Original Research Article

Molecular characterization of lactic acid organisms isolated from spontaneous fermentation of cassava- *fufu* and *gari*

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 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 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 Ethylenediamintetraacetate (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 firmus* (MK450345) and Bacillus cereus (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.

Key Words: fermented foods, lactic acid bacteria, quality assurance, safety assessment

1.0 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.

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 [12, 13]. 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 [13, 14] 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.

2.0 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 *Lactobacillus 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 µL of PBS using Quick-DNA™Fungal/Bacterial Miniprep kit. For the bacterial isolates, an aliquot of 5 µl of the extracted DNA was used as a template in a 20µl PCR reaction mixture containing illustra™ PuReTaq™ Ready-To-Go™ PCR Beads, 1µl each of forward and reverse primers, and 15µl of double distilled water. The list of primers used including information on the targeted genes and the PCR conditions are shown on Table 1.

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	CTTGGTCATTTAGAGGAAGTAA (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

2.3 Agarose gel electrophoresis and DNA Sequencing

The PCR reaction products were electrophoresed on 2% agarose gel prepared with Tris Borate Ethylenediamintetraacetate (TBE) buffers stained with ethidium bromide An aliquot of 2µl of DNA ladder (100bp) was loaded into the first well after which 4µl the PCR product was mixed with 2µl of loading dye then loaded into subsequent wells. The reaction was run at 90V and 400mA 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.

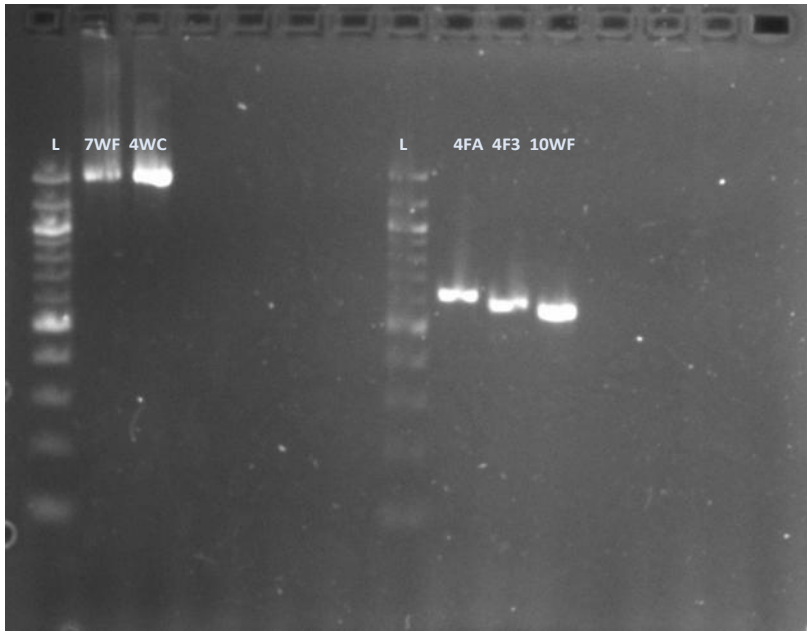
Sequence analysis of PCR products was performed at the International Institute for Tropical Agriculture, Ibadan, Nigeria. Chromatogram of the sequence was viewed using **Geneious** version 11.1.5 (www.geneious.com, [15]) and manual base calling was carried out where necessary. Following manual base calling, 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.

3.0 RESULTS

Identification of lactic acid bacterial and fungal organisms

112 Agarose gel result for bacteria specific 16S rRNA gene amplification confirmed the
113 presence of the two bacterial organisms tested in this study as shown by the presence of DNA
114 band on the agarose gel (Figure 1). Likewise, the presence of DNA bands specific for the gene
115 amplification of the ITS gene (Figure 1) confirmed the presence of the 3 fungal lactic acid tested
116 in this study.

117 As shown in Table 2, a high discrepancy in identification was observed when the results
118 of the phenotypic identification earlier reported in Ayoade et al, [4] was compared with the
119 present identification by molecular methods. With the exception of *Aspergillus oryzae*
120 MK434151 that was earlier identified as *Aspergillus fumigatus*, the identification results differed
121 even at the genus taxa for the other organisms.



Key
L- Ladder (100-1200)
7WF – Lactic acid bacterium
4WC- Lactic acid bacterium
4FA – Lactic acid fungus
4F3 – Lactic acid fungus
10WF- Lactic acid fungus

Figure 1: Picture showing agarose gel electrophoresis for 16s rRNA and ITS amplification

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)	*Saccharomyces spp*	*Schizophyllum commune*	Cassava	MK431022
WF3(8)	*Aspergillus fumigatus*	*Aspergillus oryzae*	Cassava	MK434151
WFB(9)	*Neurospora crassa*	*Aspergillus sydowii*	Cassava	MK434152
WFB(5)	*Lactobacillus brevis*	*Bacillus firmus*	Cassava	MK450345
WCA(6)	*Lactobacillus plantarum*	*Bacillus cereus*	Cassava	MK449018

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

4.0 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 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

160 associated with *fufu*/ *gari* samples [16, 17]; it is expedient to know the full identity of organisms
161 used in the deliberate inoculation of food to avoid exposure to the risk of food poisoning.

162 In a recent study, Ayoade *et al*, [4] reported the isolation and phenotypic identification of 2
163 isolates of bacteria (*Lactobacillus brevis and L plantarum*) and 3 isolates of fungi (*Aspergillus*
164 *fumigatus, Neurospora crassa and Saccharomyces spp*) as the predominant lactic acid organisms
165 isolated from the spontaneous fermentation of cassava using traditional (biochemical) methods.
166 These same organisms were identified as *Bacillus firmus, Bacillus cereus*, *Schizophyllum*
167 *commune*, *Aspergillus oryzae* and *Aspergillus sydowii* respectively in the present study using
168 molecular methods. Data from the present study showing wide disparity in identification results
169 when the results from the phenotypic (biochemical) and genotypic (molecular) methods were
170 compared confirms earlier reports that phenotypic identification of microorganisms carries a
171 high risk of misidentification and that genotypic identification using molecular methods are
172 superior to biochemical methods [14, 18].

173 The strains of lactic acid organisms now characterized in the present study are novel since they
174 were not found in the GenBank databases prior but have now been assigned accession numbers.
175 The lactic acid characteristics of similar strains of the organisms can be found in literature. For
176 example, strains of *Bacillus firmus* and *B cereus* have been recognized and approved for use as
177 probiotics and for the maintenance of gut-health in humans and animals [19]. Moreover,
178 *Schizophyllum commune* is a well known edible higher fungus Nigeria as it is valued for its high
179 nutritional composition [20]. This fungus has also been exploited in the production of cheese due
180 to its ability to produce both lactate dehydrogenase and other factors that lead to milk-clotting
181 [21].

5.0 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.

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Authors' contribution

This work was carried out in collaboration between the authors. Author A (**Dr. Femi Ayoade**) designed the study, performed laboratory analyses and wrote the first draft of the manuscript. Author B (**Scott Fayemi**) ; Author C (**Olumide Olukanni**); Author D (**Tosin Ogunbiyi**); performed laboratory analyses and worked on the manuscript Author E (**Paul Oluniyi**); performed bioinformatic analyses. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests