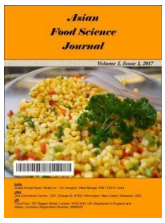

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Disparity in Phenotypic and Genetic Characterization of Lactic Acid Microorganisms Isolated from Spontaneous Fermentation of Yam (*Dioscorea rotundata*)

Femi, Ayoade[1*], Scott Fayemi[1], Tosin Ogunbiyi[1,2], Olumide Olukanni[3] and Paul Oluniyi[1,2]

[1]Department of Biological Sciences, College of Natural Sciences, Redeemer's University, P.M.B. 230, Ede, Osun State, Nigeria.
[2]African Center of Excellence for the Genomics of Infectious Diseases (ACEGID), Redeemer's University, P.M.B. 230, Ede, Osun State, Nigeria.
[3]Department of Chemical Sciences, College of Natural Sciences, Redeemer's University, P.M.B. 230, Ede, Osun State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author FA designed the study, was involved in the laboratory and wrote the first draft of the manuscript. Authors SF and TO were involved in laboratory studies while author OO was involved in manuscript processing. Author PO was involved in the analysis of bioinformatics data. All authors read and approved the final manuscript.

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ABSTRACT

Aim: In order to ensure the accurate taxonomic identification of the lactic acid organisms that were previously isolated from spontaneously fermented yam for safety assessment and quality assurance purposes, phenotypic and genetic identification data were compared.
Study Design: Using the purposive sampling method, four microorganisms were characterized using molecular methods.
Place and Duration of Study: Isolates of lactic acid microorganisms (2 bacterial and 2 fungal

**Corresponding author: Email: ayoadef@run.edu.ng;*

organisms) from spontaneously fermented yam in a previous study carried out in May 2016 were genetically identified using molecular methods.
Methodology: Genomic DNA extracted from the test lactic acid microorganisms were used as templates in a PCR reaction, then, 16s rRNA and nuclear ribosomal internal transcribed spacer (ITS) genes were amplified for the bacterial and fungal isolates respectively. The polymerase chain reaction (PCR) products were electrophoresed on 2% agarose gel prepared with Tris Borate Ethylenediamintetraacetate (TBE) buffers stained with ethidium bromide. Subsequently, the ladder was used in order to determine the sizes of the corresponding amplicons captured on gel images in comparisons. Moreover, 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 2 bacterial isolates were identified as *Bacillus subtilis* (MK448227) and *Bacillus pumilus* (MK446418), on the other hand, the fungal isolates were identified as *Aspergillus flavus* (MK433604) and *Aspergillus niger* (MK430926). Discrepancies were observed when phenotypic identification data in an earlier report were compared with the molecular data from the present study.
Conclusion: The present results underscore the limitations of phenotypic (biochemical) methods in characterizing organisms, particularly, organisms that may end up being used in food processing. Moreover, this is the first report of the novel organisms reported in the present study and makes further work into the development of starter organisms for the production of *amala* possible in the near future. In addition, proper identification helps in benchmarking the quality assurance and safety assessment of foods prepared using these organisms.

Keywords: Spontaneous fermentation; lactic acid fungi; quality assurance; safety assessment.

1. INTRODUCTION

Yam (*Dioscorea spp*) is an important staple food crop in the tropics, mainly in Africa, Americas, Caribbean, South Pacific and Asia [1]. Nigeria is the foremost yam producing country in the world accounting for over 65%, representing 38 million metric tonnes of the world production and it is more widely distributed and readily available than cereals [2,3]. White yam (*Dioscorea rotundata*) is the most cultivated yam and it is processed into a wide range of products such as yam flour (*poundo-yam*), yam gruel (*amala*), fried yam (*dundu*), fried grated-spiced yam (*ojojo*) and other products [4].

Yam is predisposed to high risk of post-harvest loss to pests and diseases due to its high moisture content, ranging between 65-85% of tuber weight [5]. In order to reduce these losses of yams in most West African countries, yams are cut into 20-30 mm slices, pre-heated in water, steeped and allowed to ferment for about 24 hours, then dried to form an intermediate form known as "*gbodo*". *Gbodo* is subsequently milled into yam flour (*elubo*) which is stirred in boiling water into a paste known as "*amala"*. *Amala* is usually eaten with different types of vegetable stew [6]. Using the conventional culture dependent method, many bacterial lactic acid fermentation organisms belonging to the genera *Lactobacillus, Weissella, Leucostoc, Lactococcus* and *Bacillus* have been isolated from yam fermentation, moreover, different species of lactic acid fungi, belonging to the following genera including *Aspergillus, Rhizopus, Neurospora* etc are found in literature [7,8,9,10].

Fermentation is a process that is used in most cultures for food preservation, in addition, the process is known to make food more flavourful while enriching the nutritional value of food [7]. In recent years, attempts have been made to isolate the fermentation organisms from spontaneously fermented indigenous African foods with the aim of using these as starter organisms and to standardize the fermentation processes [11]. On the other hand, it is important to use starter microorganisms that are "Generally Regarded as Safe" (GRAS) for fermentation in order to guarantee food safety and for quality assurance purposes [4].

In a previously published study, Ayoade et al. [8] isolated four lactic acid fermentation organisms from spontaneous yam fermentation in the process of making *elubo* and subsequently *amala*; the finished food product with specific organoleptic preferences. These organisms were identified and characterized using phenotypic methods such as cultural, morphological and biochemical methods alone. Even though these

phenotypic methods meet microbiological standards, they are limited in accuracy, particularly in the cases of novel organisms that may neither be in the databases nor found in literature [12,13]. The problems of lengthy turnaround time, delays and exorbitant cost of reagents and labour costs plague phenotypic characterization as stand-alone methods for accurate identification and characterization of microorganisms. Moreover, high discrepancy rates, in some cases, more than 75% have been reported in studies where phenotypic and molecular methods were compared for accuracy in the identification and characterization of microorganisms has been reported [13,14].

The present work is focused on characterizing lactic acid bacterial and fungal organisms previously isolated from spontaneously fermented yam using molecular techniques by sequencing the 16s rRNA genes for the bacterial and nuclear ribosomal internal transcribed spacer (ITS) genes for the fungal isolates. This work makes available the data needed to confirm the accurate taxonomic identity of the isolated fermentation organisms that were earlier reported and provides the benchmark data to assess these organisms as safe and may be used in quality assurance tests for these potential starter organisms.

2. MATERIALS AND METHODS

2.1 Source of the Lactic Acid Microorganisms

In a previous work, two lactic acid fermenting fungi isolated from 4 varieties of spontaneously fermented yam were identified (using phenotypic characteristics) as *Aspergillus flavus* and *A niger*. Similarly, 2 strains of *Lactobacillus brevis* were phenotypically identified using biochemical (phenotypic) methods in the same work. These organisms were reported to produce final food products with characteristically unique organoleptic properties.

2.2 DNA Extraction, PCR Amplification and Fragment Purification

Using Quick-DNA™Fungal/Bacterial Miniprep kit, genomic DNA was extracted from approximately 100 mg fungal or bacterial cells that have been resuspended in 200 µL of PBS. 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 for the bacterial and fungal isolates. The list of primers used including information on the targeted genes and the PCR conditions are shown on Table 1 [12].

2.3 Agarose Gel Electrophoresis and DNA Sequencing

Electrophoresis was carried out on the PCR reaction products on 2% agarose gel prepared with Tris Borate Ethylenediamintetraacetate (TBE) buffers stained with ethidium bromide 4 µl the PCR product was mixed with 2 µl of loading dye then loaded into wells and these were compared with an aliquot of 2 µl of DNA ladder (100 bp) which was loaded into the first well [12]. The reaction was run for 35 mins at 90V and 400 mA. Gel images were captured using gel documentation box and stored on file.

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

PCR products were analyzed by sequencing. The analysis 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]). The sequence was subjected to BLAST (Basic Local Alignment Search Tool) analysis to identify the organism subject to manual base calling was carried out where necessary. These sequences were subsequently submitted to GenBank and accession numbers were assigned.

3. RESULTS

3.1 Identification of Lactic Acid Bacterial and Fungal Organisms

Fig. 1 shows the presence of DNA band on the agarose gel for bacteria specific 16S rRNA gene amplification, confirming the presence of the two bacterial organisms tested in this study. Moreover, the presence of DNA bands specific for the gene amplification of the ITS gene (Fig. 1) confirmed the presence of the 2 fungal lactic acid organisms tested in this study.

Table 2 shows a conflict in the identification data when phenotypic and molecular methods were compared in the identification process. The identities of the two bacterial isolates differed completely when the molecular and biochemical results were compared. On the other hand, the phenotypic identification data correlated well with the molecular identification data for the fungal organisms, moreover, the strains of fungi were unique and were registered subsequently submitted to GenBank and accession numbers were assigned.

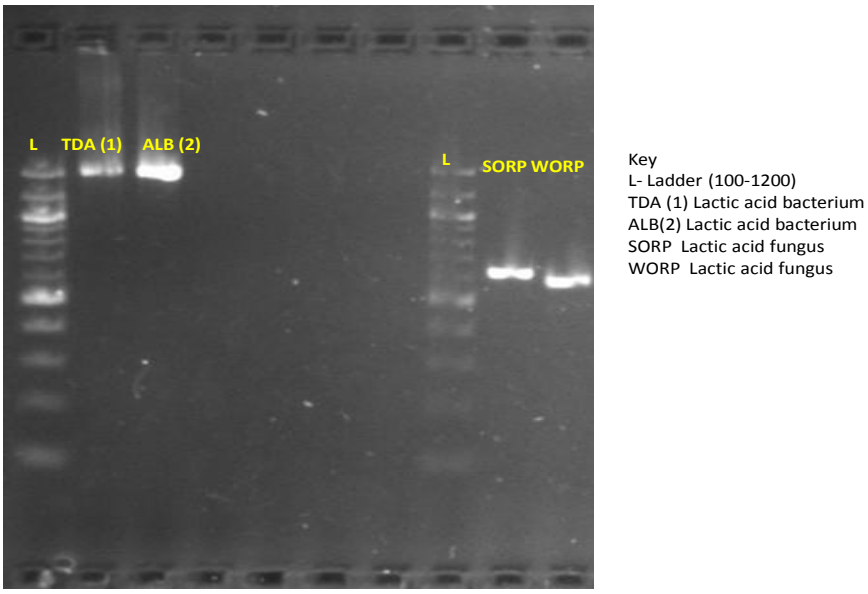

Fig. 1 Picture showing agarose gel electrophoresis for 16s rRNA and ITS amplification

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)
SORP	*Aspergillius flavus*	*Aspergillius flavus*	Yam	MK433604
WORP	*Aspergillus niger*	*Aspergillus niger*	Yam	MK430926
TDA(1)	*Lactobacillus brevis*	*Bacillus subtilis*	Yam	MK448227
ALB(2)	*Lactobacillus brevis*	*Bacillus pumilus*	Yam	MK446418

**Phenotypic identification data from Ayoade et al. [8]*

4. DISCUSSION

In an attempt to standardize the fermentation of traditional African food products such as *amala*, made from spontaneously fermented yam, research efforts into the isolation, identification and characterization of the predominant lactic acid fermenters is becoming commonplace. This is with the view of possibly developing these into viable starter organisms in processing of these food products [16,17,18,19]. However, accurate identification of these potential starter organisms is of paramount importance considering that these organisms may eventually end up in food production. Recent interest in standardizing the fermentation process has led to research efforts focused on isolation, identification and characterization of the major lactic acid organisms with a view to developing these as viable starter organisms. 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. 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 *elubo* (yam flour) samples [20,21,22] makes it necessary to ensure that the organisms that may end up being used in the preparation of food are well characterized in order to avoid the risk of food poisoning.

Ayoade et al. [8] recently reported the isolation and phenotypic identification of 2 strains of *Lactobacillus brevis* and 2 isolates of lactic acid fungi (*Aspergillus flavus and A niger*) as the predominant lactic acid organisms isolated from the spontaneous fermentation of yam using traditional (biochemical) methods. These same organisms were identified as *Bacillus subtilis, B pumilus*, *Aspergillus flavus* and *Aspergillus niger* respectively in the present study using molecular methods. Data from the present study showing 50% 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 [14,23].

The novel strains of lactic acid organisms characterized in the present study have been assigned accession numbers. Similar organisms as those reported in the present work are 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 [24,25]. Moreover, many fungal species belonging to the genus *Aspergillus* have been reported in indigenous fermented food processing and in spontaneously fermented yam in particular [26,27].

Many members of the Genus *Bacillus* have been noted to be involved in yam fermentation and in the production of *elubo* (yam flour) including other fermented indigenous African foods [28, 29]. For example, Adetunji and Olaoye [30] reported up to forty bacteriocin-producing strains of *Bacillus sp* isolated from yam flour (elubo) that showed antimicrobial activity against laboratory stocks of pathogenic bacteria including *Salmonella enteritidis*, *Micrococcus luteus* and *Staphylococcus aureus*. Moreover, lactic acid bacteria including many members of the *Bacillus sp* that were isolated from food have been demonstrated to produce antimicrobial peptides including bacteriocins and bacteriocin-like substances [31], these substances may be accountable for the preservative effect of fermentation on the fermented food.

In addition, Oboh et al. [32,33] reported the effectiveness of strains of three species of *Aspergillus*, namely, *A niger*, *A flavus* and *A fumigatus* in reducing the anti-nutrient factors in fermented cassava as the resulting final food product had lower phytate content than the unfermented samples.

5. CONCLUSION

The present work provides evidence the baseline data required for quality assurance and safety assessment of novel lactic acid organisms that may eventually be used as starter organisms for industrial scale production of *elubo* with the benefit of producing the desired organoleptic characteristics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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