Biosystems) was used with the Taq Dye Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). The full identities of the isolates were then obtained by subjecting the nucleotide sequences to searches in the Gene Bank (<http://www.ncbi.nlm.nih.gov/blast/>) with the Blast search program.

Analysis of the 16S rDNA gene sequence

The generated sequences of the 16s rDNA genes were subjected to alignment in the databases at the BLAST, Basic Local Alignment and Search Tool, Website: <http://www.ncbi.nih.gov/blast/igi>. The isolates were then identified based on the result of the analysis.

RESULTS

Table 1 shows the result of the conventional method of identification of LAB, the carbohydrate utilization pattern and biochemical characteristics of the isolates. All the 20 isolates were identified as *L. plantarum*. The result obtained agrees with the characterization pattern of other authors (Sneath et al., 2009).

Table 2 shows the comparison between the phenotypic method and the genotypic method using the 16S rDNA gene sequence of the 20 isolates that were initially identified

as *L. plantarum*. The topmost sequences producing significant alignments when the nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) in the gene bank Database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) for *L. plantarum* isolates.

Altogether, seventeen *L. plantarum* isolates that have been identified before showed a significant alignment in the gene database. The result of the PCR sequencing correlated in 17 out of 20 isolates while there was no correlation in 3 out of 20. The names and accession numbers of these seventeen isolates have significant alignments with the *L. plantarum*. All the seventeen topmost species was shown to produce significant alignment with the marker and have expected value (E value) of between $1e - 73$ and $5e - 7$ and maximum identification (Max identity) of between 95 and 100%. They were all *L. plantarum*. Three out of the twenty isolates did not have significant alignment with the others. They were identified as *L. pentosus* and one unidentified *Lactobacillus* sp. There was significant difference in the molecular method and the conventional methods. The result however did not correlate but a divergent view was presented which shows a difference in their gene sequence.

Table 3 shows the qualities and quantities of the 16S rDNA genes of the *L. plantarum* obtained by PCR using V3 primer, after purification.

The 16S rDNA of the 17 species after amplification with primers was found to belong to the *L. plantarum* group as they were identified as *L. plantarum* by partial gene sequencing. The 16S rDNA genes of the other of the three organisms were not shown because a different gene sequence was presented.

Figure 1 shows the *L. plantarum* strain 16S ribosomal RNA gene, the partial sequence alignment of 16S rDNA after amplification of the gene by PCR in the gene bank data base. Molecular characterisation of the isolates was done by extracting the DNA gene sequence using universal primers and when compared, it was identified as *L. plantarum* with alignment.

Figure 2 shows the nucleotide sizes in base pairs (bp) of the plasmids of the selected seventeen *L. plantarum* isolate that were used for further work after their identities have been confirmed by 16S rDNA. This base sequence provides significant information on the 16S rDNA gene sequence of the *L. plantarum*. The nucleotide base sequence of the 16S rDNA has provided a basis for phylogenetic identification and analysis.

DISCUSSION

Accurate and definitive microorganism identification is essential for a wide variety of application including biotechnological, industrial, biomedical, pharmaceutical and environmental studies. The 16S rDNA sequence based analysis is a central method to understand not only

Table 1. Physiological and biochemical characteristics of isolates.

Isolate code	Gram reaction	Cell morphology	Catalase	Oxidase	Casein hydrolysis	Gel HND	M.R	V.P	H ₂ SP	Growth at 15°C	45°C	pH at 3.9	pH at 9.2	pH at 5	4% NaCl	CIT.UTI	Glucose	Xylose	Rhamnose	Triammonium citrate	Raffinose	Sucrose	Lactose	Maltose	Galactose	Fructose	Arabinnose	Mannose	Dulcitol	Mannitol	Inositol	Motility	Indole	NH ₃ Arg	Nitrate red	Probable identity	
1	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	<i>Lactobacillus plantarum</i>	
2	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
3	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
4	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
5	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
6	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
7	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
8	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
9	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
10	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
11	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
12	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
13	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
14	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
15	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
16	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
17	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
18	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
19	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>
20	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	W	+	-	+	-	-	-	-	-	-	<i>L. plantarum</i>

R = Rod; + = A positive reaction; - = A negative reaction; D = A delayed reaction; W = A weakly positive reaction; M.R = methyl red test, V.P = Voges Proskauer

Table 2. Comparison of phenotypic and genotypic methods of identification of *L. plantarum*.

Isolate code	Conventional identity	Closest relative (using 16srDNA gene sequencing)	Identity	Gene bank accession no
<i>L. plantarum</i> CO1	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	GQ180906.1
<i>L. plantarum</i> CO2	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	GQ166663.1
<i>L. plantarum</i> CO3	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	GQ166662.1
<i>L. plantarum</i> CO4	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	GQ166661.1
<i>L. plantarum</i> CO5	<i>L. plantarum</i>	<i>L. pentosus</i>	91%	GQ180915.1
<i>L. plantarum</i> CO6	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	GQ180902.1
<i>L. plantarum</i> LV1	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ861114.1
<i>L. plantarum</i> LV2	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ861113.1
<i>L. plantarum</i> LV3	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ861112.1
<i>L. plantarum</i> LV4	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ861111.1
<i>L. plantarum</i> LV5	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ851111.1
<i>L. plantarum</i> LV6	<i>L. plantarum</i>	<i>L. pentosus</i>	91%	FJ851122.1
<i>L. plantarum</i> LV7	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ851116.1
<i>L. plantarum</i> LV8	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ851113.1
<i>L. plantarum</i> TV1	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ84495.1
<i>L. plantarum</i> TV2	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ844955.1
<i>L. plantarum</i> TV3	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ844949.1
<i>L. plantarum</i> TV4	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ844954.1
<i>L. plantarum</i> TV5	<i>L. plantarum</i>	<i>Lactobacillus</i> sp.	90%	FJ843956.1
<i>L. plantarum</i> TV6	<i>L. plantarum</i>	<i>L. plantarum</i>	95%	FJ844953.1

Table 3. Qualities and quantities of the 16S rDNA genes of the *L. plantarum* obtained by PCR using V3 primer, after purification.

S/N	Sample ID	16S	rDNA		
		Conc. (ug/L)	A260nm	A260/280	0.0260/230
5	H101	18.34	0.367	1.82	0.02

the microbial diversity within and across the group but also to identify new strains. Bacterial species have at least one copy of the 16S rDNA gene containing highly conserved regions together with hyper variable regions, which is used for identification of new strains. However, a considerable variation can occur between species in both the length and the sequence of 16S rDNA ITS region, therefore this region is useful in characterization of bacterial species (Mohammed et al., 2011). The 16S rDNA gene is very useful because the genome of all bacteria contains this conserved gene and any small variability in this region is unique and specific to each species. This characteristic is usually harnessed in their identification (Mohania et al., 2008).

Considering the conventional method for identifying LAB isolates, the objective of this study was to compare the phenotypic method and the 16SrDNA sequencing which is a species-specific PCR reaction for the proper identification of the twenty *Lactobacillus* sp. The genus level was however the same for all the isolates, they were

further characterized using PCR reactions to perform complete identification. The results obtained with 95% reliability and higher were considered; those lower than this were not considered because their gene sequences were identified as different organisms. Considering that species-specific PCR reactions target specific genes of genera and species, the molecular method was considered reliable. Molecular bacteria identification is based on the full length of 16S rDNA gene sequence by several studies have shown that the initial few base pair sequence provides sufficient discrimination between strains because this region shows a high genetic diversity.

Of the 20 isolates used in this work, three presented divergent results as compared to 16S rDNA sequencing and species-specific PCR reaction. This confirmed the result of 17 out of 20 isolates tested (17/20), that is, 85% and divergent result were obtained in 3 out of 20 (15%) isolates that were screened (3/20). Out of these, 2 were identified as *L. pentosus* while the last was a

GQ180905.1 *Lactobacillus plantarum* strain TJ2 16S ribosomal RNA gene, partial sequence,
 Length=182, Score = 283 bits (153), Expect = 1e-73,
 Identities = 153/153 (100%), Gaps = 0/153 (0%) Strand=Plus/Plus

Query
 GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAACTCTGTTGTT

|||||
 Sbjct
 GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAACTCTGTTGTT

Query
 AAAGAAGAACATATCTGAGAGTAACTGTTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

|||||
 Sbjct
 AAAGAAGAACATATCTGAGAGTAACTGTTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

Query CGGCTAACTACGTGCCAGCAGCCGCGGTAATAA
 |||||
 Sbjct CGGCTAACTACGTGCCAGCAGCCGCGGTAATAA

Figure 1. Alignment of 16S rDNA nucleotide sequences of *L. plantarum* against *L. plantarum* strain LpT2 (accession no GQ166663.1) and *L. plantarum* strain LpT1 (accession no GQ166662.1) in the gene bank data base.

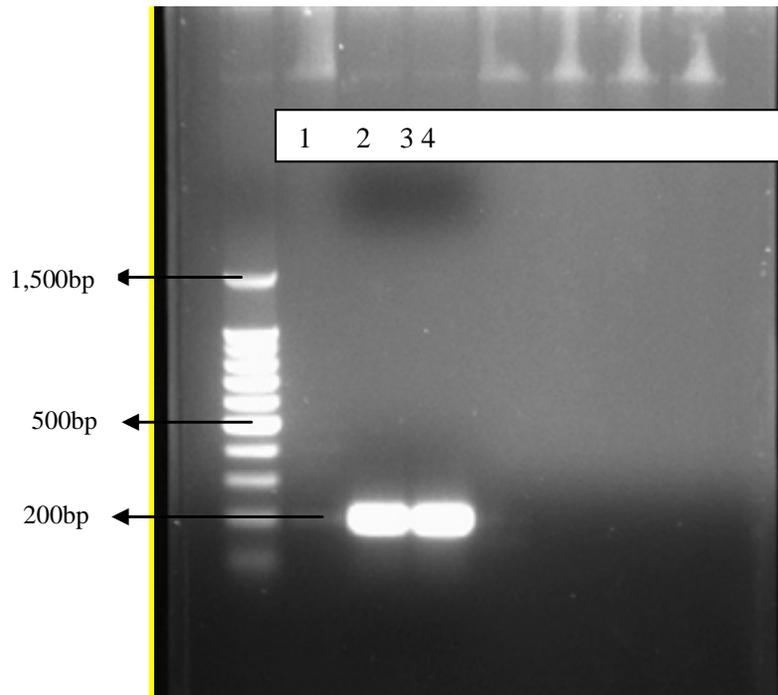


Figure 2. The nucleotide sizes of the plasmids of the selected nine *L. plantarum* isolate.

Lactobacillus sp. that could not be identified. This result agrees with the report of Marroki et al. (2011) who reported a similar view stating that *L. plantarum* and *L. pentosus* have very similar 16S rDNA sequences that only differ by 2 base pair. Other authors also reported that both organisms belong to the same phylogenetic group and they can only be differentiated when analysis of 16S-23S larger spacer is done (Ennahar et al., 2003). This may also be the same reason for the other *Lactobacillus* sp. that was not identified by this method presenting a result that did not correlate with those obtained earlier by phenotypic method. However, the result of the conventional method cannot be discarded completely but it can be regarded as giving a clue or presumptive result which can then be confirmed by molecular method.

Differences between genotypic and phenotypic tests have been identified previously not just for LAB but also for many other bacteria (Gomes et al., 2008; Paula et al., 2012). They also noted that this tool is useful for identifying microorganisms at sub species level which cannot easily be identified by other common technique. Phenotypic method may also have poor reproducibility as a result of changes that occur during the growth and metabolism of different organisms. This also agrees with the report of Mohania et al. (2008) who reported that bacterial isolates do not express their genes at the same time or they may lose some characteristics such as plasmids during culturing. This may however be responsible for the inconsistencies that are usually identified in sugar fermentation patterns and other biochemical tests that rely on physiological characteristics of different organisms for identification.

Gill et al. (2006) also stressed another importance of this molecular method being a desirable advantage of 16S rDNA over the conventional one. Apart from being rapid, the sequence could also be performed not only on bacterial culture but also on the sample so as to study the diversity of the organisms without culturing. The efficacy and efficiency of this method was clearly demonstrated in this work by differentiating strains belonging to the same species and it has been clearly identified by various authors such as Gill et al. (2006) and Morgan et al. (2009) because the results are not subjective.

The molecular method used in this work further confirmed the real identities of *L. plantarum* that were used for further work in the fermentation pattern for the formulation of a weaning food blend as earlier reported by Adeyemo and Onilude (2013). The real identities of the organisms are usually revealed by molecular methods and the results can be reproduced at any time and in different places without environmental variations. Based on the result of this study, the 16S rDNA sequencing method is specific for the gene of target and broader strategies that can characterize lactic acid bacteria without prior knowledge of genetic targets, this is however a desirable characteristics of this method, it is

thus recommended for proper identification of organisms to be used in fermented foods as starter culture or bio-preservative.

The result obtained in this work agrees with the result obtained by Parker et al. (2001). They opined that several PCR methods have subsequently been developed to overcome difficulties experienced with phenotypic methods. The method described in this work allows the amplification of specific PCR products. This enables direct sequencing of unknown regions without the need for DNA cloning but makes use of analysis of microbial genetic elements. Shittu et al. (2006) also noted the accuracy of the molecular diagnostic method in the ability to rapidly identify microorganisms isolated from clinical samples from genus level to species level using automated systems. Reduction of analysis time and reproducibility would be advantageous, especially for organisms that are fastidious, slow-growing and of medical and industrial importance.

The result obtained also solves the problem of misidentification. This agrees with the work of Woo et al. (2008) who reported that some LAB species are closely related to *Lactobacillus* sp. The importance of accurate identification need to be emphasized in LAB obtained from fermented foods that are used as probiotics or starter cultures. This is because some LAB are also involved in clinical infections such as *Leuconostoc* sp., *Pediococcus* sp. and *Enterococcus* sp. These organisms are of medical importance and should not be misidentified with other *Lactobacillus* sp. The use of 16S rDNA will lower the risk of inaccurate or poor identification of these pathogens that are also similar to other *Lactobacillus* sp.

However, in industrial microbiology for example, there are various importance of rapid methods of identification of microorganisms. First, it is of paramount importance to food/industrial microbiologists for screening and identification of organisms that are of great industrial and biotechnological purposes. Rapid detection and identification of microorganisms also allows for continuous monitoring of microbial growth in relation to various metabolites that are produced by them especially in pharmaceutical industries such as enzymes, vaccines, antibiotics, organic acids etc. Also, the ease of producing a large number of copies of a specific DNA sequence can be applied in the industry for the production of many important products from microorganisms using some specific genes from them.

Finally, the advantage of genotyping is that it is an accurate method for the identification of *L. plantarum* in that the genome is stable; the genetic composition of the organism is independent of cultural conditions and method of isolation; it can easily be subjected to automation and the results can be analysed statistically with ease. LAB are referred to as "probiotics" and it belongs to the group of organisms that are generally regarded as safe (GRAS). Its prompt and quick identification is a

useful tool in distinguishing between these probiotics and other opportunistic pathogens that may also be present as contaminant in fermented foods.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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