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Microbiological Analysis and Molecular Characterization of Bacterial and Fungal Isolates Present in Exposed and Packaged Cassava, Plantain and Yam Flour Sold in Selected Markets in Port Harcourt, Rivers State, Nigeria

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Abstract In Nigeria, increasing cases of food borne diseases especially diarrhea reported by many families has been linked to consumption of microbial contaminated flour based meals. Exposed and packaged cassava, yam and plantain flour are locally available in our markets. In this study, standard microbiological methods were used to isolate and identify bacterial and fungal isolates from the flour samples. Further characterization of the isolates was done using molecular methods. Our results shows that *Bacillus* sp. (46.67 %), *Staphylococcus* sp. (40 %), E*scherichia coli* (10 %) and *Salmonella* sp. (3.33 %) is the percentage frequency of occurrence of bacterial isolates; *Microsporum audouinii* (14.08 %), *M. canis* (2.82 %), *M. nanum* (5.63 %), *Exserohilum* sp. (9.86 %), *Trichoderma* sp. (7.04 %), *Candida tropicalis* (5.63 %), *C. rugosa* (9.9 %), *C. krusei* (2.82 %) *C. glabrata* (5.63 %), *Aspergillus fumigatus* (4.23 %), *A. flavus* (1.41 %), *A. terreus* (2.83 %), *A. versicolor* (1.41 %), *A. clavatus* (2.82 %), *A. niger* (5.63 %), *Phaeoacremonim* sp. (1.41 %), *Epicoccum* sp. (2.82 %), *Exophiala dermatitidis* (1.41 %), *Penicillium* sp. (1.41 %), *Cokeromyces* sp. (2.82 %), *Aureobasidium* sp. (1.41 %), *Rhodotorula* sp. (2.82 %), *Fonsecaea pedrosoi* (1.41 %) and *Phoma* sp. (2.82 %) are percentage frequency of occurrence of fungal isolates. Molecular characterization revealed the bacterial isolates to be *Bacillus megaterium* strain WSH10 16S, *Enterobacter* sp. strain HZ21, *Alcaligenes feacalis* strain CGAPGPBS and *Acinetobacter junii* strain SB132 while the fungal isolates are *Aspergillus niger* strain NI26, *Paecilomyces sinensis* strain Gr133 and *Tramestes polyzona* strain CNRMA14.236. It is recommended that edible flours should be produced under strict hygienic condition and packaged to prevent microbial contamination of the products.

Keywords: *packaged, exposed, flour, molecular characterization, microbiological analysis*

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1. Introduction

Plantain, cassava and yam are high starchy staple foods consumed by many families in Nigeria. These staples are highly perishable because of its high moisture content which supports microbial spoilage. Therefore, they are usually processed into edible flours which have reduced moisture content and longer shelf life than freshly harvested yam, plantain and cassava. Low moisture content of edible flour is unfavourable to support growth of microorganisms [1,2,3].

Cassava, yam and plantain as edible flours have different food applications [2,4,5]. Apart from its nutritional importance mainly in providing energy to human body, cassava and yam could be beneficial to human health as a result of hypocholesterolemic, antioxidative, hypoglycemic, immunomodulatory and antimicrobial activities of bioactive constituents in the tubers [6]. In Nigeria, some orthodox and traditional medical personnel recommend plantain flour as a diet that can help diabetic patients manage their health condition [7].

Notwithstanding low water activity of yam, cassava and plantain flour, contamination of these edible flours by pathogenic and non-pathogenic microorganisms could occur during processing [8,9]. Unhygienic handling of these edible products and undue expose to environment during retailing also predisposes edible flours to microbial contamination. In Nigerian markets, edible flours especially

the ones produced by local farmers are usually exposed during retailing while others produced by cottage industries are usually packaged. Consumption of flour-based products could lead to outbreak of diseases such as diarrhea despite application of heat in the form of baking and cooking at the point where flour is used for food production [7,10].

In most published works, microbiological quality of edible flours such as cassava, plantain and yam flour were determined using conventional methods [9,11,12]. Recently, Odu et al. [13] carried out a study that investigated the microbiological quality of packaged and exposed cassava, yam and plantain flour sold in markets and supermarkets in Port Harcourt Nigeria. They reported the presence of coliforms, *Escherichia coli*, *Salmonella* sp., *Staphylococcus* sp., *Bacillus* sp. and fungi in the flour samples. Using conventional method, Odetunde *et al.* [14] isolated *Flavobacterium* sp., *Micrococcus* sp., *Bacillus subtilis*, B. *polymyxa*, B. *cereus* and *Esherichia coli* from cassava flour. In a related study, Ajayi [15] detected *Escherichia coli*, *Klebsiella* sp., *Bacillus cereus*, *B. globisporus*, *B. circulans* and *Enterococcus* spp. in dry plantain flour. Somorin *et al.* [12] reported that *Bacillus megaterium*, *Staphylococcus saprophyticus*, *Fusarium oxysporum*, *Aspergillus niger* and *Rhizopus nigricans* were present in flour obtained from water yam and white yam. However, their method of identifying microorganisms has its limitations. Molecular identification methods have proven to be more reliable and advantageous than culture-based methods in food safety microbiology [16,17,18]. So far, there are limited studies that involved molecular methods in identification of potentially pathogenic microorganisms in cassava, plantain and yam flour placed in the markets and supermarkets for public consumption [7].

Therefore, this study is aimed at microbiological analysis and the use of molecular methods to identify bacteria and fungi present in exposed and packaged cassava, yam and plantain flour available in some open markets and supermarkets in Port Harcourt metropolis, Rivers State, Nigeria.

2. Materials and Methods

Five edible flour samples each of packaged cassava, yam and plantain flour totaling fifteen (15) samples were obtained from three supermarkets in Port Harcourt, Nigeria. Similarly, fifteen (15) plastic containers already sterilized were used to separately put five samples each of exposed cassava, plantain and yam flour purchased from fifteen retailers in three popular open markets within Port Harcourt metropolis. All the flour samples were taken to Food and Industrial Microbiology Laboratory, University of Port Harcourt where analysis were conducted.

2.1. Microbiological Analysis

Microbiological analysis of the flour samples were carried out using the methods described by Odu *et al*. [19] including that of Eman and Sarifar [20]. Presence of *Salmonella* sp. in the flour samples were ascertained using APHA method [21]. Identification of bacterial isolates was based on the methods described by Cheesbrough [22] while that of fungal isolates were made possible using the method described by Frazier and WestHoff [23].

2.2. Bacterial DNA Extraction

DNA extraction method as described by Chikere and Ekwuabu [24] was adopted. Five milliliter (5 ml) of an overnight broth culture of bacteria isolates in Luria bertani (LB) broth was spun using centrifuge at 1400 rpm for 3 min; the cell was suspended in 500 µl of normal saline and heated at 95°C for 20 min. The suspended bacteria already heated was fast cooled on ice and spun at 1400 rpm for 3 min. The supernatant containing DNA was transferred into 1.5 ml microcentrifuge tube and stored at -20°C. Nanodrop 1000 spectrophotometer was used to quantify the extracted genome.

2.3. Fungal DNA Extraction

Extraction of fungal DNA was done using a ZR fungal/bacterial DNA miniprep extraction kit supplied by Inqaba South Africa. Pure culture of fungal isolates which displayed heavy growth was suspended in 200 µL of isotonic buffer into a ZR bashing bead lysis tubes and 750 µL lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 mins. The ZR bashing bead lysis tubes were centrifuged at 10,000x*g* for 1 min. Four hundred (400) microlitres of supernatant was transferred to a Zymo-Spin IV spin filter (orange top) in a collection tube and centrifuged at 7000x*g* for 1 min. One thousand two hundred (1200) microlitres of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 µL. Exactly 800 µL was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 1 min. The flow through was discarded from the collection tube and the remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microlitre of the DNA Pre-WAS buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 1 min followed by the addition of 500 µL of fungal DNA Wash Buffer and centrifuged at 10,000x*g* for 1 min. The Zymo-spin IIC column was transferred to a clean 1.5 µL centrifuge tube, 100 µL of DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* for 30 sec to elute DNA. The ultra pure DNA was then stored at -20 ^{o}C for other downstream reaction.

2.4. Internal Transcribed Space (ITS) Amplification

Aided by ABI 9700 Applied Biosystems thermal cycler, ITS region of rRNA genes of the bacterial isolates were amplified using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers at a final volume of 50 µL for 35 cycles. The polymerase chain reaction (PCR) mix was made up of X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, dNTPs, $MgCl_2$), primers at a concentration of 0.4 M and extracted DNA which was the template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 min; denaturation, 95°C for 30 sec; annealing, 53°C for 30 sec; extension, 72°C for 30 sec and final extension, 72°C for 5 min. The product was resolved on a 1.5 % agarose gel at 120 V for 15 min and then visualized using UV transilluminator.

2.5. 16S rRNA Amplification

The 16S rRNA region of the rRNA genes of the isolates were amplified using the 27F and 1492R primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µL for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, dNTPs, $MgCl_2$), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 min; denaturation, 95°C for 30 sec; annealing, 52°C for 30 sec; extension, 72°C for 30 sec for 35 cycles and final extension, 72°C for 5 min. The product was resolved on a 1 % agarose gel at 120 V for 15 min and visualized on a UV transilluminator.

2.6. Sequencing

BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa was used for sequencing amplified 16S rRNA of the isolates.

2.7. Phylogenetic Analysis

The sequences obtained were edited using the Bioinformatics Algorithm Trace Edit. Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 [25]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed [26]. Jukes-Cantor method was used to compute the evolutionary distances [27].

3. Results

Table 1 shows the colonial morphology and biochemical characteristics of *Salmonella* sp., *Staphylococcus* sp., *Bacillus* sp. and *Escherichia coli* isolated from packaged and exposed cassava, yam and plantain flour. Frequency of occurrence of bacterial isolates from all the flour samples presented in Figure 1 shows that *Bacillus* sp. (46.67 %) was predominant but *Salmonella* sp. (3.33 %) had the least frequency of occurrence. The characteristics of fungi isolated from exposed and packaged cassava flour is presented in Table 2 and Table 3, respectively. In Table 4 and Table 5, the characteristic of fungi isolated from exposed and packaged plantain flour, respectively is reported. Similarly, Table 6 and Table 7 describes the characteristics of fungi isolated from packaged and exposed yam flour. Frequency of occurrence of fungal isolates from packaged and exposed cassava, yam and plantain flour is stated in Figure 2. Agarose gel electrophoresis showing the amplified 16S rRNA and ITS (600bp) of the bacterial and fungal isolates is reported in Figure 3 and Figure 4, respectively. Table 8 shows the result of presumptive and molecular characterization of isolates from exposed and packaged cassava, yam and plantain flour. Phylogenetic tree showing evolutionary relationship between the fungal and bacterial isolates is reported in Figure 5 and Figure 6, respectively.

Table 1. Colonial morphology and biochemical characteristics of bacterial isolate from packaged and exposed edible flour

Isolate code	Media	Cell morphology	Gram reaction	Catalase	Citrate	Oxidase	Motility	H_2S	Gas	Starch hydrolysis	Indole	MR	VP	Spore former	Glucose	Sucrose	Lactose	Slant	Butt	Probable organism
EDCP1	NA	Rod	+	+	+	-	+	-	-	+	+	-	-	+	A	A/G	-	B	B	*Bacillus* sp.
SRCP2	NA	Rod	+	+	-	+	+	-	-	+	+	+	+	+	A/G	A/G	-	B	B	*Bacillus* sp.
OMP3	NA	Rod	+	-	-	+	+	-	-	+	+	+	-	+	A	A/G	-	B	B	*Bacillus* sp.
MTY5	NA	Rod	+	+	-	+	-	-	-	+	-	+	-	+	A	A/G	-	B	B	*Bacillus* sp.
EDYP1	NA	Rod	+	+	-	+	+	-	-	+	-	+	+	+	A	A/G	-	B	B	*Bacillus* sp.
SRYP2	NA	Rod	+	-	-	+	+	-	-	+	+	-	-	+	A	A/G	-	B	B	*Bacillus* sp.
SRPP2	NA	Rod	+	+	+	+	+	-	-	+	+	-	+	+	A	A/G	-	A	B	*Bacillus* sp.
SLPP5	NA	Rod	+	-	-	+	+	-	-	+	+	+	-	+	A	A/G	-	A	B	*Bacillus* sp.
MTC2	NA	Rod	+	-	-	-	+	-	-	+	-	+	+	+	A	A/G	-	B	B	*Bacillus* sp.
EDYP2	NA	Rod	+	-	+	-	-	-	-	+	+	+	-	+	A	A/G	-	A	A	*Bacillus* sp.
SRYP2	MSA	Cocci	+	+	+	-	+	-	-	+	+	-	+	-	A/G	A/G	-	B	B	*Staphylococcus* sp.
MTC5	MSA	Cocci	+	+	-	-	+	-	-	+	+	-	+	-	A/G	A/G	-	B	B	*Staphylococcus* sp.
OMC	MSA	Cocci	+	-	-	-	+	-	-	+	+	-	-	-	A	A/G	-	A	A	*Staphylococcus* sp.
SRCP3	MSA	Cocci	+	+	-	-	+	-	-	+	+	+	-	-	A	A/G	-	B	B	*Staphylococcus* sp.
EDCP1	MSA	Cocci	+	-	-	+	-	-	-	+	+	-	+	-	-	A/G	-	A	B	*Staphylococcus* sp.
MTP2	MSA	Cocci	+	+	-	-	+	-	-	+	+	+	-	-	A	A/G	-	A	B	*Staphylococcus* sp.
ROP1	MSA	Cocci	+	+	+	+	+	-	-	+	+	+	-	-	AG	A/G	A/G	A	B	*Staphylococcus* sp.
MTY5	MSA	Cocci	+	+	-	+	-	-	-	-	+	+		-	A	A/G	-	B	B	*Staphylococcus* sp.
SRPP3	MSA	Cocci	+	+	-	-	+	-	-	+	+	-	+	-	A	A/G	-	B	B	*Staphylococcus* sp.
EDPP1	MSA	Cocci	+	+	+	+	-	-	-	+	-	+	-	-	AG	A	-	A	A	*Staphylococcus* sp.
SRYP3	MSA	Cocci	+	-	+	+	-	-	-	+	-	+	-	-	AG	A	-	B	B	*Staphylococcus* sp.
ROY1	MSA	Cocci	+	-	-	+	+	+	-	+	-	+	-	-	AG	A	-	A	B	*Staphylococcus* sp.
SRYP3	MSA	Cocci	+	+	-	+	-	-	-	+	+	-	-	-	AG	A/G	A/G	A	A	*Staphylococcus* sp.
OMP3	SS	Cocci	-	+	+	+	-	-	+	+	-	+	-	-	AG	A/G	A/G	A	A	*Salmonella sp.*
OMY3	EMB	Short rod	-	+	-	-	+	-	-	+	+	-	-	-	AG	A/G	A	A	A	*Escherichia coli*
ROY2	EMB	Short rod	-	+	-	+	+	-	+	+	-	-	-	-	AG	A/G	A/G	A	A	*Escherichia coli*
ROY	EMB	Rod	-	+	-	+	+	-	+	+	-	-	-	-	AG	A/G	A/G	A	A	*Escherichia coli*
OMC	NA	Rod	+	+	+	+	+	-	-	+	+	+	+	+	A	A/G	-	B	B	*Bacillus* sp.
MTP1	NA	Rod	+	+	-	+	+	-	-	+	-	+	-	+	AG	A/G	A/G	A	A	*Bacillus* sp.
ROY2	NA	Rod	+	-	-	-	+	-	+	-	-	+	-	+	AG	A/G	A	A	A	*Bacillus* sp.

Glucose fermentation; TSI - Triple sugar iron agar; S - Slant; B - Butt; I - Indole; M - Methyl red; V - Voges Proskauer; C - Citrate; NA - Nutrient agar; EMB - Eosin methylene blue agar; MAC - MacConkey agar, SSA - *Salmonella/Shigella* agar, AG - Acid and Gas. ED, SR and SL represent supermarkets; OM, RO and MT represent open markets; C - Exposed cassava flour; P - Exposed plantain flour; Y - Exposed yam flour; PP - Packaged plantain flour; CP - Packaged cassava flour; YP - Packaged yam flour.

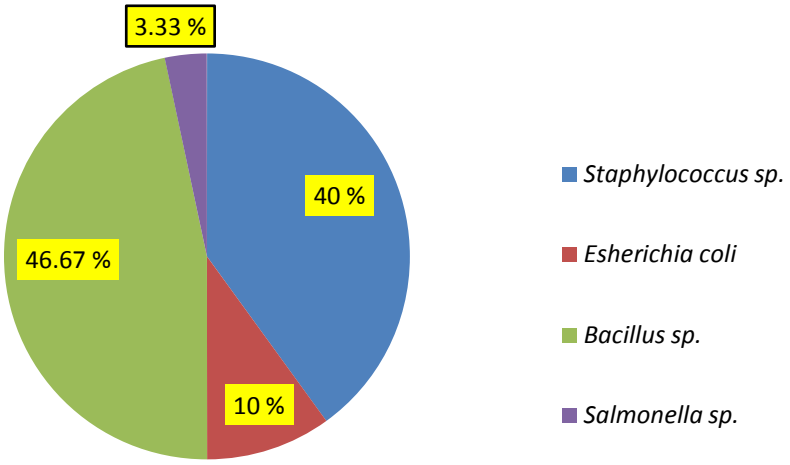

Figure 1. Percentage frequency of occurrence of bacterial isolates

Table 2. Characteristics of fungi isolated from exposed cassava flour

Isolate code	Macroscopy	Microscopy	Presumptive identification
ROC1	Creamy, velvet sough colony	Non-septate hyphae with branched rough conidiophore	*Phaeoacremonium parasiticum*
ROC 2	Black colony	Septate, cream erect hyphae with long conidiophores, black cluster conidia	*Aspergillus niger*
OMC	White dry rough colony	Oval yeast cells	*Candida krusei*
	White flat over agar plate	Septate hyphae with short branched conidiophores containing conidia	*Trichoderma* sp.
MTC 1	Creamy velvet	Septate hyphae, no conidia	*Microsporum audouinii*
	White flat over agar plate	Septate hyphae with short branches of conidiophores containing conidia	*Trichoderma* sp.
MTC2	Creamy velvet	Septate hyphae, no conidia	*Microsporum audouinii*

Key: RO, OM and MT represent open markets; C - Exposed cassava flour.

Table 3. Characteristics of fungi isolated from packaged cassava flour

Isolate code	Macroscopy	Microscopy	Presumptive identification
EDCP1	Creamy velvet	Septate hyphae, no conidia	*Microsporum audouinii*
	White velvet	Septate hyphae with large conidia rough and egg shape	*Microsporum nanum*
	Black colony	Septate cream erect hyphae with long conidiophores having black cluster conidia	*Aspergillus niger*
EDCP2	Creamy velvet	Septate hyphae, no conidia	*Microsporum audouinii*
SRCP1	Grayish velvet colony	Septate hyphae with dispersed conidia, no conidiophores	*Phoma* sp.
SRCP2	Grayish velvet colony	Septate hyphae with dispersed conidia, no conidiophores	*Phoma* sp.
	White fluffy cover on agar plate	Septate hyphae with short branches. Conidiopores containing conidia	*Trichoderma* sp.
SLCP	Darkish green with velvet colony, black reverse	Septate hyphae with branched conidiophores housing the conidia	*Fonsecaea pedrosoi*
	White velvet	Septate hyphae with large conidia rough and egg shape	*Microsporum nanum*

Key: ED, SR and SL represent supermarkets; CP - Packaged cassava flour.

Table 4. Characteristic of fungi isolated from exposed plantain flour

Isolate Code	Macroscopy	Microscopy	Presumptive Identification
ROP1	Pink colony	Elongated oval budding cells, no hyphae.	*Rhodotorula* sp.
	Grayish velvet	Septate hyphae, no conidia	*Microsporum audouinii*
	Dark gray cotton colony with black reverse.	Dark septate hyphae with elongated conidiophores housing long conidia	*Exserochilum* sp.
ROP2	Pink colony	Elongated oval budding cells, no hyphae.	*Rhodotorula* sp
OMP	White flattened cover on agar plate.	Septate hyphae with short branched conidiophore containing the conidia	*Trichoderma* sp.
	Creamy colony	Septate hyphae on conidia	*Candida rugosa*
MTP1	Pink colony	Elongated oval budding cells, no hyphae	*Rhodotorula* sp.
	White dry rough colony	Oval yeast cells	*Candida krusei*
	Shiny, smooth creamy colony	Oval yeast cells	*Candida tropicalis*
	White velvet	Septate hyphae with numerous large conidia	*Microsporum canis*
	White colony with dark reverse	Yeast like budding cells with very short hyphae.	*Aureobasidium pullulans*
	White colony with yellow at central area with brown reverse	Septate hyphae with sporangiosphores in a round vesicle	*Cokeromyces recurvatus*
MTP 2	White velvet	Septate hyphae with numerous large conidia	*Microsporum canis*

Key: RO, OM and MT represent open markets; P -Exposed plantain flour.

Table 5. Characterization of fungi isolated from packaged plantain flour

Isolate code	Macroscopy	Microscopy	Presumptive Identification
EDPP 1	Yellow/Green	Septate hyphae with brush like conidiophores	*Penicillium* sp.
EDPP 2	Grayish velvet	Septate hyphae no conidia	*Microsporum audouinii*
SRPP 1	Grayish velvet	Septate hyphae no conidia	*Microsporum audouinii*
SRPP 2	Grayish velvet	Septate hyphae no conidia	*Microsporum audouinii*
SLPP	Grayish velvet	Septate hyphae no conidia	*Microsporum audouinii*

Key: ED, SR and SL represent supermarkets; CP - Packaged plantain flour.

Table 6. Characteristic of fungi isolated from packaged yam flour

Sample code	Macroscopy	Microscopy	Presumptive identification
EDYP1	White velvet	Septate hyphae with numerous large conidia	*Microsporum canis*
	Creamy colony	Septate hyphae no conidia	*Candida rugosa*
	Creamy tiny colony	Oval yeast cell with single budding	*Candida glabrata*
	Green with yellow spots	Septate hyphae with round conidia on conidiophores	*Aspergillus versicolor*
	Green and creamy colony	Septate hyphae with long roungh conidiophores close to close versicle	*Aspergillus flavus*
EDYP2	Creamy colony	Septate hyphae no conidia	*Candida rugosa*
	Leave green	Septate hyphae with smooth conidiophores and conidia	*Aspergillus fumigatus*
	White velvet	Septate hyphae with numerous large conidia	*Microsporum canis*
SRYP1	Creamy colony	Septate hyphae no conidia	*Candida rugosa*
	Creamy smooth colony	Oval yeast cell	*Candida tropicalis*
	White velvet	Septate hyphae with numerous large conidia	*Microsporum canis*
SRYP2	Creamy colony	Budding yeast cell oval in shape	*Candida rugosa*
	Creamy smooth colony	Oval yeast cell	*Candida tropicalis*
	Leave green colony	Septate hyphae with smooth conidiophores and conidia	*Aspergillus fumigatus*
	White velvet	Septate hyphae with numerous large conidia	*Microsporum canis*
	Creamy tiny colony	Oval yeast cell with single budding	*Candida glabrata*
SLYP	White velvet	Septate hyphae with numerous large conidia	*Microsporum canis*
	White and green colony	Septate hyphae with smooth conidiophores housing large club shaped versicle	*Aspergillus clavatus*
	Creamy tiny colony	Oval yeast cell with single budding	*Candida glabrata*

Key: ED, SR and SL represent supermarkets; YP-Packaged yam flour.

Table 7. Characteristic of fungi isolated from exposed yam flour

Isolate code	Macroscopy	Microscopy	Presumptive identification
ROY 1	Black colony	Septate, cream erect hyphae with long conidiophores housing black cluster conidia	*Aspergillus niger*
	Creamy and yellow colony	Septate hyphae with round conidia on smooth conidiophores	*Aspergillus terreus*
	Creamy colony	Septate hyphae, no conidia.	*Candida rugosa*
ROY 2	White tuft over agar plate	Septate hyphae with short branches of conidiophores containing conidia	*Trichoderma* sp.
	White colony with yellow at central area, with brown reverse	Septate hyphae with sporangiophores in a round vesicle	*Cokeromyces recurvatus*
OMY	Black colony	Septate, cream erect hyphae with long conidiophores housing black cluster conidia	*Aspergillus niger*
	Creamy and yellow colony	Septate hyphae with round conidia on smooth conidophores	*Aspergillus terreus*
	White and green colony	Septate hyphae with smooth conidiophores housing large club shaped vesicle	*Aspergillus clavatus*
MTY 1	Grayish velvet	Septate hyphae, no conidia	*Microsporum audouinii*
MTY 2	White tuft cover agar plate	Septate hyphae with short branches of conidiophores containing conidia	*Trichoderma* sp.

Key: RO, OM and MT represent open markets; Y - Exposed yam flour.

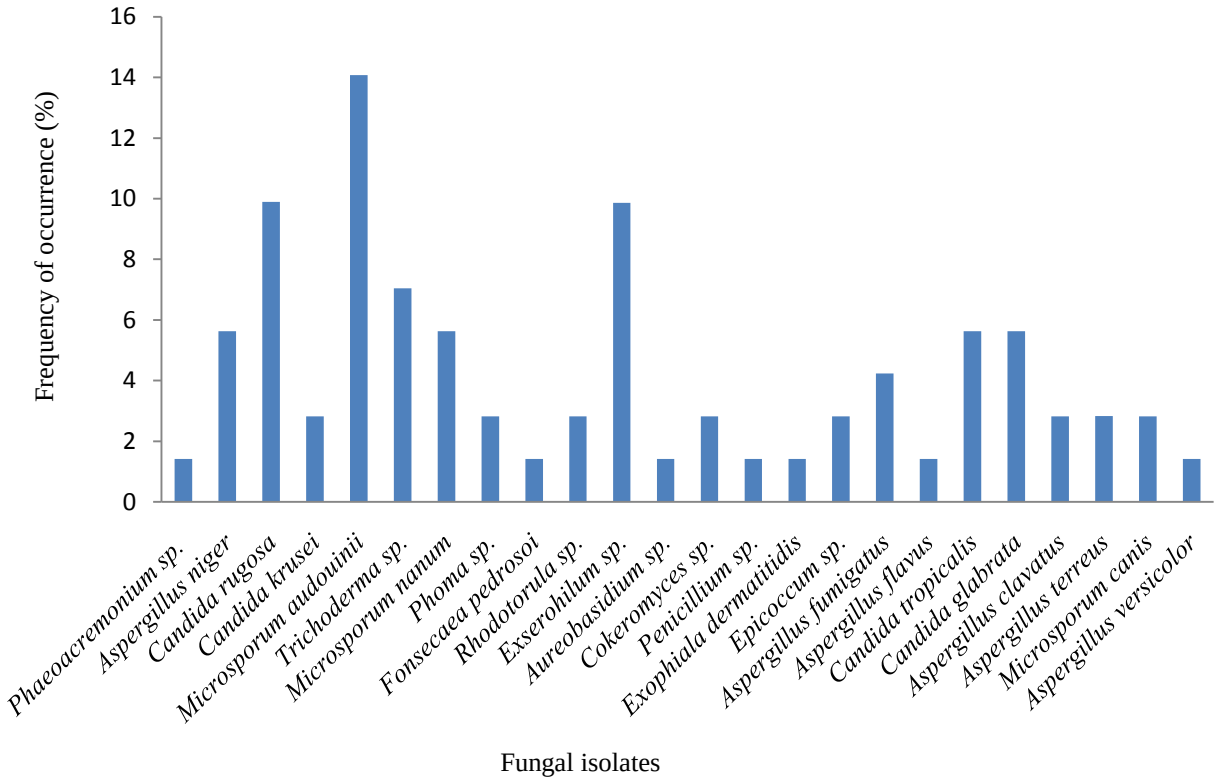

Figure 2. Frequency of occurrence of fungal isolates from exposed and packaged cassava, yam and plantain flour

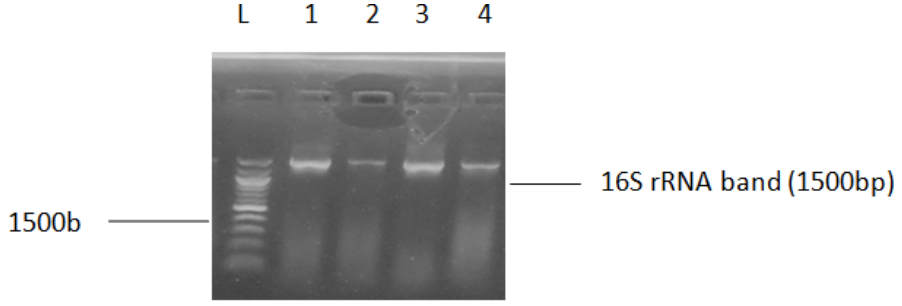

Figure 3. Agarose gel electrophoresis showing amplified 16S rRNA of the bacterial isolates. Lane L represents the 100bp molecular ladder. The lanes (1, 2, 3, 4) express the level of migration of genes on the agarose gel

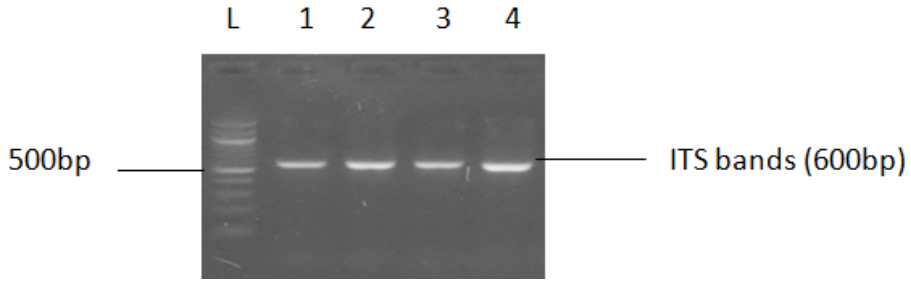

Figure 4. Agarose gel electrophoresis showing amplified ITS (600bp) of the fungal isolates. The lanes (1,2,3,4) express the level of migration of genes on the agarose gel

Table 8. Presumptive characterization of isolates and molecular characterization results

Sample Code	Percentage similarity	Presumptive organism isolated	Bioinformatics Result	Accession number
ROY2	100	*Bacillus* sp.	*Bacillus megaterium*	MG825417
OMP	100	*Salmonella* sp.	*Enterobacter* sp.	MG825418
EDPP2	100	*Staphylococcus* sp.	*Alcaligenes faecalis*	MG825419
OMP	98.8	*Aspergillus niger*	*Aspergillus niger*	MG825420
MTY2	99.6	*Candida rugosa*	*Paecilomyces sinensis*	MG825421
SRYP2	100	*Staphylococcus* sp.	*Acinetobacter junii*	MG825423
EDCP2	100	*Microsporum audouinii*	*Trametes polyzona*	MG825422

Key: RO, OM and MT represent open markets; ED and SR represent supermarkets; Y-Exposed yam flour; P-Exposed plantain flour; PP - Packaged plantain flour; CP – Packaged cassava flour.

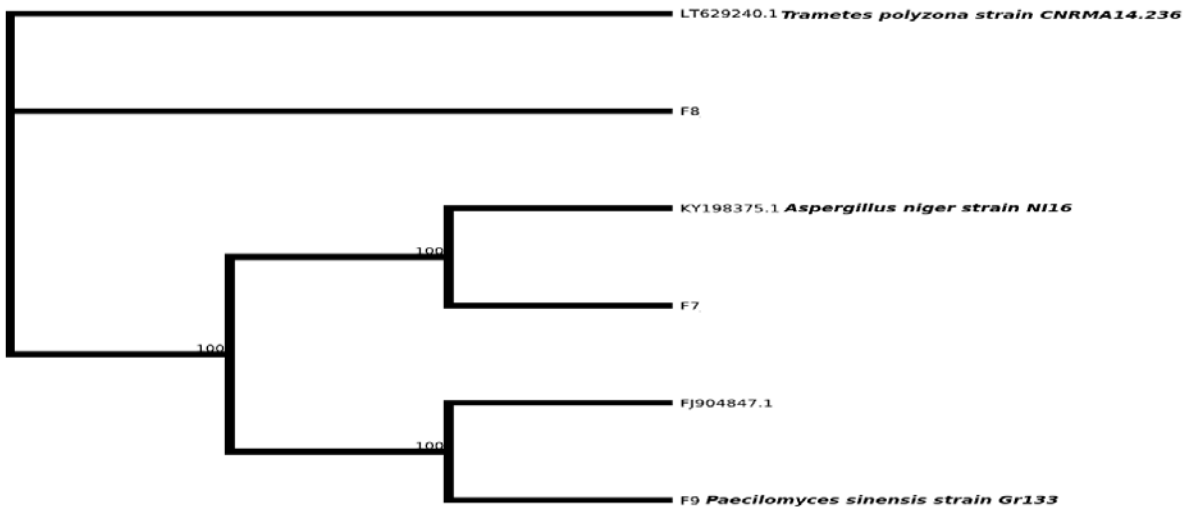

Figure 5. Phylogenetic tree showing evolutionary relationship between the fungal isolates

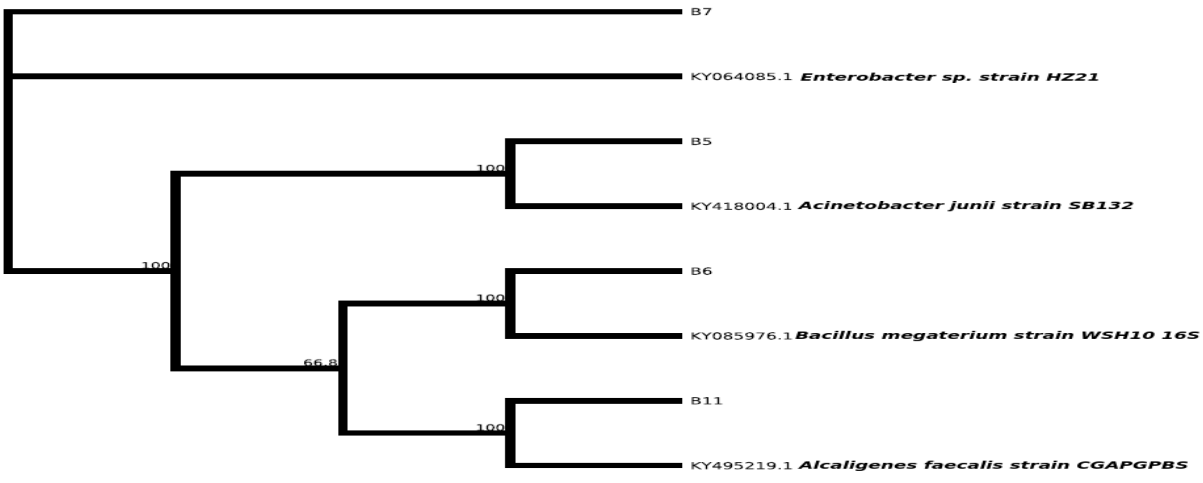

Figure 6. Phylogenetic tree showing evolutionary relationship between the bacterial isolates

4. Discussion

A total of fourteen fungal genera were isolated from the flour samples of which *Microsporum* sp., *Candida* sp. and *Aspergillus* sp. were predominant. In a related study, Ajayi [15] reported that *Aspergillus niger*, *A. fumigatus*, *Penicillium chrysogenum*, *Saccharomyces cerevisiae*, *Fusarium* spp. *Rhizopus stolonifer* and *Mucor* spp. were present in wet and dry plantain. Studies carried out by Omohimi *et al*. [2] and Odetunde *et al.* [14] also reported that *Aspergillus* sp. and *Penicillium* sp. was predominant in retailed yam and cassava flours. The presence of fungal isolates in cassava, yam and plantain flour could have resulted from contamination during processing. A related study by Somorin *et al.* [12] identified *Aspergillus niger*, *A. flavus*, *Penicillium oxalicum*, *P. citrinum* from milling machines used in yam flour processing.

This study has shown that *Escherichia coli* were present in the edible flour samples which recorded 10 % frequency of occurrence. A related study by Ojokoh and Gabriel [28] reported that *E. coli* was present in yam flour which they suggested could be as a result of improper handling of the flour samples as well as washing and steeping water used during processing. According to Gacheru *et al*. [8], *E. coli* was only detected in one sample of cassava flour out of many samples that were tested. The presence of *E. coli* in the edible flour samples is an indication of degree of feacal contamination both from human and animals. Presence of *E. coli* in plantain flour was reported by Ajayi [15]. Contamination of food by *Escherichia coli* including other food borne pathogens such as *Staphylococcus aureus* and *Bacillus cereus* poses a threat to public health [29].

Bacillus sp. was the predominant bacteria isolated from exposed and packaged cassava, plantain and yam flour. Its frequency of occurrence was 46.67 %. This result is in agreement with a similar study by Addo *et al*. [30]. *Bacillus* sp. is a spore former widely distributed in nature. The spores can withstand very high temperature and has the ability to produce enterotoxins which is poisonous to humans [31]. In a related study that involved microbiological quality of plantain flour, Ajayi [15] also isolated *Bacillus cereus* from plantain flour.

Another dominant bacterium isolated from exposed and packaged cassava, yam and plantain the flour samples was *Staphylococcus* sp. which recorded 40 % frequency of occurrence. In a related study, Somorin *et al.* [12] isolated *S. aureus*, *S. saprophyticus*, *S. epidermidis* from commercially milled white yam flour. The commonest specie of *Staphylococcus* ubiquitous in nature is *S. aureus* [32]. Enterotoxins released by *S. aureus* are responsible for staphylococcal food-borne disease (SFD). Globally, SFD is considered as one of the most common food-borne diseases. This could be as a result of improper food handling practices especially in retail food industry [33,34]. Foods such as flour extensively manipulated using hand is often associated with staphylococcal food poisoning [32].

Among the four bacterial genera isolated from cassava, plantain and yam flour samples, *Salmonella* sp. had the lowest frequency of occurrence (3.33 %). Although the frequency of occurrence is low, its presence only in exposed plantain flour obtained from OM open market should be considered as a threat to public health [13]. Although Djeri *et al*. [35], reported absence of *Salmonella* sp. in yam chips used in processing yam flour, contamination of the final product could still occur. *Salmonella* sp. is a causative agent of salmonellosis which is a food borne disease. Inappropriate storage of food and preparation of food in a very large quantity are some of the factors that increase the risk of food poisoning caused by *Salmonella* sp. [36].

The presence of *Aspergillus* sp. in the flour samples should be a thing of serious concern because it could produce aflatoxins. Poor storage conditions and traditional processing methods could encourage growth of *Aspergillus* sp. in cassava products such as cassava flour [37]. *Penicillium* sp. infection could cause rhinocerebral mucormycosis, mucocutaneous, genitourinary, gastrointestinal, pulmonary and disseminated infections [15]. *Microsporum* is among dematophyte species that cause *Tinea capitis* which is predominant disease that affect preadolescent children [38,39,40]. *Candida* sp. and *Alternaria* sp. are among common fungi that could contaminate or cause food spoilage. For example *Candida kefyr* could cause bloodstream infection [41].

Molecular characterization results revealed that *Bacillus* sp. showed 100 % similarity to *Bacillus megaterium* strain WSH10 16S, *Salmonella* sp. showed 100 % similarity to *Enterobacter* sp. strain HZ21, *Staphylococcus* sp. showed 100 % similarity to *Alcaligenes feacalis* strain CGAGPBS and *Staphylococcus* sp. showed 100 % similarity to *Acinetobacter junii* strain SB132. As for the fungal isolates, *Aspergillus* sp. showed 98.8 % similarity to *Aspergillus niger* strain NI26, *Candida* sp. showed 99.6 % similarity to *Paecilomyces sinensis* strain Gr133 and *Microsporum audounii* showed 99.6 % similarity to *Tramestes polyzona* strain CNRMA14.236. The result emanating from molecular characterization of bacterial and fungal isolates was submitted to GenBank in NCBI database using assigned accession number of *Bacillus megaterium* (MG825417), *Enterobacter* sp. (MG825418), *Alcaligenes feacalis* (MG825419), *Acinetobacter junii* (MG825423), *Aspergillus niger* (MG825420), *Paecilomyces sinensis* (MG825421) and *Tramestes polyzon*a (MG825422). Aruwa and Ogundare [9], reported the presence of *Acinetobacter* sp. *Enterobacter* sp. in cassava flour (pupuru)

5. Conclusion

Bacillus sp., *Escherichia coli*, *Salmonella* sp. and *Staphylococcus* sp. were identified as bacterial isolates while *Microsporum* sp., *Exserohilum* sp., *Trichoderma* sp., *Candida* sp., *Aspergillus* sp., *Phaeoacremonim* sp., *Epicoccum* sp., *Exophiala* sp., *Penicillium* sp., *Cokeromyces* sp., *Aureobasidium* sp., *Rhodotorula* sp., *Fonsecaea* sp. and *Phoma* sp. were fungal isolates identified in the flour samples using standard microbiological methods. Further characterization of the isolates using molecular methods revealed the bacterial isolates to be *Bacillus megaterium* strain WSH10 16S, *Enterobacter* sp. strain HZ21, *Alcaligenes feacalis* strain CGAPGPBS and *Acinetobacter junii* strain SB132 while that of fungal isolates are *Aspergillus niger* strain NI26,

Paecilomyces sinensis strain Gr133 and *Tramestes polyzona* strain CNRMA14.236. In addition to using conventional microbiological methods to identify bacteria and fungi present in packaged and exposed cassava, yam and plantain flour sampled from selected supermarkets and open markets in Port Harcourt, Rivers State, Nigeria, this study has also provided useful information by identifying the strains involved using molecular methods.

Competing Interests

Authors have declared that no competing interests exist.

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