1 ***Original Research Article***

2 Molecular characterization of lactic acid organisms isolated from spontaneous fermentation 3 of cassava- *fufu* and *gari*

4 ABSTRACT

5 **Aim:** The present work was aimed at ensuring the accurate taxonomic identification of the 6 fermentation organisms previously isolated from spontaneously fermented cassava for the 7 purpose of safety assessment and quality assurance.
8 **Study Design:** Purposive sampling method was used as all the five isolated organisms (from the 9 previous study); 2 bacterial and 3 fungal organisms were characterized using molecular methods.
10 **Place and Duration of Study:** The genotypically characterized organisms are from a previous 11 study carried out in May 2016 where lactic acid bacterial and fungal organisms were isolated 12 from spontaneously fermented cassava.
13 **Methodology:** Genomic DNA was extracted from the lactic acid microorganisms and this was 14 used as a template in a Polymerase Chain Reaction (PCR). The 16s rRNA genes were amplified 15 for the bacterial isolates while nuclear ribosomal internal transcribed spacer (ITS) genes were 16 targeted for the fungal isolates. The amplicons were electrophoresed on 2% agarose gel prepared 17 with Tris Borate Ethylenediaminetetraacetate (TBE) buffers stained with ethidium bromide. The 18 sizes of the corresponding amplicon captured on gel images were compared with that of the 19 ladder to determine their sizes. Furthermore, sequences of the PCR products were analyzed and 20 the chromatograms subjected to BLAST (Basic Local Alignment Search Tool) analyses to 21 identify the lactic acid organisms.
22 **Results:** The 3 fungal isolates were identified as *Schizophyllum commune* (MK 431022), 23 *Aspergillus oryzae* (MK434151), *Aspergillus sydowii* (MK434152), while the bacterial isolates 24 were identified as *Bacillus firmus* (MK450345) and *Bacillus cereus* (MK 449018). A high level 25 of discrepancies was observed when the results of the identification of the same isolates by 26 biochemical methods in an earlier report were compared with the results from the present study 27 using molecular methods.
28 **Conclusion:** The identification and characterization of the isolates and the discrepancies 29 between the outcomes of the biochemical and molecular methods underscore the limitations of 30 phenotypic (biochemical) methods in characterizing organisms that may eventually be used as 31 starter organisms in food fermentation. The present result makes quality assurance and safety 32 assessment of foods prepared using these organisms as starter organisms possible.
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34 **Key Words:** fermented foods, lactic acid bacteria, quality assurance, safety assessment

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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 reduce 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. *Bacillus subtilis* was long considered to be unable to grow in the absence of molecular oxygen as a terminal electron acceptor. However, as in the case of other members of the genus *Bacillus*, the ability of *B. subtilis* to utilize nitrate as an alternative electron acceptor has been described by several groups [27].

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58 On the other hand, accurate taxonomic identification of the fermentation organisms is
59 absolutely essential for safety assessment and assurance when deliberate inoculation of food with
60 starter organisms is to be carried out. The isolated lactic acid microorganisms from that previous
61 study were identified and characterized using phenotypic methods, such as cultural,
62 morphological and biochemical methods alone. Although the use of phenotypic and biochemical
63 tests for identification of microbes have been the traditional standard for many years it is fraught
64 with many problems such as inaccuracy especially in the case of novel organisms that may not
65 be in the databases [12, 13]. For example, in many cases, more than 75% discrepancy rates have
66 been reported in studies where phenotypic and molecular methods were compared for accuracy
67 in identification and characterization of microorganisms [13, 14] due to erroneous results and the
68 interpretation of such results. Other problems of phenotypic identification as a stand-alone
69 method of characterization include lengthy turnaround time and delays and the exorbitant cost of
70 reagents and supplies and labour.

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

78 **2.0 MATERIALS AND METHODS**

79 *2.1 Source of the lactic acid microorganisms*

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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 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 in 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	95°C for 3 min, 94°C for 30 sec, 55°C for 40 sec

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		3' (R)	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

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99 *2.3 Agarose gel electrophoresis and DNA Sequencing*

100 The PCR reaction products were electrophoresed on 2% agarose gel prepared with Tris-
101 Borate Ethylenediaminetetraacetate (TBE) buffers stained with ethidium bromide An aliquot of
102 2µl of DNA ladder (100bp) was loaded into the first well after which 4µl the PCR product was
103 mixed with 2µl of loading dye then loaded into subsequent wells. The reaction was run at 90V
104 and 400mA for 35 mins. Gel images were captured using a gel documentation box and the
105 corresponding amplicon size compared with that of the ladder to determine their size.

106 Sequence analysis of PCR products was performed at the International Institute for
107 Tropical Agriculture, Ibadan, Nigeria. Chromatogram of the sequence was viewed using
108 **Geneious** version 11.1.5 [15] and manual base calling was carried out where necessary.
109 Following manual base calling, the sequence was subjected to BLAST (Basic Local Alignment

110 Search Tool) analysis to identify the organism. These sequences were subsequently submitted to
111 GenBank and accession numbers were assigned.
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113 **3.0 RESULTS**

114 *Identification of lactic acid bacteria and fungal organisms*

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

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

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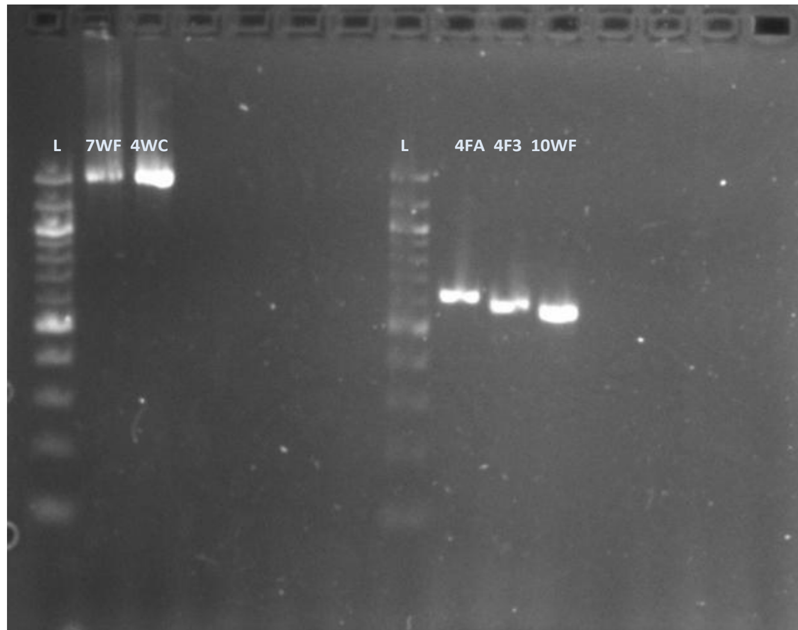

Key
L- Ladder (100-1200)
7WF – Lactic acid bacterium
4WC- Lactic acid bacterium
4FA – Lactic acid fungus
4F3 – Lactic acid fungus
10WF- Lactic acid fungus

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

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

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150 *Phenotypic identification data from Ayoade *et al* [4].
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153 **4.0 DISCUSSION**

154 Traditionally *fufu* and *gari*, food products made from cassava are spontaneously fermented
155 without consideration for the addition of starters. Recent interest in standardizing the
156 fermentation process has led to research efforts focussed on isolation, identification and
157 characterization of the major lactic acid organisms with a view to developing these as viable
158 starter organisms [2, 5]. On the other hand, correct identification of the potential starter
159 organism is vital to any quality assurance and safety assessment plan to ensure the safe use of
160 such live cultures in mass food production. Quality assurance and safety assessment are
161 paramount issues *in fufu/gari* production as a result of recent reports of the detection of major
162 mycotoxins such as aflatoxin B1 and G1, fumonisin B1 and B2 and zearalenone found to be

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

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

176 The strains of lactic acid organisms now characterized in the present study are novel since they
177 were not found in the GenBank databases prior but have now been assigned accession numbers.
178 The lactic acid characteristics of similar strains of the organisms can be found in the literature.
179 For example, strains of *Bacillus firmus* and *B cereus* have been recognized and approved for use
180 as probiotics and for the maintenance of gut-health in humans and animals [19]. Moreover,
181 *Schizophyllum commune* is a well known edible higher fungus Nigeria as it is valued for its high
182 nutritional composition [20]. This fungus has also been exploited in the production of cheese due
183 to its ability to produce both lactate dehydrogenase and other factors that lead to milk-clotting
184 [21-24]. Bacteria isolated from cassava-derived food items and cassava by-products have
185 interesting properties and could potentially be used as probiotics [25]. The use of effluents from

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186 gari and fufu processing led to the isolation of different species of L. fermentum; other strains,

187 however, could be isolated from different effluents [26].

188 5.0 CONCLUSION

189 The present study provides evidence of accurate and full characterization of potential starter

190 organisms for industrial-scale production of gari with the benefit of producing the desired

191 organoleptic characteristics. Moreover, the present study provides the baseline data required for

192 quality assurance and safety assessment of foods in which these organisms may be used in the

193 future.

194 Authors' contribution

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

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201 Competing Interests

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203 The authors declare that they have no competing interests

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