

Full Length Research Paper

Aflatoxigenic fungi in Nigerian poultry feeds: Effects on broiler performance

Tochukwu E. Ejioffor¹, Anthony C. Mgbeahuruike^{2*}, Emanuela I. Nwoko³, Obianuju N. Okoroafor⁴, Chinwe J. Aronu⁵, Nwaigwe O. Chukwuemeka⁵, Adiong A. Felix⁶, Adong F. Atawal⁶ and Onwumere O. S. Idolor⁷

¹Department of Agricultural Education, Faculty of Vocational Technical Education, University of Nigeria, Nsukka, Enugu State, Nigeria.

²Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.

³Department of Physical and Health Education, Public Health Education Unit, Faculty of Education, University of Nigeria, Nsukka, Enugu State, Nigeria.

⁴Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Nigeria Nsukka, Enugu State, Nigeria.

⁵Department of Animal Health and Production, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.

⁶Department of Zoology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

⁷Department of Agriculture Technology, Delta State Polytechnic, Ozoro, Delta State, Nigeria.

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Aflatoxigenic fungi common to poultry feeds from Nigerian feed mills were investigated and their effects on broiler production were determined. Sixty two weeks old Abor-acre broilers were randomly divided into 5 treatment groups. Each treatment group had 3 replicates of 4 birds each. Birds in each treatment group were fed feed from one of five companies. Feed intake and body weights were determined. Blood samples were analyzed for hematological parameters. Feeds were analyzed for aflatoxin concentration using reverse phase High Performance Liquid Chromatography (HPLC). Aflatoxigenic fungi were identified by sequencing of the fungal ITS region. The major fungal contaminants identified in most of the feed samples were *Aspergillus* and *Rhizopus* species. Total aflatoxin content (AFB1+AFB2+AFG1 +AFG2) of the feeds ranged from <0.8 to 370±120 µg/kg. Feeds from three companies had aflatoxin concentrations above the European Community Regulatory Limits (ECRL, 20 µg/kg). Aflatoxin level was positively related to packed cell volume (PCV) and hemoglobin (Hb), although the relationship was not statistically significant. PCV and Hb were positively related. A negatively significant relationship was observed between the aflatoxin levels and WBC of the birds, feed intake and reduced body weight possibly because of the high aflatoxin concentrations in the feeds. The study has highlighted the effects of long storage time and poor processing of feed on intake and broiler performance.

Key words: Aflatoxin, broilers, feed, hematology, fungi.

INTRODUCTION

Contamination of food crops and feed ingredients by aflatoxigenic fungi is a common occurrence in animal

feed production (Probst et al., 2010; Diedhiou et al., 2011). Cereals, concentrate, hay and other animal feeds

have been reported to be substrates for the growth of fungi such as *Aspergillus* species (Scudamore and Patel, 2000; Firdous et al., 2012; Majeed et al., 2013). Aflatoxins are difuranocoumarin derivatives produced from the polyketide pathway of some strains of *Aspergillus*. *Aspergillus flavus* and *A. parasiticus* are the two widely known aflatoxin producing fungi, although other species such as *A. bombycis*, *A. ochraceoroseus*, *A. nomius* and *A. pseudotamari* have been reported to produce aflatoxin but at a relatively low level (Klich et al., 2000; Peterson et al., 2001). Within each aflatoxigenic fungal species, different strains show qualitative and quantitative differences in their aflatoxin producing abilities (Klich and Pitt, 1988). *Aspergillus flavus* contaminated groundnut meal was linked to the mysterious turkey X disease that claimed the lives of over 100,000 turkey poults near London, England (Blout, 1961). Contamination of feed crops with aflatoxin is common in the fields before harvest, where such crops are usually associated with drought stress (Diener et al., 1987). However, the most important variables that affect the contamination of such feed crops are the moisture contents of the substrate and the relative humidity of the surroundings during storage, which can promote fungal growth (Wilson and Payne, 1994).

Aflatoxin contamination has been linked to increased mortality in farm animals and thus significantly lowers the value of grains as animal feed and as export commodity (Smith and Moss, 1985). Rearing birds with aflatoxin contaminated feed can increase veterinary care costs and reduce livestock production which may cause significant economic losses to the poultry industry (Hussein and Brasel, 2001). Consumption of aflatoxin-contaminated diet by broilers has been shown to induce haematological, biochemical and liver physiological changes as well as growth depression (Che et al., 2011). In addition, haematological values of avian species are equally influenced by poultry diseases (Kokosharov and Todorova, 1989; Branton and May, 1997; Burnham et al., 2003).

Measurement of haematological parameters provides valuable information on an individual's health status. However due to lack of information, blood profile has not been widely used in avian medicine as an index to determine the health status of birds (Mushi et al., 1999). Avian blood differs in cellular composition from their mammalian counterpart (Smith et al., 2000). Some factors like physiological (Alodan and Mashaly, 1999), environmental conditions (Vecerek et al., 2002), diet contents (Odunsi et al., 1999), water and feed restriction (Galip, 1999), fasting (Lamosova et al., 2004), administration of drugs (Khan et al., 1994) and anti-aflatoxin premixes (Oguz et al., 2000) have been

reported to affect the haematological parameters of birds. For example, processes such as blood-cell formation, haemoglobin synthesis, coagulation process, cellular and serum composition of blood are common haematological parameters that are easily affected by changes in the environments and physiology of broilers (Kassirskii, 2010).

Aflatoxin-contamination of poultry feeds results in increased mortality of the affected birds, decrease blood cell count, lower egg production, lower feed consumption rate, impaired resistance to infectious diseases, reduced vaccination efficiency and induced pathological damage to the liver and other organs (Kamalavenkatesh et al., 2005). Aflatoxins can act as immunosuppressive agents affecting cell-mediated and humoral immune compartments (Braz, 2005). Studies have shown that dietary aflatoxin has both genotoxic and mutagenic effects on male Swiss albino mice (Ezekiel et al., 2011). Varying doses of aflatoxin B1 (AFB1) has been reported to affect the hematological parameters of broiler chicks resulting in depressed cellular immunity due to suppression of the phagocytic activity of macrophages and decrease in T-lymphocyte (Celik et al., 2000). Other studies showed that AFB1 concentration in the liver caused a considerable liver damage resulting in deficiency in humoral immunity (Fung and Clark, 2004).

Some studies have demonstrated the use of medicinal plants and biological methods in reducing aflatoxin level in feed samples (Hassan et al., 2017a, b, c, d)

In Nigeria, commercial livestock feed companies depend solely on feed ingredients which are produced locally. Feed mill owners purchase large quantities of grains and other feed ingredients during the production seasons, and these feed stuff are used for feed production throughout the year, without any regulatory measures to control the effects of fungal growth and aflatoxin production. Furthermore, the long post-harvest periods and improper storage coupled with the tropical climate in Nigeria are known to favor fungal growth. As a result, the level of aflatoxin contamination in most livestock feeds in Nigeria may be on the increase from the delivery of grains from harvesting point, to storage for feed manufacturing and finally to poultry farms in the form of feed.

There is limited information on the growth performance and hematological parameters of Arbor-acre broilers fed aflatoxin contaminated feeds. Thus, the present study was designed to identify the major fungal agents responsible for aflatoxin production in poultry feeds in Nigeria and evaluate the level of aflatoxin contamination of poultry feeds from different feed mills. Finally, the study will investigate the effect of the aflatoxin contaminated feeds on the growth performance and

*Corresponding author. E-mail: Anthony.mgbeahuruike@unn.edu.ng. Tel: +2348039253623.



Figure 1. The birds used in this study. The experimental birds were Arbor-acre broilers purchased from St Anthony's farm Akpugo and were reared in pens within the faculty of veterinary medicine animal farm, University of Nigeria, Nsukka.



Figure 2. Feed samples used in this study. (a) Feed sample with long storage time (6 months); (b) Feed sample with shorter storage time (3 months).

hematological parameters of Arbor-acre broilers in Nigeria. The findings from this study followed guidelines for reporting in vivo experiment in animal research (Kilkenny et al., 2010).

MATERIALS AND METHODS

Broilers and feed treatments

The birds used in this experiment were purchased from St Anthony's farm Akpugo Nike in Enugu State (Figure 1). The birds were handled following the Animals Scientific Procedures Act of 1986. The 2-week old birds were acclimatized for three weeks in the Faculty of Veterinary Medicine Poultry Farm, University of

Nigeria, Nsukka. During this period, the birds were given routine vaccination in addition to antibacterial and anticoccidial drugs. Poultry feeds with storage time ranging from 3 to 6 months were purchased from five commercial feed companies in Nigeria: A2, B2, C2, D2 and E2 (Figure 2). The nutrient contents of the feed samples were obtained from the information provided by the company that produced each feed. Crude protein content, fat/oil, crude fibre and metabolizable energy (ME) were recorded for each sample. The birds were randomly divided into 5 treatment groups according to the feed source. Each treatment group had a total of 12 birds divided into 3 replicates of 4 birds/replicate. The birds were wing marked and birds in the same replicate were housed in pens of 1x1 m² size. At the 5th week, the birds were given feeds from the corresponding companies. Ambient temperature, lighting, ventilation and other environmental conditions were fully met according to the requirements in the technical instructions for Arbor-

Table 1. Aflatoxin concentrations for the five feed samples analyzed in this study.

Feed samples	AFB ₁ (µg/kg)	AFB ₂ (µg/kg)	AFG ₁ (µg/kg)	AFG ₂ (µg/kg)	Aflatot
A2	<0.2 ± 0.0	<0.2 ± 0.0	<0.2 ± 0.0	<0.2 ± 0.0	<0.8
B2	71.0 ± 23.0	8.3 ± 2.7	12.0 ± 3.7	1.0 ± 0.3	92 ± 30
C2	300.0 ± 94.0	41.0 ± 13.0	32.0 ± 10.0	3.0 ± 1.0	370 ± 12
D2	11.0 ± 3.5	1.7 ± 0.5	1.0 ± 0.3	<0.2 ± 0.0	14 ± 4.4
E2	130.0 ± 42.0	17.0 ± 5.4	11.0 ± 3.4	0.8 ± 0.3	160 ± 51

The concentration of aflatoxin in each feed sample was expressed as means ± SD. Aflatot = Total aflatoxin.

acre broiler breeding. The birds were fed ad libitum. Feed intake was determined by subtracting the quantity of feed left after one day of serving birds in each group from the total quantity served to the birds. The average for each group was calculated and expressed as mean.

Sampling and hematological analysis

The birds were weighed at the 5th week before they were placed on the feed treatments. Blood samples were collected from the jugular veins of each bird in EDTA anticoagulant treated syringes, transferred to 2 ml Eppendorf tubes containing anticoagulants and stored for further use. Subsequent blood samples were collected from the brachial vein of the birds from each group at the 6th, 7th and 8th week (Bermudez and Stewart-Brown 2003). Hematological analysis included counts of red blood cell (RBC), white blood cells (WBC), packed cell volume (PCV) and hemoglobin (Hb). In addition, the absolute counts of heterophils, lymphocytes, monocytes, eosinophils and basophils were determined following routine procedures. For the PCV and RBC counts, microhaematocrit method was used (Cole, 1986). Total Hb was determined using cyanmethemoglobin method (Brown, 1984). Total WBC count was determined as described previously by Schlam et al. (1975). Other hematological parameters were determined using routine methods (Campbell, 1988). Body weight measurements and feed intakes were also determined on individual bird at weekly intervals from the 6th to 9th week during the course of the treatments.

Fungal isolation and identification by PCR amplification of the ITS region

About 2 g of the feed samples fed to the birds in each group was ground to a fine powder using a mortar and pestle. The ground feed samples were transferred to labeled test tubes and 8 mL of sterile distilled water was added to each tube. The mixture was homogenized with a vortexing machine. Aliquots of 0.2 mL were collected from each tube and spread on freshly prepared SDA and PDA media (Oxoid, Cambridge, UK) and incubated for 5 days at 25°C. To produce a pure culture of each fungus, fungal colonies with homogenous morphology were picked from each plate and transferred to new PDA plates and incubated for another 5 days at 25°C. Genomic DNA was extracted from the pure cultures (Oxoid, Cambridge, UK) using a hexadecyltrimethylammonium bromide (CTAB-based method; Nygren et al., 2008). DNA concentrations were determined spectrophotometrically using NanoDrop (Thermo Scientific, Wilmington, DE). Dream Taq DNA polymerase (Thermo Scientific, Wilmington, DE) was used for PCR amplification of the highly conserved fungal internal transcribed spacer (ITS) region using primer pairs ITS1F and ITS4 as previously described by White et al. (1990). Amplified PCR products were purified using

ethanol-sodium acetate precipitation protocol and sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands). Sequence data were used for BLAST searches in GenBank at the National Center for Biotechnology Information (NCBI-www.ncbi.gov).

Aflatoxin analysis by reverse phase HPLC

Aflatoxins in the feed samples were analyzed following the procedure described by Barmark and Larsson (1994). Briefly, 10 g of the feed samples from each treatment group was weighed and ground to a fine powder using a mortar and pestle. The ground feed samples were transferred quantitatively to extraction tubes containing 80% (v/v) aqueous acetonitrile. The tubes were placed on a rotary shaker for 45 min for extraction. The extract was filtered through folded filter paper (Schleicher and Shuell 597½); a 1 mL aliquot was diluted with 40 mL de-ionized water and mixed thoroughly. The diluted extract was purified on immuno affinity columns (Vicam AflaTest, Waters Corp.). The purified extract was analyzed by reversed phase HPLC (Shimadzu Corp.) with isocratic elution and fluorescence detection after post column derivatization with bromine by the KOBRA CELL® (Rhone Diagnostics, Glasgow UK). Each feed sample was analyzed for the four known aflatoxins-aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁) and aflatoxin G₂ (AFG₂).

Statistical analysis

'R' statistical package was used for data analysis. A generalized linear model (glm) with Gaussian error structure was used, with the significance level set at $P < 0.05$ (Mathsoft, 1999). The effect of aflatoxin was treated as a dependent variable while weight gain, feed intake, RBC, PCV, WBC and Hb were independent variables. Furthermore, the analyses included the interactions of Hb with WBC and Hb with PCV. Model selection was carried out using step-wise backward single term deletion of non-significant variables, starting with the interactions.

RESULTS

Aflatoxin analysis

The four aflatoxins were detected in the feed samples but at varying concentrations (Table 1). Feed samples B2, C2 and E2 had high concentrations of AFB₁ higher than the recommended limits in livestock feeds. The same trend was maintained for these feed samples for AFB₂ and AFG₁ but at relatively lower levels. Feed samples A2

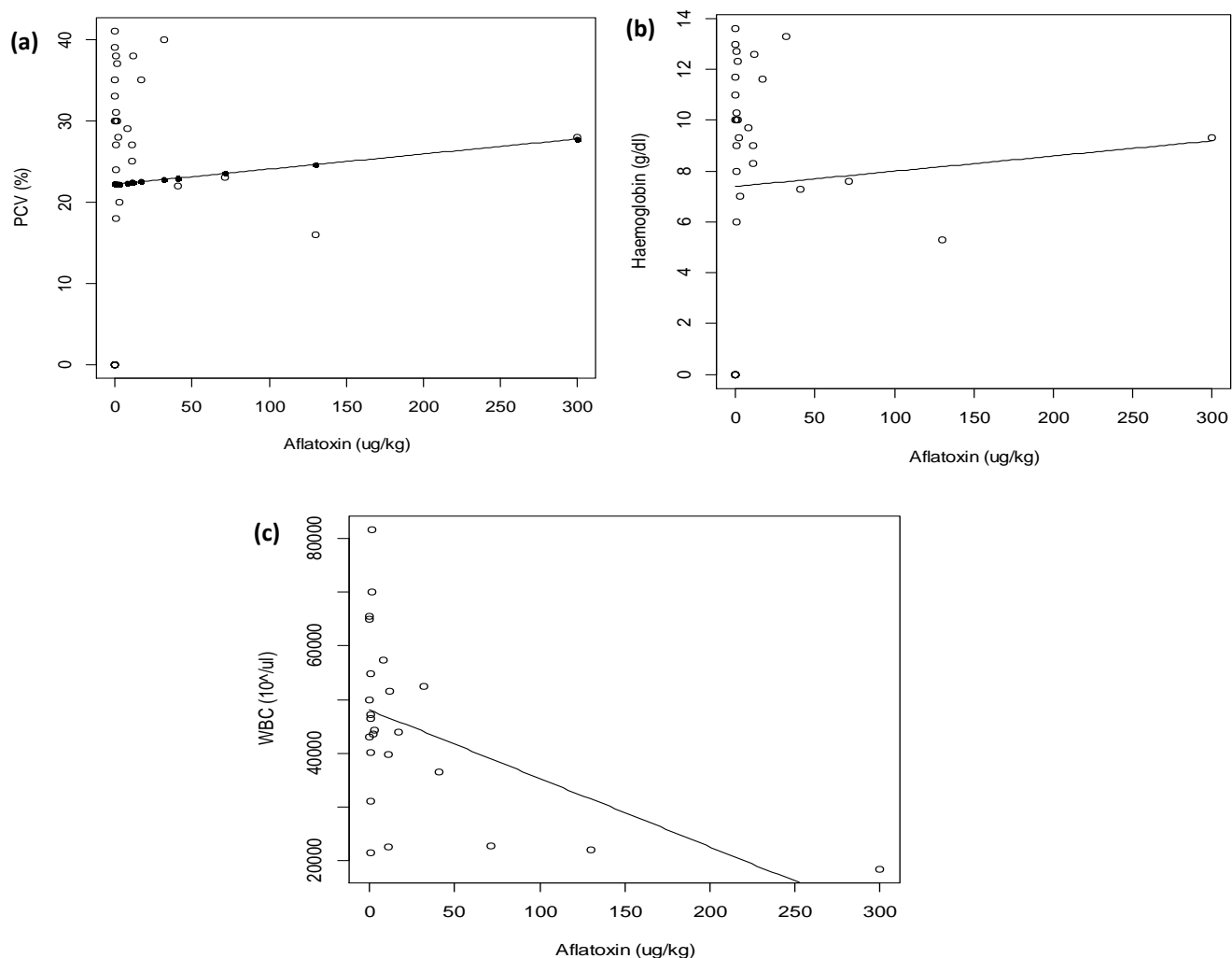


Figure 3. Relationships between aflatoxin concentration of the feeds and some tested blood parameters; (a) Relationship between aflatoxin and PCV ($P=0.45$, $B=3.85$); (b) Relationship between aflatoxin and Hb ($P=0.54$, $B=0.92$); and (c) Relationship between aflatoxin and WBC ($P=0.03$, $B=-0.01$).

Table 2. Summary table for relationship between aflatoxin and body condition of the experimental birds.

Parameter	Estimate	Std. error	t value	Pr(> t)
Intercept	8.10	1.82	0.05	0.96
Hb	9.20	1.50	0.62	0.54
PCV	3.85	5.00	0.77	0.45
WBC	-0.01	0.01	-2.27	0.03
Hb:PCV	0.41	0.18	2.24	0.03

Significant values are in bold. Although Hb and PCV are not significant, they are however left in the model because the interaction between them is significant.

and D2 were the only samples that had aflatoxins below the recommended level. There was no statistically significant relationship between the aflatoxin level in the feed and weight gain; aflatoxin level and feed intake and aflatoxin level and RBC of the birds. Aflatoxin level was

positively related to PCV but the relationship was not statistically significant (Figure 3a and Table 2; $P=0.54$, $B=0.92$). Furthermore, the aflatoxin level was positively related to Hb but also was not statistically significant (Figure 3b and Table 2; $P=0.45$, $B=3.85$). A negative

Table 3. Nutrient composition of the feeds utilized in this study.

Nutrient	Feed A2	Feed B2	Feed C2	Feed D2	Feed E2
Crude protein (%)	18.0	17.0	23.0	18.0	19.0
Fat/oil (%)	6.0	10.0	10.0	6.0	2.9
Crude fibre (%)	5.0	15.0	10.0	5.0	6.0
M.E (kcal/kg)	2900	3000	2830	3000	2875

The nutrient composition of each feed was obtained from the information provided by the companies sampled in this study.

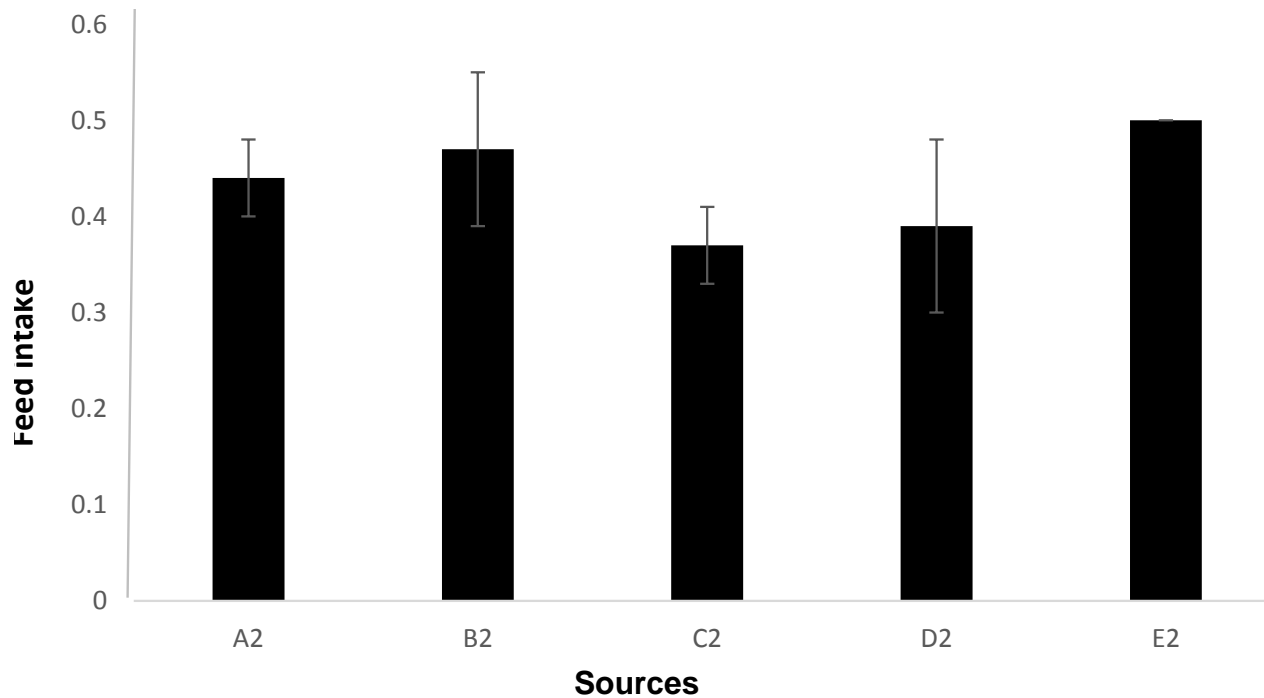


Figure 4. Feed intakes of the Abor-acre broiler birds given feeds from five different sources. The birds were fed ad libitum. Feed intake was determined by subtracting the quantity of feed left after one day of serving birds in each group from the total quantity served to the birds. The average for each group was calculated and expressed as means. The bars are averages of 4 different measurements taken at different time intervals.

statistically significant relationship was observed between the aflatoxin level in the feeds and the WBC of the birds (Figure 3c; $P = 0.03$, $B = -0.01$).

Nutrient composition of the feeds, intake and broiler performance

The nutrient composition of the 5 feed samples is presented in Table 3. The fat/oil content and crude fibre content were highly variable between the feed samples. The feed samples had different levels of mould contamination (Figure 2) and this negatively affected the feed intake. Birds in groups C2, D2 and E2 had relatively reduced feed intakes whereas birds in groups A2 and B2 had better feed consumption rates (Figure 4). A similar

trend was observed in the body weights of the broilers in each group. The broilers in groups C2, E2, B2 and D2 had reduced weight gain (Figure 5); whereas, the birds in Group A2 had relatively higher weight gain.

Haematological analysis

The haematological parameters are presented in Table 4. Birds in each group showed increased WBC counts, PCV values and hemoglobin values. Although the lymphocyte counts were relatively high; they varied among the groups. The average total RBC count in all the groups were low while the mixed blood populations, heterophils, monocytes, eosinophils and basophils were scantily represented in some of the groups. However, a

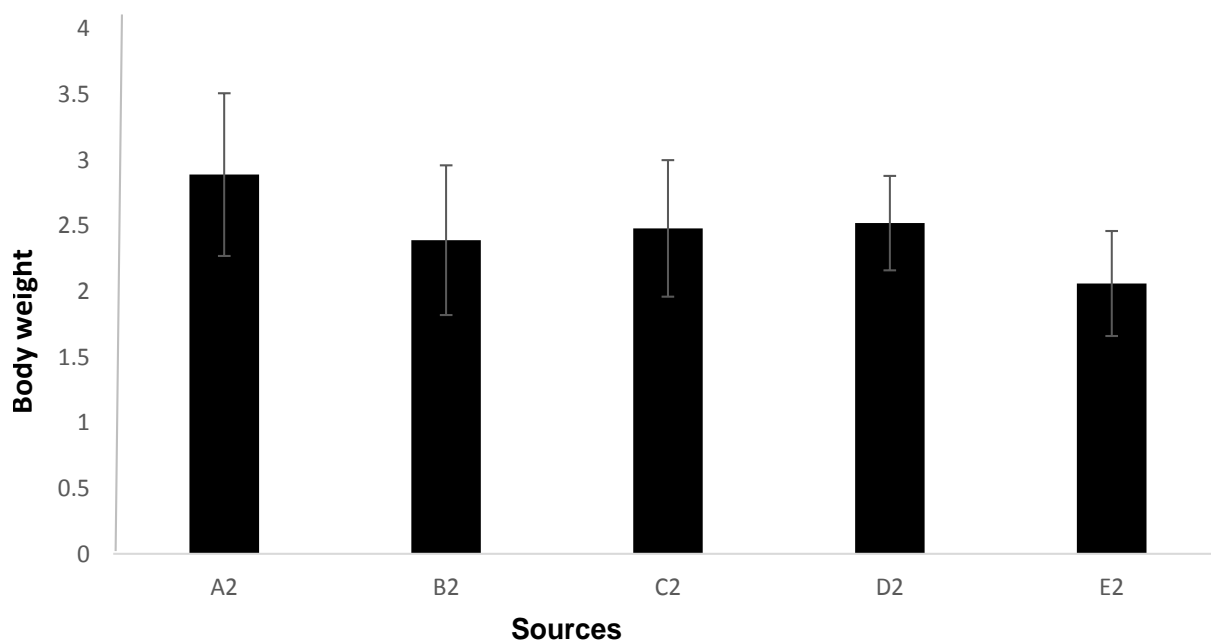


Figure 5. Body weights of Abor-acre broilers fed feed from five different sources. Body weight of individual bird in each group was measured at different time intervals and the average for each group was determined and expressed as means. The bars are averages from 4 different measurements taken at different time intervals.

Table 4. Hematological parameters of Abor-acre broilers fed feeds with different aflatoxin concentrations.

Feed sample ^a	RBC count ($10^6/\mu\text{l}$)	WBC count ($10^6/\mu\text{l}$)	PCV (values %)	Haemoglobin (g/dl)	Lymphocytes	Mixed
A2	7.1 ± 6.6	47.8 ± 9.4	37.6 ± 5.2	12.5 ± 1.7	nm	nd
B2	7.2 ± 4.6	47.7 ± 3.4	33.8 ± 7.5	11.3 ± 2.4	28.8 ± 5.5	nd
C2	3.4 ± 0.7	49.9 ± 7.8	34.0 ± 5.5	11.2 ± 1.8	20.0 ± 4.4	nd
D2	2.9 ± 1.2	56.0 ± 14.4	33.0 ± 4.7	10.0 ± 1.5	16.8 ± 9.0	nd
E2	2.6 ± 0.7	43.1 ± 7.8	32.5 ± 5.3	10.8 ± 1.8	8.5 ± 1.9	nd

The hematological values of individual bird in each group were determined and expressed as means ± SD; ^a = Feed samples fed to birds in different group. nd = not detected; nm = not measured.

statistically significant relationship between PCV and Hb was observed ($P = 0.03$, $B = 0.41$).

Identification of aflatoxigenic fungi by ITS sequencing

Heterogeneous growth of fungi with mixed morphological appearance was observed. However, after sub culturing, 2 pure cultures were obtained from the feed samples in each group. *Aspergillus* species were common in the feed samples fed to birds in Groups B2 and E2. *Rhizopus oryzae* was isolated from feed samples A2, E2 and C2. Other fungal species identified in this study include *Trichosporon asahii* from feed sample D2. All the identified fungal species had between 99 and 100%

sequence identities with existing sequences in the Genbank. However, some of the samples were contaminated during the PCR reaction and could not proceed to the sequencing and identification stage.

DISCUSSION

Feed contamination by aflatoxins is a major problem in the tropics and the major fungal organisms implicated in this problem are the *Aspergillus* species, especially *A. flavus* and *A. parasiticus* (Donner et al., 2009; Diedhiou et al., 2011). Among the isolated fungal species, *Aspergillus* species (*A. flavus* and *A. oryzae*) and *Rhizopus* species (*R. oryzae*) were the most common contaminants found in the feeds in the present study.

From our analysis, the four known aflatoxins (AFB₁, AFB₂, AFG₁ and AFG₂) were present in the feeds at varying concentrations. Feeds C2 and E2 had the highest aflatoxin contents; 58.1 and 25.1%, respectively. The high concentration of aflatoxin in these feed samples could be as a result of long storage time or poor processing methods in the source companies. It is also possible that the concentration of aflatoxins in these feed samples could be as a result of problems with the feed ingredients. Other studies have reported heavy contamination of feed ingredients used in poultry feed production with *Mucor* and *Rhizopus* species due to the high carbohydrate content of the ingredients (Infeanyi et al., 2007; Ariyo et al., 2013). Studies have also reported high contamination of common feed ingredients such as maize, rice, peanut meals and barley used in livestock feed production (Firdous et al., 2012; Sherazi et al., 2014). These feed crops mature in the seasons characterized by hot temperatures and high humidity, as a result, the chances of contamination with fungal infections are quite high (Ratnavathi et al., 2012). Furthermore, *A. flavus* can infect maize crops prior to harvest and remain viable during storage (D'Mello, 2000).

In this study, AFB₁ was found in concentrations far beyond the recommended limits in most of the feed samples analyzed. For example, feed samples B2, C2 and E2 had 71.0, 300.0 and 130.0 µg/kg average concentrations of AFB₁ respectively which are doses far much higher than the recommended limits of 100 ppm in poultry feeds by the United States (FDA, 2003). The European community recommended a maximum AFB₁ content level of 20 and 10 µg/kg for whole feed in poultry and chicks, respectively (Jewers, 1987). AFB₁ is a toxic aflatoxin that induces hepatic cell necrosis, haemorrhage and hepatocellular carcinomas in animals (Khan et al., 2010). At levels of even less than one part per million (ppm), AFB₁ is capable of damaging cells within an organism (Van Kessel and Hiang-Chek, 2004). The acute toxicity of AFB₁ in poultry varies from species to species. For ducklings and chickens, the LD₅₀ single dose (mg/kg body weight) is 0.3 and 6.0 to 16.0, respectively. Poultry diets containing 250 to 500 µg/kg of aflatoxins have been shown to predispose birds to attacks by bacteria and viruses (Edds et al., 1973).

Feed intake and body weight differed among the different groups of birds. For example, birds in groups C2, D2 and E2 had reduced feed intake and consequently reduced body weight possibly because of the high aflatoxin concentrations in the feeds, although this could not be statistically proven in the study's analysis. The differences in feed intake and body weight could also be explained by the nutrient contents of the feeds. Although, the variation in the nutrient contents of the feeds was not high; feeds from companies C2, D2 and E2 had relatively high protein contents. Feed intake was relatively higher for the birds in group B2 and the corresponding body weight of the birds in this group was

also high. It could be that the feed given to this group of birds was more palatable possibly because of its high crude fibre content. Although the study's analysis in this study was limited to aflatoxin; it is possible that other mycotoxins in the feed may have partly contributed to some of the observed conditions in the birds. In a different study, deoxynivalenol was reported to have reduced feed intake and weight gain in birds by 26%; the same study also demonstrated a 16% reduction of feed intake in response to aflatoxin B1 (AFB1) (Andretta et al., 2012).

Hematological values of avian species are used as performance index in determining the health condition of birds (Vecerek et al., 2002). Hematological values are influenced by poultry diseases and other health related conditions (Kokosharov and Todorova, 1987). There were positive relationships between the aflatoxin level in the feeds and the following hematological parameters, PCV and Hb, but the relationships were not statistically significant. The lack of statistically significant relationship between the aflatoxin level and Hb and aflatoxin level and PCV, does not reduce the physiological or clinical values of the result. However, the interaction between PCV and Hb in the birds fed aflatoxin contaminated feed was statistically significant. Furthermore, the aflatoxin contaminated feed decreased the WBC level of the birds and this was statistically significant. These results indicate alteration in haemostasis and blood system damage possibly induced by the aflatoxin. In contrast Che et al. (2011) reported increased WBC level induced by mold contaminated feed. Also, Abbès et al. (2006) reported a significant increase in WBC after some mice were treated with 500 mg/kg ZEN. The differences in results from the three independent studies could possibly be explained by the storage period of the feeds, level of contamination by other mycotoxins and perhaps by the level of consumption of the contaminated feeds. From the study, it could be concluded that poultry feeds stored for a long time can negatively affect the general performance of Abor-acre broilers and this could have negative economic impact on the farmer. Also the study showed variations in feed processing methods, this calls for a more standardized approach that will ensure the production of quality feeds that can withstand the deteriorative effects of environmental agents during storage.

Compliance with ethical standards in experiments involving the use of animals

The authors declare that during the course of this experiment, no animal was treated in an inhumane manner. Animal management and facilities were in accordance with the ethical standards involving the use of animals for *vivo* experiment. All authors who participated in this experiment read the manuscript and

gave their consents for submission.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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