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Isolation and molecular identification of lactic acid bacteria by sequencing the 16S Rrna from newly bred yellow and white cassava fufu flour . A traditional fermented food

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Abstract

Fufu flour is a fermented wet-paste made from cassava. It is ranked next to *garri* as an indigenous food of most Nigerians in the South. Dried *fufu* flour have a longer shelf life, convenient to store and less bulky. It is a product of naturally fermented dough. Fermentation is done by consortia of microorganisms, of which the majority of which belong to lactic acid bacteria (LAB) genera. The indigenous LAB of cassava mash could be a potential source of starter cultures and probiotics. The aim of this study was to isolate the LAB from the newly bred yellow cassava and white cassava mash and identify them by 16s rRNA sequencing.

Method: The newly bred cassava were fermented and 8 (eight) mash/dough samples were collected from each sample at different fermentation stages at National Root Crop Research Institute, Umudike. The bacterial isolates obtained from the fermenting mash samples were selected on the basis of their been positive to acid production. The LAB were identified by morphology (Gram staining reaction and cell form) biochemistry (catalase-negative, oxidase-negative motility-non motile, glucose-positive, lactose positive and mannose negative). Genomic DNA of bacterial isolates were extracted using the zymo kit (zymo research group California, USA). 16s rRNA of the bacterial isolates were amplified using the broad-range 16s rRNA

PCR27F:5'-AGAGTTTGATCCTGGCTCAG-3'. 1492R:5'-GGTTACCTTGTTACGACTT-3'. And ITS for the fungi isolate: ITS 1F:5'-TCCGTAGGTGAACCTGCGG-3' ITS 2R:5'-GCTGCGTTCTTCATCGATGC-3'.The amplified product was sequenced at Ingaba Biotech West Africa (IBWA) South Africa Sequence analysis and comparison within the resources at the database were carried out to identify the isolated microorganisms.
The results showed that 56 and 64 purified bacterial isolates were identified as LAB with Gram-positive, catalase – negative oxidase-negative motility-non motile, glucose-positive, lactose positive and mannose negative) and rod/oval cocci shaped characteristics. Identification by its rRNA gene sequencing showed all the LAB isolates. The bacterial LAB isolates were identified as *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus pentosus, Lactococcus lactis, and Saccharomyces cerevisae,* with a similarity index of approximately 98 – 100%. The identified fermenting strains may be vital in the formulation of starter cultures and for use commercially in the production of *fufu* flour as probiotics.

Key words: Traditional fermented *fufu* flour, yellow cassava, lactic acid bacteria, yeast, 16s rRNA gene, PCR.

Introduction

Cassava (*Manihot esculenta* Crantz) is an important root crop in African, South America India and Asia, providing energy to over 500 million people as reported by (FAO, 2000). These has been stable increase in the production of cassava worldwide. Hence world production has doubled according to Plucknett *et al,* (2001). The production of cassava for human consumption has been approximated to be 65% of cassava products, while 25% is for industrial use, mainly starch (6%) or animal feed (19%) and (10%) lost as waste Maziya-Dixon *et al,* (2007) cassava can be consumed either boiled or in a variety of processed forms depending on local customs and preferences and are known by different names. Cassava can be processed into different forms and are the cheapest source of staple food in Nigeria. In Nigeria cassava root tubers are processed in different ways which vary from regions to region which result to different products such as *fufu, lafun, garri,* flour and others. Cassava can be processed by soaking, fermentation, cooking, steaming and chipping, frying, drying and roasting. Yellow cassava is a bio-fortified root with beta-carotene. The excesses of bio-fortification is to increase the nutritional value with β-carotene for the rural poor, who do not have access to commercially fortified foods, according to Yassir (2007). The successful bio-fortification efforts around the world have result in the release of cassava varieties rich in β-carotene a precursor of vitamin A. Birol *et al.* (2015).

The new bred varieties acts as vehicle of conveying pro-vitamin A to vitamin deficient (VDA) population as reported by Tanumihardjo *et al.* (2008). The fresh bio-fortified cassava roots are also referred to as yellow cassava. The yellow cassava according to Egesi (2011) is a newly released bio-fortified crop which is similar to the white varieties in terms of utilization for man and animal, though the pulp colour differs. Cassava is traditionally processed into different forms of fermented products such as *fufu, garri, lafun, attieke* and chips which is transported easily trade and suitable for fast preparation of meals as stated by Kouame, (2012). Over the years, demand for the production and consumption of fermented foods has extremely increased and are substantial of the part of the diet world wide as reported by (Elyas *et al,* 2015). In Nigeria the most popular food derived from fermented cassava are *fufu* and *garri* whose quality is dependent on technology of processing which consist of different steps like peeling, steaming, boiling,

slicing, punding, grating, roasting, soaking, pressing and fermentation according to Kouame, (2012). Fermentation of cassava dough by bacteria and yeast not only profer detoxification, it also improves food quality and safety by product preservation, flavor development, cyanide reduction and changes in functional properties as stated by Obilie *et al*, 2003.

Lactic acid bacteria (LAB) are constituted of a heterogeneous group of Gram-positive bacteria and widely consumed along with fermented foods and beverages due to their utilization as starter cultures in fermentation processes. They are present in foods like fermented meat, daily products, vegetables are fruits. They colonize the gastrointestinal and urogenital tracts of human and animals according to Adegoke and Babalola, (1988).

Lactic acid bacteria (LAB) generally regarded as safe (GRAS), play an essential role in the majority of food fermentations and preservation, and a wide variety of strains are used as starter cultures in making of food such as dairy, meat and bakery products (Gemechu, 2015). The help to enhance the sensory quality and safety features of fermented foods (Holzapfel and Wood, 2014). They produce a metabolities such as organic acids, carbon dioxide, hydrogen, peroxide, diacetyl and bacteriocins which can inhibit pathogenic and spoilage microorganism and extend the shelf life and safety of the food product as reported Piard and Desmazeanud, (1992). Lactobacilli represent one of the major microbial groups involved in these desirable fermentations. Among them, *Lactobacillus plantarum* is known among cassava dough fermentative germ (Kostinek *et al.,* 2007). The LAB can also be developed into probiotics for functional food. Probiotics are living micro-organisms that if in enough amount in human or animal will confer health benefits to the host by improving the intestinal microbiota balance (Alegria *et al.,* 2016). Phenotype characterization of some fermenting microbial flora having been employed such as the phenotypic characterization of fermenting microbial flora of fermenting dough during cassava fermentation using biochemical identification. It is important to identify microorganisms with molecular approaches which have been developed to provide rapid and accurate identification of bacteria using 16s rRNA gene sequences as reported by Jand and Abbott (2007). The study was aimed at conducting molecular identification of microorganisms found during the fermentation of newly bred yellow and white cassava to have a better understanding of LAB found during fermentation of the food and to isolate the LAB from the mash and identify them by 16S rRNA sequencing, which will help to select LAB strains as potential microbial starters for cassava dough fermentation for instant *fufu* flour and other products.

Material and Methods

Preparation of fermented yellow and white cassava root

About 30kg each of the two cassava varieties (Umucass 45 and TME 419) were obtained from National Root Corps Research Institute Umudike and used for production of instant *fufu* flour. The freshly harvested cassava roots were processed using the peeled, cut methods described by Aniedu and Oti (2008) into small sizes of about 8cm average size, washed with clean water and were subject to natural fermentation at different fermentation regimes of 0 h, 24 h, 48 h and 72 h respectively, in plastic container of same volume and diameter (25 litres and 54 cm) respectively and properly labeled. They were soaked in (1:2 ratio) and allowed to ferment for 72 h at ambient temperature (28 $\pm 2^{o}$ C).

Isolation of lactic acid bacteria (LAB)

At 0 h and 24 h intervals, 5 ml of the fermenting yellow and white cassava mash was collected from each of the samples and dispersed into 45ml of sterile distilled water in the test tube as aliquots. The mixtures were shaken to homogenize. Serial dilutions were carried out using 10-fold dilutions. The pour plate method as descried by Ezeama, (2007) was used. Aliquot (1.0 ml) of the samples were introduced into each sterile petri dish, then 15ml of liquefied MRS agar medium (Himedia, Mumbai, India) at 45^0c was poured into each plates and mixed by rotating the plate first in one direction and then in the opposite direction. The MRS agar plates were allowed to solidify on a level surface and incubated anaerobically at 37^0c using the anaerobic jar (Gas pak system, BBL) at 30^0C for 72 h. The dominate colonial morphology of the isolates on MRS agar were milky white, circular, non- pigmented, convex and elevated. The colonies were further sub-cultured and purified on MRS agar by streaking. The pure cultures were overlaid with glycerol and preserved for further use according to Pal *et al.* (2005).

Phenotypic characteristic

The cells were positive to gram staining reaction, catalase test, oxidase and sugar fermentation, when examined biochemically. Molecular characterization was carried out at International Institute of Tropical Agriculture (IITA). Microbiological Unit Ibadan, Nigeria and DNA sequencing was done at Ingaba Biotech West Afroca (IBWA) South Africa in Collaboration with IITA. Ibadan Nigeria. Sample processing, laboratory isolation and identification of (LAB) were carried out at National Root Crops Research Institute Umudike, Nigeria. Acid production test were done by the method described by (Dicks and Van-Vuren, 1987) with slight modification. Each strain was inoculated at 30^0C in 5ml MRS agar medium without beef extract and with 0.004 g/l of bromocresol purple in tubes. Acid production was monitored for three days, for yellow area formation in the tube and the acidification ability was analyzed by a visual examination of the yellow area's spread.

DNA extraction of lactic acid bacteria (lab) and yeast isolated during natural fermentation of cassava varieties.

DNA extraction

Whole genomic DNA was extracted from the LAB samples using the ZYMO kit (Zymo research group California USA). Using the following steps:

1. Microorganisms (LAB) were cultured on MRS and Potatoes dextrose agar for yeast and incubated for 24 h.
2. Hundred milligrams (100mg) of the cultured microorganisms was collected and suspended in 200µl of water in an eppendorff tube and 750 ml of bashing bead lysis solution was added.
3. Bashing bead was added and the Eppendorf tube was secured in a 2 ml tube holder Distruptor Genie (cat no. s6001-2120 from Zymo Research corp.) and processed at maximum speed for 5mins.
4. The bashed sample was centrifuged at 10,000 Xg (r.p.m) for 1 min
5. 400µl of the supernatant was transferred to a Zymo-spin filter in a collection tube and centrifuged for 1min at 8,000X g (r.p.m).
6. Genomic lysis buffer (1200µl) was added to the filtrate in the collection tube

7. An aliquot of 800µl of the above mixture was transferred into the zymo –spin IIC Column in A collection tube for 1min at 10,000X g (r.p. m).
8. The flow thru was discarded and 200 µl of DNA pre-wash buffer was added to the above column after which it was spinned for 1min at 10,000X g (r.p.m).
9. 500µl of g-DNA wash buffer was then added to the column and spinned at 10000Xg for 1 min.
10. The column was then transferred to a new Eppendorf tube and 100µl of DNA elution buffer was added to elute the DNA. The ultra –pure DNA which is suitable for PCR and other purposes) was then transfered into a -20° fridge for storage.

(Zymo research group California USA).

Aliquots of the Extracted DNA was mixed with loading dye (Thermo scientific Loading dye) and was loaded on 1% agarose gel (Fig 1 and Fig 2) using SB buffer and run using the following electrophoresis conditions 121v, 120 mA for 1 hour after which it was placed in a gel doc (Multi Doc –it digital imaging system) so as to visualize the bands.

DNA Gel pictures below in fig 1:

Extracted DNA was then amplified using the broad –range 16s rRNA PCR (Amani *et al*., 2017) on the V4 region using the primers:

For Bacterial samples (LAB strains):

27F: 5'-AGAGTTTGATCCTGGCTCAG-3';

1492R: 5'-GGTTACCTTGTTACGACTT-3' (Yi-Lin *et al*., 2015)

And ITS for *the fungi isolate* :

ITS1F:5'-TCCGTAGGTGAACCTGCGG-3'

ITS2R:5'-GCTGCGTTCTTCATCGATGC-3' (White *et al*., 1990**)**

PCR CONDITIONS for 16 s rRNA

Initial den.	Den.	Ann. Tempt	Extension	No. of circles	Final extension	Hold tempt
94˚C	94˚C	50˚C	72˚C	40	72˚C	4˚C
3min	45sec	60sec	90sec		10min	∞

PCR **CONDITIONS for ITS**

Initial den.	Den.	Ann. Tempt	Extension	No. of circles	Final Extension	Hold tempt
94˚C	94˚C	58˚C	72˚C	45	72˚C	4˚C
4min	30sec	40sec	1 min		10min	∞

(Tao *et al*., 2016)

PCR Cocktail mix

The DNA was subjected to the following cocktail mix and condition for the PCR

10× PCR buffer	1.0
25mM Mgcl2	1.0
5pMol forward primer	0.5
5pMol reverse primer	0.5
DMSO	1.0
2.5Mm DNTPs	0.8
Taq 5u/ul	0.1
10ng/µl DNA	2.0
H2O	3.1
	10µL

The amplicon from the 16s rRNA PCR reaction above was loaded on 1.5% agarose gel and the gel picture is attached as PCR. The ladder used is hyper ladder (50bp) from BIOLAB. The expected base pair of the amplicon is around 750bp). (Fig 3 and 4).

DNA Sequencing and BLAST Search

DNA sequencing was sent to Inqaba Biotech West Africa (IBWA) (a subsidiary of Inqaba Biotechnical Industries (Pty) Ltd. P. O Box 14356, Hartfield 0028 Pretoria, South Africa) for sequencing which was carried out using the Sanger method with an Applied Biosystems (Foster City, CA, USA) automatic DNA sequencer (ABI PRISM 3130x l Genetic Analyzer) and an Applied Biosystems BigDye (ver. 3.1) kit. Blast search (Altschul *et al*., 1990) was carried out using the NCBI nucleotide database "16S rRNA sequences (Bacteria and Archaea)" with the program selection optimized for "Highly similar sequences (megablast)."This was done by a manual blast search against the NCBI 16S isolate database for the top 10 operational taxonomic unit species (OTUS) for exploratory purposes (NCBI BLAST, RRID: nlx_84530). Results from the operational taxonomic unit (OTU) clustering were matched to the Greengenes data base using an RDP classifer within the QIIME pipeline and used for further analysis.

Results and Discussion

The present study deal with molecular characterization of LAB isolates from fermented cassava dought, and to obtain strains with many beneficial properties as starter culture for commercial uses.

Phenotypic characterization of lab isolates

Table 1 shows the morphological and physiological properties of the lactic acid bacteria isolated from both cassava mash. The pH profile of the cultivation decreased as fermentation progressed. It is clear that lactic acid bacteria grow and remain viable within the pH range of 4.5-6.5. Similar results for cell forms of lactobacillus genus which grow until a final pH of 4.6 have been reported by MacDonald *et al.* (1990). All the isolates of lactic acid bacteria fermented the cassava roots into it's end product. Lactic acid bacteria have been consistently responsible for the traditional fermentation of cassava even the newly bred yellow variety.

Table 1: Morphological and physiological properties of the lactic acid bacteria isolated from both cassava mash

Cell formed	Gram stain	Catalase	Oxidase	Motility	Glucose	Lactase	Probable genus
Short rod in tetrades or pairs	+	-	-	-	+	+	Lactobacillus
Long/short rod in pairs or singles	+	-	-	-	+	+	Lactobacillus
Oval cocci	+	-	-	-	+	+	Lactococcus
White circular cocci oval	+	-	-	-	+	+	Lactobacillus
Short rod in pairs or single	+	-	-	-	+	+	Lactobacillus
Oval cocci in pairs	+	-	-	-	+	+	Lactococcus

Molecular identification of isolates during natural fermentation

The identification of the lactic acid bacteria (LAB) isolated during natural fermentation was done based on 16s rRNA gene. The isolates from the fermenting mash were identified as *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus pentosus and Lactococcus lactis*. The most commonly isolated yeast was identified as *Saccharomyces cerevisae* from both cassava fermenting mash. All the LAB isolated in this study were found to be members of Lactobacilli Genus. A total of 64 lactic acid bacteria were isolated from (TME 419) cassava mash while 56 isolates were obtained from the (Umucass 45) cassava mash and they were mainly genus of lactobacillus. In (TME 419) mash, among them were 96.8% (62 isolates) which were long/short rod shaped cluster, tetrads which were in pairs or occurred singly when observed in microscopy. From the fermenting mash of (UMUCASS 45) a total of 56 lactic acid bacteria was isolated and 76.7% (43 isolates) were short rods, which occurred singly or in pairs, tetrads as observed in the microscopy and belong to the genus *Lactobacillus*. While 19.6% (11 isolates) were cocci in shape which showed well rounded morphology, a typical feature of *Lactococcous* . The most frequently isolated LAB species from both mash were *L. plantarum* followed by *L. fermentum.* The 16s rRNA gene sequences of 750bp of all isolates when compared with related bacteria using the BLAST Program at NCBI (http://www.ncbi.nih.gov/) showed that the isolates were closest relatives of *L. plantarum, L. fermentum, L. pentosus and L. lactis* which showed 98-100% proximity based on NCBI BLAST. This is applicable to the isolated yeast *Saccharomyces cerevisae* . This confirm the presences of *L . plantarum, L. fermentum,* during the fermentation of cassava starch as stated by Lacerda, *et al*;(2011) and *L. lactis , L . pentosus* , according to Guira, *et al.*2016).

Electrophoresis for DNA bands are shown in fig 1 and 2 using the primers
For Bacterial samples (LAB strains):
27F: 5'-AGAGTTTGATCCTGGCTCAG-3';
1492R: 5'-GGTTACCTTGTTACGACTT-3' (Yi-lin *et al*.,2015**)**
And ITS for *the fungi isolate* :
ITS1F:5'-TCCGTAGGTGAACCTGCGG-3'
ITS2R:5'-GCTGCGTTCTTCATCGATGC-3' (White *et al*., 1990**)**

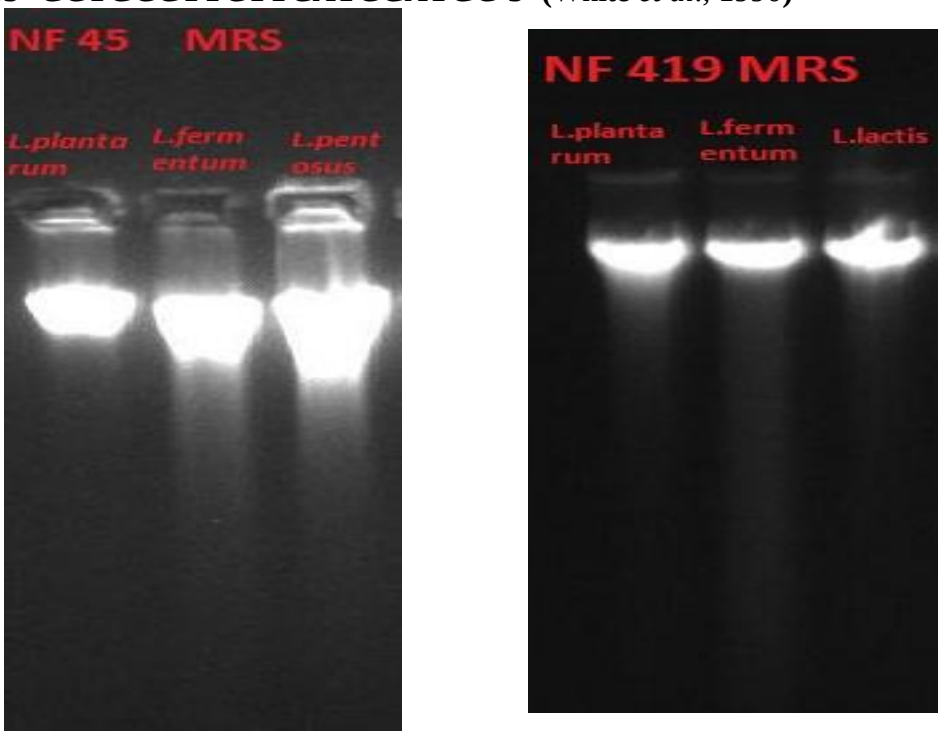

Fig. 1: gDNA Gel picture NF45 & 419 MRS

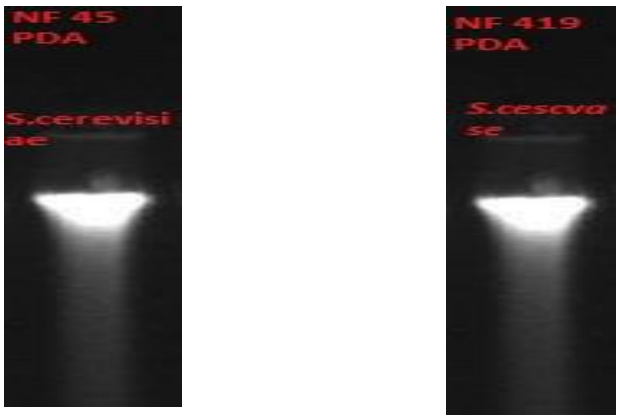

Fig. 2: gDNA gel for NF45; 419 (PDA)

The PCR amplification was performed to obtain more copies of the 16s rRNA gene for the sequencing process as shown below indicating 99-100% sequence similarity to *L. platarum, L. fermentum, L. Pentous, L. lactics* and *S. cerevisiae.*

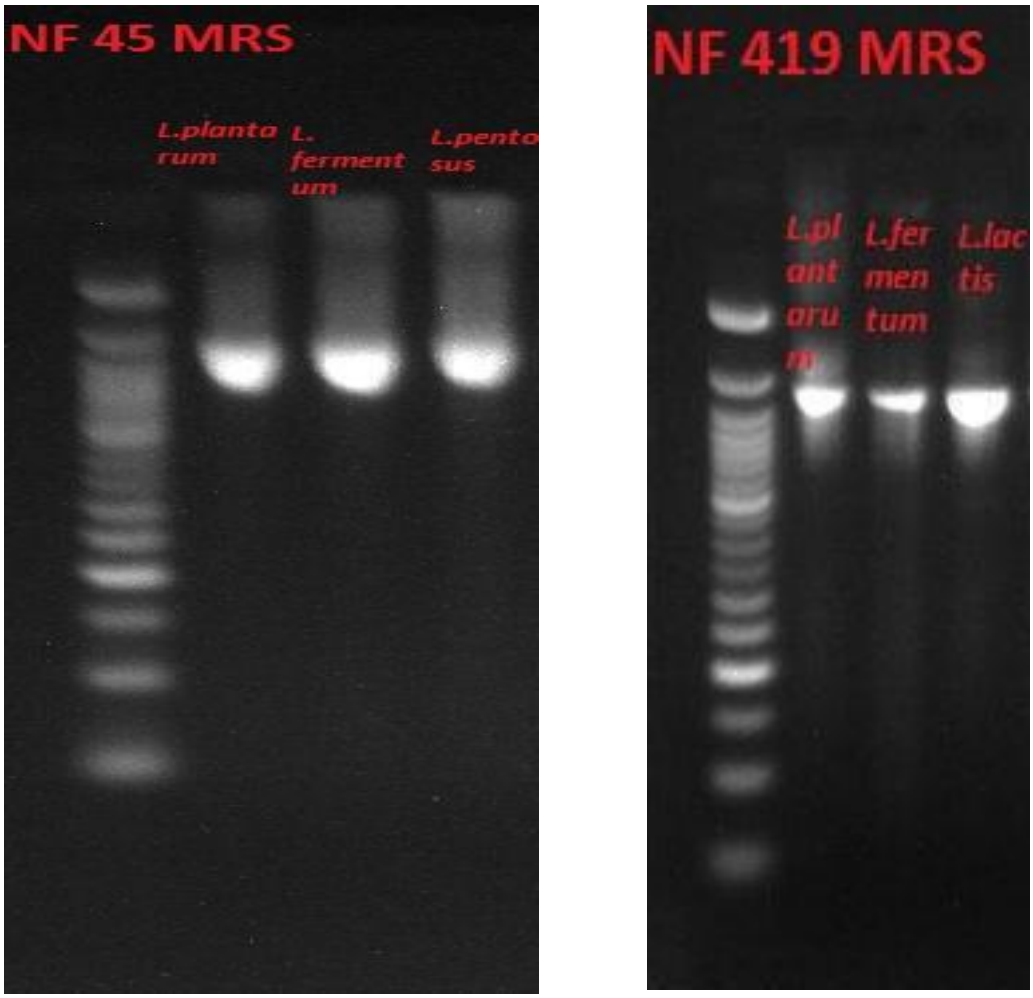

Fig. 3:PCR reaction gel picture (750kb)

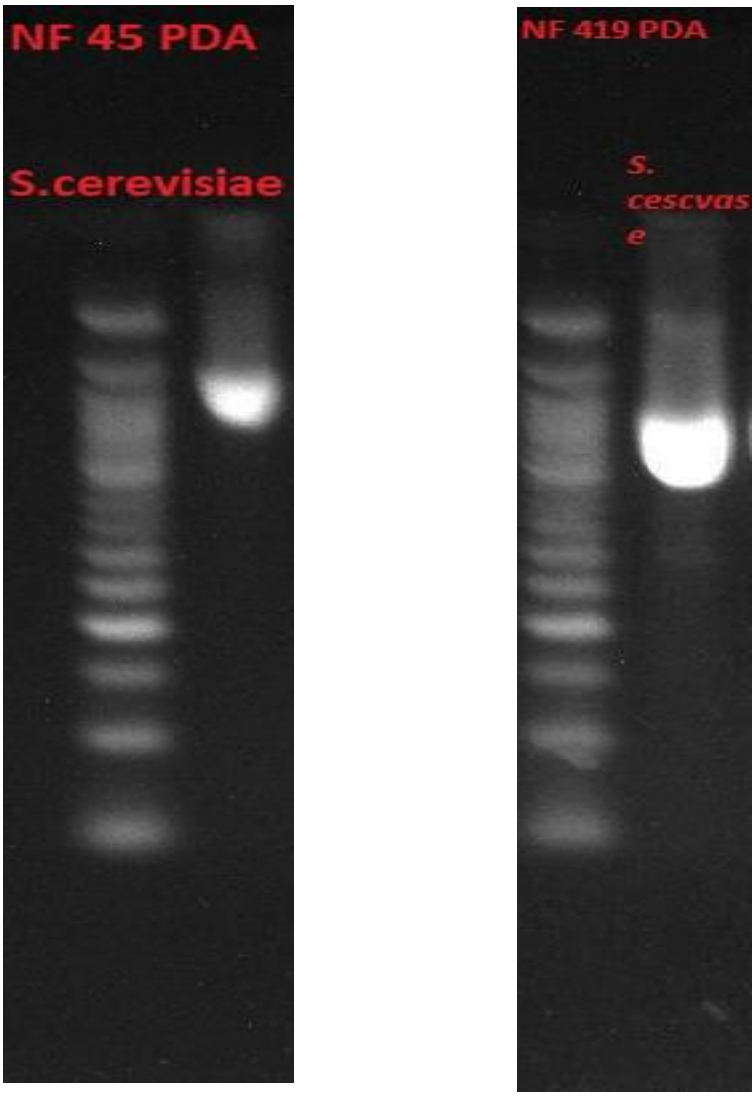

Fig. 4: PCR reaction gel picture 750kb

16s rDNA gene sequence similarity

The molecular identification of the isolated strains were done based on 16s rRNA sequence analysis. The partial sequences of 16s rRNA obtained from isolates were aligned with all available 16s rRNA sequences in the Gen Bank data base to obtain the similarity of the isolates. The 16s rRNA partial sequencing of isolated strains are shown in Figure 5, 6, 7, 8, 9, 10, 11,12, 13 below;

GKRRYCGYGAGKTACYAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGAT
TAGCTTACTCTCGCGAGTTCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATT
TGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCATGTCCCCGA
AGGAMCGTCTAATCTCTTAGATTTGCATAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTAGYTTCGAATTAAACCACATGCT
CMACCGCTKGTGCGGSCCCCCGYCAATTCCTTTGAGTTTCAGCCTTGSGGCCGTACTCCCCRGGCGGAATGCTTAATGCGTTA
GYKGCRGCACTGAARGGSGGGAAACCCYYCCAACACTTRGCATTCATCSGTTTASGGWAKGGACTACCRGGGTATYCTAATC
CTGTTTGCYWMCCCATACTTTTCRAGCCTCASCGTCAGTTAMMRGACMARAMAGCCGCCTTTCGCCACTGGKGTTCTTCCAW
ATATCTMCGCATTTCAMCSGCTACACATGRAGTTCCACTGTCCTYTTTCTGCACTCAAGTTTCCCAGTTTCCGRATGCACTTCT
TCGGTTGAGCGRAGKTTTCACATCARACTTAAAAAAACCGCCTGCGCTCGCTTACGCCAATAATCCGGAMAACGCTTGCACT
ACGTTACGCGGGGCGYGYGSRRGMAMAAAAAAA

Fig. 5: *L. plantarum*
NF 45 MRS

TTGACTGAGCTCGATTCTCTTTGGGAAGCGATCCAAAATTCATTCCGTAAGTCTAAGGGCGATACGACTT
ACAAAAACTTAATTGCTCCGGCAAAACCCCTCGACTTCGCTAACGGCAGGCTTCGCATCCAGCT
CCAGTACCACCGCGACTTTTGGGAGCAGCTGACCGACCAGGTCGTCGAAATCGTCTACCAACGCACCGGC
CAAGAAATTCGCCCGGACTACGTGTTGGCAACCGATCCGACCCCCCTGGCCCAAACGCCGCCCCGGCCAC
AGTCGACCTTTAAAGAGGAAACGCCCCTCAACCCGGAGTACACCTTCCAAACCTTCATTGAAGGACGTAG
CAACATGATGGCCTACGCTTCAGCCTTTGCGGCTTCCGAGTCCCCGGGTGACCAGTACAACCCGCTCCTG
ATTTACGGGGGCGTCGGCCTAGGCAAGACCCACTTGATGCAAGCCATCGCTAACAACATGAAGTTTCACA
ACCCTAGCGTACGGATCAAGTACGTAACCAGCGAAAACTTCATGAACGACTTCGTTAACTCTATTAAATC
CGGGACCCAAGAGGAGTTCCGCCGCGAATACCGCGACCTCGACGCCCTCTTGGTCGACGATATTCAGTTC
TTCGCCTCCAAGGGGGAGACGCAAACCGAATTTTTCAATACCTTTAACGTCTTATACGACAACAAAAAGC
AGATTGTCCTGACCTCCGATAAGGATCCCCGCGAAATCCCCAACCTGACGGAACGGTTGGTCTCCCGCTT
CATGTGGGGGGTCCCGGTCGAAATTACCTCGCCGGCACTGGAAACCCGGATTGCGATTTTAAAGTCCAAG
GCTGAAGAAGAGCACCTCGACGTGCCAAACGACGTCTTAAACTTCGTTGCCCAACGCATCAACACCAACG

Fig. 6: *L. fermentum*
NF45 MRS

GCGATAATTAATAACGATTTGATGGCTTTCACGTTTGTCCATGGCTTCATAGGCTGCTTGAACATGGTCG
AGGTCAAAGCGTTTCGTAAAGACCTTTCCGGGATGAATCTGACCATTTAAAACAGCATCTAATAGGACTT
TACGATCATAGGTGGTGACTGAGGCAATCCCGCCACGTAGACCAATATTGCGCCAGAATAAGTTATTGGT
GTTCATTTCTGCCTTCTGTGGGACGCCGACTCGACCAACAACGGCACCCGGACGGCCAACCTTCACTGCA
GTATCGACCGATTGTTCAGTACCGACACATTCCAATACTGCGTCAGCCCCCTTGCCATCAGTCAATTCAA
AGACTTTTTTAACCCGCTTCGTCGCCCCGTTCAGCAATAATGTCAGTCGCACCAAATTCTTTAGCGAGGG
CTTGCCGATCCGCATGGCGACTCATGGCAATAATGCGACTCGCACCACGTAACTTGGCAGAAATGACACC
GCAAAGGCGACTGCCCCATCACCCATGACAACGACCGTATCACCTTGTTTAACTTCAGCCGTTGCAGCCG
CGTGGTAACCAGTCGCCATGACATCTGATAGCGTCAGTAGATCATTTAACTGCGCGTCGGTGTAATCACT
TGGTTGGCCAGGTACTTTAACCAGTGACCAGTCCGCATTGGTGAAACGTAAATATTCACCCTGATAGCCA
ACCATTTCATCGGGATGATTGTTGAGACAGTTACCTTCAAAACCGGCCAAACAAGCGGCACAATGCCCAC
AACCATGCGTAAACGGTGCAATAACAAAATCACCTGGTTTGACATGGGTCACTTCATCGCCGACAGATTC
AACCACCCCGATGGCTTCATGGCCGACCGCTGACGGGTGCTTACGATCTGAAATTCCGCGGAACCACCAC
AAATCTGATCCACAGACACAAGCCCGAACAATCCGTAAAACGGCGTCTGTGGGTTGTTTAATGGTCGCCT
TTGGCAATTCGCGTGCTTCAACTTTTCCTGGTTCAACAAATACTGCTGTTTTCATAGTTAATAGCCTCCT
AAAGCTGTTTAAAATTGTTAGTGATTTAATTGATTCAATTATCGTGGCTCAAAATTTAGTCTGATCAGCA

Fig. 7: *L.pentosus*
NF45 MRS

ATTCWATTGCCAGCGCTTAATTGCGCGGCGATAAACCTTCTACACACATTGCTTATTCTTTTTGAATATTGCTTTGGGT
TGAGCCTAGCTCAGCCCAGAGGTCTTAAACACAAAGATTTATTCTTTTTTTATAAAACCATAGTCATGAAAATTTTAA
CAAAATTAAAATATTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCAACGATGAAGAACGCAGCGAATTGCGAT
AAGTATTGTGAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTGCACCCTCTGGTATTCCAGAGGGTA
TGCCTGTTTGAGCGTCATTTCTCTCTCAAAGCTTCGGTTTTGGTATTGAGTGATACTCTGCTCTCCAGGGTTAACTTGA
AATAAGATATGGCAAGAGTGTGCTAGGCGCAGCTTGAGCTTGTGTAATGTATTAGGTTCTACCAACTCATTAAACGAC
TCTTGTGAAACCCAGCATATTTGGCTCGGCCTAACAACATCTTCATAAAGTTTGACCTCAAATCAGGTAGGACTACCC
GCTGAACTTAAGCATATCAAAAGGCGGAGGAA

Fig. 8: *S. cerevisiae*
NF45 PDA

GTATGCAGTACATGACTTAGTTCATGGGCTGTCTGAGTGTCAGACACCGAAAAGCGCCGCTCATTTTCAGAAAA
GCCCTTACCAAAAAATCCTTTACAAGTGATCCGTTTCCCAGCATAATGTAGTCAATTAAATAAATAATTTCTTCG
GGGCAGGGTGAAAATCCCGACCGACGGTGACAAGCAATGCTTGAAGTCCGTGACCCGCAAATTTGCGGCTGAA
CCAGTGCGATTCTGGTACCGACAGTGAAAGTCTGGATGGGAGAAGAAAAGGCAGGGGGAAGTCCCCTGCTACC
TTTTTGAACACGCTTTCAACCAGGGGTCCCGGAATTTTTCCGGGGCCCCTTTTAAATTTCCCGGGGAAATGGGA
ATGGCAACCTCCTCGGCGGGCCGAGGAGGTTGGTTCCCACTCACCTATGAAAGGAAGTCATTACCTTGAGTTCA
GACGAACAGTTCATGCAACTGGCGCTCGCTGAAGCGGCCAAGGGCGGGATCGCCACCTGGAAGAACCCCCAGG
TCGGCGCCGTGATAGTCAAAGAACGGCCAAATCGAATCGGGGTGGTCGTACGAGTGTTACACCGGGCGAAGAA
GAATCCGCGTGCGCACGGGATTAATTGGAGGACTTCAACGCTGGTTGCTCCAATACAACTG

Fig. 9: *L. fermentum*
NF 419 MRS

ATTCAAGAAAGGAATCAGAGCAAAAAAATAAGTGAGAACTCATTCCCTTCAAGAAATACGAGTTCTCACT
AATGTTTGTTCTGTCAAGGCGATTATAACAAATGGCAAAAGAAAAGGCGAGATACTTCACTTTTTTACTT
TATCCAGAATCGATACCACAAGATTGGCAAACAAAACTTGAAACTTTAGGCGTTCCAATTGCAATTAGCC
CGCTTCATGATAAAGATTTGAGCAGTGTTGAGGGGCAAAAGTATAAAAAAGCCCATTATCACGTTATTTA
TATCTCTAAAAATCCTGTCACAGCAGAAAGCGTTCGCTTGAAAATCAAGCGTGCTTTAGGCGATAAGAGT
GTTGCTAAAGTACAAATTGTTGTCCAGAGCATGGAAAATATGTATTTGTATCTGACACATGAATCTAAAG
ACGCTATTGCTAAAAATAAGCACAAGTACAGCAAGCATGACATTGCTGTACTGAACAATTTTGATATTGA
TCGCTATATTACGCTTGATGTTGAAGATAAAGACGACATGCTGAATGATGTTTGCGATTTGATTGATGAC
CATAATTTGGCGAATATGCGTGAACTGAGACGCTTTTTAAAAGCTCATGGTTCAGAATATGGCATGCCCG
GTATTAAAGTCGTTAATTCGGTTTTACGTGCTCATACTGGACTGATAAGGCTGTATTTCGATGCTGTTTA
TCAGGAACGCAAGTATGGCAGAGGCGATATAAACAAAGAGACTGGCGAGATACAAGATTAATTAGCGAAT
GGAAAGTAGGTGTTCGATTTGAGCACCTTTTTTGTTGTCGGCTAGCCGACTTCTGATACAAGTCTTTAGC
ACAATAACACAATCAACTTTAGTTGTTGTGTGTAAGTGCGCCCTTAGGAATATAATTTGAATATATTTCA
GATTTTCAATCTGACTGCTACTATCATCAAGATAAGAAATAACTAATTTGTGTCAAAAATTTTCTCTAAA
TCTGTTTCATCAACATTTTCTTCAATTTCCAAATCTCTATACATCTCAACAAAAATATTCACTAATTTTT

Fig. 10: *L. plantarum*
NF419 MRS

CTTAAAATTTAACAATCTATTTTCAAATTCTTAGATTCTATTACATTAGAGATAAAATATCCCCAAAGAT
AGAAAAGACTGTCTAAAAGCTGTGGTAAATTTTTCTTAATTTTTTCTTTTTCCACAGTAAATAATCTATA
AATTTGACTTTTTTTGATTTGTGGATAAGTCTGTATGCATTTGATATTACTCATTTTTTTCTGTGGATAA
GAAAACTTTTACACATTATCCACAGTTTTGTTAATAACTTTTTCTTATGGTGGAAAACTTTCTTTTGTTA
ATTTCAACAGACTGTGGAAAAATATAATGTGAAATGATATAATGAGTTATCAACAAACTAAAAAGTAAAG
GAGTAATATGGCATCCCTTAATGAAAATCAAAAATTTTGGGCCCGTGTAACCGAACTAGCTCAGCAGAGT
ATCGGTAAACAAGCTTATGATTTTTTTATTGAGCCTGCTCAATTAATGTCTGTCGAGCAAGATACTGCCA
ATATCTTGCTTGATAGTGGAATGAAAAAAGATTATTGGAAAAAACAGTCCGATTTGATCACAACGGCGGG
ATTTGAGGTCTTTGGTCGGATGATTGATTATGAACTCTATGCAAATGATGAGTTAACTGAACTGGAACTT
CATCGTTTAAATAATCAATCTTCTATTGAAGAGCAGCCGCGTTCTACTGCTAAACCCGCTTCTCCTTTGG
TTAGTGGTCTCAACGAGAAATATAATTTTGAAAATTTTGTGCAAGGTCCTGGTAACCGTTGGACTTTGGC
TGCGGCAATTGCTGTTGCTGATAAACCTGGTGATACCTATAATCCACTATTTATTTATGGTGGAGCTGGT
CTAGGTAAGACGCATTTAATGCATGCAATTGGAAATCAGATTTTGACTGACAATCCGACTGCTCGAATTA

ACACCACACCCACACCACACCCACACACACCACACCCACACACCACACCCACACCCACACACCCACACCC
ACACACCACACACCACACCACACCACACCCACACCCACACCACACCCACACCCACACACCACACCCACAC
CCACACACCACACACTACCCCTAACACTACCCTATTCTAACCCTGATTTTACCTGTCTCCAAACCTACCC
TCACATTACCCTACCTCCCCACTCGTTACCCTGCCCCACTCAACCATCCACTCCCAACCACCATCCATCT
CTCTACTTACCACTAACCACCGTCCACCATAACCGTTACCCTCCAATTACCCATATCCAACTCCACTACC
ACTTACCCTACTATTACCCTACCATCCACCATGTCCTACTCACTGTACTGTTGTTCTACCCTCCATATTG
AAACGTTAACAAATGATCGTAAATAATACATACATACTTACCCTACCACTCTATACCACCACTACCACCA
CCGCCACTTGCCACACTCACCTTCACTTCTACTGATATGTCATACGCACACGGATGCTACAGTATATACC
ATCTCAAACTTACCCTACTTTCATATTCCACACCATGGCCCCATTCTCACTAAATCAGTACCAAATGCAC
TCACATCATTATTCACGGCACTTGCCTCAGCGGTCTATACCCTGTGCCATTTACGCATAACTCCCACGAT
TATCCACATTTTATTACCTATATCTCATTCGGCGGCCCCAAATATTGTATAACTGCTCTTAATACATACG

Fig. 12: *S. cerevisiae*
NF419 PDA

CCACACCACACCCACACACCCACACACCACACCACACACCACACCACACCCACACACACACATCCTAACA
CTACCCTAACACAGCCCTAATCTAACCCTGGCCAACCTGTCTCTCAACTTACCCTCCATTACCCTGCCTC
CACTCGTTACCCTGTCCCATTCAACCATACCACTCCGAACCACCATCCATCCCTCTACTTACTACCACTC
ACCCACCGTTACCCTCCAATTACCCATATCCAACCCACTGCCACTTACCCTACCATTACCCTACCATCCA
CCATGACCTACTCACCATACTGTTCTTCTACCCACCATATTGAAACGCTAACAAATGATCGTAAATAACA
CACACGTGCTTACCCTACCACTTTATACCACCACCACATGCCATACTCACCCTCACTTGTATACTGATTT
TACGTACGCACACGGATGCTACAGTATATACCATCTCAAACTTACCCTACTCTCAGATTCCACTTCACTC
CATGGCCCATCTCTCACTGAATCAGTACCAAATGCACTCACATCATTATGCACGGCACTTGCCTCAGCGG
TCTATACCCTGTGCCATTTACCCATAACGCCCATCATTATCCACATTTTGATATCTATATCTCATTCGGC
GGTCCCAAATATTGTATAACTGCCCTTAATACATACGTTATACCACTTTTGCACCATATACTTACCACTC
CATTTATATACACTTATGTCAATATTACAGAAAAATCCCCACAAAAATCACCTAAACATAAAAATATTCT
ACTTTTCAACAATAATACATAAACATATTGGCTTGTGGTAGCAACACTATCATGGTATCACTAACGTAAA

Fig. 13: *S. cerevisiae*
NF36 PDA

Many authors have reported the presence of *L. plantrum and L. breris and L. fermentum* among the lactic acid bacteria in natural cassava fermentation according to (Kostine *et al.*, 2007; Obilie *et al.*, 2004). *Lactobacillus plantarum* and *L. fermentum* have been reported by Lacerda *et al.*(2011) as the predominant lactic acid bacteria during sour cassava starch fermentation isolated from cassava flour manufactures. The LAB isolated and identified in this study are suitable source for use of test selection of starter cultures. They have ability to lower pH with increase in titratable acidity. Yeasts and lactic acid bacteria are implicated in the fermentation of wide variety of beverage fermentation and traditional food as stated by Obinna-Echem *et al.*, (2013). Yeast help in facilitating alcoholic fermentation while lactic acid bacteria produces lactic acid as a major by product from carbohydrate fermentation according to Steinkrans (2002).

Conclusion

The study has shown that different lactic acid were involved in the fermentation process of both cassava mash. The isolates were successfully identified by 16s rRNA gene sequencing. The analysis of the 16s rRNA gene sequence indicate that the LAB isolates were identified as *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus pentosus* *Lactococcous lactis* and the yeast as *Saccharomyces cerevisae*, with a similarity index of approximately 98-100%. The LAB isolated from the cassava mash can be used as probiotics and will help in the formulation and development of starter cultures and for use in the development of small-scale commercial production of instant *fufu* flours.

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