



Aflatoxins and aflatoxigenic fungal contamination of common poultry feed products in Katsina State, Nigeria

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Abstract

This study was conducted to screen the common poultry feed products sold within Katsina State, Nigeria, for the presence of aflatoxigenic fungi and aflatoxins. A total of 15 poultry feed samples from 3 major producing companies were purchased from retailers, and then cultured for fungal isolation on potato dextrose agar (PDA) supplemented with Cycloheximide. Fungal colonies were identified using standard mycological techniques. The samples were further extracted using a methanol-water solvent (v/v) 1:2, and the extracts were analyzed for the presence of aflatoxins using Fourier Transform Infrared Spectroscopy (FTIR). A total of 30 fungal isolates comprising *Aspergillus* spp. (30%), *Mucor* spp. (30%), *Rhodotorula* spp. (13.3%), *Rhizopus* spp. (10%), *Saccharomyces* spp. (10%), *Geotrichum* spp. (3.3%) and *Endomyces* spp. (3.3%) were recovered. The FTIR results showed that 10% of the samples were contaminated by aflatoxins as evidenced by the presence of peaks/wavenumbers specific for aflatoxins' functional groups in their FTIR spectra (2850 to 3100 cm^{-1} , 1650 to 2000 cm^{-1} , 1670 to 1820 cm^{-1} , 1400 to 1600 cm^{-1} , 1364 to 1369 cm^{-1} and 1040 to 1050 cm^{-1} , for aromatic –C–H and CH_2 , in-plane aromatic –CH bending, Carbonyl C=O, aromatic C=C, – CH_3 adjacent to epoxy ring, and symmetric stretching of =C–O–C, respectively). The poultry feeds analyzed were contaminated by aflatoxigenic fungi and aflatoxins, in addition to other potentially pathogenic fungi. Therefore, more stringent prevention and control methods are required to reduce the contamination levels, to avoid loss of poultry lives and possible transfer of carcinogenic mycotoxins to humans along the food chain.

Keywords: Aflatoxins, *Aspergillus* sp., Carcinogenic, FTIR, Poultry feed

1. Introduction

Mycotoxins are poisonous biomolecules that are produced as secondary metabolites by some fungal

species, as they grow on various substrates under suitable growth conditions (Tola and Kebede, 2016).

Several mould fungi are capable of contaminating various foods and feeds with these toxic secondary metabolites, which have adverse effects on human and animal consumers following consumption of these contaminated food or animal feed (WHO, 2006; Maciorowski *et al.*, 2007; Mostafa *et al.*, 2012). Moreover, Liu and Wu, (2010); Salim *et al.*, (2011) added that they are responsible for many acute and chronic diseases in humans and animals such as; liver damage, esophageal cancer, reduced digestive enzyme activity, immune suppression, and various effects on children including stunted growth with many annual mortality cases. In addition to causing diseases, mycotoxigenic fungi and mycotoxins affect feed quality by reducing its nutritive value and producing an unpleasant smell. Monson *et al.*, (2014) reported that they also affect poultry performance and health, leading to severe economic losses.

Several studies of Magnoli *et al.*, (2011); Ghadeer and Al Delamiy, (2012) revealed that *A. flavus* and *A. parasiticus* are of significant concern in poultry contamination, being the most common producers of aflatoxins. Varga *et al.*, (2011) stated that out of these two *Aspergillus* spp., *A. flavus* is found frequently in contaminated feeds. Generally, aflatoxins are the most common and most toxic primary mycotoxins of concern in poultry feedstuffs. It is composed of several types such as; Aflatoxin B₁ (AFB₁), Aflatoxin B₂ (AFB₂), Aflatoxin G₁ (AFG₁), and Aflatoxin G₂ (AFG₂), which are the most commonly encountered (Monbaliu *et al.*, 2010; Lereau *et al.*, 2012). Of these aflatoxins, AFB₁ is the most toxic (Habib *et al.*, 2015; Haruna *et al.*, 2017), and is the most commonly encountered natural carcinogens produced by these *Aspergilli*. Xu *et al.*, (2000) reported that it is metabolized in the body into its hydroxylated form called Aflatoxin M₁ (AFM₁), which is excreted in the breast milk of humans and animals following the ingestion of contaminated food or feed with AFB₁. The most chronic form of aflatoxin exposure manifestation is hepatocellular carcinoma (HCC, or liver cancer), which has been described by WHO as the third-leading cause of cancer death globally

(WHO, 2008), and with about 550,000 to 600,000 reported new cases annually (Habib *et al.*, 2015). Approximately, 83% of these deaths occur in East Asia and sub-Saharan Africa. It has been estimated that more than five billion people in developing countries are at risk of chronic exposure to aflatoxins, through contaminated foods/feeds (Habib *et al.*, 2015).

Unfortunately, many of the people in developing countries such as Nigeria are not even aware of the detrimental effects associated with aflatoxin-contaminated livestock products. Zaki *et al.*, (2012) documented that this lack of awareness and other socio-economic factors make consumers prefer cheap commodities over premium quality products. It is of concern that little or no effort has been made to study mycotoxins contamination in the feed chain in Nigeria, considering the public health implications (Zaki *et al.*, 2012). The objectives of the present study, therefore, were to screen the common poultry feed products sold within Katsina State, Nigeria for the presence of aflatoxigenic fungi and aflatoxins. To the best of our knowledge, this is the first study reporting the incidence of aflatoxins and mycoflora contaminating poultry feed products in Katsina state, Nigeria.

2. Material and methods

2.1. Samples collection and study area

A total of 15 poultry feeds samples of various categories including; Broiler Starter (4), Broiler Super Starter (1), Broiler Finisher (4), Grower's Chick Mash (4), and Layer Finisher (2) feeds, belonging to three selected poultry feed companies (Top Feeds, Vital Feed, and Soviet), were collected from retailers in Katsina state. Each sample was collected in triplicates, and mixed thoroughly to make a single composite sample. Samples were analyzed in the Microbiology Laboratory of Umaru Musa Yar'adua University, Katsina, and in the Biochemistry Laboratory of Bayero University, Kano, Nigeria.

2.2. Isolation of mycoflora from the poultry feed samples

About 10 g of each feed sample was added into 90 ml of sterile dist. water, and then serially diluted. About 0.2 ml of each dilution was inoculated individually into plates of potato dextrose agar (PDA), supplemented with cycloheximide to prevent the bacterial, and incubated at $27 \pm 2^{\circ}\text{C}$ for 5d. After incubation, plates were examined macroscopically for development of separate fungal colonies (Habib *et al.*, 2015). These colonies in each plate were counted, and then expressed as total cfu/ g (colony- forming units per gram) of each feed sample.

2.3. Identification of the fungal isolates

Fungal isolates were grown as single spore colonies and then identified based on their macroscopic and microscopic characteristics according to Alexopoulos *et al.*, (2011); Habib *et al.*, (2015). Each isolate was sub-cultured on Sabouraud dextrose agar (SDA) and incubated for 5 d at $27 \pm 2^{\circ}\text{C}$. Colonies were examined macroscopically for both sides of the plates to investigate their cultural characteristics. For the microscopic characterization, a loopful of the fungal mycelium was teased out on a glass slide, and then stained with lactophenol cotton blue (LPCB). A coverslip was placed on the stained preparation and then examined under X10 and then X40 objective lens of a microscope, for investigating the microscopic features.

2.4. Extraction of aflatoxins

Each of the 15 composite poultry feed samples was defatted with n-hexane, and subsequently, 10g of the defatted sample was added into Erlenmeyer flasks. About 90 ml of 30/60 (v/v) methanol-water was added to each sample, sealed and then shaken for 24 h. The mixture of each sample was allowed to settle down for 2 h, and the supernatant was carefully filtered using Whatman's No. 1 filter paper. The filtrates were then concentrated using a rotary evaporator, and finally dried residue was added into sterile air-tight bottles and kept at 4°C until use (Sule *et al.*, 2014). Similarly, 1 ml of standard AFB₁ (2 μg / ml) was added into

aflatoxin free poultry feed, and extracted using the same method as control.

2.5 Determination of the aflatoxins level in the poultry feed extracts

To evaluate the level of aflatoxins in the poultry feed extract, Attenuated Total Reflectance-Fourier Transformed Infrared Spectroscopic analysis (ATR-FTIR) was carried out using Shimadzu, Japan, FT-IR spectrometer, as described by Salisu *et al.*, (2017). Approximately, 5 mg/ml solution of each extract was prepared in 30/60 (v/v) methanol-water solvent. An aliquot of 100 μl of each extract was applied directly on pre-cleaned FTIR diamond, scanned from 550 to 4000 cm^{-1} for 32 times, and then averaged as a single spectrum. A background scan from 400 to 4000 cm^{-1} was carried out before loading each sample, and the background spectrum was subtracted from the sample spectrum. The FT-IR spectra were recorded in the absorption range between 550 to 4000 wavenumbers cm^{-1} at a resolution of 8 cm^{-1} . Each sample spectrum was normalized, smoothed, and then automatic peak picking was carried out at 90%. Finally, the absorption peak values of each spectrum were screened for the presence of peaks that represent the functional groups specific for aflatoxins (i.e. aromatic =CH, -C-H, C=C and phenyls, carbonyl C=O group, CH₃ adjacent to epoxy ring, in-plane -CH bending of phenyl, symmetric stretching of =C-O-C, and peaks for isolated H group), in reference to Mirghani *et al.*, (2001); Fischer *et al.*, (2006); Santos *et al.*, (2010); Bhat, (2013); Salisu *et al.*, (2019).

3. Results

3.1. Bio-burden of mycoflora in the poultry feed samples

A total of 30 different fungal species belonging to seven fungal genera were obtained from the 15 poultry feed samples. The phenotypic characteristics (i.e. morphological and microscopic) were used to identify the isolates as presented in Table 1.

Table 1: Mycoflora contaminating poultry feed samples collected from Katsina state

Isolate Number	Macroscopic Characteristics	Microscopy	Species	Frequency of Isolation	% of Occurrence
1	Pin like greenish-yellow growth with sclerotia and milk reverse side.	Hyaline septate hyphae bearing non-branched long rough conidiophores with bulb end containing conidia.	<i>Aspergillus flavus</i>	6	20.0
2	Pin like black colonies with white border and milk revers sides.	Hyaline septate hyphae bearing non-branched smooth conidiophores with bulb end bearing conidia like sub rays.	<i>Aspergillus niger</i>	3	10.0
3	Large white colonies.	Yeast-like with mycelium and cocci or ovoid ascospores, the mycelium divided into cylindrical arthrospores.	<i>Endomyces</i> spp.	1	3.33
4	Colonies are fast-growing, flat, white to creamy, dry and finely suede-like with no reverse pigment	Chains of hyaline, smoothed, one-celled, subglobose to cylindrical, slimy macroconidia by the haloathric fragmentations of undifferentiated hyphae.	<i>Geotrichum</i> spp.	1	3.33
5	Cotton like white growth spotted with black colour	Large globose sporangia borne on aseptate hyphae, devoid of rhizoids.	<i>Mucor racemosus</i>	9	30.0
6	Cotton like white growth spotted with black colour.	Large globose sporangia borne on aseptate hyphae with rhizoids.	<i>Rhizopus stolonifer</i>	3	10.0
7	Cream to orange/red colonies	Unicellular cocci or ovoid shape, larger than bacterial cells.	<i>Rhodotorula</i> spp.	4	13.33
8	Flat, smooth, moist, glistening and cream to tannish cream coloured colonies.	Unicellular cocci, larger than bacterial cells, globose to ellipsoidal cells with blastoconidia.	<i>Saccharomyces cerevisiae</i>	3	10.0
Total				30	100%

where; The macroscopic characteristics were based on the cultural characteristics of the isolates on SDA medium. The frequency column showed the total number of times each species was isolated in the poultry feed samples, while the % of occurrence indicates the prevalence rate of each species among the 30 isolates.

Of the seven fungal genera, *Aspergillus* spp. and *Mucor* spp. exhibited the highest percentage of occurrences in the feed samples (30% each), while *Geotrichum* spp. and *Endomyces* spp. recorded the least frequency of occurrence (3.3% each) (Table 1). On the other hand, Fig. 1 showed the phenotypic appearance of the *Aspergillus* spp. (*A. flavus* and *A. niger*), whereas, Fig. 2 (a and b) showed the

distribution of the 30 fungal species in the various poultry feed categories analyzed. Broiler starter feed has the highest number of isolates (11 isolates) belonging to 5 different fungal species, followed by Grower chicken mash feed with 8 isolates belonging to 6 species. Both of the Broiler finisher feed and Layer finisher feed had 7 and 4 isolates, belonging to 4 and 3 different fungal species, respectively (Fig. 2).

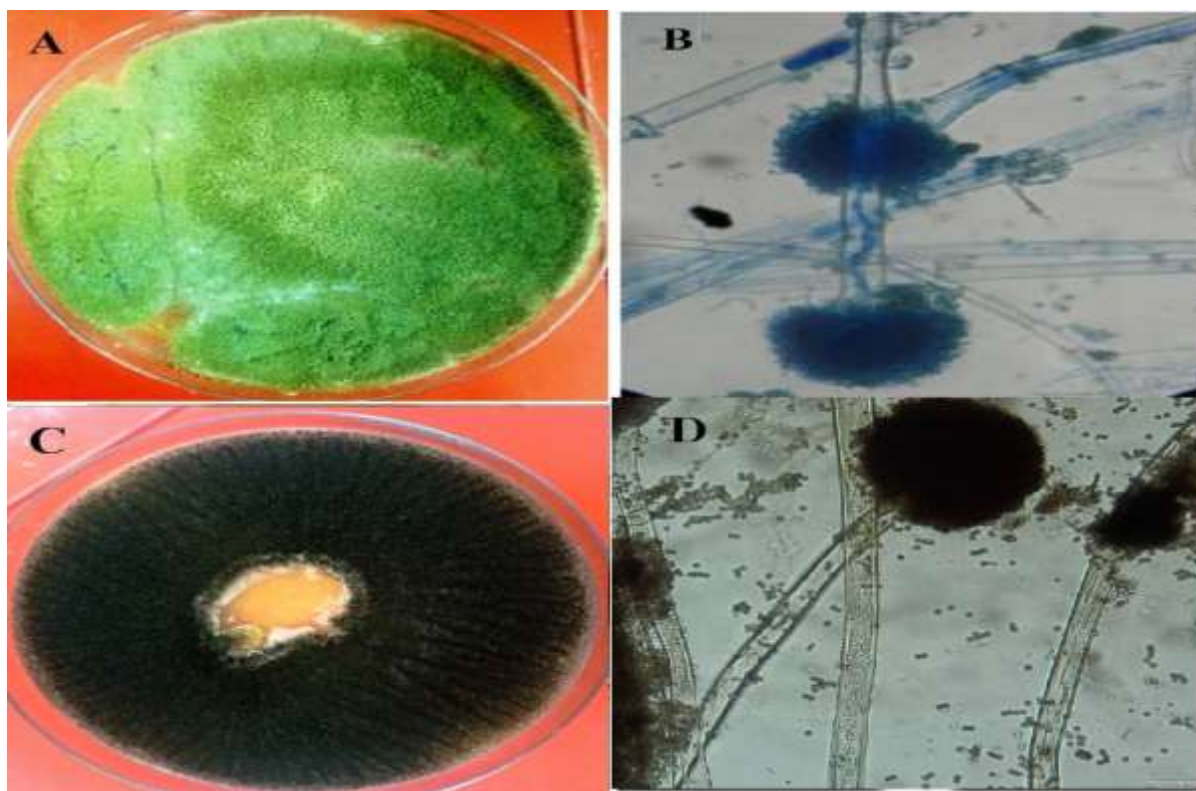
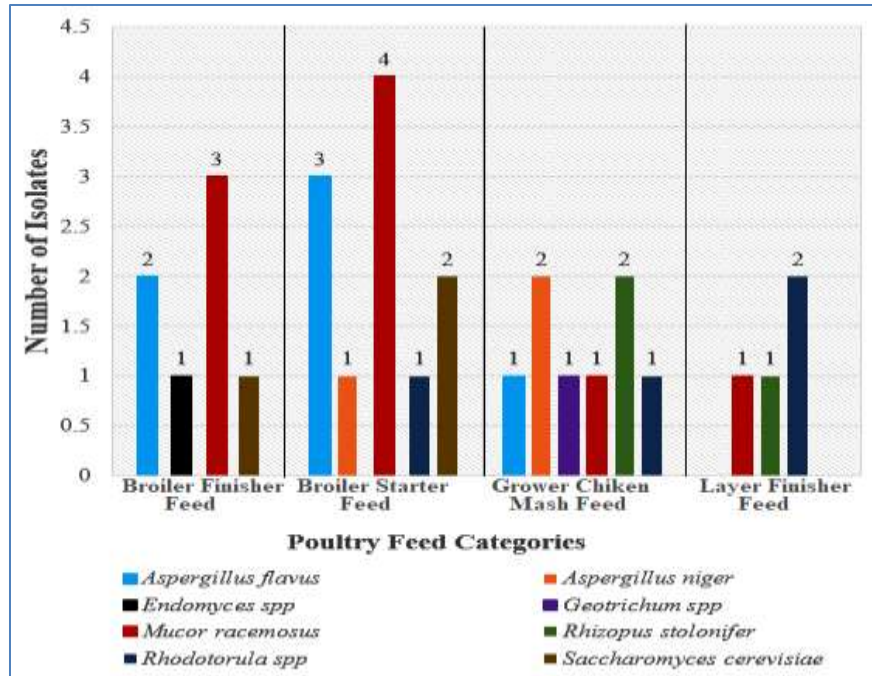
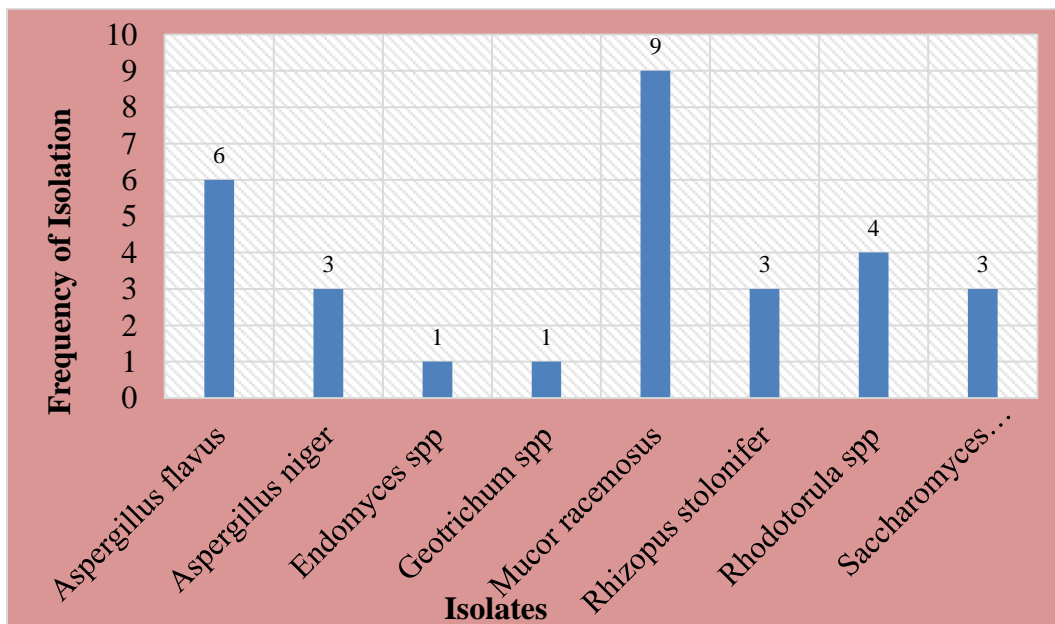


Fig. 1: Macroscopic and microscopic appearance of the *Aspergillus* spp. A and C are the macroscopic appearance of *A. flavus* and *A. niger* on SDA medium, respectively. While, B and D are the respective microscopic appearance of *A. flavus* and *A. niger* stained with LPCB, Mg x400.



(a)



(b)

Fig. 2: a)-Distribution of the 30 fungal species in the various poultry feed categories of Katsina state. Each species is represented by a unique color, b)-Frequency of isolation of the fungal spp. from the different poultry feed samples. The number on the top of each bar denotes the total frequency of isolation of this species in the respected poultry feed category.

3.2. Fourier transform infrared (FTIR) spectroscopic analysis

The results of the FTIR analysis of the feed extracts revealed that 3 out of the 15 extracts analyzed recorded the presence of aflatoxins, as evidenced by the presence of peaks characteristic for aflatoxins' functional groups (i.e. aromatic =CH, -C-H, C=C and

phenyls, carbonyl C=O group, CH₃ adjacent to epoxy ring, in-plane -CH bending of phenyl, symmetric stretching of =C-O-C, and peaks for isolated H group) in their FTIR spectra (Table 2, Fig. 3). On the other hand, Fig. (4) shows the FTIR spectra of the standard AFB1, and the three positive poultry feed extracts.

Table 2: Specific FTIR spectroscopic wavelengths showed by the positive standard and aflatoxin positive feed extracts

Wavenumber cm ⁻¹	Peak intensity	Functional group
2850 – 3100	Medium	Aromatic -C-H and CH ₂
1650 – 2000	Weak	In-plane aromatic -CH bending
1670 – 1820	Strong	carbonyl C=O group
1400 – 1600	Medium	Aromatic C=C
1364 – 1369	medium	-CH ₃ adjacent to epoxy ring
1040 – 1050	Strong	Symmetric stretching of =C-O-C

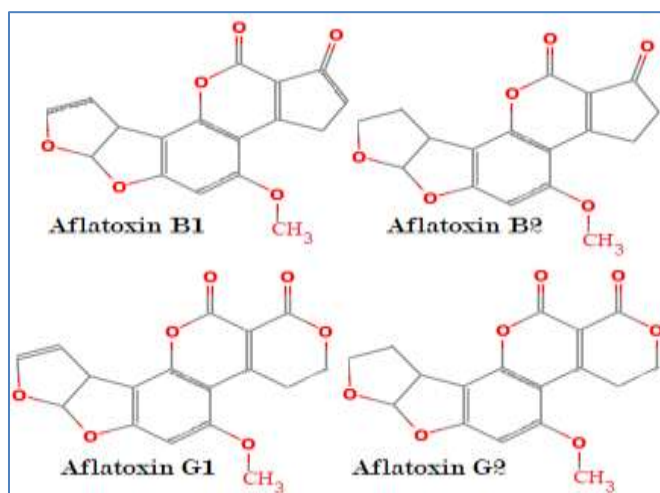


Fig. 3: Chemical structure of aflatoxins showing the specific functional groups used for their identification by FTIR

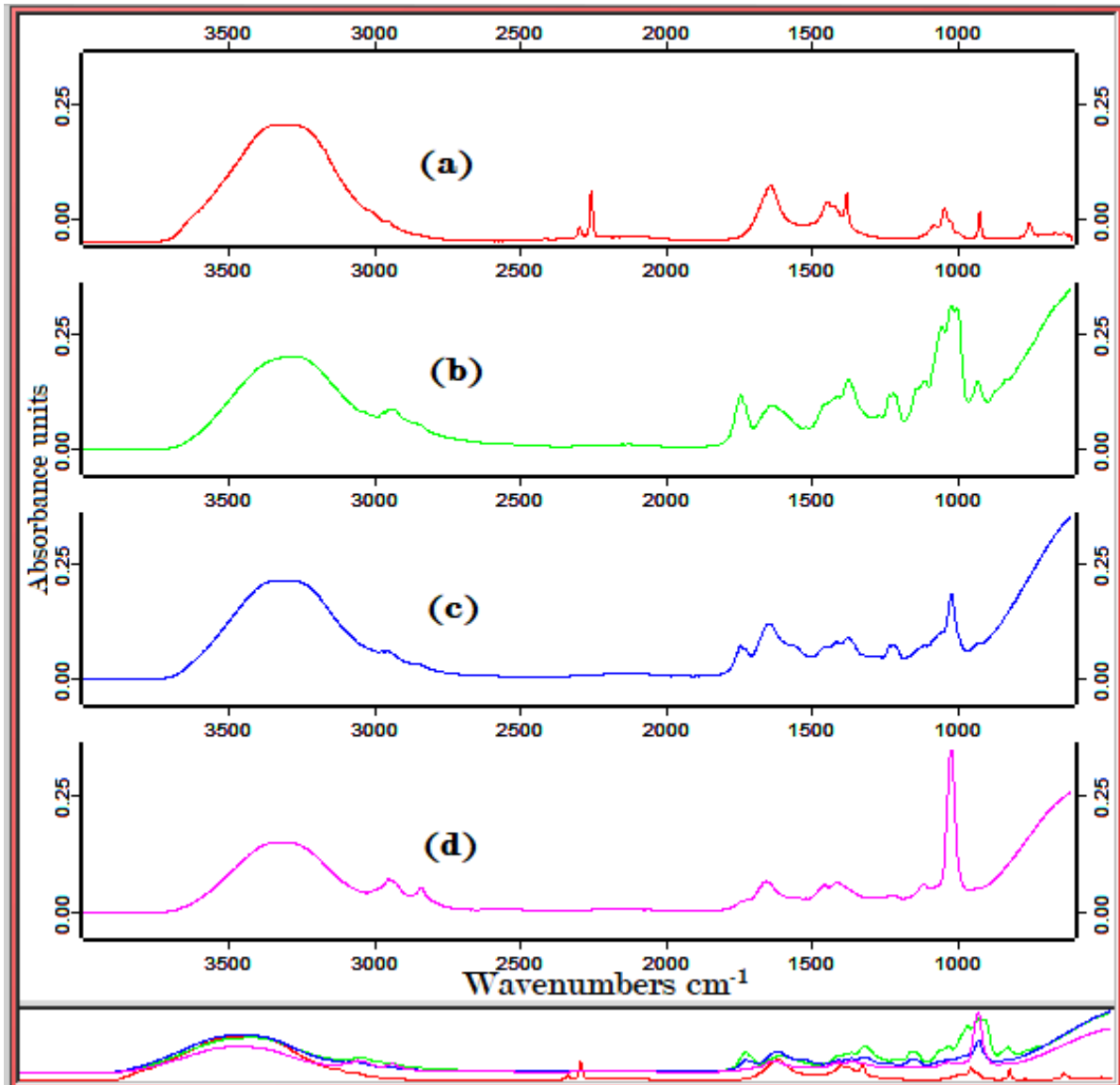


Fig. 4: FTIR spectra of the standard aflatoxin B₁ and the three aflatoxin positive poultry feed extracts, recording the presence of the aflatoxins functional groups. The upper most spectrum labelled as " (a) " is for the for the aflatoxin standard B₁, while " (b) ", " (c) " and " (d) " are the spectra of the 3 aflatoxin positive poultry feed samples.

4. Discussion

Poultry feeds represent a complex ecosystem that favors the proliferation of mycotoxigenic fungi, due to their multicomponent raw materials from a variety of agricultural crops, coming from various environments with different fungal loads (Xu *et al.*, 2018). The current results indicated the presence of 30 fungal isolates, belonging to 7 genera. This study is inconsistent with the previous reports of high fungal contamination of poultry feeds in Nigeria (Obi and Ozugbo 2007; Osho *et al.*, 2007; Uwazuoke and Ogbulie 2008; Ezekiel *et al.*, 2014; Habib *et al.*, 2015; Aliyu *et al.*, 2016), and other parts of the world such as; Argentina (Dalcero *et al.*, 1997), Brazil (Oliviera *et al.*, 2006; Rossa *et al.*, 2006), Serbia (Krnjaja *et al.*, 2007) and Pakistan (Saleemi *et al.*, 2010).

In the present work, the prevalence of *Aspergillus* spp. is 9(33%). The prevalence of *A. flavus* reported in this study is less than those reported 55% by Diba *et al.*, (2007), 91.8% by Ezekiel *et al.*, (2014), 83.3% by Sivakumar *et al.*, (2014), 50% by Fakruddin *et al.*, (2015), 51.6% by Gherbawy *et al.*, (2016), 35% by Fapohunda *et al.*, (2012), 41.2% by Aliyu *et al.*, (2016) and 64.3% by Ghaemmaghami *et al.*, (2016). However, the prevalence in this study is higher than others such as 22.64% by Greco *et al.*, (2014), 17.27% by Davari *et al.*, (2015) and 20% by Habib *et al.*, (2015).

The current results show that the fungal species most prevalent in the poultry feeds belong to *Aspergillus* and *Mucor* spp., whereas *Geotrichum* and *Endomyces* spp. are the least. *Aspergillus* spp. are the most toxic among the 30 fungal isolates, as they have been previously implicated in several respiratory illnesses in human and animals. The conidia of these fungi might have contaminated the food grains used to produce the poultry feed products, especially during drying of these grains and of the oil seeds by farmers. Habib *et al.*, (2015) attributed these results to the fact that many subsistent farmers in Nigeria used to dry

their grains by the roadside where all sorts of debris may enter into these grains, rodents and animals go to eat, defecate and urinate, and/or in closed rooms without proper ventilation thus creating favorable environment for fungal contamination.

Currently, broiler starter and broiler finisher are the most contaminated feeds. Previous studies of Hell *et al.*, (2000); CDC. (2004) attributed these results to the feed miller who stored broiler starter for a longer period, due to less demand from the farmers. Meanwhile, it is to be noted that the longer the storage period of the farm products, the higher will be the level of fungal and aflatoxin contamination of such products.

Attenuated total reflectance Fourier transformed infrared spectroscopy (ATR – FTIR) has been shown to be one of the most rapid, sensitive and reliable tools for the qualitative and quantitative determination of aflatoxins in foods and feeds (Mirghani *et al.*, 2001; Pearson *et al.*, 2001; Berardo *et al.*, 2005; Bhat, 2013; Kaya-Celiker *et al.*, 2014; Kaya-Celiker *et al.*, 2015; Lee *et al.*, 2015; Wang *et al.*, 2015). In this study, 10% of the feed extracts analyzed were contaminated by aflatoxins, as evidenced by their ATR-FTIR spectra. The wavelength and absorption pattern of aflatoxins in the positive extracts were similar to those reported by Kaya-Celiker *et al.*, (2014); Lee *et al.*, (2015).

Conclusion

The poultry feeds analyzed were contaminated by aflatoxigenic fungi and aflatoxins, in addition to other potentially pathogenic fungi. Therefore, more stringent prevention and control methods are required to reduce the feed contamination levels, to avoid loss of poultry lives and possible transfer of carcinogenic mycotoxins to humans along the food chain.

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Conflict of interest

The authors declare no conflict of interests.

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