



## Molecular Characterization of Lactic Acid Organisms Isolated from Spontaneous Fermentation of Cassava- *fufu* and *gari*

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### Authors' contributions

This work was carried out in collaboration among the authors. Author FA designed the study, performed laboratory analyses and wrote the first draft of the manuscript. Authors SF, OO and TO performed laboratory analyses and worked on the manuscript. Authors OO and PO performed bioinformatic analyses. All authors read and approved the final manuscript.

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### ABSTRACT

**Aim:** The present work was aimed at ensuring the accurate taxonomic identification of the fermentation organisms previously isolated from spontaneously fermented cassava for the purpose of safety assessment and quality assurance.

**Study Design:** Purposive sampling method was used as all the five isolated organisms (from the previous study); 2 bacterial and 3 fungal organisms were characterized using molecular methods.

**Place and Duration of Study:** The genotypically characterized organisms are from a previous

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study carried out in May 2016 where lactic acid bacterial and fungal organisms were isolated from spontaneously fermented cassava.

**Methodology:** Genomic DNA was extracted from the lactic acid microorganisms and this was used as a template in a polymerase chain reaction (PCR) reaction. The 16s rRNA genes were amplified for the bacterial isolates while nuclear ribosomal internal transcribed spacer (ITS) genes were targeted for the fungal isolates. The amplicons were electrophoresed on 2% agarose gel prepared with Tris Borate Ethylenediaminetetraacetate (TBE) buffers stained with ethidium bromide. The sizes of the corresponding amplicon captured on gel images were compared with that of the ladder to determine their sizes. Furthermore, sequences of the PCR products were analyzed and the chromatograms subjected to BLAST (Basic Local Alignment Search Tool) analyses to identify the lactic acid organisms.

**Results:** The 3 fungal isolates were identified as *Schizophyllum commune* (MK 431022), *Aspergillus oryzae* (MK434151), *Aspergillus sydowii* (MK434152), while the bacterial isolates were identified as *Bacillus spp* (MK450345) and *Bacillus spp* (MK 449018). A high level of discrepancies was observed when the results of identification of the same isolates by biochemical methods in an earlier report were compared with the results from the present study using molecular methods.

**Conclusion:** The identification and characterization of the isolates and the discrepancies between the outcomes of the biochemical and molecular methods underscore the limitations of phenotypic (biochemical) methods in characterizing organisms that may eventually be used as starter organisms in food fermentation. The present result makes quality assurance and safety assessment of foods prepared using these organisms as starter organisms possible.

**Keywords:** Fermented foods; lactic acid bacteria; quality assurance; safety assessment.

## 1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a major staple food crop in the developing world, it is reputed to constitute the basic diet for about 500 million people globally and the fifth most cultivated plant in the world [1]. Spontaneous fermentation of cassava is widely used in these resource-challenged countries as means of processing the harvested tubers into less easily perishable food products, to reduce the cyanide content and to improve the nutritional status of the food [2,3]. Fermentation is also known to confer some sensory characteristics on food (such as colour, taste and aroma) to the delight of the consumers [4].

The factors that determine the desirable food characteristics include the type of the fermenting (lactic acid) organisms [5-7]; the length of the fermentation process [8]; the conditions of the fermentation process- wet or dry [4]; the type of substrate, maize, cassava and yam being the most popular substrates for food fermentation in Nigeria [9-11].

In a previous study, Ayoade et al, [4] demonstrated the proof of principle that fermentation of cassava actually reduces the cyanide content in fermented cassava products, namely, fufu and gari. Moreover, the reports indicated that apart from increasing the

nutritional value of the final product, the organoleptic characteristics of the final food product is consistently maintained in a reproducible manner due to the action of the lactic acid fermenting organisms. Lactic acid bacterial isolates belonging to the genera *Lactobacillus*, *Weissella*, *Leuconostoc* and *Lactococcus* including aerobic mesophiles including species of the genera *Bacillus*, *Clostridium*, *Staphylococcus*, *Serratia*, *Acinetobacter* and *Raoutella* have been isolated from spontaneous fermentation of cassava in previous studies [12-14].

On the other hand, accurate taxonomic identification of the fermentation organisms is absolutely essential for safety assessment and assurance when deliberate inoculation of food with starter organisms is to be carried out. The isolated lactic acid microorganisms from that previous study were identified and characterized using phenotypic methods, such as cultural, morphological and biochemical methods alone. Although the use of phenotypic and biochemical tests for identification of microbes have been the traditional standard for many years it is fraught with many problems such as inaccuracy especially in the case of novel organisms that may not be in the databases [15,16]. For example in many cases, more than 75% discrepancy rates have been reported in studies where phenotypic and molecular methods were

compared for accuracy in identification and characterization of microorganisms [16,17] due to erroneous results and the interpretation of such results. Other problems of phenotypic identification as a stand-alone method of characterization include lengthy turnaround time and delays and exorbitant cost of reagents and supplies and labor.

In the present work, previously isolated bacterial and fungal lactic acid organisms from spontaneously fermented cassava were characterized using molecular techniques by sequencing the 16s rRNA genes for the bacterial and nuclear ribosomal internal transcribed spacer (ITS) genes for the fungal isolates. The present data provides the necessary data required to confirm the accurate taxonomic identity of the isolated fermentation organisms that were earlier reported and serves as a veritable tool for subsequent assurance and safety assessment of foods prepared using these organisms as starter organisms.

## 2. MATERIALS AND METHODS

### 2.1 Source of the Lactic Acid Microorganisms

Two lactic acid fermenting bacterial and fungal organisms from a previous study [4] were isolated and identified from a previous study where 2 varieties of cassava were spontaneously fermented and processed under 2 different conditions, namely, wet and dry to produce two distinctly different final food products with characteristically different organoleptic properties. The bacterial organisms were identified as *L. brevis* and *L. plantarum* while the fungal organisms were presumptively identified as *Neurospora crassa*, *Aspergillus fumigatus* and *Saccharomyces spp.*

### 2.2 DNA Extraction, PCR Amplification and Fragment Purification

Genomic DNA was extracted from approximately 100 mg fungal or bacterial cells that have been resuspended in 200  $\mu$ L of PBS using Quick-DNA™ Fungal/Bacterial Miniprep kit. For the bacterial isolates, an aliquot of 5  $\mu$ l of the extracted DNA was used as a template in a 20 $\mu$ l PCR reaction mixture containing illustra™ PuReTaq™ Ready-To-Go™ PCR Beads, 1 $\mu$ l each of forward and reverse primers, and 15 $\mu$ l of double distilled water [12]. The list of primers used including information on the targeted genes

and the PCR conditions are shown on Table 1. All reactions were performed in duplicates with negative control.

### 2.3 Agarose Gel Electrophoresis and DNA Sequencing

The PCR reaction products were electrophoresed on 2% agarose gel prepared with Tris Borate Ethylenediaminetetraacetate (TBE) buffers stained with ethidium bromide. An aliquot of 2  $\mu$ l of DNA ladder (100 bp) was loaded into the first well after which 4 $\mu$ l the PCR product was mixed with 2  $\mu$ l of loading dye then loaded into subsequent wells. The reaction was run at 90 V and 400 mA for 35 mins. Gel images were captured using gel documentation box and the corresponding amplicon size compared with that of the ladder to determine their size.

Sequence analysis of PCR products was performed at the International Institute for Tropical Agriculture, Ibadan, Nigeria. Chromatogram of the sequence was viewed using Geneious version 11.1.5 (www.geneious.com, [18]) and manual base calling was carried out where necessary. Following manual base calling, the sequence was subjected to BLAST (Basic Local Alignment Search Tool) analysis to identify the organism. These sequences were subsequently submitted to GenBank and accession numbers were assigned.

### 2.4 Evolutionary Relationship of Taxa

The evolutionary history was inferred using the Neighbor-Joining method [19]. The evolutionary distances were computed using the Maximum Composite Likelihood method [20] and are in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 157 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [21].

## 3. RESULTS

### 3.1 Identification of Lactic Acid Bacterial and Fungal Organisms

Agarose gel result for bacteria specific 16S rRNA gene amplification confirmed the presence of the two bacterial organisms tested in this study as

shown by the presence of DNA band on the agarose gel (Fig. 1). Likewise, the presence of DNA bands specific for the gene amplification of the ITS gene (Fig. 1) confirmed the presence of the 3 fungal lactic acid tested in this study.

As shown in Table 2, a high discrepancy in identification was observed when the results of the phenotypic identification were compared with the present identification by molecular methods. With the exception of *Aspergillus oryzae* MK434151 that was earlier identified as *Aspergillus fumigatus*, the identification results differed even at the genus taxa for the other organisms.

### 3.2 Evolutionary Relationship of Taxa

Figs. 2a and b shows the evolutionary history of the test organisms inferred using the Neighbor-Joining method [19]. The optimal tree with the sum of branch length = 7.88457907 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [20]. The tree is drawn to scale, with branch lengths in the same units as those of

the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [20] and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 179 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [21].

### 4. DISCUSSION

Traditionally *fufu* and *gari*, food products made from cassava are spontaneously fermented without consideration for the addition of starters. Recent interest in standardizing the fermentation process has led to research efforts focussed on isolation, identification and characterization of the major lactic acid organisms with a view to developing these as viable starter organisms [2, 5]. On the other hand, correct identification of the potential starter organism is vital to any quality assurance and safety assessment plan to

**Table 1. List of targeted genes, the primers used and the PCR conditions**

Type of Isolate	Targeted gene	Primer used	PCR conditions
Bacterial	16s rRNA	pA 5' AGAGTTTGATCCTGGCTCAG 3' (F) pH 5' AAGGAGGTGATCCAGCCGCA 3' (R)	95°C for 3 min, 94°C for 30 sec, 55°C for 40 sec 72°C for 1 min 30 sec, and 72°C for 10 min for 35 cycles
Fungal	ITS 1 and 2	CTGGTCATTTAGAGGAAGTAA (F) TCCTCCGCTTATTGATATGC (R)	95°C for 2 min, 95°C for 30 sec, 55°C for 40 sec 72°C for 1 min, and 72°C for 10 min for 40 cycles

**Table 2. BLAST results in comparison with earlier reported results from phenotypic identification of lactic acid microorganisms associated with the spontaneously fermented cassava**

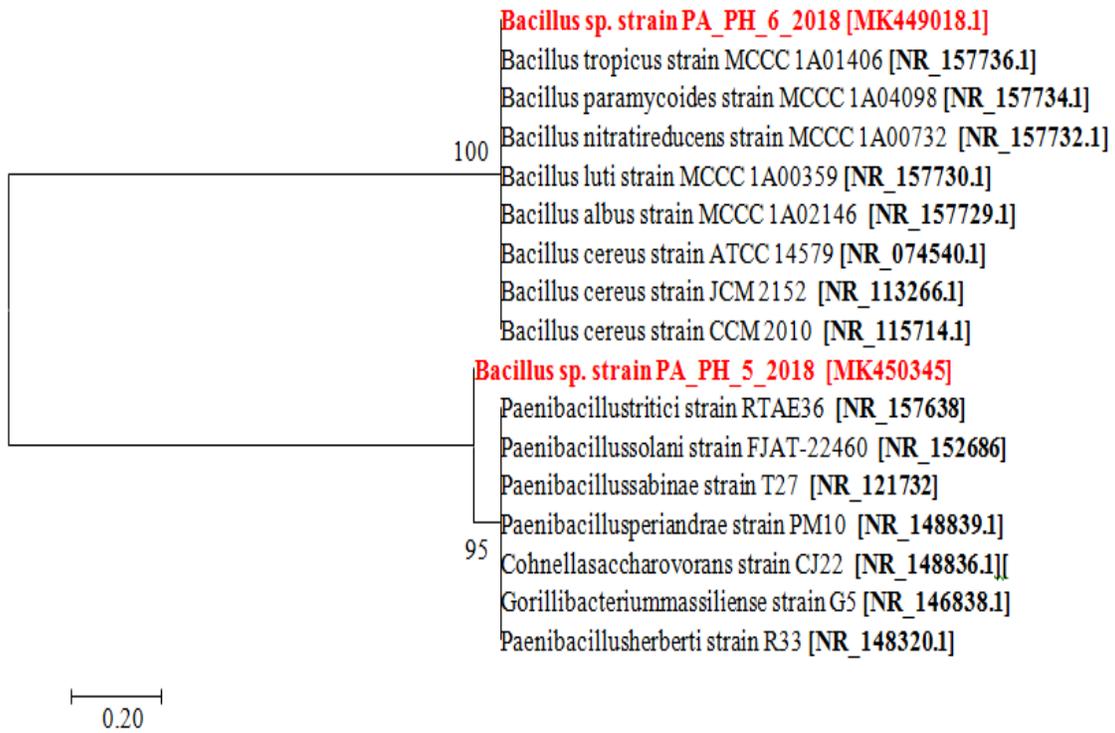
Sample ID	Phenotypic identification result*	BLAST Result(s)	Source	GenBank Accession Number(s)
YFA(7)	<i>Saccharomyces spp</i>	<i>Schizophyllum commune</i>	Cassava	MK431022

WF3(8)	<i>Aspergillus fumigatus</i>	<i>Aspergillus oryzae</i>	Cassava	MK434151
WFB(9)	<i>Neurospora crassa</i>	<i>Aspergillus sydowii</i>	Cassava	MK434152
WFB(5)	<i>Lactobacillus brevis</i>	<i>Bacillus spp</i>	Cassava	MK450345
		Strain PA-PH 5_2018		
WCA(6)	<i>Lactobacillus plantarum</i>	<i>Bacillus spp</i>	Cassava	MK449018
		Strain PA-PH 6_2018		

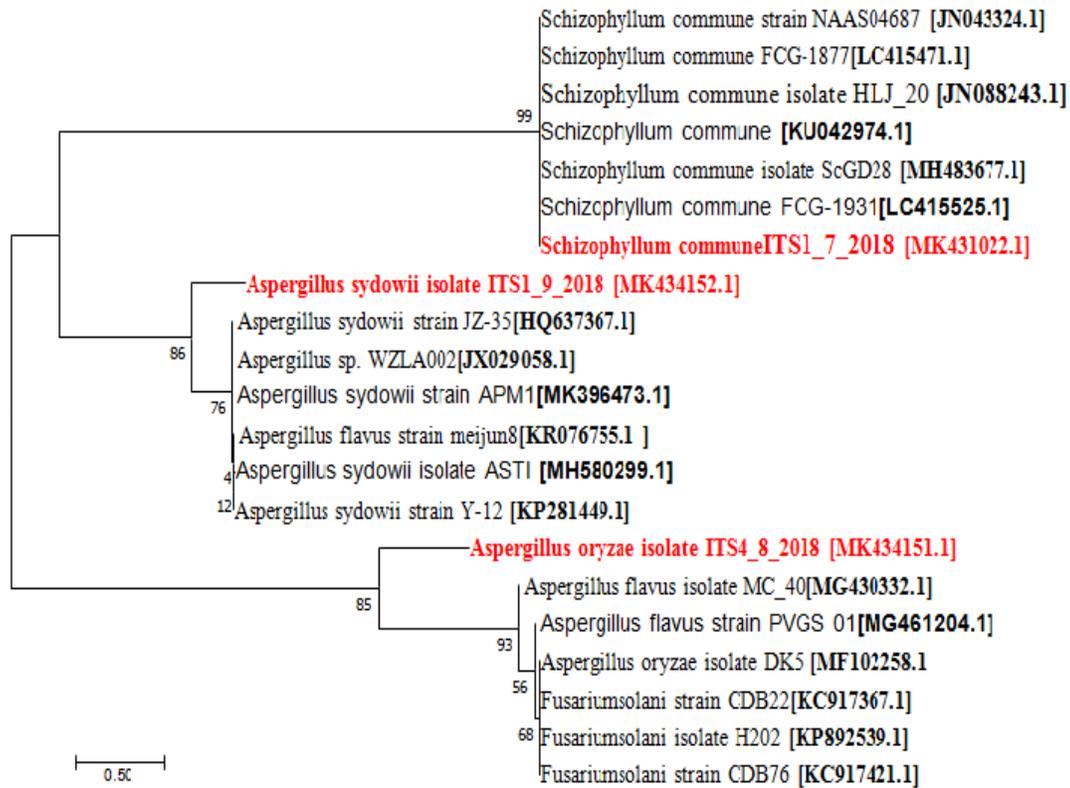
\*Phenotypic identification data from Ayoade et al [4]



Fig. 1. Picture showing agarose gel electrophoresis for 16s rRNA and ITS amplification



**Fig. 2a. Phylogenetic tree showing the relationship between the two distinct *Bacillus* species identified and characterized in the study**



**Fig. 2b. Phylogenetic tree showing the relationship between the three distinct fungal species identified and characterized in the study**

ensure the safe use of such live cultures in mass food production. Quality assurance and safety assessment are paramount issues *in fufu/gari* production as a result of recent reports of the detection of major mycotoxins such as aflatoxin B1 and G1, fumonisin B1 and B2 and zearalenone found to be associated with *fufu/gari* samples [22,23]; it is expedient to know the full identity of organisms used in the deliberate inoculation of food to avoid exposure to the risk of food poisoning.

In a recent study, Ayoade et al, [4] reported the isolation and phenotypic identification of 2 isolates of bacteria (*Lactobacillus brevis* and *L. plantarum*) and 3 isolates of fungi (*Aspergillus fumigatus*, *Neurospora crassa* and *Saccharomyces spp*) as the predominant lactic acid organisms isolated from the spontaneous fermentation of cassava using traditional (biochemical) methods. These same organisms were identified as *Bacillus firmus*, *Bacillus cereus*, *Schizophyllum commune*, *Aspergillus oryzae* and *Aspergillus sydowii* respectively in the present study using molecular methods. Data

from the present study showing wide disparity in identification results when the results from the phenotypic (biochemical) and genotypic (molecular) methods were compared confirms earlier reports that phenotypic identification of microorganisms carries a high risk of misidentification and that genotypic identification using molecular methods are superior to biochemical methods [17,24].

The strains of lactic acid organisms now characterized in the present study are novel since they were not found in the GenBank databases prior but have now been assigned accession numbers. The lactic acid characteristics of similar strains of the organisms can be found in literature. For example, strains of *Bacillus firmus* and *B. cereus* have been recognized and approved for use as probiotics and for the maintenance of gut-health in humans and animals [25]. Moreover, *Schizophyllum commune* is a well known edible higher fungus Nigeria as it is valued for its high nutritional composition [26]. This fungus has also been exploited in the production of cheese due to its

ability to produce both lactate dehydrogenase and other factors that lead to milk-clotting [27].

Fermentation as a metabolic process is known to convert carbohydrates to organics acids and has been used in many cultures over time to increase protein and vitamins content and improve the balance of essential amino acids and to also increase the volatiles organic content and reduce anti-nutrients content in food [14]. Apart from the stated uses, fermentation is known to enhance the aroma and flavor of food by improving the organoleptic properties of fermented food. The aroma and flavor compounds and the peculiar organoleptic properties is known to depend on the microorganisms involved in the fermentation process. The aroma and flavor compounds found in fermented African cassava have reported to include aldehydes, organic acids, alcohol, alkanes, terpenes, ketones, nitrogen compounds among others [28,29]. The results from the present study make it possible to replicate the unique organoleptic properties already established through the use of the organisms now typed by the molecular methods used in this study.

## 5. CONCLUSION

The present study provides evidence of accurate and full characterization of potential starter organisms for industrial scale production of *gari* with the benefit of producing the desired organoleptic characteristics. Moreover, the present study provides the baseline data required for quality assurance and safety assessment of foods in which these organisms may be used in the future.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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