

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

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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, Purification and Sequencing of generated Nested PCR products (Macrogen Inc., USA).

Results: The acquired partial sequences obtained were identified by submission to the non-redundant nucleotide database of National Centre for Biotechnology Information (NCBI). By the sequence similarity, the isolates were identified molecularly as *Weissella cibaria* 5 (5 of the 18 isolates; 27.78%), *Weissella kimchi* 5 (5, 27.78%), *Weissella paramensisenteroides* 3 (3, 16.67%), *Lactobacillus plantarum* 2 (2, 11.11%), *Pediococcus pentosaceus* 2 (2, 11.11%) and *Lactobacillus pentosus* 1 (1, 5.56%) from fresh vegetable; while *Weissella cibaria* 4 (4 of the 18 isolates, 18.18%), *Weissella confusa* 3 (3, 13.64%), *Weissella paramensisenteroides* 4 (4, 18.18%), *Leuconostoc paramensisenteroides* 1 (1, 4.55%), *Lactobacillus plantarum* 8 (8, 36.36%), *Lactobacillus paraplantarum* 1 (1, 4.55%) and *Lactobacillus pentosus* 1 (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.

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1. INTRODUCTION

Lactic acid bacteria (LAB) have been and is still being extensively studied for their potential commercial [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. MATERIAL 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, fluted 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 37 °C 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 -20 °C.

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.

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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'-TACGGYTACCTTGTACGACTT-3') (Integrated DNA Technologies). Nested PCR using primers 27F and WLAB2R (5'-TCGAATTAAACCACATGCTCCA-3') [10] amplified a smaller, more variable region of the 16S rRNA gene (~~This is mostly useful in distinguishing bacterial strains from one another~~) with the TC-412 PCR Thermal Cyclor machine, which is mostly useful in distinguishing bacterial strains from one another. The PCR reaction mixture (20 µl) consisting of 10 µl 2× PCR master mix (Norgen biotek Corporation, Canada), 1 µl of each primer (2.5 µM), 6.5 µl nuclease free water and 1.5 µl template DNA. The thermocycler program was as follows: 94°C for 1 min; 30 cycles of 95°C for 30 s, 44°C for 30 s, and 72°C for 2 min; and a final extension step at 72°C for 4 min. The nested PCR products were analyzed by electrophoresis on a 1.0 % agarose gel stained with ethidium bromide in 1× TAE buffer at 100 V for 45 min. The bands were visualized under UV trans-illuminator (Cleaver Scientific Ltd). The sizes of DNA fragments were estimated using a standard 1 kb DNA ladder ((Norgen biotek Corporation, Canada), and the gels were documented using the gel documentation apparatus (Cleaver scientific Ltd). The 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. Bacterial identification was done by searching the homology in the Genbank DNA database using BLAST.

3. RESULTS AND DISCUSSION

A total of 105 bacteria were isolated from fresh fruits and vegetable, in which 40 presumptive LAB colonies were found. Most of the LABs were to be non motile, catalase negative and gGram positive tiny rods which occurring in pairs and chains, while few were cocci and they occurring 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 1500 bp fragments (result not shown) which was in line with the reference work of [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), and generated PCR products of about 900 bp was obtained as predicted for LAB group by [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% sequence identity with *L. plantarum* though with different accession numbers. Isolates AT4, CT3 and CC8 had 99-100% sequence identity with *W. confusa*. CT5 proved to be a 100% match to *L. paraplantarum*. AC4, AC6, CC2 and CC6 gave a result of 99-100% sequence identity with *W. cibaria*. AC5, AB1, BB2 and BB7 proved to share 99-100% sequence identity with *W. paramesenteroides*. AC8 was found to be 99% match to the species *L. paramesenteroides* and AB4 shared 99% identity with *L. pentosus*.

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

S/N	Isolate	Reference from NCBI database	Nucleotide	Percentage	NCBI
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	code of organisms identified		number	similarity (%)	accession number
<u>Isolates from vegetables/fruits</u>					
1	AT2	<i>Lactobacillus plantarum</i>	1457	99	FJ386491.1
2	AT4	<i>Weissella confusa</i>	1404	99	DQ321751.1
3	AT5	<i>Lactobacillus plantarum</i>	1213	100	HM218724.1
4	AT7	<i>Lactobacillus plantarum</i>	1176	99	HQ853454.1
5	AT9	<i>Lactobacillus plantarum</i>	1265	100	HQ853454.1
6	BT7	<i>Lactobacillus plantarum</i>	286	99	FR745401.1
7	CT3	<i>Weissella confusa</i>	1407	99	DQ321751.1
8	CT5	<i>Lactobacillus paraplantarum</i>	1176	100	HQ834497.1
9	CT8	<i>Lactobacillus plantarum</i>	1416	99	GQ131206.1
10	CT9	<i>Lactobacillus plantarum</i>	426	99	FR745401.1
11	AC4	<i>Weissella cibaria</i>	675	100	AB593356.1
12	AC5	<i>Weissella paramesenteroides</i>	1397	99	AB362621.1
13	AC6	<i>Weissella cibaria</i>	1175	99	GU138616.1
14	AC8	<i>Leuconostoc paramesenteroides</i>	1416	99	DQ523483.1
15	CC2	<i>Weissella cibaria</i>	1229	99	AB494716.1
16	CC6	<i>Weissella cibaria</i>	1450	100	AB362617.1
17	CC8	<i>Weissella confusa</i>	1482	100	DQ321751.1
18	AB1	<i>Weissella paramesenteroides</i>	1470	99	FJ405229.1
19	AB4	<i>Lactobacillus pentosus</i>	1491	99	AB362758.1
20	BB2	<i>Weissella paramesenteroides</i>	1459	99	FJ405229.1
21	BB7	<i>Weissella paramesenteroides</i>	1474	99	AB425970.1
22	BB8	<i>Lactobacillus plantarum</i>	1004	100	CP002222.1
<u>Isolates from vegetables</u>					
23	AU2	<i>Weissella paramesenteroides</i>	1414	99	FJ405229.1
24	AU3	<i>Weissella cibaria</i>	1484	100	AB362617.1
25	AU4	<i>Lactobacillus plantarum</i>	1471	100	GU552552.1
26	AU5	<i>Lactobacillus plantarum</i>	1472	99	FJ386491.1
27	AU7	<i>Weissella paramesenteroides</i>	1431	99	AB362621.1
28	BU2	<i>Pediococcus pentosaceus</i>	1481	100	AB481102.1
29	BU3	<i>Weissella cibaria</i>	1488	100	AB362617.1
30	BU8	<i>Weissella paramesenteroides</i>	1480	99	FJ405229.1
31	CU2	<i>Lactobacillus plantarum</i>	1363	100	GU552552.1
32	AA2	<i>Weissella cibaria</i>	1482	100	AB362617.1
33	AA3	<i>Lactobacillus plantarum</i>	1475	100	FJ386491.1
34	AA8	<i>Weissella cibaria</i>	1484	99	AB362614.1
35	AA10	<i>Weissella kimchi</i>	1484	99	AF312574.1
36	BA3	<i>Lactobacillus plantarum</i>	1471	100	DQ239698.1
37	BA4	<i>Weissella kimchi</i>	1480	99	AF312874.1
38	BA7	<i>Pediococcus pentosaceus</i>	1481	100	FM179610.1
39	BA8	<i>Weissella cibaria</i>	1474	99	GU138579.1
40	CA6	<i>Lactobacillus pentosus</i>	1477	100	AB362758.1

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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 sheared 99-100% identity with *L. plantarum* though with different accession numbers. AA10 and BA4 sheared 99% match to a known sequence obtained for *W. kimchi*, BU2 and BA4 were considered 100% identical to *P. pentosaceus*. AU3, BU3, AA2, AA8 and BA8 had 99-100% identity with *W. cibaria*. AU2, AU7 and BU8 showed 99% identity with *W. paramesenteroides* and CA6 had a 100% 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 42. *L. plantarum* had the highest percentage abundance for fresh fruit samples. Some genera of LAB isolated from in this work, like *Lactobacillus*-spp, *Weissella*-spp, *Leuconostoc*-spp, and *Pediococcus*, spp were also isolated in the previous study by [7] in which involving the use of 16S rRNA gene sequencing analysis was used to identify LAB diversity from fermented kimchi (a vegetable dish in Korea). *Leuconostoc* spp. was described as long been known to be quite common among micro flora of vegetables.

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On the basis of 16S rRNA gene sequence similarities, [11] identified their some selected strains from fresh fruits and vegetables were identified as *L. plantarum*, *Leuconostoc* spp., and *W. cibaria* [11] which is in line with the findings from this our research. [42] reported that *W. kimchi* and *W. cibaria* were reported as the most predominant in kimchi fermentation [12], which were also obtained. This is in the present study accordance with this study because for these two isolates (both *W. kimchi* (27.27%) and *W. cibaria* (27.27%) were the most predominant in the vegetable samples. [13] also described *Pediococcus* spp. were described as often being associated with plant materials [13] and that is in line with this the data of present 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 previously [14, 15], they reported that, *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 by [16] during fermentation of Bode an Ethiopian cereal beverage [16]; while [17] isolated *W. confusa* and *Pediococcus* spp. were isolated during fermentation of Som-fak prepared from minced fish fillet [17]. [18] isolated *W. confusa* was isolated from Boza [18] which is related to this work as *W. confusa* and *Pediococcus* were also isolated in this study. *L. plantarum*, *W. paramesenteroides* and *Leuconostoc* isolated from this study is in accordance with the a previous work of [9], in which isolated *L. plantarum*, *W. paramesenteroides* and *Leuconostoc* were isolated from the soil.

L. pentosus identified in this work was also found to be isolated by [19] from Malaysian fruits [19]. *W. paramesenteroides* and *W. confusa* were also isolated from Guinea Grass by [20], as just were isolated and in this study, indicating that they may be associated with various plants. *L. paraplantarum* was isolated by [21] from tea [21] and using 16S rRNA partial gene sequencing. In addition, [7, 22] isolated *L. paraplantarum* 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	Proportion in the isolates from	
	Fruits	Vegetables
<i>Lactobacillus plantarum</i>	36.36%	27.77%
<i>Weissella confusa</i>	13.63%	0%
<i>Weissella cibaria</i>	18.18%	27.88%
<i>Weissella paramesenteroides</i>	18.18%	16.66%
<i>Lactobacillus paraplantarum</i>	4.54%	0%
<i>Leuconostoc paramesenteroides</i>	4.54%	0%
<i>Lactobacillus pentosus</i>	4.54%	5.55%
<i>Weissella kimchi</i>	0%	11.11%
<i>Pediococcus pentosaceus</i>	0%	11.11%

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.

LAB with good beneficial properties was characterized for further use as anti-microbial agents for food preservation and storage.

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