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Isolation and Molecular Characterization of Lactic Acid Bacteria Isolated from Fresh Fruits and Vegetables Using Nested PCR Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ORA and AKA designed and supervised the study. Author ECE wrote the protocol, and wrote the first draft of the manuscript. Authors ECE and PIO managed the analyses of the study, performed the statistical analysis. Author ECE managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The study investigated the diversity and identities of Lactic Acid Bacteria (LAB) isolated from different fresh fruits and vegetables using Molecular Nested PCR analysis with the view of identifying LAB with anti-microbial potentials.
Study Design: Nested PCR approach was used in this study employing universal 16S rRNA gene primers in the first round PCR and LAB specific Primers in the second round PCR with the view of generating specific Nested PCR products for the LAB diversity present in the samples.
Place and Duration of Study: Biotechnology Centre of Federal University of Agriculture, Abeokuta, Ogun State, Nigeria, between January 2011 and February 2012.
Methodology: Forty Gram positive, catalase negative strains of LAB were isolated from fresh fruits and vegetables on Man Rogosa and Sharpe agar (Lab M) using streaking method. Standard molecular methods were used for DNA extraction (Norgen Biotek kit method, Canada), Polymerase Chain Reaction (PCR) Amplification, Electrophoresis,

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Purification and Sequencing of generated Nested PCR products (Macrogen Inc., USA).
Results: The partial sequences obtained were deposited in the database of National Centre for Biotechnology Information (NCBI). Isolates were identified based upon the sequences as *Weissella cibaria* (5 isolates, 27.78%), *Weissella kimchi* (5, 27.78%), *Weissella paramensenteroides* (3, 16.67%), *Lactobacillus plantarum* (2, 11.11%), *Pediococcus pentosaceus* (2, 11.11%) and *Lactobacillus pentosus* (1, 5.56%) from fresh vegetable; while *Weissella cibaria* (4, 18.18%), *Weissella confusa* (3, 13.64%), *Leuconostoc paramensenteroides* (1, 4.55%), *Lactobacillus plantarum* (8, 36.36%), *Lactobacillus paraplantarum* (1, 4.55%) and *Lactobacillus pentosus* (1, 4.55%) were identified from fresh fruits.
Conclusion: This study shows that potentially LAB can be quickly and holistically characterized by molecular methods to specie level by nested PCR analysis of the bacteria isolate genomic DNA using universal 16S rRNA primers and LAB specific primer.

Keywords: Nested PCR; Molecular characterization; 16S rRNA gene; Lactic acid bacteria.

1. INTRODUCTION

Lactic acid bacteria (LAB) have been extensively studied for their commercial potential [1], food preservation and health benefits [2]. They are industrially important microorganisms used worldwide mainly in the dairy industry for manufacturing fermented milk products and cheese. Industrial importance of LAB is based on their ability to ferment sugars readily into different metabolites and provide an effective method for preserving fermented food products. These bacteria are gram positive, non-spore forming and naturally present in media rich in organic products such as food products [2]. LAB is, however, a genetically diverse group of bacteria encompassing widely recognized genera which include: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* [3]. Some authors include the genus Bifidobacterium because of its probiotic role, although it belongs to a different phylogenetic group [4]. Moreover, although many representatives of LAB are perfectly safe and used for generations in food, some species are pathogens such as pathogenic Streptococci [5]. Identification of LAB based on carbohydrate fermentation patterns is unreliable and not accurate enough to distinguish closely related strains due to their similar nutritional requirements [6]. Owing to the considerable economical importance of LAB, many researchers are now actively working on these bacteria using an array of genetic tools. Many chromosomal genes of interest have been characterized providing a new insight into the genetic organization of LAB. Sequencing analysis of the 16s RNA genes has been used to determine the diversity and dynamics of LAB in food [7,8,9]. This in turn will lead to a better understanding of the physiology of LAB, in particular by the application of new genomic technologies such as proteomics, global transcription analysis and comparative genomics. It may be expected that in depth understanding of the genetics and physiology of these bacteria will give rise to new working hypotheses and facilitate strain use, selection and improvement. In this present study, we examined the diversity of LAB present in some fresh fruits and vegetables using DNA extraction, 16s rRNA gene amplification, nested PCR amplification, purification of nested PCR products and sequencing.

2. MATERIALS AND METHODS

Fresh fruits and vegetables were collected from three different retail market locations (Adatan, Kuto and Osiele) in Abeokuta town, Ogun state western part of Nigeria. About 300g each of tomatoes, citrus, banana, flutted pumpkin vegetable (*Telfairia occidentalis*) and green vegetable (*Amaranthus spinosus*) were obtained. The samples were wrapped separately in sterile polyethylene bags, and transported to the laboratory for analysis.

2.1 Lactic Acid Bacteria Isolation

Ten gram each, of fresh fruits and vegetables samples were soaked in 90 ml of normal saline solution (8.5 g NaCl / L), homogenized for 2 min, appropriately diluted in normal saline, pour plated onto de Man Rogosa and Sharpe agar (LAB M) and were incubated at 37ºC anaerobically for 48-72h. Distinct colonies were sub-cultured twice and pure cultures were stored in MRS agar slants overlaid with 20% glycerol and stored at -20ºC.

2.2 Characterization of the LAB Isolates

Overnight cultures of LAB isolates were Gram stained and examined microscopically for morphology and phenotype. Catalase test was carried out by adding few drops of freshly prepared 3% hydrogen peroxide (Analar) to each plate containing 18h old culture of each isolate.

2.3 Molecular Characterization

2.3.1 Bacteria isolates genomic DNA extraction

Total genomic DNA was extracted from overnight culture of bacteria isolates using Bacterial Genomic DNA extraction kit (Norgen Biotek Corporation, Canada). Fragments of the gene of interest, the 16S ribosomal gene, were amplified using standard PCR protocol and the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Integrated DNA Technologies). Nested PCR using primers 27F and WLAB2R (5'-TCGAATTAAACCACATGCTCCA-3') [10] amplified a smaller, more variable region of the 16S gene (This is mostly useful in distinguishing bacterial strains from one another) with the TC-412 PCR Thermal Cycler machine. The PCR reaction mixture (20 µl) consisting of 10µl 2x PCR master mix (Norgen biotek Corporation, Canada), 1 µl of each primer (2.5µM), 6.5µl nuclease free water and 1.5 µl template DNA. The thermocycler program was as follows: 94ºC for 1min; 30 cycles of 95ºC for 30 s, 44ºC for 30 s, and 72ºC for 2 min; and a final extension step at 72ºC for 4 min. The nested PCR products were analyzed by electrophoresis on a 1.0 % agarose gel stained with ethidium bromide in 1X TAE buffer at 100 V for 45min. The bands were visualized under UV trans-illuminator (Cleaver Scientific Ltd). The sizes of DNA fragments were estimated using a standard 1kb DNA ladder ((Norgen biotek Corporation, Canada), and the gels were documented using the gel documentation apparatus (Cleaver scientific Ltd). 16S rDNA gene amplicons were purified using EXOSAP-IT kit (Affymetrix, Inc. USA) according to the manufacturer's instructions. Nucleotide sequences were determined by analysis of fluorescently labeled DNA products generated by AmpliTaq DNA Polymerase on an AB 3730x DNA Analyzer. Primers: 518F and 800R were used in all sequencing reactions.

2.3.2 Sequence alignments and phylogenetic inference

Sequence similarity was estimated by searching the homology in the Genbank DNA database using BLAST. The sequence information was then imported into the MEGA 5 software program for assembly and alignment. The 16S rDNA sequences of isolated bacterial strains were compared to sequences from type LAB strains held in GenBank (Fig. 1). Nucleotide substitution rates were calculated, and phylogenetic trees were constructed by the neighbor-joining method. The topologies of trees were evaluated by bootstrap analysis of the sequence data with MEGA 5 software based on 100 random resamplings.

2.3.3 Nucleotide sequence accession numbers

The nucleotide sequences obtained in this report were deposited with GenBank under the following accession numbers: KF023193, KF023194, KF023197, KF023198, KF023201, KF023202, KF023203, KF023204, KF023207, KF023208, KF023210, KF023215, KF023216, KF023217, KF023218, KF023225, KF023226, KF023227, KF023229, KF023230, KF023232, KF023236, KF023238, KF023240, KF023241, KF023242, KF023243, KF023247, KF023248, KF023252, KF023253, KF023254, KF023257, KF023258, KF023266, KF023267, KF023268, KF023269, KF023270.

3. RESULTS AND DISCUSSION

In a total of 105 bacteria isolated from fresh fruits and vegetable, 40 presumptive LAB colonies were found to be non motile, catalase negative and gram positive tiny rods which occur in pairs and chains, few were cocci and they occur singly. This study was performed to reveal the diversity in the LAB community present in some fresh fruits and vegetable using nested PCR analysis. PCR using 16S rRNA gene primers generated amplicons of around 1500bp fragments (result not shown) which was in line with the results of previous study [7] as theoretically predicted for bacteria family. The amplicon from the first round of PCR which were thereafter, used as templates to run a nested PCR (to narrow it down to LAB genera), generated PCR products of about 900bp as predicted for LAB group [10].

The data in Table 1 show that isolates from fresh fruits (s/n. 1 - 22) belong to the LAB family. Eight isolates (AT2, AT5, AT7, AT9, BT7, CT8, CT9 and BB8) had 99-100% similarity with *L. plantarum* though with different accession numbers. Isolates AT4, CT3 and CC8 had 99-100% similarity with *W. confusa*. CT5 proved to have a 100% similarity match to *L. paraplantarum*. AC4, AC6, CC2 and CC6 gave 99-100% similarity to *W. cibaria*. AC5, AB1, BB2 and BB7 proved to shared 99-100% similarity with *W. paramesenteroides*. AC8 was found to have 99% similarity match to the strain *L. paramesenteroides* and AB4 shared 99% similarity with *L. pentosus*.

Table 1. Identification of Bacteria Isolates from fresh fruit and vegetables

S/N	Isolate code of organisms identified	Reference from NCBI database	Percentage similarity (%)
1	AT2	*Lactobacillus plantarum*	99
2	AT4	*Weissella confusa*	99
3	AT5	*Lactobacillus plantarum*	100
4	AT7	*Lactobacillus plantarum*	99
5	AT9	*Lactobacillus plantarum*	100
6	BT7	*Lactobacillus plantarum*	99
7	CT3	*Weissella confusa*	99
8	CT5	*Lactobacillus paraplantarum*	100
9	CT8	*Lactobacillus plantarum*	99
10	CT9	*Lactobacillus plantarum*	99
11	AC4	*Weissella cibaria*	100
12	AC5	*Weissella paramesenteroides*	99
13	AC6	*Weissella cibaria*	99
14	AC8	*Leuconostoc paramesenteroides*	99
15	CC2	*Weissella cibaria*	99
16	CC6	*Weissella cibaria*	100
17	CC8	*Weissella confusa*	100
18	AB1	*Weissella paramesenteroides*	99
19	AB4	*Lactobacillus pentosus*	99
20	BB2	*Weissella paramesenteroides*	99
21	BB7	*Weissella paramesenteroides*	99
22	BB8	*Lactobacillus plantarum*	100
23	AU2	*Weissella paramesenteroides*	99
24	AU3	*Weissella cibaria*	100
25	AU4	*Lactobacillus plantarum*	100
26	AU5	*Lactobacillus plantarum*	99
27	AU7	*Weissella paramesenteroides*	99
28	BU2	*Pediococcus pentosaceus*	100

29	BU3	*Weissella cibaria*	100
30	BU8	*Weissella paramesenteroides*	99
31	CU2	*Lactobacillus plantarum*	100
32	AA2	*Weissella cibaria*	100
33	AA3	*Lactobacillus plantarum*	100
34	AA8	*Weissella cibaria*	99
35	AA10	*Weissella kimchi*	99
36	BA3	*Lactobacillus plantarum*	100
37	BA4	*Weissella kimchi*	99
38	BA7	*Pediococcus pentosaceus*	100
39	BA8	*Weissella cibaria*	99
40	CA6	*Lactobacillus pentosus*	100

Isolates from vegetables(s/n. 23 - 40) that were sequenced were also found to belong to LAB family as shown in Table 1. The results of isolates identification based on the NCBI database were as follows: AU3, AU5, AA3, BA3 and CU2 shared 99-100% similarity with *L. plantarum* though with different accession numbers. AA10 and BA4 shared 99% match to a known sequence obtained for *W. kimchi*, BU2 and BA4 were considered 100% similar to *P. pentosaceus*. AU3, BU3, AA2, AA8 and BA8 had 99-100% similarity with *W. cibaria*. AU2, AU7 and BU8 showed 99% similarity with *W. paramesenteroides* and CA6 had a 100% similarity match to a known sequence obtained for *L. pentosus*. The percentage abundance of the identified LAB in relation with each other was indicated in Table 2. *L. plantarum* had the highest percentage abundance for fresh fruit samples.

Some genera of LAB isolated from this work like *Lactobacillus*, *Weissella*, *Leuconostoc*, and *Pediococcus* were also isolated in the previous study [7] involving the use of 16S rRNA gene sequencing analysis to identify LAB diversity from fermented kimchi (a vegetable dish in Korea). *Leuconostoc* was described as long been known to be quite common among micro flora of vegetables.

On the basis of 16S rRNA gene sequence similarities, some strains isolated from fresh fruits and vegetables were identified as *L. plantarum, Leuconostoc* spp, *W. cibaria* [11] which is in line with the findings from this research. It has been reported that *W. kimchi* and *W. cibaria* were the most predominant in kimchi fermentation [12]. This is in accordance with this study for these two isolates (*W. kimchi* 27.27% and *W. cibaria* 27.27%) were the most predominant in the vegetable samples. *Pediococcus* spp. have been described as often being associated with plant materials [13] and that is in line with this research as *P. pentosaceus* was found to be isolated from fresh fruits and vegetable which are also of plant source.

According to the work carried out by other authors [14,15], *L. plantarum* in plant materials was dominating the LAB flora. It was however, observed that generally *L. plantarum* was the dominating specie in this work. *W. confusa* and *P. pentosaceus* were also Isolated during fermentation of Bode an Ethiopian cereal beverage [16] while *W. confusa* and *Pediococcus* spp. were isolated during fermentation of Som-fak prepared from minced fish fillet [17]. The isolation of *W. confusa* from Boza [18] is related to our results as *W. confusa* and *Pediococcus* were also isolated in this study. *L. plantarum, W. paramesenteroides* and *Leuconostoc* isolated in this study is in accordance with the results of a previous work [9], in which *L. plantarum*, *W. paramesenteroides* and *Leuconostoc* were isolated from soil.

L. pentosus identified in this work was also found from Malaysian fruits [19]. *W. paramesenteroides* and *W. confusa* were also isolated from Guinea Grass [20] as just were isolated in this study indicating that they may be associated with plant. *L. paraplantarum* was isolated from tea [21] using 16S rRNA partial gene sequencing. In addition, *L. paraplantarum* was isolated from kimchi [7,22] which is in line with this work where *L. paraplantarum* was also isolated.

The isolation frequency of LAB from both fruit and vegetable samples were shown in Table 2, *L. plantarum* has the highest percentage of isolation from the fruit samples while *W. cibaria* is reported as the highest for the vegetables.

Table 2. Isolation frequency of LAB

Isolate Identity	Fruits	Vegetables
Lactobacillus plantarum	36.36%	27.77%
Weissella confusa	13.63%	Nil*
Weissella cibaria	18.18%	27.88%
Weissella paramesenteroides	18.18%	16.66%
Lactobacillus paraplantarum	4.54%	nil
Leuconostoc paramesenteroides	4.54%	nil
Lactobacillus pentosus	4.54%	5.55%
Weissella kimchi	nil	11.11%
Pediococcus pentosaceus	nil	11.11%

**. Not isolated.*

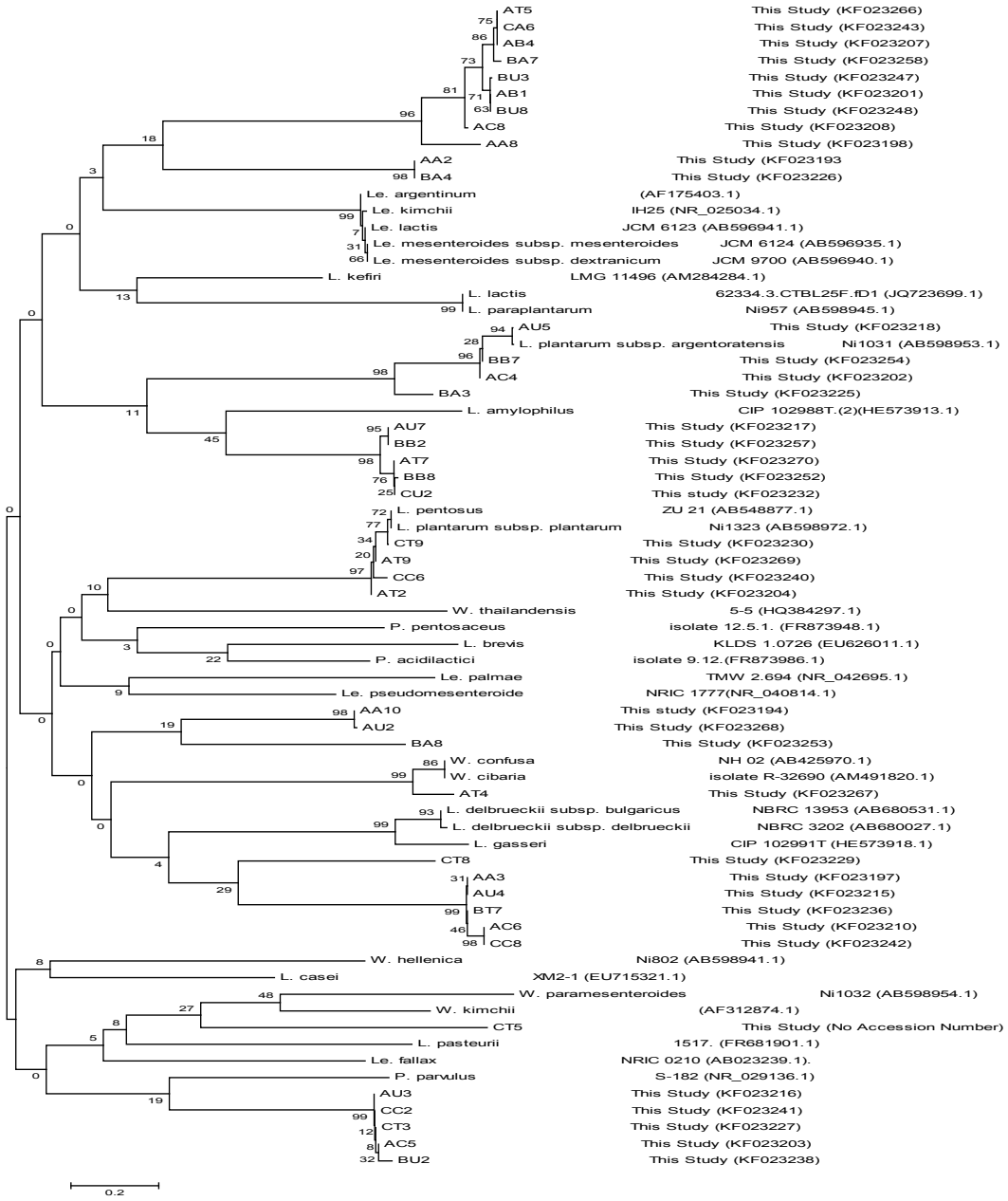

Fig. 1. Phylogenetic tree showing the relative positions of identified Isolates from fresh fruits and vegetables as inferred by the neighbor-joining method of partial 16S rDNA sequences. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the accession numbers for all 16S rDNA sequences (between brackets). The bar indicates 5% sequence divergence

4. CONCLUSION

The data obtained from this work provided useful framework for further studies on profiling for antimicrobial activity of LAB, their proteolytic activities and lipolytic activities. Therefore, the findings of this research offers real time information about the LAB genera and a better understand of their genetic diversity. Molecular identification of possible beneficial LAB creates holistic and faster method of microbial characterization.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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