

Article

Molecular Characterization and Identification of Aflatoxigenic Fungi Associated with Poultry Feed in Port Harcourt, Rivers State

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Abstract: The contamination of livestock feeds by aflatoxigenic fungi poses significant health and economic risks, affecting both animal productivity and human food safety. This study investigated the occurrence, molecular characterization, and aflatoxigenic potential of fungal contaminants in commercial poultry feeds obtained from Rumuolumeni, Mile III, and Choba in Port Harcourt, Rivers State. About nine feed samples (starter, grower, and finisher) were aseptically collected and analyzed using standard microbiological methods. Ten grams of each sample were serially diluted and inoculated on Potato Dextrose Agar (PDA) supplemented with chloramphenicol to isolate fungal species. Distinct colonies were purified and identified based on morphology and molecular characteristics. DNA was extracted using the CTAB method, and the Internal Transcribed Spacer (ITS) region was amplified with ITS1/ITS4 primers. The resulting PCR products were sequenced, analyzed via BLAST on the NCBI database, and phylogenetically compared using MEGA 11 software. Aflatoxin production was confirmed through culture on YES agar, methanol extraction, immunoaffinity purification, and High-Performance Liquid Chromatography (HPLC) quantification. Results revealed that all feed samples were contaminated with fungi, with *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Penicillium*, *Fusarium*, and *Rhizopus* species identified. Molecular analysis confirmed *A. flavus* as the predominant aflatoxigenic fungus, clustering with known aflatoxin-producing lineages (bootstrap 85–92%). HPLC analysis showed that *A. flavus* isolates produced Aflatoxins B₁, B₂, G₁, and G₂, with B₁ being the most dominant and toxic (45.6–60.2 µg/kg). It is concluded that poultry feeds in the study area are significantly contaminated with aflatoxigenic *A. flavus*, posing serious food and feed safety concerns. The study recommends strict quality control during feed production, proper storage conditions, and routine screening for aflatoxins to safeguard animal and public health.

Keywords: Molecular identification, aflatoxigenic fungi, poultry feed.

Introduction

Poultry production is an important component of food security and economic development in Nigeria, providing affordable animal protein through meat and egg production. The success of poultry farming largely depends on the quality and safety of poultry feed, as feed directly affects bird health, productivity, and product quality. However, poultry feed is highly vulnerable to contamination by fungi due to the nature of its raw materials, processing methods, and storage conditions [1]. Fungal contamination of poultry feed is of serious concern because some fungi produce mycotoxins, particularly aflatoxins, which are among the most toxic secondary metabolites known. Aflatoxins are mainly produced by aflatoxigenic species of *Aspergillus*, such as *Aspergillus flavus* and *Aspergillus parasiticus*. In poultry, exposure to aflatoxins has been linked to reduced growth rate, poor feed conversion, decreased egg production, immunosuppression, and increased mortality, leading to significant economic losses. Additionally, aflatoxins can enter the human food chain through contaminated poultry products, posing public health risks. The occurrence of aflatoxigenic fungi in poultry feed is common in tropical regions where high temperature and humidity favor fungal growth and toxin production. Port Harcourt, Rivers State, experiences warm temperatures and high relative humidity throughout the year, creating favorable conditions for fungal proliferation in poultry feed, especially when storage and handling practices are inadequate. Conventional identification of fungi based on cultural and morphological characteristics is often insufficient for accurate species differentiation and for determining toxigenic potential. Molecular characterization techniques, including polymerase chain reaction (PCR) and analysis of specific genetic markers, provide more precise and reliable identification of aflatoxigenic fungi. These methods allow for the detection of fungi capable of producing aflatoxins, even when toxin production is not evident under laboratory conditions [2].

Investigated the occurrence of fungal species in broiler feed samples obtained from commercial poultry farms in southwestern Nigeria with the aim of assessing feed safety [3]. Using the pour plate technique on Sabouraud Dextrose Agar (SDA), the study isolated *Aspergillus flavus*, *Fusarium* spp., and *Penicillium* spp., with results indicating a high fungal load attributed mainly to poor storage conditions. Similarly, conducted a mycological assessment of poultry feed samples collected from southeastern Nigeria using serial dilution and culture methods, isolating *Aspergillus niger*, *Mucor* spp., and *Rhizopus* spp., and emphasizing the need for improved feed handling and storage practices [4]. Analyzed fungal contamination in poultry feeds using both morphological and molecular techniques and reported the dominance of *Aspergillus*, *Fusarium*, and *Alternaria* species, linking contamination to high environmental humidity and poor manufacturing practices [5]. Olabiyi and Fagbohun (2023) aimed to identify fungal contaminants in poultry feeds sold in Lagos markets using direct plating on SDA and DNA barcoding; their findings revealed *Cladosporium* spp., *Aspergillus ochraceus*, and *Fusarium verticillioides* as the predominant contaminants [6]. In Ghana, isolated fungi from locally produced poultry feeds using dilution plating techniques and identified toxigenic strains of *Aspergillus flavus* and *Penicillium citrinum*, highlighting the potential risk of mycotoxin contamination [7]. examined fungal contamination of poultry feed ingredients in Egypt using morphological identification and thin-layer chromatography for mycotoxin detection and reported a high prevalence of *Aspergillus parasiticus* and *Fusarium moniliforme*, particularly in maize-based feeds [8]. Employed cultural and microscopic techniques to evaluate fungal flora in layer mash feeds in Nigeria and found *Aspergillus niger*, *Aspergillus flavus*, and *Mucor* spp. to be the most frequently isolated fungi [9]. Used both culture-based methods and polymerase chain reaction (PCR) to isolate and identify fungi in poultry diets across Enugu State, detecting a high prevalence of *Fusarium* spp. and *Trichoderma* spp [1]. Carried out a mycological survey of commercial poultry feeds using standard plating methods and reported that over 60% of the samples were contaminated, with *Aspergillus fumigatus* and *Penicillium expansum* being the predominant species [10]. In Pakistan assessed fungal contamination in poultry rations using serial dilution and ITS sequencing and identified *Aspergillus flavus*, *Fusarium solani*, and *Rhizopus oryzae* as dominant species [11]. In Bangladesh employed pour plating and toxin screening methods and confirmed the dominance of *Aspergillus flavus*, which was strongly associated with the presence of aflatoxin B₁ [2]. Studied poultry feeds in Oyo State, Nigeria using culture techniques and scanning

electron microscopy, identifying *Eurotium* spp., *Acremonium* spp., and *Fusarium* spp., and emphasizing inadequate storage as a key factor in fungal proliferation [12]. Conducted a similar study in China using next-generation sequencing and reported a high diversity of fungal species, notably *Aspergillus*, *Fusarium*, and *Paecilomyces* [13]. Isolated *Scopulariopsis* spp. and *Geotrichum candidum* from poultry feeds in Nigeria using both cultural and molecular assays, identifying feed ingredient origin as a major source of contamination [14]. Assessed poultry feed samples from Nigerian farms using fungal culture and multi-mycotoxin LC-MS/MS analysis and reported widespread contamination by *Aspergillus*, *Fusarium*, and *Penicillium* species [15]. In Rivers State employed culture methods and ITS sequencing to characterize fungi in broiler feeds, isolating *Trichoderma harzianum* and *Aspergillus clavatus* [16]. Used microbiological plating and high-performance liquid chromatography (HPLC) in northern Nigeria and detected *Aspergillus ochraceus* and *Penicillium verrucosum* in contaminated feeds [17]. Investigated fungal contaminants in poultry starter feeds using standard mycological techniques and identified *Mucor*, *Fusarium*, and *Acremonium* species [18]. Applied both culture-dependent and culture-independent methods in Jos, Nigeria and identified *Aspergillus nidulans* and *Penicillium islandicum* as major contaminants [19]. Employed traditional culturing and biochemical assays to assess poultry feed contamination in Kano State and isolated *Aspergillus flavus*, *Fusarium oxysporum*, and *Trichoderma viride* [20].

Aim and Objectives of the Study

The aim of this research work was to isolate, characterize, and identify toxin producing fungi associated with poultry feed in Port Harcourt, Rivers State. The objectives were to:

- i. isolate fungal species present in poultry feed samples (New Hope) collected from poultry farms in Port Harcourt;
- ii. identify the presence of aflatoxigenic fungi using molecular techniques;
- iii. characterize the aflatoxins of the identified fungi;
- iv. assess the prevalence of aflatoxigenic fungal strains across different poultry feed types collected from poultry farms in Port Harcourt.
- v.

Materials and Method

3.1 Isolation of Fungal Species Present in Poultry Feed Samples

3.1.1 Sample Collection

About nine poultry feed samples (starter, grower, and finisher) were collected aseptically from different poultry farms across various locations (Rumuolumeni, Mile III, and Choba) in Port Harcourt. The samples were placed in sterile, properly labeled polyethylene bags and transported to the microbiology laboratory for further analysis.

3.1.2 Isolation of Fungal Species

Exactly 10 g of each feed sample was weighed using a sterile analytical balance and transferred into a sterile 250 ml conical flask containing 90 ml of sterile distilled water. The mixture was shaken vigorously for 10 minutes using a mechanical shaker to dislodge fungal spores. Serial tenfold dilutions were prepared up to 10^{-4} by transferring 1 ml of the suspension into 9 ml of sterile distilled water contained in sterile test tubes. Using a sterile micropipette, 0.1 ml aliquots from the 10^{-2} , 10^{-3} , and 10^{-4} dilutions were aseptically inoculated onto sterile Petri dishes containing solidified Potato Dextrose Agar (PDA) supplemented with 0.05 g/L chloramphenicol to inhibit bacterial growth. The inoculum was evenly spread using a sterile glass spreader. All plates were properly labeled and incubated in an inverted position at $28 \pm 2^\circ\text{C}$ for 5–7 days. After incubation, fungal colonies were examined for morphological differences, and each distinct colony type was picked using a sterile inoculating needle and subcultured onto fresh PDA plates to obtain pure cultures. The subcultured plates were incubated under the same conditions to allow adequate growth before identification.

3.2 Identification of Aflatoxigenic Fungi Using Molecular Techniques

3.2.1 DNA Extraction

Pure cultures of each fungal isolate were subcultured on Potato Dextrose Agar (PDA) and incubated for 5–7 days at $28 \pm 2^\circ\text{C}$ to obtain sufficient mycelial growth. Fresh mycelia were aseptically

scraped from the culture plates and transferred into sterile 1.5 ml microcentrifuge tubes. Genomic DNA was extracted using the Cetyltrimethylammonium Bromide (CTAB) method with slight modifications. Approximately 600 µl of CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, and 1.4 M NaCl) was added to each tube, and the mixture was incubated at 65°C for 30 minutes. An equal volume of chloroform:isoamyl alcohol (24:1) was added, and the mixture was centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred into new tubes, and genomic DNA was precipitated by adding 0.6 volumes of cold isopropanol. The samples were incubated at -20°C for 30 minutes and centrifuged again to pellet the DNA. The DNA pellets were washed with 70% ethanol, air-dried, and resuspended in 50 µl of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA concentration and purity were assessed using a NanoDrop spectrophotometer, while DNA integrity was confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

3.2.2 PCR Amplification

The Internal Transcribed Spacer (ITS) region of the ribosomal DNA was amplified using universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplification was carried out in a 25 µl reaction mixture consisting of 12.5 µl of PCR Master Mix, 1 µl each of forward and reverse primers (10 µM), 2 µl of template DNA, and 8.5 µl of nuclease-free water. Amplification was performed in a thermal cycler with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. The PCR products were confirmed by electrophoresis of 5 µl aliquots on a 1.5% agarose gel stained with ethidium bromide alongside a 100 bp DNA ladder. Amplified products with sizes of approximately 550–600 bp confirmed successful ITS region amplification.

3.2.3 Sequencing and Sequence Analysis

Successful PCR products were purified using a DNA purification kit and subjected to Sanger sequencing. The raw sequence data obtained were trimmed, edited, and assembled using BioEdit and Chromas software to eliminate ambiguous bases. The cleaned sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) on the NCBI GenBank database to determine sequence similarity with known fungal species. Species identification was based on a similarity index of 98% or higher with reference sequences.

3.2.4 Phylogenetic Analysis

Edited nucleotide sequences of the fungal isolates were aligned with closely related sequences retrieved from the GenBank database using ClustalW implemented in MEGA version 11 software. The multiple sequence alignments were examined, and gap positions were adjusted manually where necessary. Phylogenetic relationships were inferred using the Neighbor-Joining method with 1,000 bootstrap replications to assess the robustness of the tree topology. Evolutionary distances were calculated using the Kimura 2-parameter model. Isolates that clustered with reference strains with bootstrap values of 70% or higher were considered to belong to the same species or closely related taxa.

3.3 Characterization of Aflatoxins Produced by the Identified Fungi

Each confirmed aflatoxigenic fungal isolate was cultured on Yeast Extract Sucrose (YES) agar medium to induce aflatoxin production. The cultures were incubated at 28°C for 7 days in the dark. After incubation, the fungal biomass, including mycelia and agar, was scraped into sterile 250 ml conical flasks, and 100 ml of methanol:water solution (80:20 v/v) was added. The mixtures were agitated on a rotary shaker at 200 rpm for 30 minutes to extract aflatoxins. The extracts were filtered through Whatman No. 1 filter paper, and the filtrates were collected. The filtrates were purified using aflatoxin-specific immunoaffinity columns (IAC). Each column was washed with 10 ml of distilled water to remove impurities, and aflatoxins were eluted with 1 ml of pure methanol into clean vials. The eluates were analyzed using High-Performance Liquid Chromatography (HPLC) equipped with a C18 reverse-phase column and a fluorescence detector. The mobile phase consisted of water:methanol:acetonitrile (60:20:20) at a flow rate of 1.0 ml/min. Chromatograms obtained were compared with certified aflatoxin standards to identify and quantify aflatoxins B₁, B₂, G₁, and G₂ based on retention times and peak areas.

3.4 Prevalence of Aflatoxigenic Fungal Strains Across Different Poultry Feed Types

The prevalence of aflatoxigenic fungal strains in each poultry feed category was calculated by dividing the number of aflatoxigenic isolates by the total number of fungal isolates recovered from each feed type and multiplying the result by 100.

Results

The results in table 4.1 indicated that fungal contamination was present across all feed samples regardless of feed type, location, or dilution plated. Starter feeds (S1, S4, S7) generally showed a slightly higher number of fungal isolates (2–3) compared to grower and finisher feeds, suggesting that early-stage feeds may be more susceptible to fungal growth due to their higher nutrient and moisture content. The consistent detection of fungi across all samples and dilutions implies that contamination is widespread and may originate from common sources such as raw materials, handling, or storage conditions.

4.1: Isolation of fungal species

Sample	Feed Type	Location	Dilution Plated	Number of Fungal Isolates Identified
S1	Starter	Rumuolumeni	10 ⁻³	3
S2	Grower		10 ⁻²	2
S3	Finisher		10 ⁻⁴	2
S4	Starter	Mile III	10 ⁻³	3
S5	Grower		10 ⁻²	2
S6	Finisher		10 ⁻³	2
S7	Starter	Choba	10 ⁻²	2
S8	Grower		10 ⁻³	2
S9	Finisher		10 ⁻⁴	2

The results from table 4.2 revealed that *Aspergillus flavus* was the predominant aflatoxigenic fungus among the isolates, showing high-quality DNA amplification with clear ITS bands (around 560 bp) and strong BLAST similarity ($\geq 99\%$) to reference aflatoxigenic strains. The clustering of *A. flavus* isolates (S1, S4, S6, S8) with known aflatoxin-producing lineages at high bootstrap values (85–92%) confirms their genetic potential to produce aflatoxins, particularly of the B-series. In contrast, *Aspergillus niger*, *A. fumigatus*, *Penicillium*, *Fusarium*, *Candida*, and *Rhizopus* species were identified as non-aflatoxigenic, clustering with non-toxicogenic or unrelated groups. This implies that while several fungal genera are present in the feed samples, *A. flavus* poses the major toxicological risk due to its aflatoxin-producing ability.

Sample	DNA Extraction	PCR Amplification (ITS1/ITS4)	Expected Band Size (bp)	BLAST Identified Species (≥98% Similarity)	Aflatoxigenic Potential	Phylogenetic Relationship (Bootstrap ≥70%)
S1	High-quality DNA (A260/A280 ≈ 1.9)	Clear, intense band	560 bp	<i>Aspergillus flavus</i> (99%), <i>Penicillium chrysogenum</i> (98%), <i>Fusarium oxysporum</i> (99%)	<i>A. flavus</i> – Aflatoxigenic; others – Non-aflatoxigenic	<i>A. flavus</i> clustered with reference aflatoxigenic strains (bootstrap 85%)
S2	Moderate yield (A260/A280 ≈ 1.8)	Distinct band	570 bp	<i>Aspergillus niger</i> (99%), <i>Candida tropicalis</i> (98%)	<i>A. niger</i> – Non-aflatoxigenic	<i>A. niger</i> grouped with non-aflatoxigenic <i>Aspergillus</i> spp. (bootstrap 82%)
S3	Good yield (A260/A280 ≈ 1.9)	Clear band	560 bp	<i>Aspergillus fumigatus</i> (99%), <i>Penicillium citrinum</i> (98%)	Non-aflatoxigenic	Clustered with <i>A. fumigatus</i> complex (bootstrap 90%)
S4	High yield (A260/A280 ≈ 1.9)	Strong bands	550–600 bp	<i>A. flavus</i> (99%), <i>A. niger</i> (99%), <i>F. oxysporum</i> (98%)	<i>A. flavus</i> – Aflatoxigenic	<i>A. flavus</i> cluster supported (bootstrap 88%)
S5	Moderate yield (A260/A280 ≈ 1.7)	Faint but visible band	560 bp	<i>A. fumigatus</i> (99%), <i>Rhizopus arrhizus</i> (98%)	Non-aflatoxigenic	Formed cluster with <i>A. fumigatus</i> group (bootstrap 83%)
S6	High yield (A260/A280 ≈ 1.9)	Strong band	560 bp	<i>A. flavus</i> (99%), <i>P. chrysogenum</i> (98%)	<i>A. flavus</i> – Aflatoxigenic	<i>A. flavus</i> clustered with reference aflatoxin-producing strains (bootstrap 90%)
S7	Good yield (A260/A280 ≈ 1.8)	Clear band	570 bp	<i>A. niger</i> (99%), <i>F. oxysporum</i> (98%)	<i>A. niger</i> - Non-aflatoxigenic	<i>A. niger</i> grouped with non-toxicogenic cluster (bootstrap 84%)
S8	High yield (A260/A280 ≈ 1.9)	Strong band	560 bp	<i>A. flavus</i> (99%), <i>Penicillium citrinum</i> (98%)	<i>A. flavus</i> – Aflatoxigenic	<i>A. flavus</i> formed monophyletic clade with aflatoxin B ₁ -producing strains (bootstrap 92%)
S9	Moderate yield (A260/A280 ≈ 1.8)	Clear band	560 bp	<i>A. fumigatus</i> (99%), <i>Fusarium proliferatum</i> (98%)	Non-aflatoxigenic	Clustered with <i>A. fumigatus</i> reference isolates (bootstrap 89%)

4.2: Molecular Identification of Aflatoxigenic Fungi

The results in table 4.2.1 revealed that all *Aspergillus flavus* isolates tested were capable of producing aflatoxins, with variations in the types and diversity of toxins produced. Specifically, all isolates synthesized Aflatoxin B₁ and B₂, confirming their strong toxigenic potential, while isolates from samples S4 and S8 also produced G-series aflatoxins (G₁ and G₂), suggesting a higher metabolic capacity and toxicity level. The presence of both B- and G-type aflatoxins indicates a broad aflatoxin biosynthetic profile among these strains.

4.2.1. Fungal Species and types of Aflatoxin.

Sample Code	Confirmed Fungal Species	Aflatoxin Types Detected
S1	<i>Aspergillus flavus</i>	B ₁ , B ₂
S4	<i>Aspergillus flavus</i>	B ₁ , B ₂ , G ₁
S6	<i>Aspergillus flavus</i>	B ₁ , B ₂
S8	<i>Aspergillus flavus</i>	B ₁ , B ₂ , G ₁ , G ₂

The results in table 4.3 showed that all analyzed *Aspergillus flavus* isolates produced B-series aflatoxins (B₁ and B₂), while some also synthesized G-series aflatoxins (G₁ and G₂), indicating significant toxigenic diversity among the strains. The strong and sharp HPLC peaks, especially for Aflatoxin B₁, confirm its dominance and high concentration across samples, making it the most potent and hazardous toxin detected. The detection of G₁ and G₂ in S4 and S8 further suggests that certain isolates possess a broader aflatoxin biosynthetic capability. Overall, these results imply that the feed samples are heavily contaminated with multiple aflatoxin types, posing serious health risks to animals and humans through consumption or bioaccumulation.

Table 4.3. Characterization of Aflatoxin.

Aflatoxin Type	Chemical Group	Detected in Samples	Retention Characteristics (HPLC)	Peak Area (Relative Fluorescence Intensity)	Estimated Concentration (µg/kg)	Aflatoxigenic Status
Aflatoxin B ₁ (AFB ₁)	B-series	S1, S4, S6, S8	Retention time ≈ 10.4–10.6 min	Strong, sharp peaks	B ₁ : 45.6; B ₂ : 12.4	Highly aflatoxigenic
Aflatoxin B ₂ (AFB ₂)	B-series	S1, S4, S6, S8	Retention time ≈ 12.2–12.4 min	Strong peaks for B ₁ and G ₁	B ₁ : 52.3; B ₂ : 10.8; G ₁ : 8.7	Highly aflatoxigenic
Aflatoxin G ₁ (AFG ₁)	G-series	S4, S8	Retention time ≈ 16.7–16.8 min	Moderate peaks	B ₁ : 35.4; B ₂ : 8.5	Aflatoxigenic
Aflatoxin G ₂ (AFG ₂)	G-series	S8 only	Retention time ≈ 18.5 min	Very strong peaks	B ₁ : 60.2; B ₂ : 14.7; G ₁ : 20.5; G ₂ : 11.2	Strongly aflatoxigenic

Discussion

The findings presented in Table 4.1 indicated that fungal contamination was evident across all poultry feed samples irrespective of feed type, location, or dilution plated. Such a pattern aligns with the report by Adebiyi, who observed that starter feeds often harbor more fungal species because their higher energy density and less processed ingredients enhance microbial proliferation during storage. Similarly, Okafor and Nwachukwu noted that early-stage feeds, rich in carbohydrates and lipids, tend to support the rapid growth of fungi such as *Aspergillus* and *Penicillium* species, especially under humid tropical conditions. The consistent detection of fungal isolates across

all samples and dilution levels implies that contamination was not isolated but systemic, affecting both raw materials and finished feed products. This observation corroborates the findings of Ezekie, who reported ubiquitous fungal contamination in Nigerian poultry feeds, attributing it to poor storage hygiene, use of contaminated raw materials, and improper drying processes. The occurrence of

fungi even at higher dilution levels (10^{-4}) in some samples (e.g., S3 and S9) indicates a relatively high fungal load, suggesting that the contamination is not superficial but well established. Akinola similarly reported that fungal spores can persist even after pelleting due to their heat-resistant structures, thus surviving standard feed processing conditions.

Furthermore, the uniformity of fungal occurrence across locations Rumuolumeni, Mile III, and Choba suggests that environmental factors such as humidity, temperature, and poor handling practices are key contributors rather than location-specific differences. According to Onyeke and Eze, fungal contamination in feed production environments is heavily influenced by regional humidity levels and ventilation quality. Their study found that urban and peri-urban feed stores in Rivers State share similar contamination profiles due to overlapping environmental and logistic factors, such as shared supply chains and open-air storage. The higher occurrence of fungi in starter feeds aligns with the report of Oluwafemi, who identified *Aspergillus flavus*, *Penicillium chrysogenum*, and *Mucor spp.* as common contaminants in nutrient-dense poultry feeds. They explained that such fungi thrive in organic substrates rich in nitrogen and amino acids, which are abundant in starter formulations designed for early chick growth. The results from this study also correspond with those of Bankole and Adebajo, who documented that *Aspergillus* species dominate fungal communities in poultry feeds due to their rapid growth rate, ability to tolerate low water activity, and production of resilient spores. The detection of multiple fungal isolates (2–3 species) per sample indicates a mixed fungal community rather than a single dominant contaminant, which may increase the risk of mycotoxin production. This observation corroborates the findings of Ezekiel, who reported co-occurrence of *Aspergillus*, *Fusarium*, and *Penicillium* species in poultry feeds across Southern Nigeria, often leading to the simultaneous production of aflatoxins and fumonisins. The presence of such mixed populations implies that feed safety concerns extend beyond fungal presence to include the potential for synergistic toxin effects. According to Fapohunda, co-contamination by multiple fungi can enhance the overall toxicity of feed through combined mycotoxin production, thereby posing serious risks to poultry health and productivity.

Moreover, the uniform fungal counts across grower and finisher feeds (mostly two isolates) suggest that feed processing and pelleting reduce but do not completely eliminate fungal presence. This finding is in agreement with Kehinde, who noted that mechanical pelleting and heat treatment reduce microbial load by about 60–70%, yet spores of *Aspergillus* and *Fusarium* often survive due to their thermotolerant nature. The persistence of these fungi even after processing supports the argument by Nwosu, that contamination may occur post-production during packaging, transportation, or storage, particularly when relative humidity exceeds 65%. Additionally, the presence of fungi in all samples across dilutions (10^{-2} to 10^{-4}) reflects high contamination intensity. According to Oladipo and Ojo, high colony counts across dilutions suggest that contamination occurred both pre- and post-production. They emphasized that inadequate drying of feed ingredients such as maize and soybean meal often introduces spores before mixing, while poor aeration during storage promotes fungal proliferation. The implications of these findings are significant for feed safety and poultry health. Fungal contamination can lead to nutrient loss, reduced feed palatability, and production of mycotoxins detrimental to both poultry and humans. This aligns with the findings of Abiodun, who linked fungal contamination in commercial poultry feeds to reduced growth rates, immunosuppression, and increased mortality in broilers. Similarly, Ezekiel and Falade emphasized that mycotoxin exposure through contaminated feed can result in economic losses due to feed spoilage and decreased productivity. This means that the study demonstrates that fungal contamination is widespread across all feed types and locations, with starter feeds showing slightly higher susceptibility. The consistency of contamination across dilutions and sampling points indicates systemic contamination possibly linked to raw materials, processing, and storage conditions. These results corroborate earlier reports by Oluwafemi and Ezekiel, affirming that fungal presence in poultry feeds remains a persistent challenge in humid tropical environments like Rivers State.

The findings presented in Table 4.2 indicated that molecular identification using ITS1/ITS4 primers successfully amplified fungal DNA across all feed samples, producing distinct bands ranging between 550 and 600 bp, which are consistent with the expected ITS region sizes for filamentous fungi.

The high DNA quality ($A_{260}/A_{280} \approx 1.8-1.9$) and clear PCR bands obtained from most samples confirm that the extraction and amplification procedures were effective, producing amplifiable genomic DNA suitable for molecular characterization. This observation aligns with the report of Akinyemi who demonstrated that ITS1/ITS4 primers reliably amplify fungal DNA across multiple genera, including *Aspergillus*, *Penicillium*, and *Fusarium*, due to their high specificity for the conserved regions of fungal ribosomal DNA. Similarly, Chukwura and Nwosu reported that ITS-based sequencing provides a robust approach for distinguishing aflatoxigenic from non-aflatoxigenic fungal isolates in feed samples. The BLAST analysis results revealed that *Aspergillus flavus* was the predominant aflatoxigenic fungus, detected in four of the nine samples (S1, S4, S6, and S8) with high sequence similarity ($\geq 99\%$) to reference strains. These isolates also exhibited strong phylogenetic clustering with known aflatoxin B₁-producing lineages, supported by high bootstrap values (85–92%). This strongly indicates that the *A. flavus* isolates possess the genetic potential for aflatoxin biosynthesis. This finding corresponds with the report of Ezekiel, who observed that *A. flavus* is the most frequently encountered aflatoxigenic species in Nigerian poultry feeds, accounting for over 60% of total mycotoxigenic isolates. Likewise, Bankole and Adebajo documented that *A. flavus* dominates fungal communities in stored feed ingredients due to its adaptive spore structure and ability to thrive in humid tropical conditions conducive to aflatoxin production. The strong amplification and clustering of *A. flavus* isolates with reference aflatoxigenic strains further corroborate the findings of Omotayo who reported that phylogenetic grouping with bootstrap support $\geq 80\%$ often confirms toxigenic potential among *A. flavus* isolates. The present study's bootstrap values (85–92%) thus provide strong molecular evidence of toxigenicity. Similarly, Kehinde demonstrated that ITS-based phylogenetic analyses can clearly differentiate between aflatoxigenic and non-aflatoxigenic lineages within the *Aspergillus* genus, confirming its suitability as a diagnostic tool for mycotoxin risk assessment.

In contrast, *Aspergillus niger*, *A. fumigatus*, *Penicillium chrysogenum*, *Penicillium citrinum*, *Fusarium oxysporum*, *Candida tropicalis*, and *Rhizopus arrhizus* were identified as non-aflatoxigenic species, clustering with non-toxicogenic or unrelated fungal groups. These results suggest that while these fungi are present in poultry feeds, they do not pose significant aflatoxin-related threats. This observation aligns with Fapohunda who reported that most *A. niger* and *A. fumigatus* strains isolated from animal feeds are non-toxicogenic, though some may cause spoilage or allergic reactions. Similarly, Oluwafemi found that *Penicillium* and *Fusarium* species are frequently isolated from stored feeds but often contribute to other mycotoxins (such as citrinin or fumonisins) rather than aflatoxins. The predominance of *A. flavus* among the isolates underscores its ecological advantage and toxigenic dominance in feed environments rich in carbohydrates and lipids. This finding supports the work of Ezekiel and Falade, who highlighted that *A. flavus* thrives in nutrient-dense substrates under conditions of moderate humidity and poor aeration, conditions common in tropical feed storage systems. The presence of aflatoxigenic *A. flavus* in multiple samples also implies potential cross-contamination during feed mixing or handling. According to Oladipo and Ojo, shared equipment, improper cleaning of storage facilities, and reuse of contaminated sacks are major pathways for fungal cross-contamination in Nigerian feed mills. Moreover, the coexistence of *A. flavus* with other non-aflatoxigenic species such as *Penicillium* and *Fusarium* suggests a complex microbial interaction within feed matrices. Such co-occurrence may influence toxin biosynthesis either synergistically or antagonistically. Fapohunda reported that mixed fungal communities can either suppress or enhance aflatoxin production depending on interspecies competition for nutrients. The phylogenetic clustering of *A. flavus* with aflatoxin-producing lineages in this study, despite cohabitation with other fungi, suggests that these interactions did not inhibit its toxigenic expression potential. The identification of *A. flavus* isolates with aflatoxigenic potential in 44% of samples (S1, S4, S6, S8) raises significant public health and economic concerns. Aflatoxins, particularly aflatoxin B₁, are potent hepatotoxins and carcinogens, posing risks to poultry health, feed quality, and food safety. Abiodun reported that aflatoxin-contaminated poultry feeds cause liver damage, reduced growth rate, and immunosuppression in broilers. Furthermore, Ezekiel highlighted that aflatoxin residues can enter the human food chain through contaminated meat and eggs, emphasizing the need for molecular surveillance in feed safety monitoring programmes. However, the molecular identification results

confirm that *Aspergillus flavus* is the principal aflatoxigenic species in the analyzed poultry feeds, showing clear genetic affiliation with known toxigenic lineages based on ITS sequencing and phylogenetic analysis. Other isolated species, though non-aflatoxigenic, may still contribute to feed spoilage or produce secondary toxins. These findings corroborate previous studies by Ezekiel and Omotayo affirming that ITS-based molecular tools provide accurate and reliable identification of aflatoxigenic fungi. The results highlight the importance of routine molecular monitoring and improved feed storage practices to mitigate aflatoxin contamination risks in poultry production systems.

The findings presented in Table 4.2.1 revealed that all *Aspergillus flavus* isolates obtained from the analyzed poultry feed samples were aflatoxigenic, producing different combinations of aflatoxin types. All isolates synthesized aflatoxin B₁ and B₂, while isolates from samples S4 and S8 additionally produced G-series aflatoxins (G₁ and G₂). This variation in aflatoxin profiles among *A. flavus* isolates indicates strain-specific differences in biosynthetic capacity, which may be attributed to genetic, environmental, and substrate-related factors influencing the aflatoxin pathway. The consistent detection of aflatoxin B₁ across all isolates underscores its dominance as the most toxic and prevalent aflatoxin in contaminated feed. This observation corroborates the findings of Amaike and Keller, and Bennett and Klich, who reported that aflatoxin B₁ (AFB₁) remains the most potent hepatocarcinogen among naturally occurring mycotoxins due to its high stability and bioaccumulative potential in animal tissues. Furthermore, the co-occurrence of both B- and G-series aflatoxins in isolates S4 and S8 suggests the presence of *A. flavus* strains possessing a complete aflatoxin biosynthetic gene cluster. According to Perrone and Gallo, such strains often contain functional *aflR* and *aflS* regulatory genes that enhance the expression of downstream enzymes responsible for the production of both B- and G-type aflatoxins. Chang and Ehrlich also emphasized that the presence of both toxin types in a single isolate signifies a more metabolically versatile *A. flavus* genotype, capable of synthesizing a broader range of secondary metabolites. This finding is consistent with the reports of Shehu and Adebajo, who identified multi-aflatoxin-producing *A. flavus* genotypes in poultry feeds across Southwestern and Northern Nigeria, linking their occurrence to environmental and substrate diversity that favors aflatoxin biosynthesis.

The universal production of aflatoxin B₁ and B₂ among the isolates further confirms the widespread prevalence of toxigenic *A. flavus* in feed materials, raising significant concerns for feed and food safety. Aflatoxin B₁, in particular, poses serious health risks due to its mutagenic and hepatocarcinogenic effects, as well as its capacity to enter the food chain through animal products such as eggs, milk, and meat Tola & Kebede, Omotayo. The detection of both G₁ and G₂ in isolates from S4 and S8 implies favorable environmental and nutritional conditions that promote extended aflatoxin biosynthesis. Okoth observed that factors such as high moisture content, nutrient-rich substrates, and warm, humid climates typical of tropical regions enhance the activity of aflatoxin biosynthetic enzymes. Similarly, Mohammed and Yusuf reported that optimal growth conditions, particularly temperature and pH, stimulate gene expression within the *afl* cluster, leading to increased production of multiple aflatoxin types in stored feed ingredients.

Moreover, the variability in aflatoxin types observed among the *A. flavus* isolates in this study reflects underlying genetic heterogeneity within the species. Anike and Dutton noted that *A. flavus* populations are genetically diverse, and this diversity determines differences in aflatoxin yield and type among isolates obtained from similar ecological sources. Razzaghi-Abyaneh and Shams-Ghahfarokhi similarly reported that adaptive mutations and environmental pressures can shape the metabolic expression of the aflatoxin gene cluster, resulting in diverse toxin profiles. Such strain-level variation is critical in risk assessment because isolates capable of producing multiple aflatoxins pose compounded health hazards compared to those limited to only B-series toxins. The occurrence of AFB₁, AFB₂, AFG₁, and AFG₂ in the *A. flavus* isolates underscores the significance of poultry feed as a reservoir for diverse aflatoxigenic strains. These findings align with the reports of Atanda and Cardwell, who documented multiple aflatoxin types in commercial feeds and raw materials, particularly under tropical storage conditions that favor fungal growth and toxin synthesis. The implications are substantial for livestock health, productivity, and public health, as chronic exposure to aflatoxins can result in reduced feed efficiency, immunosuppression, and contamination of animal-derived foods.

Therefore, the findings of this study reaffirm the need for continuous monitoring, implementation of proper storage practices, and adoption of mitigation strategies such as biological control and detoxification to minimize aflatoxin hazards in animal feeds. The observed variability in aflatoxin types thus not only demonstrates the metabolic diversity of *A. flavus* isolates but also highlights the critical importance of maintaining stringent quality control across the feed production and supply chain to safeguard animal and human health.

The findings presented in Table 4.3 revealed that all *Aspergillus flavus* isolates analyzed produced aflatoxins of the B-series (Aflatoxin B₁ and B₂), while some strains also synthesized aflatoxins of the G-series (Aflatoxin G₁ and G₂). This demonstrates a significant level of toxigenic diversity among the isolates. The consistent detection of AFB₁ and AFB₂ in all positive samples (S1, S4, S6, and S8) confirms the high aflatoxigenic capacity of *A. flavus*, indicating its dominance in aflatoxin biosynthesis. The high-performance liquid chromatography (HPLC) retention times recorded for these toxins approximately 10.4–10.6 minutes for AFB₁ and 12.2–12.4 minutes for AFB₂ fall within the expected chromatographic range for B-series aflatoxins, validating the precision and accuracy of the analytical procedure. These findings are consistent with those of Ali, who reported similar retention characteristics for aflatoxins extracted from *A. flavus* isolates in feed and grain samples. The strong and sharp fluorescence peaks observed for AFB₁ indicate its high concentration and stability within the analyzed feed matrix. Estimated concentrations of AFB₁ ranged between 45.6–60.2 µg/kg, while AFB₂ concentrations ranged from 10.8–14.7 µg/kg. These values exceed the maximum tolerable limits (20 µg/kg) set by the U.S. Food and Drug Administration (FDA) and the Food and Agriculture Organization (FAO) for animal feed, suggesting substantial contamination and high exposure risk FAO/WHO. The detection of G-series aflatoxins (AFG₁ and AFG₂) in samples S4 and S8 further indicates that certain *A. flavus* strains possess an expanded biosynthetic potential, enabling the co-production of both B- and G-type aflatoxins. This aligns with the findings of Matumba, who reported that dual-type aflatoxin producers exhibit greater genetic diversity and adaptability under humid storage conditions.

The peak areas and retention characteristics observed in this study confirm the strong aflatoxigenic potential of the isolates. The very strong fluorescence intensity recorded for AFG₂ in sample S8 suggests a higher biosynthetic activity, possibly due to environmental or nutritional factors influencing secondary metabolite production. Similar observations were made by Omotayo, who emphasized that moisture levels, temperature, and substrate composition significantly affect aflatoxin biosynthesis in tropical feed environments. The combination of high AFB₁ levels with detectable G-series aflatoxins demonstrates the coexistence of highly toxigenic strains, which poses increased toxicological hazards for both animal and human health. The findings also imply that the analyzed feed samples are heavily contaminated with multiple aflatoxin types, representing a cumulative toxic burden. Adekoya reported comparable results in Nigerian poultry feed samples, where high AFB₁ concentrations were frequently associated with poor storage and inadequate moisture control. Moreover, the concurrent production of B- and G-series aflatoxins indicates that certain isolates may carry multiple gene clusters responsible for aflatoxin biosynthesis, as suggested by Guchi. It implies that the HPLC characterization confirmed the dominance of Aflatoxin B₁ as the most abundant and toxic compound in the analyzed feed samples, followed by AFB₂, AFG₁, and AFG₂. The coexistence of multiple aflatoxin types reflects a high-risk contamination scenario that threatens feed quality and food safety. These results corroborate earlier reports by Ezekiel and Omotayo, who found that *A. flavus* remains the principal producer of aflatoxins in animal feed within humid tropical environments. Therefore, continuous monitoring, improved feed storage practices, and the adoption of antifungal control strategies are essential to reduce aflatoxin contamination and safeguard both animal health and public safety.

Conclusion

The study demonstrates that fungal contamination is widespread in all analyzed poultry feeds, with starter feeds showing slightly higher fungal diversity, likely due to higher nutrient and moisture content. Molecular analysis confirmed *Aspergillus flavus* as the predominant aflatoxigenic species, showing strong DNA amplification, high sequence similarity (≥99%) with reference strains, and

clustering with aflatoxin-producing lineages (bootstrap 85–92%). HPLC results revealed that all isolates produced B-series aflatoxins (AFB₁ and AFB₂), while some also produced G-series aflatoxins (AFG₁ and AFG₂), highlighting the presence of highly toxigenic strains. The high concentrations of AFB₁ underscore its toxicological significance.

Recommendations

Based on the result of the findings, the following were recommended;

- i. Feed manufacturers and farmers should adopt proper storage conditions such as maintaining low moisture levels (<12%), using clean, dry, and well-ventilated facilities to prevent fungal growth and aflatoxin formation.
- ii. Regular screening of feed and feed ingredients for fungal contamination using both conventional and molecular techniques (ITS-based PCR) should be institutionalized.
- iii. Feed industries should explore biocontrol agents such as non-aflatoxigenic strains of *A. flavus*, as well as post-harvest interventions like the use of organic acids, ozone treatment, or adsorbents to reduce aflatoxin levels in contaminated feeds.
- iv. Government agencies such as NAFDAC and SON should strengthen enforcement of feed safety standards to ensure compliance with international aflatoxin limits (≤ 20 $\mu\text{g}/\text{kg}$ for AFB₁).

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