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Molecular Characterization of Microorganisms Associated with Melon (*Colocynthis citrullus* L.) Seeds Sold in Three Markets in Delta State, Nigeria

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Abstract: Microorganisms that relate to *Colocynthis citrullus* (L.) seeds collected from three markets: Igbudu, Kpolokor and Ugborikoko within Uvwie Local Government Area in Delta State, were studied. The isolation of the fungi that were attached to the seeds was done using Standard Blotter and Agar Methods. Isolation of bacteria was done using Serial Dilution and Nutrient Agar Methods. Total Heterotropic Bacteria Counts (THBC) were determined at the dilution factors of 10^{-3}, 10^{-5} and 10^{-7}. Based on the morphological, cultural, colour of the individual colonies and number of viable count of bacteria, the 10^{-5} dilution factor was subjected to different biochemical tests and basic molecular techniques to identify the bacteria to the species level. Ten microorganisms were identified and isolated: *Aspergillus flavus* link Ex fr, *Fusarium oxysporum* Schlecht, *Penicillium spp* Thom, *Rhizopus stolonifer* Enhreb ex Link, *Aspergillus niger* Van Tiegh (fungi); *Bacillus flexus, Bacillus infantis, Escherichia coli, Pseudomonas xiamenensis* and *Pseudomonas aeruginosa* (bacteria). The outcome showed that Melon seeds collected in the Igbudu market had the highest fungal load of (38%) and Kpolorkor and Ugborikoko had (31) and (31) respectively. The overall number of heterotrophic bacteria counted in each of the markets was maximum 16.2×10^4 cfu-g at Igbudu market, minimum 8.7×10^4 cfu-g at Kpolokor and Ugborikoko had the lowest 5.5×10^4 cfu-g. There is also a need to sensitize vendors on the need to maintain high standards of handling such seeds to avoid microbial contamination.

Keywords: *Colocynthis citrullus, Microorganism, Polymerase Chain Reaction, Sequencing.*

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I. INTRODUCTION

Melon *Colocynthis citrullus* (L.), a creeping annual of the Cucurbitaceae, many of which have a tropical distribution. The melon herein spoken of, is a bad kind of a melon, when compared with the more popular melon: the watermelon. Despite its relative unknownness to the western world, it is grown to a very large scale in tropical Africa, whereby the seeds form a significant source of dietary protein and oil. Small scale farmers dominate agriculture in Nigeria and contribute approximately 80% of the total food demand. Adequate production of most Nigeria staple crops such as cereals, yam, cassava, cash crops and some vegetables like melon, which is consumed in many parts of the country will contribute massively to the agricultural sector. Other vegetables such as melon (*C. citrullus*), which is also crucial in the diet of any part of Africa. It is grown in the tropical, temperate and subtropical region where rain and irrigation is adequate (Fayinka, 2004). Melon is an ingredient of several delicious Nigerian cuisines e.g Egusi soup, the seeds of the melon gourd are used in the soup, the melon gourd fruit is bitter and inedible, therefore, the gourds are cultivated as a source of seeds only. It is also versatile and can be either a snack, a flavoring agent or even pressed to obtain its oil. It is known by various common names such as Egusi in Yoruba, Egwusi in Igbo, Agushi in Hausa and white-seed melon in English. It is made up of proteins and vitamins and can be planted easily because it's relatively easy to cultivate. High resistance to pests and diseases is one of its reasons why it can be easily cultivated as opposed to other crops. Also, its mode of growth is such that it is able to suppress other weeds. It creates a blanket-like shadow on the ground which does not give sufficient moisture and sunlight to the weeds. About 40% of melon seed comprises of oil which can also be used as animal fodder. The advantage of the oil is that it is cholesterol free. The oil contains unsaturated fatty acid that constitutes approximately 78% and is crucial in heart protection and inhibition of cardiovascular disease. The oil is made up of four fatty acids that are; linoleic, oleic, palmitic and oleic acids with the former being the most common. It

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has a relatively high fibre content which makes it easy to digest. The vitamin E element is useful in keeping the skin of a young person smooth and it also enhances fertility. The oil is a useful component which can be extracted and used for soap making. The various components of the melon plant are seeds, roots and stems, possess qualities that reduce blood sugar, thus they can prevent or manage diabetes. Recent records have indicated however that plant parts like fruits and seeds also harbour certain bacteria. The assumption over the years is that the primary niche in which bacteria can colonize is the outer part of the seeds; bacteria have seldom been localized to other tissues of the seeds. *Staphylococcus aureus*, *Escherichia coli, Xanthomonas spp*, *Lactobacillus spp*, *Pseudomonas spp, Klebsiella spp* and *Bacillus spp* are bacteria that is most likely to attack melon seeds. But *Bacillus spp* is usually located in the ground and this could have been of vegetable source. Over consumption of these organisms may cause gastrointestinal disease but others such as toxins of *Staphylococcus aureus* may result in vomiting, stomach cramps and diarrhoea when consumed. (Dinges *et al*, 2000). The presence of *Escherichia coli* shows faecal contamination of seeds since it is an indicator organism.

S. aureus has been linked to toxins that cause toxic shock syndrome (TSS), Staphylococcal Foodborne Disease (SFD), scalded skin syndrome. (Plata *et al*., 2009). These toxins impact the lower respiratory tracts, bloodstream, skin and soft tissues since it may lead to osteomyelitis and endocarditis among other infection (Schito, 2006). Also, the presence of *Klebsiella spp* and *Pseudomonas spp* can cause gastroenteritis as these microorganisms are often associated with gastrointestinal infections. Aflatoxin is a poisonous secondary metabolite of *Aspergillus parasiticus* that can be found in the melon seeds that have been long-stored. Markets are locations of buying and selling. In most market places more so the rural area, it is thought that the environment and cleaning up of the drainages is seldom done which contributes to the occurrence of foodborne epidemics. In most cases, the market places are usually crowded meaning that particulate matter bearing microorganisms could have settled on important areas. Microorganisms contributes to degrading of food quality, because they are excellent sources of nutrients, microorganisms grow rapidly and makes what once was appealing to a sour, foul smelling, unattractive mass only suitable for the garbage can. Melon seeds should be avoided to the activity of the pathogenic microorganisms on them. This ultimately results in poor nutrition value, not to mention the market value. Melons are oilseeds, hence needs to be kept at a moisture content that does not encourage heating within the seed pile or the growth of fungi or bacteria. The oil that is pressed from the seed may not be fit to be consumed by humans as it may grow mold or bacteria. When harvesting melons, scooping the melon out of the fruit is intensive and extremely heavy to move it to another location. The seeds of the melons are also not easy to carry to a source of water particularly when it is in high number. Washing of melon seeds is very important after harvest which is highly labor intensive. Without having the melon seeds near a water source, then it will have to be transported which ultimately raises the cost of production. The blenders that are used to chop the melon seeds into small pieces are also vital since the build-up of microorganisms unless washed immediately can be a possible threat. The degradation of melon seeds causes a decline in nutritive content, color changes, rise in peroxide value, low germination of seeds and generation of mycotoxins (Bankole *et al.,* 2004). Unless proper sorting of seeds is performed prior to milling, it could be a health risk to consumers. The presence of trace metals affects the safety of food in the accumulation since they disrupt the normal operations of the nervous system, kidney, as well as other crucial organs. Spoilage of melon seeds occurs when physical and chemical changes such as the influences of air, heat, light and moisture fosters the growth of microorganisms. The aim of this study was to determine the distribution of microorganisms in melon (*C. citrullus*) seeds sold in three markets in Delta State.

Isolation of these microorganisms is very crucial in identifying ways of preventing or managing their effects on plants since the microorganisms have an effect on their growth and yield. The use of modern molecular methods that rely on amplification of specific regions of the genome of an organism are gaining more and more popularity in identification and taxonomy of microorganisms. This has proved to be more precise as compared to the traditional cultural approach.

II. MATERIALS AND METHODS

➢ *Study Area*

Delta State is located between 5º 42’14.40” N, 5º 56’2.04” E. It is located in South and South geopolitical zone in Nigeria. This experiment was performed at Edwin Clark University Microbiology Laboratory, Delta State. Molecular studies was conducted at Nucleometrix Laboratory, Bayelsa State. Sequencing of the PCR products was done at Inqaba Biotechnological, Pretoria South Africa.

➢ *Sample Collection*

Seeds of *Colocynthis citrullus* supposed to be healthy were collected in the three markets respectively, Ugborikoko, Kpolokor and Igbudu markets in Uvwie Local Government Area of Delta State.

➢ *Preparation of Culture Media*

Media used were prepared as per the instructions of the manufacturer. They were potato dextrose agar (PDA) and nutrient agar (NA).

- *Potato Dextrose Agar:*

A total of 39 grams (39 g) of potato dextrose agar (PDA) powder was dissolved in 1 litre of distilled water placed in a conical flask which was covered with cotton wool and aluminium foil paper. It was thoroughly mixed and autoclaved at 121ºC for a duration of 15min. The medium was allowed to cool to 45-50ºC and then aseptically transferred into sterile Petri dishes.

- *Nutrient Agar:*

The nutrient agar (NA) powder (28 g) was dissolved in 1 litre of distilled water using conical flask with a cotton wool and aluminium foil paper cover. The mixture was thoroughly

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mixed and sterilized in an autoclave at 121°C, for a duration of 15 minutes and allowed to cool to 45-50°C, then 20 ml of it was transferred aseptically into sterile Petri dishes.

➢ *Isolation and Identification of Fungi from Diseased Melon Seeds*

The fungi related to the melon seeds were isolated by application of the standard blotter technique according to International Seed Testing Association (ISTA, 1996). Seeds of melon were sterilized by 70 percent ethanol solution for 5 minutes period and then using sterile distilled water, three changes were used to remove the surface contaminant. Melon seeds sterilized on the surface were then dried between blotters and then plated on three layers of moistened Whatman filter paper (no 1, diameter 9cm) in sterilized Petri dishes and left to incubate at (28°C ± 2°C) over 7 days.

- *Isolation of Fungi on Colocynthis citrullus Seeds*

Fungal organisms were isolated by the traditional use of media and simple molecular methods. Standard Blotter Method as Recommended by International Seed Health Testing Association (ISTA, 2016) was used to isolate fungi. The forceps, filter paper, distilled water (in a conical flask) and Petri dishes were autoclaved at 121°C for a duration of 15mins. The seeds of *Colocynthis citrullus* were teased into small pieces, surface sterilized in a beaker with ethanol (70%) for 2-3minutes and rinsed with sterile distilled water three times. Sterilized 9cm Whatman filter paper was put in 3 layers on the Petri-dishes. Petri-dishes of 9cm diameter containing 10 seeds each were placed in the laboratory under equidistant placement of the seeds and the Petri-disks were allowed to incubate 7days at 27±2°C. Sub-cultures of the isolated fungi were further cultured in Potato Dextrose Agar after incubation. Frequency of occurrence of each fungus was calculated.

- *Inoculation and Incubation of Fungal Organisms.*

The individual colonies which were distinguished by colour and had been isolated off the *Colocynthis citrullus* seeds were then picked up using a sterile inoculating loop and transferred onto the sterilised Petri-dishes which contained Potato Dextrose Agar medium. To avoid contamination the inoculating loop was sterilized by dipping in 70% ethanol and flaming in a Bunsen burner and then waving slightly to cool down. All Petri-dishes had codes along with dates, were sealed with masking tape and put upside down to avoid contamination by any moisture that might be in them. Incubation of the Petri dishes at room temperature (27°C) of 7 days was done to obtain pure cultures of the fungal organisms. Contaminated plates due to other organism (s) were discarded and further sub culturing of fungus was performed to get pure cultures. A total of 3 Petri dishes were used for each fungus.

➢ *Isolation of Bacteria from Colocynthis citrullus*

Isolation of bacterial organisms was carried out using traditional media culturing. Serial dilution and spread plate method was used to find the microbial population of the sample as outlined by Aneja (2003). *Colocynthis citrullus* seeds with disease symptoms were surface sterilized with 70% ethanol (1gram each) at a time (3minutes). Three sequential rinses of the seeds with sterile distilled water were performed and the seeds moved into different sterile mortar. The samples were crushed and afterwards, they were transferred into 100ml conical flasks. 10mls of sterile normal saline was placed in each conical flask and the mixture shaken for a period of 1 minute. 1ml of the stock solution was pipette out into 9ml of sterile normal saline in a 20ml test tube to make 10^{-1} dilution, 1ml of 10^{-1} was then aseptically transferred to another 9ml of sterile normal saline to make 10^{-2} dilution and it was continued in that sequence until 10^{-10} dilution was obtained. Aliquots (0.1ml) of 10^{-3}, 10^{-5} and 10^{-7} diluents were inoculated into nutrient agar and evenly spread using sterile bent glass rod. The plates were kept at 37°C incubation of 48hours then the number of bacteria colonies counted. Following the enumeration; the various morphological types that emerged on the plates were handpicked using sterile wire loop and sub-cultured by streaking the colonies on prepared nutrient agar and incubated at 37°C over a period of 24hours to obtain pure cultures of bacteria. Based on the results obtained pure cultures were inoculated on sterile nutrient agar in Bijou bottles and incubated at 27°C by streaking the culture on the sterile and solidified nutrient agar. The stock culture was stored in the refrigerator to be further identified or used. Gram staining and motility tests were carried to test the isolates to determine whether they are gram positive or gram negative bacteria as well as test the presence of locomotive organelles respectively.

➢ *Molecular Characterization*

- *DNA Extraction*

ZR fungal/bacterial DNA mini prep extraction kit available at Inqaba South Africa was used to extract. The heavy growth of the pure culture of the suspected isolates was suspended in 200 microliters of isotonic buffer in a ZR Bashing Bead Lysis tubes, 750 microliters of the lysis solution was added to the tube. The tubes were clamped in a bead beater with 2ml tube holder assembly and clamped at maximum speed during 5 minutes. ZR bashing bead lysis tube were centrifuged at 10,000xg/1 minute. Four hundred (400) microliters of the supernatant were added to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 xg at 1 minute. The fungal/bacterial DNA binding buffer was 1000-200 (1200) microliters added to the filtrate in the collection tubes bringing the end volume to 1600 microliter, 800 microliters was then transferred into a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000xg The rest of the volume was transferred to the Zymo-spin and spun. Two hundred (200) microliter of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000xg for 1 minute followed by the addition of 500 microliter of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000xg for 1 minute. The column matrix of Zymo-spin IIC column was transferred to 1.5 microliter centrifuge tube, 100 microliter DNA elution buffer was added to the column matrix and centrifuged at 10,000xgmicroliter and eluted the DNA. The purest DNA was then kept at -20 degree to be used in other downstream reaction.

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- *DNA Quantification*

The extracted genomic DNA was measured by means of Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The sterile distilled water (2 ul) and normal saline (blank) were used to start the equipment. Two microliters of the extracted DNA was placed on the lower pedestal, upper pedestal was lowered to touch the extracted DNA in the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

- *Extended Spectrum Beta- Lactamaes Detection*

✓ *Amplification of SHV Genes*

The isolates in the SHV genes were amplified using the SHV F: 5' CGCCTGTGTATTATCTCCCT-3' and SHV R: 5'-CGAGTAGTCCACCAGATCCT-3' primers on an ABI 9700 Applied Biosystems thermal cycler in a total volume of 30 microlitres under 35 cycles. The PCR mixture consisted of: the X2 Dream taq Master mix provided by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 56ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. It was purified and resolved in a 1% agarose gel at 120 V with visualization on a UV transilluminator with a product size of 281bp.

✓ *The Amplification of the TEM Genes:*

The TEM genes which are gotten from the isolates were amplified with the TEMF: 5'-ATGAGTATTCAACATTTCCGTG-3' and TEMR: 5'-TTACCAATGCTTAATCAGTGAG-3' primers on an ABI 9700 Applied Biosystems thermal cycler with a final volume of 40 microlitres at. The PCR mix contained: X2 Dream taq Master mix provided by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was cast (resolved) on a 1% agarose gel and 120 V and incubated at 120 V, 25 minutes and visualized under a UV transilluminator with a 850bp product size.

✓ *OXA Genes Amplification:*

TEM genes of the isolates were amplified with the OXA-1F: 5'- AGCCGTTAAAATTAAGCCC-3' and OXA-1R: 5'-CTTGATTGAAGGGTTGGGCG-3' primers on an ABI 9700 thermal cycler at a final volume of 40 microliters in 35 cycles. The PCR mix contained: the X2 Dream Taq Master mix provided by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 47ºC for 30 seconds; extension, 72ºC for 40 seconds for 35 cycles and final extension, 72ºC for 5 minutes.es. A 10% agarose gel was resolved at 120 V and allowed to run 25 minutes to visualize a 911bp sized product.

✓ *CTX-M Gene Amplification:*

The amplification of CTX-M genes from the isolates using the CTX-MF: 5'-CGCTTTGCGATGTGCAG-3' and CTX-MR: 5'-ACCGCGATATCGTTGGT-3' primers on a final volume of 40 microliters was done in the ABI 9700 Applied Biosystems thermal cycler in 35 cycles. The final PCR mixture consisted of: the X2 Dream Taq Master mix, which has been provided by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers having concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. 1% agarose gel was used to resolve the product at 120 V for a period of 25 minutes and visualized on a UV transilluminator in case of a 550bp product size.

✓ *16S rRNA Amplification:*

The 16s rRNA region of the rRNA gene of the isolates were amplified by using the 27F: 5-AGAGTTGATCMTGGCTCAG-3 and 1492R: 5-CGGTTACCTTGTTACGACTT-3 primers in an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mixture contained: the X2 Dream Taq Master mix provided by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at 0.5uM concentration and the extracted DNA as template. The PCR conditions were as follows: First denaturation, 95 o C in 5 minutes, denaturation, 95 o C in 30 seconds, annealing, 52 o C in 30 seconds, extension, 72 o C in 30 seconds in 35 cycles and last extension, 72 o C in 5 minutes.es. The product was run on a 1 percent agarose gel at 130 V and 30 minutes and visualized in a blue light transilluminator.

➢ *Sequencing*

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

➢ *Phylogenetic Analysis*

Sequences that were gotten, were edited by using the bioinformatics algorithm Trace edit, sequences that are similar were downloaded from the National Center for Biotechnology Information (NCBI) data base with the aid of BLASTN. MAFFT was used to align these sequences. The Neighbor-Joining method was used to infer an evolutionary history with MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree that was obtained after the 500 replicates (Felsenstein, 1985) is considered to reflect the evolutionary history of the taxa under analysis. The Jukes-Cantor method was used to compute the evolutionary distances (Jukes and Cantor 1969).

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➢ *Statistical Analysis*

Data were statistically analyzed by adopting one-way ANOVA. The difference between the means was divided where necessary with the help of the Duncan Multiple Range Test, which was set at a 95% confidence interval (SPSS 17.0.1, SPSS Inc.). Demographic information was given in percentages.

III. RESULTS

➢ *Fungi on Colocynthis citrullus Seeds:*

Samples of melons in various markets of Delta States, Nigeria were tested on fungi. Melon seeds were determined to have five organisms of fungi (Table 1 and Plate 1). Figure 1 below shows the incidence of the fungi that is exhibited in the various markets. The most prevalent was found to be *Fusarium oxysporum* and *Rhizopus stolonifer* and least prevalent was *Penicillium spp* in all the markets surveyed in Delta State and the disease incidence was different. The disease incidence was maximum for *F. oxysporum* (56%) in Kpolokor while Ugborikoko (26%) recorded the least. The highest record was *R. stolonifer* in Igbudu (40%) and Ugborikoko (26) with no record in Kpolokor (0%). *Aspergillus niger* was highest in Kpolokor (10%), Ugborikoko (6%) and none in Igbudu (0) markets. The highest incidence was found in Ugborikoko (8%), Kpolokor (4%) in which there is no growth of *Penicillium spp* and Igbudu market respectively. The current associations were found to have more fungal load on melon seeds (38 %) of melon seeds sourced in Igbudu and (31) melon seeds sourced in Ugborokoro and Kpolokor markets in Delta State respectively Figure 2.2. The difference in the occurrence of the fungi among the different market is blamed on poor sanitation of the market, handling and poor personal hygiene of the vendors.

Table 1 Macroscopic and Microscopic Identification of Fungi Associated with Colocynthis citrullus Seeds from Three Markets in Delta State

MACROSCOPIC DESCRIPTION	MICROSCOPIC	DESCRIPTION
S/N Colony description on PDA	Conidia shape	Fungi isolated
1. Black mycelia growth	Globose	*Aspergillus niger*
2. White fluffy mycelia growth with black spots	Globose	*Rhizopus stolonifer*
3. White cottony growth	Sickle-shaped	*Fusarium oxysporum*
4. Grey mycelia growth	Conidia in chains	*Penicillium* spp
5. Green mycelia growth	Globose	*Aspergillus flavus*

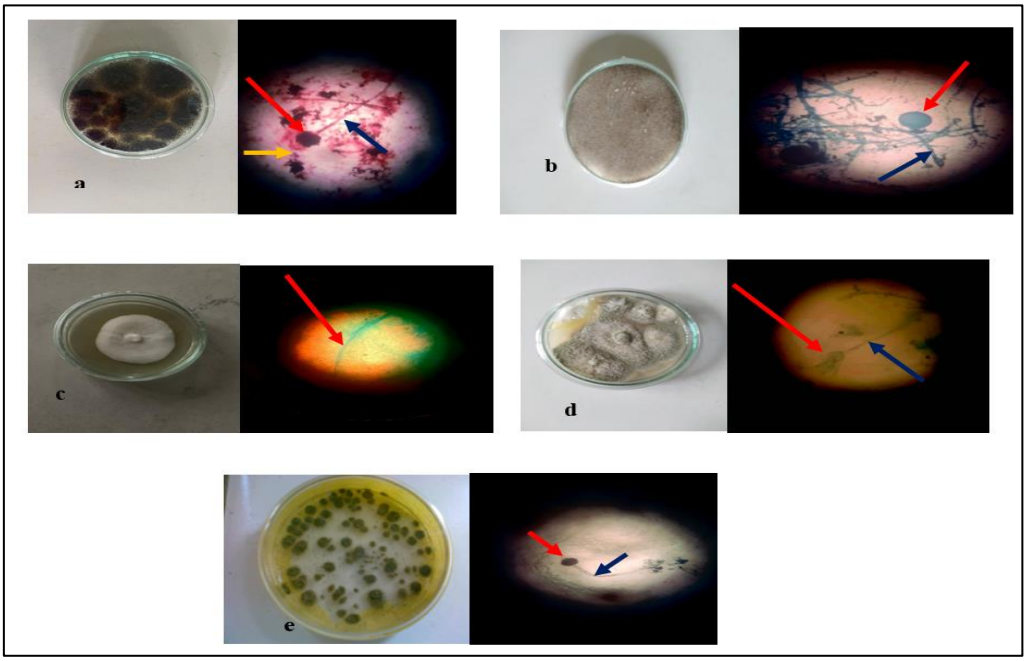

Plate 1: (a) Pure culture of *Aspergillus niger* with microscopic view of *Aspergillus niger* at (100X magnification). Red arrow= Highlights the conidiophores with a large vesicle, Blue arrow= Hypha, Orange arrow= Conidia (b) Pure culture of *Rhizopus stolonifer* with Microscopic view of *Rhizopus stolonifer* at 100X magnification. Red arrow= Oval-shaped sporangium containing asexual sporangiophore, Blue arrow= thread-like structures present in the background are broken hyphae (c) Pure culture of *Fusarium oxysporum* with microscopic view of *Fusarium oxysporum* at 100X magnification. Red arrow= showing macroconidia (d) Pure culture of *Penicillium* spp with Microscopic view of *Penicillium* spp at 400X magnification. Red arrow= Conidia, Blue arrow= Conidiophore (e) Pure culture of *Aspergillus flavus* with microscopic view of *Aspergillus flavus* at 100X magnification (Red arrow= Globose conidia, Blue arrow= Colourless conidiophore.

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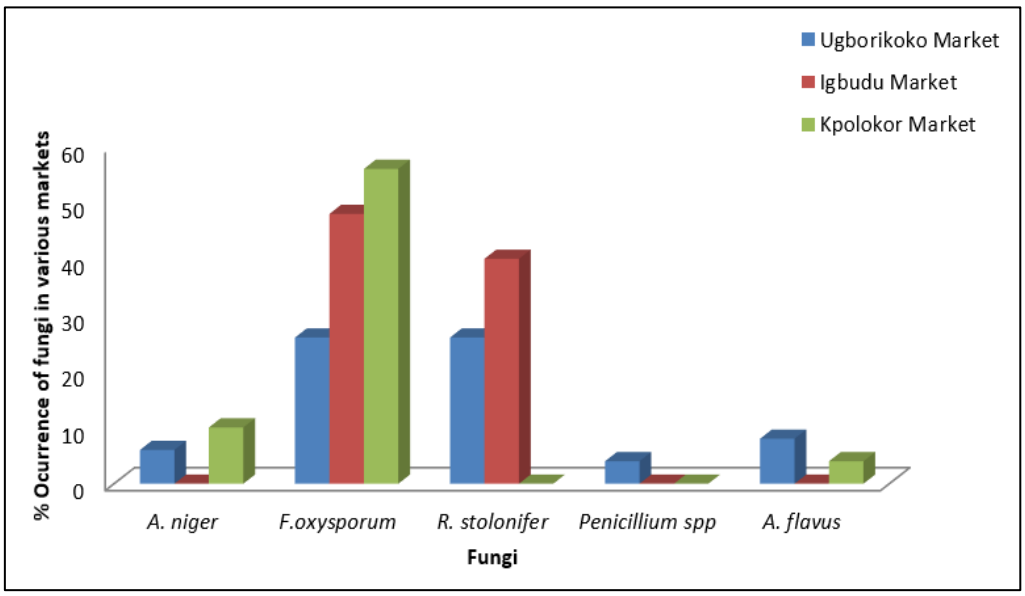

Fig 1 Fungi Isolated from Melon Seeds Distribution Across the Various Markets in Delta State

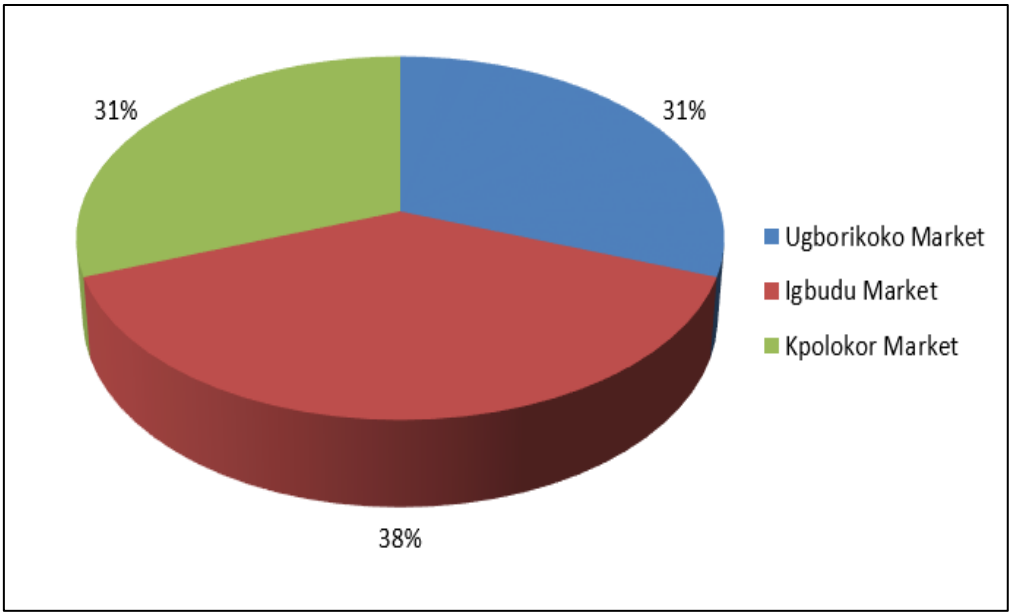

Fig 2 Percentage Occurrence of Fungi Isolated from Melon Seeds in Markets in Delta State

➢ *Total Population Count of Bacteria from Colocynthis citrullus*

The bacteria colonies on Petri dish with ground Colocynthis citrullus seeds were counted on nutrient agar (Table 1). The market with the highest bacterial count of the seeds was Igbudu market, (16.2 x 104 cfug-1), then Kpolokor market (8.7x 104cfug-1) and finally Ugborikoko market (5.5 x 104cfug-1). Colocynthis citrullus seeds produced five bacterial organisms (Table 2). Samples 1 had opaque creamish white surface, sample 2 white circular surface, sample 3 had circular straw, raised entire surface, sample 4 had smooth white surface with regular edges, non-pigmented and slightly raised in the centre while sample 5 had smooth colonies, with flat edges and an elevated appearance.

Table 2 Total Population Count of Bacteria from *Colocynthis citrullus* from Three Markets in Delta State

Sample ID	Markets	Colony count ($cfug^{-1}$) Seeds
IgM	Igbudu	$16.2 \times 10^{4*}$
KpM	Kpolokor	8.7×10^{4}
UgM	Ugborikoko	5.5×10^{4}

*Values Presented Here are Expressed as Mean of Three Replicates (n=3)

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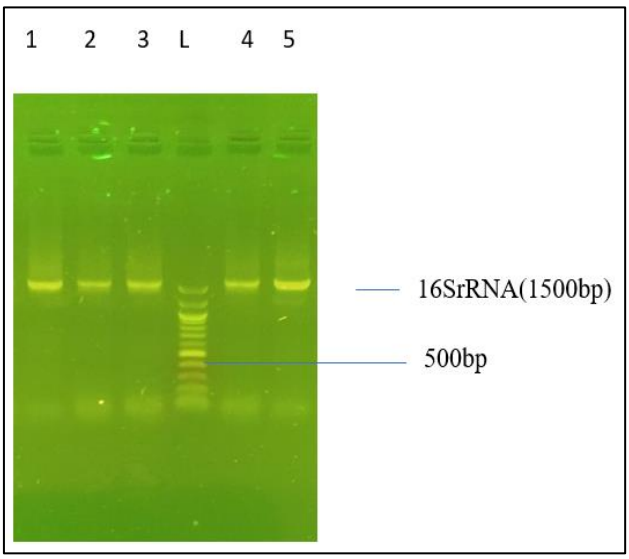

Plate 2 Agarose Gel Electrophoresis Showing the Amplified 16S rRNA Fragment. Lanes 1-5 Represent the Amplified 16S rRNA Bands at 1500bp while L Represents the 100bp Molecular Ladder.

➢ *Phylogenetic Analysis of Bacteria Associated with Colocynthis citrullus Seeds*

The visualization of the 16S rRNA region of the rRNA gene of the isolates results of the 1% agarose gel electrophoresis indicated that the PCR products were amplified to have a molecular weight of each equivalent to 1500 base pairs. The purified lanes 1-5 that are depicted on the Plate 1 are the 16S rRNA gene bands (1500bp) and lane L is the 100 base pair (bp) molecular ladder. The sequence of 16S rRNA that was obtained of the bacterial isolates gave a perfect match on the Megablast search of highly similar sequences to the NCBI non-redundant nucleotide (nr/nt) database. After sequencing, the blast results revealed the species identity of the bacterial isolates to be *Bacillus flexus*, *Bacillus infantis*, *Escherichia coli*, *Pseudomonas xiamenensis* and *Pseudomonas aeruginosa*. The 16S rRNA of the isolates had a percentage similarity of 100 to other species. The phylogenetic position of the 16S rRNA isolates 1, 2, 3, 4 and 5 against *Escherichia*, *Pseudomonas* and *Bacillus sp* evolutionary distances derived by the JukesCantor method were comparable to the phylogenetic position of *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus flex.* Using visual observation, the sample of the 16S rRNA of each DNA sample (melon seeds samples in Igbudu, Kpolokor and Ugborikoko markets) was observed to align themselves at 1500 base pairs (1500bp) that demonstrates the presence of bacterial spp in the *C. citrullus* samples in line with past researches that show bacteria are approximately 1 To supplement this, molecular identification of the DNA samples taken out of the different markets sampled (Igbudu, Kpolokor and Ugborikoko markets) showed that three genus of bacterial species were present as illustrated in Table 2, viz *Bacillus, Escherichia and Pseudomonas*.

- *Bacteria Sequences were Submitted to GenBank and Accession Numbers were Obtained:*

✓ *Bacillus infantis* (OR473067)
✓ *Bacillus flexus* (OR473068)
✓ *Escheriscia coli* (OR473069)
✓ *Pseudomonia xiamensis* (OR473070)
✓ *Pseudomonia aeruginosa* (OR473071)

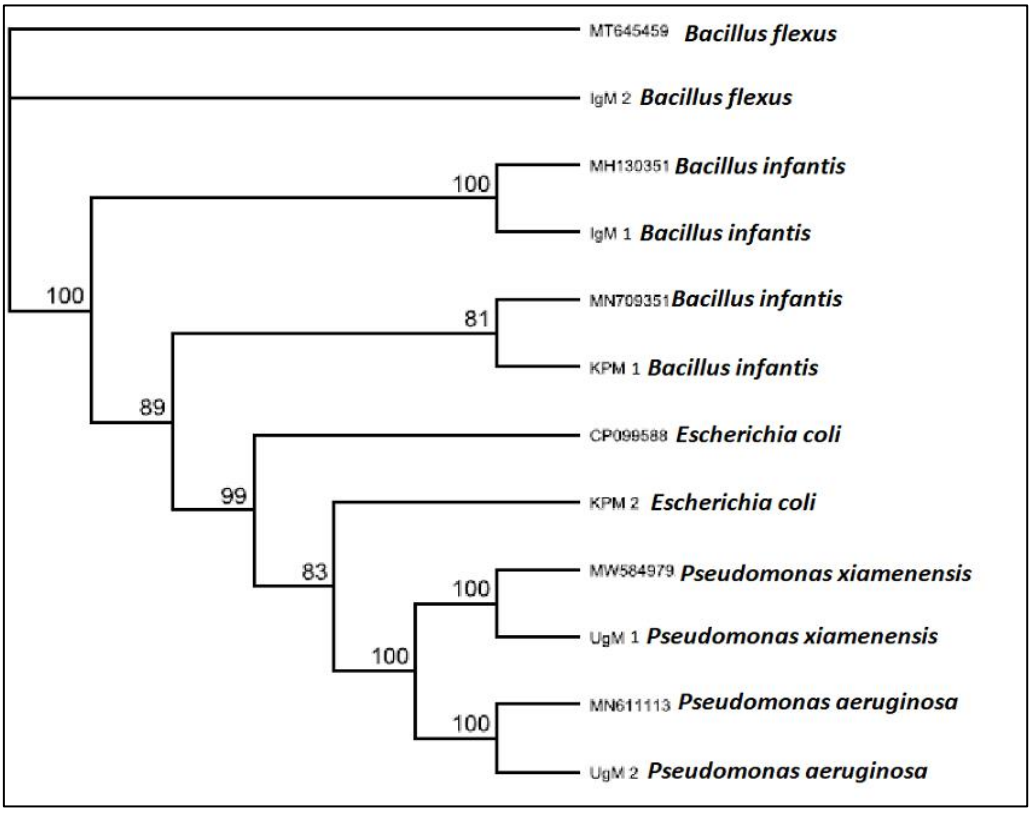

Fig 3 Phylogenetic Tree Showing the Evolutionary Distance Between the Bacterial Isolates

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IV. DISCUSSION

The following microorganisms were identified and isolated to be related to *Colocynthis citrullus* seeds: *Aspergillus flavus* link Ex fr, *Fusarium oxysporum* Schlecht, *Penicillium spp* Thom, *Rhizopus stolonifer* Enhreb ex Link, *Aspergillus niger* Van Tiegh, *Bacillus flexus*, *Bacillus infantis*, *Escherichia coli*, *Pseudomonas xiamenensis* and *Pseudomonas aeruginosa*. The results of this experiment have shown that the most prevalent fungi were *Fusarium oxysporum*, *Rhizopus stolonifer* and *Aspergillus niger* and the most prevalent bacteria were *Bacillus spp* and *Pseudomonas spp* that were found to be associated with melon seeds.

Isalar *et al.*, (2021) extracted similar organisms by their research on the topic of Fungal contaminants associated with Groundnut (*Arachis hypogaea*) seeds. Damage to the oilseeds has been reported to be caused by fungi associated with them. Fagbohun *et al.*, (2011) who gave a total of seven fungi isolated on the stored sundried melon seeds and include *Fusarium sp. Rhizopus sp. Penicillium sp.*, *Mucor sp., A. niger, Aspergillus tamari* and *Penicillium sp*. Etaware (2019) also reported *A. flavus*, *Cladosporium spp*, *A. niger*, *Penicillium chrysogenum* and *Rhizopus oryzae* had severe spoilage effects on the inoculated healthy melon seeds which corroborate with our findings. *Aspergillus spp* was among the most common fungi found in *C. citrullus* in the different markets whereas *Penicillium sp* was the least common fungi found in this study this goes in line with the report of Bankole *et al.*, (2005), that stored *C. citrullus* contains numerous species of *Aspergillus* and some of *Penicillium*. This could be explained by inappropriate postharvest handling like threshing and drying that could leave wounds on the seeds thus making them easy to be entered by these pathogens. The results also showed there were more spoilage organisms from Igbudu market followed by those purchased from Kpolokor and Ugborikoko markets respectively in Delta State, this is because the vendors from Igbudu markets sold the melon in a tray close to a gutter by the roadside with so many commuters, vendors from Kpolokor sold their melon from a sack while those from Ugborikoko markets sold their melon from a basket. This may be due to the fact that the melon sold on tray is more exposed to humidity, rain and dirt than the other two markets. This was due to the poor hygienic condition of the vendors with dirty clothes and most of them used their dirty hands to sell the melon. The market environments were observed to be untidy which can serve as a reservoir of microorganisms with flies and other insects around the markets can also aid transfer of microorganisms to goods sold in the markets.

These isolates of this study are devastating pathogens of melon, cereal crops and other commodities, and synthesize mycotoxins prior, or shortly after harvest, drying and storing. Among the metabolites that Adetoun *et al.*, (2020) discovered in their study, were 16 mycotoxins and other metabolites produced by various fungal genera, including but not limited to, *Aspergillus, Alternaria, Fusarium and Penicillium*. All the fungi detected in this current research is known to cause massive damages leading to change in colour, texture and flavour that make the seeds unacceptable because of rancidity, direct loss in case of too mouldy seeds, lowered germination ability of seeds and poisoning in man because of the release to mycotoxins. On the same note, there are other fungi that are related to stored products, and which can generate toxin metabolites or chemicals that are harmful to the health of consumers. Aflatoxins are produced by *A. flavus* which are hepatotoxic and immune suppressive in humans, Ochratoxin A produced by *Penicillium chrysogenum* are carcinogenic, teratogenic, nephrotoxic and cause urinary tract disease, *F. oxysporum* produce a wide range of different toxins such as fumonisins as well as zearalenone which causes hormonal imbalance, reproductive effects and have been related to oesophageal cancer in human.. (Steyn, 1995; Soriano and Dragacci 2004; Miller and Trenholm 1996). However, consumption of excess amount of these chemicals in the stored products can cause illness or death (Anon, 1993; *Mirocha et al.*, 2003).

Molecular characterization is a rapid and quick procedure which requires minimal handling and able to distinguish even morphologically, similar bacterial species. Recent molecular advances in microbiology have greatly enhanced the ability to detect bacterial pathogens in the environment. Unlike the traditional culture-based detection systems, the development of molecular detection systems and its cost reduction trend have enhanced the occurrence of pathogenic microorganism's detection. Thus, the molecular characterization was conducted in this work as well to determine five isolated bacteria based on the 16S rRNA gene sequence.

The most commonly identified bacteria to *C. citrullus* seeds are bacteria of *Bacillus species* viz; these are two strains of *B. infantis* (MH130351 and MN709351), one of *B. flexus*, *Pseudomonas species* viz; *Pseudomonas xiamenensis* and *Pseudomonas aeruginosa* and *Eschericia coli*. The greatest population was that of *Bacillus spp* and the lowest was *E.coli.* This coincides with the results of David and Aderibigbe (2010) who had noted high population of *Bacillus spp.* when fermenting melon seed. Three genera of bacteria species according to the report of Rose *et al.*, (2014) was also presented in this study and included *Staphylococcus aureus*, *Esherichia coli*, *Bacillus species*, *Staphylococcus epidermidis*, *Klebsiella species*, *Micrococcus* and *Pseudomonas species* that were the bacteria isolated in *C. citrullus*. It was also noted that *Bacillus spp* was common in Igbudu and Kpolokor markets, *Pseudomonas spp* was common only in Ugborikoko market whereas *E. coli* was common only in Kpolokor market. *Bacillus spp*. has the potential risk as these organisms may produce toxins that are harmful to human beings when ingested. Dinges *et al.*, (2000) state that *Bacillus spp*. is a common soil contaminant that could have a vegetable source. High amounts of these organisms, which is probably more than 100 cfu/ml, might cause gastrointestinal illness, but the intensity and seriousness of the infection in a person might differ based on the specific immune response capability and integrity of the immune system.

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Escherichia coli are environment, food, and intestinal bacteria of people and animals. *E. coli* are a very big and heterogeneous bacteria. Although most strains of *E. coli* are harmless, others can make you sick. Certain types of *E. coli* may lead to diarrhea, whereas some types are associated with urinary tract infection, respiratory disease and pneumonia among other diseases. (CDC, 2022)

Pseudomonas aeruginosa is a widespread microorganism, which can live in a broad range of environments. It does not only lead to disease in plants and animals but also to severe infections in immunocompromised cancer patients, severe burn and cystic fibrosis (Weihui, *et al.*, 2015). There are several causes of the contamination of melons during harvesting, packing, and shipping. The melons may come in direct contact with the soil as they grow and develop and may be contaminated with human pathogens that may be in the soil (Richards and Beuhat, 2005b).

Traditional and molecular methods were used to collect samples in all the sampled markets and found spoliation microorganisms in the sampled markets. The observation suggests that *C. citrullus* seeds from these markets will reduce seed viability and have a reduced shelf life.

V. CONCLUSION

This research revealed that the samples of *C. citrullus* seeds that were sold in the Delta State in selected markets were of low microbiological quality. The levels of contamination of both fungi and bacteria in Igbudu market were very high as compared to Ugborikoko and Kpolokor markets. Potentially toxic microorganisms such as *Aspergillus spp, F. oxysporum, R. stolonifer, Penicillium sp, Bacillus spp., E. coli* and *Pseudomonas spp*. were found in the almost all samples of the three markets raising concern over their usage in human consumption due to the harmful effects these toxins have on human health. Microbiological contamination could be profoundly caused by storage practices, which may pose a threat to the safety and health of the consumer. The seeds are packed in trays, sacks and baskets according to the survey results which brought out the poor standard of hygiene of the vendors. It is easy to contaminate the food product with microbes during the process, putting the product at risk of contamination.

To the knowledge of us, this is the first report in Nigeria of bacterial organisms (*B. flexus, B. infantis* and *P. xiamenensis*) to be linked to *Colocynthis citrullus* seeds. The isolation of these microorganisms is critical towards the process of finding mechanisms of preventing or managing their effects on plants since the microorganisms affect their growth and yield. Modern molecular techniques that are based on the amplification of particular regions of an organism's genome are increasingly being used in the identification and taxonomy of microorganisms compared to the conventional cultural method, this has shown to be more accurate.

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