Characterization of Pathogens and Mycotoxins Associated with *Cyperus Esculentus L.* (Tiger Nuts) Sold in Ibadan, Oyo State, Nigeria

Adenugba Aderonke Adenugba*, Obadina Adewale Olusegun

Babcock University, Ilishan, Ogun, Nigeria

***Corresponding Author**

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ABSTRACT

Tiger nuts (*Cyperus esculentus*) are a minor but important tuber crop noted mostly by their aphrodisiac properties that are consumed as a snack, due to their rich flavor and texture. Though consumed raw, if exposed to microbial contamination when handling impose public health. This present study therefore was aimed at determining the mycotoxin level and distribution of parasitic, bacterial, and fungal contaminants associated with tiger nuts (*Cyperus esculentus*) sold in ten major markets in Ibadan, Oyo state Nigeria. Two types of intestinal parasites were identified, *Entamoeba Histolytica* (37.5%) and *Ascaris Lumbricodes* (62.5%). Nuts contaminated with parasites were found only in six markets (Bodija, Oja-Oba, Molete, Oje, Agbeni and Ojo). Bacteriological examination showed five different groups of bacterial isolates (*Pseudomonas putida, Klebsiella pneumoniae, Bacillus cereus, Escherichia coli,* and *Staphylococcus aureus*) with the most prevalent being *Staphylococcus aureus*. Unlike parasites, bacteria isolates were found among samples from all the ten major markets. Antibiogram of bacterial isolates revealed a varying level of resistance and susceptibility to the antibiotics tested. In this current study, *Bacillus cereus* and *Escherichia coli* were the only isolates seen to be highly susceptible to all the antibiotics tested. However, *Pseudomonas putida* was seen resistant to ampicillin and *Staphylococcus aureus* was seen resistant to iminipem. Based on the result of these findings, it can be concluded that the bacterial isolates had varying levels of resistance to the antibiotics tested. The antibiotic resistance pattern expressed by the isolates from the tiger-nut samples is suggestive of possible abuse of antibiotics and that the consumption of this nut poses a potential public health threat. The fungal analysis from the test tiger nuts (*Cyperus esculentus*) samples revealed the presence of three fungal species: *Aspergillus flavus, Aspergillus fumigatus and Aspergillus niger. Aspergillus niger* and *Aspergillus fumigatus* had the same percentage occurrence (31.25%) while the highest occurrence was seen in *Aspergillus flavus* (37.50%). The highest TFC (17 log10 CFU/mL) was recorded from tiger nut samples from Orita-Aperin market while the lowest TFC (6 log10CFU/mL) was obtained in samples from Oje market. The occurrence of these fungi may cause diverse effects on human health, as they have the potential to produce mycotoxins. In this study, twenty-four different mycotoxins were analyzed in the tiger nuts samples. Of all the mycotoxins tested, Aflatoxin B1 had the highest occurrence which was seen only in two of the major markets; Gbagi market sample (95.7ug/kg) and the Ojo market sample (33.03ug/kg), and they are seen to be above the permissible limits set up by NAFDAC (20ug/kg). In conclusion, buying and eating nuts as well as other fruits taken raw from street vendors and marketplaces could pose a significant public health threat. There is a need for efficient monitoring systems for food-borne pathogens in Ibadan, Oyo state.

Keywords: Tiger nut, *Cyperus esculentus L.*, Antibiogram, Mycotoxins, Food borne pathogens, Antibiotic resistance, Aflatoxin, Aphrodiasic properties

INTRODUCTION

Tiger Nut (*C. esculentus L.*) originates from the Mediterranean Coast in North Africa, and mainly includes three parts: underground tubers, roots, and leaves above the ground. Tiger nut is a perennial monocotyledon plant that grows to a maximum height of 24-55 cm and has a fibrous, upright erect root system. It has a dimension ranging from 6 –10mm and turn out in distinct varieties. The botanical name of tiger nut is *Cyperus esculentus L.* which is a high-quality wholesome crop that contains lipids, protein, starch, fiber, vitamins, minerals and bioactive factors. The tiger nut is the tiny tuber of *Cyperus esculentus L.*, also known as the "underground walnut", it grows all over the world because of its high yield and broad prospects for comprehensive utilization. Tiger nuts

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exist in three diversities (black, brown and yellow) amongst which the yellow one is the most seek for human and animal consumption in Africa, Europe and America (Fabrice et al, 2022). According to the three varieties cultivated, that is yellow, brown and black. Only two varieties; yellow and brown are readily available in Nigeria markets. The yellow variety is preferred over others due to its basic qualities such as large size, attractive color and fleshier nature. It also produces more milk upon extraction, contains lower fat and higher protein and less anti-nutritional factors especially polyphenol (Gambo & Dau, 2014). It has long been recognized as one of the best nutritional crops used to augment diets, since a substantial intake decreased reported cases of various health related conditions such as cardiovascular disease, diabetes, cancer, and obesity, and also ideal for children, older persons and sportsmen, as well as an excellent source of iron and calcium for body growth and development (Bamishaiye & Bamishaiye, 2011). It was found to be rich in myristic acid, oleic acid, linoleic acid. Again, tiger nuts with its inherent nutritional and therapeutic advantage, could serve as alternative to cassava in baking industry (Ade-Omowaye et. al., 2008). Microbial contamination of tiger nuts by *Salmonella species, Bacillus cereus, Clostridium species,* and *Staphylococcus species Acinetobacterspecies, Enterobacter species, Corynebacterium species, Neisseria species,* and *Aeromonas species* e.t.c. is often not discovered before the nuts are consumed, this has led to food intoxication among the consumers (Ugbo et al, 2022). In Nigeria, mycotoxin contamination of millet, sorghum, peanut and tiger nut have been reported; a disturbing finding is the detection of 10 different mycotoxins present in tiger nut; with this insinuating that tiger nut contributes to the levels of mycotoxins produced during the preparation of kunu (Rubert et al., 2013). Mycotoxins are toxic secondary metabolites produced by aerobic, mycelial, microscopic fungi, especially from the genera *Aspergillus, Fusarium,* and *Penicillium.* Aflatoxins are highly toxic, mutagenic, and carcinogenic compounds that are produced as secondary metabolites by fungi belonging to several species, *mainly Aspergillus flavus* and *Aspergillus parasiticus* as well as *Aspergillus nominus*. These fungi appear largely in soil and decaying vegetation. Aflatoxins often occur in crops in the field prior to harvest. Postharvest contamination can arise during storage of the crop and also if drying is hindered. Insect or rodent infestations also facilitate mold invasion if water is allowed to exceed critical values which will enhance the mold growth. Due to the importance of these tiger nuts in daily living, this research work sought to evaluate the microbial species associated with vended Tiger Nuts, obtain information concerning the level of mycotoxin present in the product as an indication of what consumers might be exposed to.

MATERIALS AND METHODS

Sample Collection Procedure

Tiger nuts from vendors at each market were put together and labelled by the name of the site, giving a total of ten (10) groups of about 10g of nuts each. The nuts were examined macroscopically for the presence of worms and larval forms of insects. The nuts were soaked in 150 ml of sterile normal saline, in sterile bottles for one hour. After the hour, the nuts were washed by shaking the bottles vigorously. The saline was then transferred into a sterile beaker. The process was repeated for three additional washes and all the washes pooled. Aliquots of the pooled saline washes were dispensed into centrifuge tubes and centrifuged at 500rpm for 5 minutes. The supernatant was decanted and the sediments were re-suspended in 1ml of saline for parasitological examination while the other aliquot was used for bacteriological and fungal examination. The processes were repeated for each group of nuts collected. Sterile normal saline, in which washing of the nuts have not been done was also centrifuged, supernatant decanted, and used as control for both the parasitological, fungal and bacteriological examinations.

Parasitological Examination

The tiger nut samples were immediately transferred to the laboratory, where 10 g of each of the tiger nut samples was weighed and placed in sterile beakers with a saline solution (0.85% NaCl) and then washed. New gloves were used for each sample analyzed. The parasitological analysis was carried out using the Zinc sulfate floatation sedimentation technique (Wengenack, 2023). Briefly, in order to remove eggs, larvae, and cysts from the tiger nut samples, the procedure involved the tiger nut samples being soaked into a sterile saline solution and agitated them five times in the span of 30 minutes. The suspension was filtered through sterile, clean fine mesh gauze to remove larger debris. The filtrate was centrifuged at 5000rpm for 5 minutes, the supernatant was removed after centrifugation without shaking, and the sediment was meticulously examined for parasite ova, larvae, and cysts

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using a light microscope with an objective lens of 10 and 40. Parasite ova, larvae, and cysts were compared to and identified using known features (Idahosa, 2011; Gaspared & Schwartzboad, 2002).

Isolation, Enumeration, Identification, and Susceptibility Testing of Bacteria

Detection of bacteria and species identification was carried out using four (4) primary media (MacConkey, Sheep Blood Agar, Chocolate agar and Uriselect agar). The wash from each location (part of which was used previously in the parasitological examination) was dispensed aseptically in 0.1 ml aliquots into separate petri dishes and pour plated with the primary media. As a control, equal volumes (0.1 ml) of sterile normal saline were also dispensed aseptically into Petri dishes and pour plated with the media. The plates were kept for incubation at 37°C for 24 hrs. Bacterial colonies were counted on each plate after incubation to determine colony-forming units (CFU). Bacteria isolates was further sub-cultured to obtain pure cultures and the colonies were observed. Characterizations of isolates was made by microscopy, gram staining, morphologic examination, oxidation-fermentation tests and other biochemical tests including catalase test, urease test, triple sugar iron test, indole test and citrate utilization tests (Harrigan & McCance, 1993; Buchanan & Gibbon, 1974; Abbey, 2007).

Antibiogram of Pathogenic Bacteria Isolated from Tiger Nuts

Identified bacterial isolates were refined and their susceptibility patterns were determined for various antibiotics using a modified form of the Kirby Bauer method (Bauer et al., 1966). The antibiotics tested include; Penicillin (ampicillin 10µg), Aminoglycoside (gentamicin 10µg), Quinolone (ciprofloxacin 5µg), Phenicol (chloramphenicol 30µg), Carbapenem (imipenem 10µg), Sulfonamide (cotrimoxazole 25µg) (Oxoid Ltd., Basingstoke, UK). These antibiotics are among the common antibiotics found in the market. 5 ml fresh culture colony of bacteria suspension was prepared to equivalent of 0.5 McFarland standards. 1 ml of the suspension was transferred onto the agar plate; the surface of the agar was evenly swabbed using sterile cotton swab stick and allowed to dry. Sterile forceps were used to implant the antibiotic discs to the surface of the agar plate and allowed for 30 minutes for the antibiotics to diffuse before incubation. The plates were then incubated at 37°C for 18–24 hours. Zone diameters around the antibiotic discs was measured and later interpreted as sensitive or resistant based on the North Country Library System (NCLS) break point (Barry & Jones, 1988).

Isolation and Identification of Molds Present in the Tiger Nut Samples

Potato Dextrose Agar plates were aseptically prepared using standard methods. Streptomycin sulphate (0.05mg/L) was added to the agar after sterilization to prevent bacterial growth (Segalin & Reis, 2010). Using the pour plate method, 1ml of resulting mixture from the wash from each location (part of which was used previously in the parasitological examination) was inoculated into the cooled Potato dextrose agar (PDA) plates in triplicates. As a control, equal volumes (1 ml) of sterile normal saline were also dispensed aseptically into Petri dishes and pour plated with the media. After, it was then incubated at room temperature (30 ± 2°C) for 5-7 days. Fungal growths were obtained; it was further sub-cultured on fresh plates of PDA to obtain a pure culture. Each fungal isolates were placed on a microscopic slide with few drops of 0.1% Lactophenol cotton blue and observed under the microscope. The fungal isolate was identified using the fungal family of the world mycological monograph under microscopic observation. (Uzeh & Adebowale, 2021).

Molecular Characterization of Tiger Nuts Samples

The steps described by Okeke et al, (2015) for molecular identification of DNA of pure isolates was adopted in identifying bacterial isolates from the tiger nut samples. The steps involved are extraction of DNA, quantification of the extracted DNA, amplification of the 16S rRNA, amplification of internal transcribed space (ITS), sequencing and phylogenetic tree analysis (Murray et al, 1994). The nucleotide sequences obtained for each strain was edited using bioinformatics algorithm trace edit after which similar sequences was downloaded from NCBI database using BLASTIN. The nucleotide sequences obtained were aligned using Clustal X and Neighbor-Joining method described by Saitou & Nei, (1987) were used to deduce evolutionary history of the isolates using Felsenstein, (1985) as a guide. Computing of evolutionary distances was carried out using the Jukes-Cantor method (Jukes & Cantor, 1969).

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Determination of Mycotoxin from Tiger Nut Samples

The method of Gammoh, et. al. (2023) was followed for purification of mycotoxins from collected tiger nut samples. The tiger nuts samples were finely grounded and thoroughly mixed using a mill and mixer before a portion is removed for the analysis. About 5g of the homogenized and finely ground sample is transferred into a conical flask and 20ml of the extraction mixture (80% acetonitrile: 19% water: 1% Formic acid) is added to it. It was then shaken vigorously in an orbital shaker at 250rpm for 90minutes at room temperature. After extraction, the portion was centrifuged at 4200 x g for 5min, and 450µl of the supernatant was removed to glass vial. About 750 µl of ultrapure water was then added to the supernatant and Vortexed well. 1200 µl of the prepared filtered aliquot was introduced in a membrane syringe into a glass vial and vortexed again. It was then analyzed using the Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) to determine the quantity of mycotoxins present in the tiger nuts sample.

DETECTION OF MYCOTOXIN GENES

DNA Extraction

Fungal Genomic DNA was extracted according to the protocol described in Quick-DNATM Miniprep kit (Zymo research, Catalogue No. D6005). Fungal species isolated from Tiger Nut samples was inoculated on fresh Potatoe Dextrose Agar (PDA) plates and incubated at 25°C for 72hours. A small amount of each fungus was transferred into sterile mortar and crushed with pestle in phosphate buffer solution (this preliminary step was to lyse the cell wall). A solution made up of 95µl of water, 95µl of solid tissue buffer and 10µl of proteinase K was added and mixed thoroughly using a vortex mixer. It was then incubated at 55ºC for 2 hours and centrifuged to remove insoluble debris. Then 200µl of supernatant was transferred to a tube and 400µl of genomic binding buffer was added to it. The mixture was transferred to a Zymo-spin™ IIC-XL column in a collection tube and centrifuged (≥12000×g) for 1 minute, the collection tube was later discarded with the flow through. To the column in a new collection tube, 400µl DNA Pre-wash buffer was added and centrifuged and the collection tube was then emptied. Seven hundred microlitres (700µl) of g-DNA wash buffer was added and centrifuged for 1 minute and the collection tube was discarded with the flow n tube was emptied. Two hundred micro litres (200µl) of g-DNA wash buffer was added through. The Zymo-spin™ IIC-XL column was transferred to a 1.5ml Eppendorf tube and 50µl of elution buffer was used to elute the genomic DNA and it was stored at -20ºC (Zymo Research Group, USA). The DNA quality was verified by running the DNA samples by electrophoresis in 1.5% agarose gel stained with 0.04% (v/v) ethidium bromide for 25 mins then documented under the UV light prior to PCR amplification (Oetari et al., 2018).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed using a commercial kit (Quick-DNA miniprep plus kit). Universal primers 27F and 1492R are used to amplify the 16S target region according to Galkiewicz and Kellogg (2008). The PCR products were run on a gel and cleaned up enzymatically using the Exosap Method. The extracted segments were sequenced in the forward and reverse direction and purified. The purified fragments were analysed on the ABI3500xl Genetic analyzer (Applied Biosystems, Thermo Fisher Scientific) for each reaction for the entire the sample. The amplification reactions were performed according to Oetari et al. (2018); in a thermocycler Techne TC-312 (England) in PCR tubes containing 25 µL of the following reaction mixture.; 0.5 µl of both the forward and reverse primers (10mM), 2.0µl of extracted fungal DNA, sterile distilled water added to One Taq Quick-Load 2X Master Mix Buffer (New Eng-land Bio Labs). The initial denaturation was at 94°C for 5 minutes, denaturation at 940C for 30seconds, annealing at 500C for 30seconds, extension at 680C for 30seconds and there was a final extension at 68°C for 5 mins for 30 cycles. Ten (10 µl) of amplicon was resolved by 1.5% agarose gel electrophoresis. All amplicons were sent to Inquaba in south Africa for proper identification.

Table 1: Reaction volume for PCR Amplifications

Mix	1x
DNA	2.0ul

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Primer forward(1um/ul)	0.5ul
Primer reverse(1um/1ul)	0.5ul
Master Mix	12.0ul
Total	25.0ul

Agarose Gel Electrophoresis

Agarose gel (1.5%) was prepared by stirring 1.5g of agarose in 100ml 1 x TAE buffer and heating in microwave oven; the solution was allowed to cool and a drop of ethidium bromide (2mg/ml) was added; the solution was poured into a tray fixed with comb to create wells upon solidification. After cooling, the comb was carefully removed. Loading dye was added to the amplified PCR product, and carefully loaded into each well. Gels were run at a voltage of 120 for 30 minutes.

Statistical Analysis

All data were analyzed by general statistics and one-way ANOVA using the Statistical Product and Service Solutions (SPSS) software package 21.0.

RESULT AND DISCUSSION

Parasitological investigations discovered in life form of two different parasites from the tiger nuts samples collected (Table 2). The parasites identified were in the following percentage; *Entamoeba Histolytica* oocysts (15/40, 37.5%) and Ascaris Lumbricodes (25/40, 62.5%). *Ascaris Lumbricodes* recorded the highest percentage. *Entamoeba Histolytica* was found to be present on nuts from four (4) of the ten (10) markets studied, namely, Bodija. Molete, Agbeni and Ojo while *Ascaris Lumbricodes*, was found to be present on tiger nuts from three (3) markets; Oja-Oba, Agbeni and Ojo (Table 1). Interestingly, the two parasites were found to be associated with nuts bought from Ojo markets. This is consistent with results obtained elsewhere in previous studies where *Entamoeba histolytica* was seen to occur at a higher percentage (36.89%) and *Ascaris Lumbricodes* at lower percentage (0.24%) from nuts vendored in Jos, Plateau State Nigeria (Dawet et.al., 2019). The results of the study showed that samples collected from "Ojo" (13) had the highest frequency, followed by samples collected from "Oje" (10), and "OjaOba" (7) markets. The parasitic contaminants found on the nuts are associated with gastrointestinal infections. The most prevalent parasite, *Ascaris Lumbricoides* (62.5%), is known to be present in soil, they contaminate uncooked fruits or vegetables that have been grown in contaminated soil. and causes malnutrition and growth retardation in human (Ayeh-Kumi et al., 2014). *Ascaris Lumbricoides* and *Entamoeba histolytica* has also been reported to be contaminated with fresh fruits and vegetables sold in open market in South-West Ethiopia (Bekele & Shumbej, 2019). The presence of these parasites in the tiger nut samples could be due to inappropriate agricultural practices during cultivation, with cultivated tiger nuts coming into direct contact with soil and water contaminated with human and animal faeces. Therefore, further action should be taken to minimize parasitic contamination in tiger nuts by implementing good agricultural practices and improving water treatment capability.

In this study, five bacteria species were isolated from tiger nut tubers collected from the ten locations studied. The percentage of occurrences amongst all locations was recorded. Among the bacteria isolates the most predominant were *Pseudomonas putida* (3/19, 15.79%), *Klebsiella pneumoniae* (2/19, 10.53%), *Bacillus cereus* (3/19, 15.78%), *Escherichia coli* (5/19, 26.32%), and *Staphylococcus aureus* (6/35, 17.14%). *Klebsiella pneumoniae* recorded the lowest frequency (2/35, 5.71%) while *Staphylococcus aureus* recorded the highest frequency (6/19, 31.58%). This observation is comparable to the report of Ugbo *et al*. (2022) who reported higher percentage occurrence of *Staphylococcus aureus* (11/25.0%) and *Escherichia coli* (10/ 22.7%) from exposed tiger nuts. The high rate of *Staphylococcus aureus* (25.0%) and *Escherichia coli* (22.7%) is of public health concern because the organisms are implicated as causes of food-borne illnesses. It has been traced to cause food spoilage, food intoxication and food-borne diseases; hence, they should be of paramount interest when food hygiene is being evaluated. This is similar to the report that the intake of contaminated food can lead to food borne illnesses such as diarrhoeal-associated illnesses with bacteria being one of the major causes (Akhondi &

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Simonsen, 2023). The presence of *Escherichia coli* in tiger nut tubers could be associated with improper health hygiene of exposing the nut to dust and sanitary practices of using bare hands by vendors to measure and sell the tiger nuts to the public. This finding is supported by the report of study on microbial evaluation of tiger nut (*Cyperus esculentus*) sold in Abakaliki (Ugbo et al., 2022). Another research reported that *Escherichia coli* has been recognized and serve as an indicative microorganism for fecal contamination and its presence in tiger nuts tuber indicates the practice of poor hygiene and sanitary practices by vendors or hawkers (Ike et al., 2017). Highest frequency of occurrence of *Staphylococcus aureus* (31.58%) in the tiger nuts could be due to poor hygienic practices of the personnel handling the product which contaminated it with the bacterium to be a normal flora of the skin, palms, hair, nose and mucus membrane. This result partly agrees with Ire *et al*. (2020) which reported that *Staphylococcus* spp (37.3%) accounted for highest incidence of bacterial isolates from samples of tiger nut drink (kunu aya) sold within Port-harcout metropolis, Rivers state, Nigeria. Maduka *et al*. (2022) reported the presence of bacterial genera which include *Pseudomonas* spp and *Klebsiella* spp. in Tiger nut milk sold in Benin city, Nigeria. *Pseudomonas* spp (30 %) and *Bacillus* spp (30 %) accounted for the bacterial species which had the highest percentage occurrence as compared to our finding which recorded the least percentage among the bacterial species reported. The presence of *Pseudomonas* spp., *Bacillus* spp., and *Klebsiella* spp. in samples of tiger nut drink sold in Wukari, Nigeria metropolis was also reported by Ogodo *et al*. (2018*). Klebsiella species* (60%) and *Bacillus species* (40%) recorded a higher percentage occurrence while *Pseudomonas species* (20%) recorded the least percentage occurrence. The presence of coliform bacteria in tiger nuts as established in this study is of health concern. The prevalence of *Escherichia coli* in the tiger nut from this study could be harmful to consumers because it is known to be the indicator of unfavorable hygienic conditions and fecal contamination in foods. The risk is magnified with *Staphylococcus* spp which has been implicated in food-borne disease since staphylococcal toxins are common cause of food poisoning (Ugbo *et al*., 2022). The single band obtained in this study after DNA amplification of the ITS region confirmed that the isolates were obtained from pure cultures. The Antibiogram of the isolates revealed varying levels of resistance and susceptibility to the antibiotics tested. The results show that *Bacillus cereus* showed a zone of inhibition at 40mm for Ampicillin, 39mm for chloramphenicol, 38mm for Iminipem, 32mm for Cotrimoxazole, with the same zone of inhibition in Ciprofloxacin (31mm) and Gentamicin (31mm) respectively. *Escherichia coli* showed a zone of inhibiton at 34mm for Ampicillin, 20mm for chloramphenicol, 26mm for Gentamycin, 19mm for Cotrimoxazole, with the same zone of inhibition in Ciprofloxacin (31mm) and Iminipem (31mm) respectively. *Klebsiella pneumoniae, Pseudomonas putida,* and *Staphylococcus aureus* shows susceptibility to both cotrimoxazole and Gentamycin. *Klebsiella pneumoniae* was seen as Intermediate in Ciprofloxacin (25mm), Ampicillin (15mm), Cloramphenicol (15mm) and Iminipem(20mm). *Pseudomonas putida* was susceptible to both Ciprofloxacin (25mm) and Chloramphenicol (18mm) and was seen as intermediate in Iminipem (16mm). *Staphylococccus aureus* was also susceptible to both Ciprofloxacin (28mm) and Chloramphenicol (19mm) and was seen as resistant to Iminipem (16mm). This observation is in line with the report on bacteria isolates from exposed tiger nut sold by street vendors in Abakaliki, Nigeria (Ugbo et al., 2022). All the Isolates were seen to be susceptible to cotrimoxazole and Gentamycine. Amongst all the isolates tested it was only *Pseudomonas putida* and *Staphylococcus aureus* that were resistant. In this current study, *Bacillus cereus* and *Escherichia coli* were the only isolates seen to be highly susceptible to all the antibiotics tested. The discovery agrees with the finding of other researchers who worked on bacteriological and nutritional quality of tiger nut consumed by students of Nasarawa State University, Keffi Nigeria (Adesakin & Obiekezie, 2020). This finding suggests that clinicians and medical officers can have various options to select from, when treating bacterial infection acquired as a result of consumption of contaminated tiger nuts by individuals from the markets in Ibadan, Oyo state, Nigeria. However, there should be constant awareness of the public health implication of bacteria contaminated tiger nuts sold in Ibadan by environmental health officers and other government health agencies. Screening for fungal isolates present in food material gives awareness into the number and types of toxigenic microbes present there in, providing information of the sample's quality and the consequence possible risk to susceptible consumers. Fungal isolates were reported to be present in the tiger nut samples, a finding in accordance with report of Okwelle, (2020). As shown in Table 4, this study identified three fungal strains from the test tiger nuts (*Cyperus esculentus*) samples collected from ten major markets in Ibadan city; they include *Aspergillus flavus, Aspergillus fumigatus* and *Aspergillus niger*. Based on the percentage incidence, *A. flavus* was the most dominant fungal specie associated with the studied tiger nut samples. Obasi and Mani (2023) recorded the occurrence of *Aspergillus fumigatus* and *Aspergillus niger* amongst others in their work on tiger nuts from Taraba state, Nigeria. *Aspergillus niger* and *Aspergillus fumigatus* had the same percentage occurrence (14.29%) while the

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highest occurrence was seen in *Aspergillus flavus* (17.24 %). The total fungal counts (TFC) of tiger nut tubers from different market locations are shown in Table 5. The results indicated that the TFC of tiger nuts sold was within the range of 6 log10 CFU/mL to 17 log10 CFU/mL. Highest TFC (17 log10 CFU/mL) was recorded from tiger nut samples from Orita-Aperin Market while the lowest TFC (6 log10 CFU/mL) was obtained in samples from Oje market. It was informative that in all the samples of the different markets, the presence of fungi was detected. The results indicated that all were beyond the maximum acceptable limit as recommended by the International Commission on Microbiological Specifications for Food (ICSMF) of less than 105. The presence of fungal contamination can be attributed to factors such as contaminated soil, improper handling practices or procedures, substandard personal hygiene of vendors, and air-borne fungal particles. The occurrence of these fungi may cause diverse effects on human health as they have the potential of producing mycotoxins (Ikechi-Nwogu et al., 2020). The result of the mycotoxin analysis showed that majority of the samples was all contaminated with mycotoxins. Of all the mycotoxins tested, Aflatoxin B1 had the highest occurrence in only two of the major markets, that is, Gbagi market sample (95.7ug/kg) and Ojo market sample (33.03ug/kg) and they were seen to be above the permissible limits set up by NAFDAC (20ug/kg). The present findings of high aflatoxin occurence in tiger nut agree well with reports of Shamsudeen and Aminu, 2016. Both aflatoxins B1 and B2 recorded no value (that is, it was below detection limit) in Oja-Oba market sample while the other market samples were also seen to be below the permissible limit set up by NAFDAC (20ug/kg) which makes the tiger nuts sold in those markets fit for consumption). It was informative that in all the samples of the different markets, the presence of aflatoxin B2, G1 and G2 was seen to be below the permissible limit set up by NAFDAC (20ug/kg). This is consistent with the result obtained elsewhere in previous studies (Jallow et al, 2021; Omoniyi et al, 2014). Zearalenol and its derivatives were seen to be above the permissible limit set by Food and Agriculture Organization in United nations (1ug/kg). This could be a threat to human as well as animal health because zearalenone produces oestrogen effect leading to hyperstrogen (Imade et al, 2021). Ingestion of zearalenone has exhibited symptoms such as the enlargement of mammary glands, vulvovaginitis (vaginal swelling), infertility, reduced sexual drive, stillbirths, abortion, and reduced litter size (Chika. 2017). Diacetoxyscirpenol was also seen to be above the permissible limit set up by Food and Agriculture organization in United Nations (0. 1ug/kg). All samples from the ten major markets in Ibadan were seen well above the permissible limit for fuminosin B1, B2 and B3 (range 24.23ug/kg – 24.39ug/kg). The present findings of high fuminosin occurrence in tiger nut samples agree well with reports of Okwelle et al.,2020. These levels are well above the provisional maximum tolerable daily intake of 2μg/kg of body weight/day set by the joint FAO/WHO Expert Committee on Food Additives and the permissible limit set up by Food and Agriculture organization in United nations, FB1 is the most toxic of the group of fuminosins, it causes liver cancer and have also been linked with high incidence of human oesophageal cancer. In 2022, International agency research on cancer (IARC) classifies FB1 as possibly carcinogenic to humans (Ahmed Adam, 2017). Deoxynnivalenol (DON) and its derivatives was within the range of 19.34ug/kg –19.86ug/kg which is above the maximum permissible limit set by world health Organization (9ug/kg) and Food and Agriculture Organization (0.5-2ug/kg). This finding aligns with result obtained elsewhere in previous studies (Haidukowski et al.,2022). DON exposure has been associated with the occurrence of acute gastrointestinal diseases which include diarrhea, vomiting, and gastroenteritis in humans. Total Ochratoxin A(OTA) concentrations was seen in all the samples ranging from 1.20 to 1.50μg/kg and this was seen to be above the tolerable limit set by Food and Agriculture Organization (0.05ug/kg). The present findings of high ochratoxin A occurrence in tiger nut samples agrees well with result obtained elsewhere in previous studies (Dauo et al., 2023). Various studies have linked OTA exposure with the human diseases like Balkan endemic nephropathy (BEN) and chronic interstitial nephropathy (CIN), as well as other renal diseases (Bui-Klimke & Wu, 2015). Fusaric Acid (range 2.47to 2.83ug/kg) was seen to be above the permissible limit set by Food and Agriculture Organization (0.01ug/kg). Enniatin (ENN), Beauvericin (BEA) and Moniliformin are lesser known toxins, called emerging mycotoxins. Enniatin B occurrence in Oje market was seen at 57.20ug/kg, Enniatin B2 were reported in both Ojo (0.37ug/kg) and Gbagi (0.33 ug/kg) market samples below the permissible limit set by FAO, Enniatin A was seen in Bodija samples (0.36 ug/kg), Agbeni samples (0.37 ug/kg), Gbagi samples (0.39ug/kg), and Olomi samples (0.36ug/kg) to be below permissible limit set by FAO (0.5ug/kg). Enniatin A1 was also reported to be below permissible limits set by FAO (0.5ug/kg) in Bodija (0.40ug/kg), and Orita Aperin (0.47 ug/kg) market samples. Others were seen in the range of 0.50 ug/kg to 17.82ug/kg within the permissible tolerable limit set by FAO. Beavuricin was seen in the range of 0.50 ug/kg to 3.29ug/kg. Monilоformin was also seen in the range of 2.47 ug/kg to 2.93ug/kg across the ten market samples. The Emerging mycotoxins were also seen to be present in the tiger nuts samples and above the permissible limit set by Food

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and Agriculture Organization (0.5ug/kg).

Table 2: Frequency of occurrence of parasites in Tiger nuts from major markets in Ibadan metropolis

Parasites	**Market Locations**										
	Bodija	**Ojaoba**	**Molete**	**Oje**	**Agbeni**	**Ogunpa**	**Gbagi**	**Ojo**	**Oloomi**	**Orita Aperin**	**Total No (%)**
Entamoeba Histolytica oocyst	**4**	-	**2**	-	**4**	-	-	**5**	-	-	**15 (37.5)**
Ascaris Lumbricodes Ova	-	**7**	-	**10**	-	-	-	**8**	-	-	**25 (62.5)**
Total No (%)	**4**	**7**	**2**	**10**	**4**	-	-	**13**	-	-	**40 (100)**

* No parasite was found in the tiger nuts bought from market locations (Ogunpa, Gbagi, Oloomi and Orita-Aperin)

Table 3: Morphological and microscopic characteristics of the fungal strains associated with Tiger nuts (*Cyperus esculentus*)

Fungal Species	**Morphological/microscopic feature on PDA**
Aspergillus flavus	Appear yellowish-green becoming green with age but creamish yellow reverse. Appears to have radiating conidial heads, long verrucose and hyaline stripe with a small metulae.
Aspergillus niger	Appear Blackish-brown often with yellow mycelium. Conidial head appears to be globose, dividing with age. They produce dark or dark brown spores.
Aspergilus fumigatus	Appears Bluish-green at upper and creamish-yellow in its reverse. Its head is columnar in shape, metulae is absent. The conidia are flobose and greenish.

Table 4: Frequency of occurrence of fungal isolates in Tiger nut from major markets in Ibadan metropolis

Microbial Isolates	**BA**	**OA**	**MA**	**JA**	**AA**	**GA**	**YA**	**ZA**	**LA**	**WA**	**TOTAL NO (%)**
Aspergillus Niger	+	+	-	+	-	+	-	+	-	-	**5 (31.25)**
Aspergillus flavus	+	-	+	-	+	-	+	-	+	+	**6 (37.50)**
Aspergillus fumigatus	+	-	+	-	-	+	-	+	+	-	**5 (31.25)**
Total	**3**	**1**	**2**	**1**	**1**	**2**	**1**	**2**	**2**	**1**	**100**

Key: + = Positive; - = Negative; BA = Bodija; OA = Oja Oba; MA = Molete; JA = Oje; AA=Agbeni; GA= Ogunpa; YA= Gbagi; ZA= Ojo; LA= Olomi; WA= Orita Aperin

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Table 5: Frequency of occurrence of Bacterial isolates in Tiger nut from major markets in Ibadan metropolis

Microbial Isolates	(BA)	(OA)	(MA)	(JA)	(AA)	(GA)	(YA)	(ZA)	(LA)	(WA)	TOTAL NO (%)
P. putida	+	-	+	-	-	+	-	-	-	-	**3 (15.79)**
K. pneumoniae	-	+	-	+	-	-	-	-	-	-	**2 (10.53)**
B. cereus	-	-	+	-	+	-	+	-	-	-	**3 (15.78)**
E. coli	+	-	-	+	+	-	-	+	+	-	**5 (26.32)**
S. aureus	+	+	-	-	+	-	+	-	+	+	**6 (31.58)**
TOTAL	**3**	**2**	**2**	**2**	**3**	**1**	**2**	**1**	**2**	**1**	**100**

Key: + = Positive; - = Negative; BA = Bodija; OA = Oja Oba; MA = Molete; JA = Oje; AA=Agbeni; GA= Ogunpa; YA= Gbagi; ZA= Ojo; LA= Olomi; WA= Orita Aperin

Table 6: Antibiogram of bacteria isolates from tiger nuts.

Antibiotics	Conc. (µg)	*Bacillus Cereus*	*Klebsiella Pneumoniae* (%)	*Pseudomonas Putida* (%)	*Staphlococcus Aureus* (%)	*Escherichia Coli* (%)
Ciprofloxacin	**5**	**S 31mm**	**I 25mm**	**S 25mm**	**S 28mm**	**S 30mm**
Cotrimoxazole	**25**	**S 32mm**	**S 16mm**	**S 20mm**	**S 19mm**	**S 19mm**
Gentamycine	**10**	**S 31mm**	**S 26mm**	**S 15mm**	**S 27mm**	**S 26mm**
Ampicillin	**10**	**S 40mm**	**I 15mm**	**R 17mm**	**I 15mm**	**S 34mm**
Chloramphenicol	**30**	**S 39mm**	**I 15mm**	**S 18mm**	**S 19mm**	**S 20mm**
Iminipem	**10**	**S 38mm**	**I 20mm**	**I 16mm**	**R 16mm**	**S 30mm**

* Key: S = Sensitive, R = Resistant, I= Intermediate

Table 7: The molecular Identification of microbial isolates through 16S rRNA Analysis

Sample ID	Organism	Sequence length(bp)	% Identity	Gene Bank Accession No	Alignment score	Highest query coverage (%)
BA	***Bacillus cereus***	**1518**	**99.60%**	**PP819519**	**≥200**	**99%**
MA	***Klebsiella pneumoniae***	**1367**	**98.68%**	**PP819520**	**≥200**	**99%**
YA	***Klebsiella pneumoniae***	**864**	**96.99%**	**PP819521**	**≥200**	**99%**
OA	***Bacillus cereus***	**1521**	**98.41%**	**PP819522**	**≥200**	**99%**
AA	***Pseudomonas putida***	**1504**	**95.46%**	**PP819523**	**≥200**	**97%**
JA	***Bacillus cereus***	**1524**	**99.80%**	**PP819524**	**≥200**	**99%**

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*Key BA=Bodija, MA= Molete. YA=Ogunpa, OA=Oja-Oba, AA=Agbeni, JA=Oje

Table 8: Total Fungal plate count of fungal isolates from Tiger nuts tubers (*Cyperus esculentus)*

Sample Code	Isolate name	Fungal Count(cfu/ml)
BA	***Aspergillus niger***	**1.3×10^7**
OA	***Aspergillus niger***	**1.0×10^7**
MA	***Aspergillus flavus***	**8.0×10^6**
JA	***Aspergillus niger***	**6.0×10^6**
AA	***Aspergillus flavus***	**1.1×10^7**
GA	***Aspergillus fumigatus***	**6.5×10^6**
8YA	***Aspergillus flavus***	**$1.4 \times 10^{\cdot}$**
ZA	***Aspergillus niger***	**1.5×10^7**
LA	***Aspergillus fumigatus***	**8.5×10^6**
WA	***Aspergillus flavus***	**1.7×10^7**

*KEY: BA = Bodija; OA = Oja Oba; MA = Molete; JA = Oje; AA=Agbeni; GA= Ogunpa; YA= Gbagi; ZA= Ojo; LA= Olomi; WA= Orita Aperin.

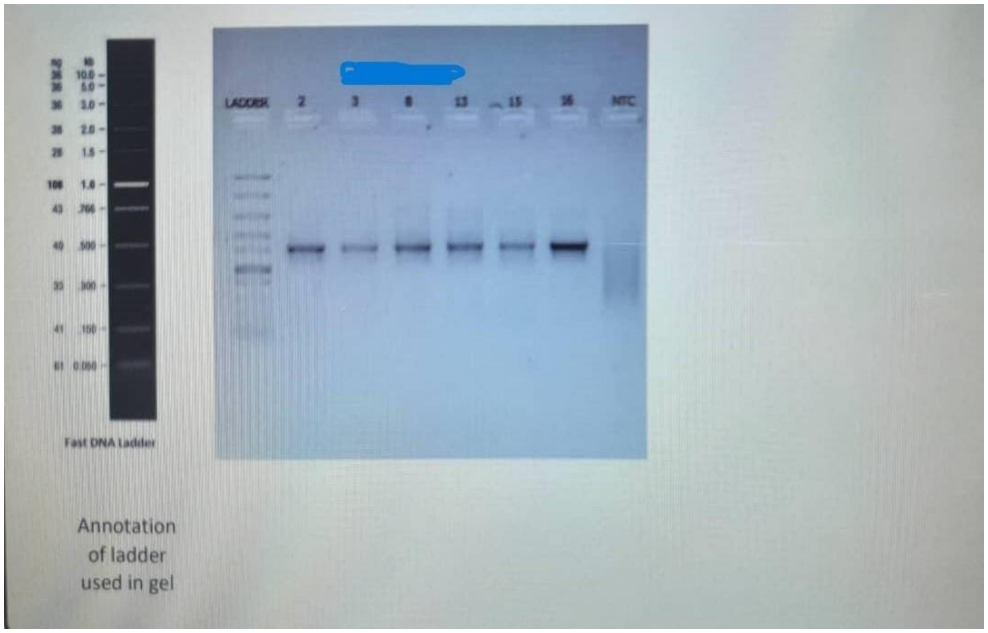

Fig 1: Picture of DNA Bands obtained indicting the amplification of the 16S target region

Table 9: Aflatoxin, Zearelenone and Diacetoxyscirpenol concentrations (µg/kg) in Tiger nuts (*Cyperus esculentus*).

Sample Code	AFB1	AFB2	AFG1	AFG2	α-Zeara lenol	β – Zeara lenol	Zeara lanone	Zeara lenone	Diacetoxy scirpenol
BA	**0.38**	**0.25**	**0.30**	**0.35**	**9.35**	**8.74**	**9.85**	**4.67**	**2.09**
OA	**0.00**	**0.00**	**0.32**	**0.37**	**9.65**	**9.54**	**9.47**	**4.86**	**2.90**

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MA	**0.33**	**0.32**	**0.37**	**0.33**	**9.41**	**9.40**	**9.61**	**4.65**	**2.51**
JA	**0.36**	**0.36**	**0.34**	**0.35**	**9.64**	**9.93**	**9.71**	**4.58**	**2.68**
AA	**3.37**	**1.38**	**0.35**	**0.37**	**9.84**	**9.69**	**9.51**	**4.87**	**2.81**
GA	**0.31**	**0.34**	**0.35**	**0.35**	**9.33**	**9.56**	**9.53**	**4.75**	**2.81**
YA	**95.70**	**13.68**	**0.38**	**0.35**	**9.49**	**9.55**	**9.48**	**4.37**	**2.70**
ZA	**33.03**	**6.84**	**0.35**	**0.39**	**9.50**	**9.42**	**9.33**	**4.47**	**2.95**
LA	**0.316**	**0.33**	**0.31**	**0.38**	**9.48**	**9.37**	**9.46**	**4.79**	**2.75**
WA	**0.33**	**0.34**	**0.32**	**0.35**	**9.84**	**9.65**	**9.55**	**4.61**	**2.93**

*KEY: BA = Bodija; OA = Oja Oba; MA = Molete; JA = Oje; AA=Agbeni; GA= Ogunpa; YA= Gbagi; ZA= Ojo; LA= Olomi; WA= Orita Aperin.

Table 10: Deoxynivalenol, Nivalenol, Fuminosin, Fusaric Acid and Moniliformin concentrations (µg/kg) in tiger nut (*Cyperus esculentus*).

Sample Code	**3-Acetyl Deoxy. nivalenol**	**15-Acetyl Deoxy-nivalenol**	**Deoxy-Nivalenol**	**Nivalenol**	**FB1**	**FB2**	**FB3**	**Fusaric Acid**	**Monili Formin**
BA	**19.54**	**19.47**	**19.53**	**19.58**	**24.27**	**24.28**	**24.33**	**2.48**	**2.93**
OA	**19.47**	**19.47**	**19.59**	**19.55**	**24.39**	**24.32**	**24.32**	**2.60**	**2.47**
MA	**19.46**	**19.55**	**19.50**	**19.39**	**24.30**	**24.30**	**24.29**	**2.70**	**2.54**
JA	**19.39**	**19.60**	**19.42**	**19.60**	**24.23**	**24.34**	**24.32**	**2.51**	**2.66**
AA	**19.34**	**19.68**	**19.86**	**19.60**	**24.27**	**24.29**	**24.27**	**2.63**	**2.65**
GA	**19.44**	**19.75**	**19.36**	**19.77**	**24.37**	**24.31**	**24.28**	**2.83**	**2.43**
YA	**19.55**	**19.42**	**19.62**	**19.42**	**24.35**	**24.32**	**24.45**	**2.55**	**2.66**
ZA	**19.52**	**19.53**	**19.39**	**19.38**	**24.26**	**24.34**	**24.23**	**2.80**	**2.54**
LA	**19.55**	**19.67**	**19.71**	**19.64**	**24.29**	**24.31**	**24.28**	**2.47**	**2.61**
WA	**19.51**	**19.50**	**19.61**	**19.42**	**24.25**	**24.38**	**24.31**	**2.62**	**2.58**

*KEY: BA = Bodija; OA = Oja Oba; MA = Molete; JA = Oje; AA=Agbeni; GA= Ogunpa; YA= Gbagi; ZA= Ojo; LA = Olomi; WA= Orita Aperin.

Table 11: Ochratoxin A, Beauvericin and Enniatin concentrations (µg/kg) in tiger nut (*Cyperus esculentus*).

Sample Code	**Ochratoxin A**	**Beauvericin**	**Enniatin A**	**Enniatin A1**	**Enniatin B**	**Enniatin B2**
BA	**1.50**	**0.58**	**0.36**	**0.40**	**1.69**	**0.64**
OA	**1.30**	**1.52**	**0.58**	**0.62**	**4.97**	**0.56**
MA	**1.33**	**0.62**	**0.57**	**0.69**	**2.23**	**0.59**

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JA	**1.33**	**0.71**	**0.51**	**0.52**	**57.20**	**3.44**
AA	**1..20**	**1.25**	**0.37**	**0.52**	**17.82**	**0.56**
GA	**1.33**	**0.52**	**0.56**	**0.54**	**1.74**	**0.53**
YA	**1.39**	**3.29**	**0.39**	**0.54**	**1.68**	**0.33**
ZA	**1.30**	**0.60**	**0.57**	**0.70**	**1.71**	**0.37**
LA	**1.33**	**0.63**	**0.36**	**0.60**	**2.06**	**0.74**
WA	**1.27**	**0.78**	**0.66**	**0.47**	**4.63**	**0.57**

*KEY: BA = Bodija; OA = Oja Oba; MA = Molete; JA = Oje; AA=Agbeni; GA= Ogunpa; YA= Gbagi; ZA= Ojo; LA= Olomi; WA= Orita Aperin

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