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Distribution of aflatoxigenic *Aspergillus* section *Flavi* in commercial poultry feed in Nigeria

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ABSTRACT

The distribution and aflatoxigenicity of *Aspergillus* section *Flavi* isolates in 58 commercial poultry feed samples obtained from 17 states in five agro-ecological zones (AEZs) in Nigeria were determined in order to assess the safety of the feeds with respect to aflatoxin-producing fungi. Correlation was also performed for incidence of species, aflatoxin-producing ability of isolates in vitro, and aflatoxin (AFB_1) concentrations in the feed. A total of 1006 *Aspergillus* section *Flavi* isolates were obtained from 87.9% of the feed samples and identified as *Aspergillus flavus*, unnamed taxon S_{BG}, *Aspergillus parasiticus* and *Aspergillus tamarii*. *A. flavus* was the most prevalent (91.8%) of the isolates obtained from the feed in the AEZs while *A. parasiticus* had the lowest incidence (0.1%) and was isolated only from a layer mash sample collected from the DS zone. About 29% of the *Aspergillus* isolates produced aflatoxins in maize grains at concentrations up to 440,500 μg/kg B and 341,000 μg/kg G aflatoxins. The incidence of toxigenic isolates was highest (44.4%) in chick mash and lowest (19.9%) in grower mash. The population of *A. flavus* in the feed had positive ($r = 0.50$) but non significant ($p > 0.05$) correlations with proportion of toxigenic isolates obtained from the feed while S_{BG} had significant ($p < 0.001$) positive ($r = 0.99$) influence on AFB_1 concentrations in the feed. Poultry feed in Nigerian markets are therefore highly contaminated with aflatoxigenic *Aspergillus* species and consequently, aflatoxins. This is a potential threat to the poultry industry and requires urgent intervention.

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1. Introduction

Aspergillus section *Flavi* is a group of *Aspergillus* species with yellow–green to brown colonies, dark sclerotia when present and usually biseriate conidia heads (Varga et al., 2011). Species within this group are known to contaminate a range of crops used as feed ingredients, including cereals and nuts (Bandyopadhyay et al., 2007; Atehnkeng et al., 2008; Kpodo and Bankole, 2008; Pildain et al., 2008; Probst et al., 2010; Diedhiou et al., 2011). They have also been reported in finished animal feed including poultry feed (Accensi et al., 2004; Saleemi et al., 2010; Azarakhsh et al., 2011; Astoreca et al., 2011). In these substrates, extrolite production and matrix deterioration follow *Aspergillus* colonization, and several mycotoxins including aflatoxins, cyclopiazonic acid and ochratoxins have been implicated (Frisvad and Samson, 2000; Peterson et al., 2001; Frisvad et al., 2005; Pildain et al., 2008; Varga et al., 2009).

The notable species responsible for synthesis of aflatoxins include *Aspergillus arachidicola*, *Aspergillus bombycis*, *Aspergillus flavus*, *Aspergillus minisclerotigenes*, *Aspergillus nomius*, *Aspergillus parasiticus*, *Aspergillus parvisclerotigenus*, *Aspergillus pseudocelatus*, *Aspergillus pseudonomius* and *Aspergillus togoensis* (Varga et al., 2011). Among these species, *A. flavus*, *A. parasiticus* and the unnamed taxon S_{BG} are the most widely distributed in West Africa (Cotty, 1989; Cotty and Cardwell, 1999; Atehnkeng et al., 2008; Donner et al., 2009; Diedhiou et al., 2011). *Aspergillus tamarii* also occurs in sub-Saharan Africa though it is regarded as a non-aflatoxin producing species. The *A. flavus* isolates from West Africa are known to produce large sclerotia when present and biosynthesize only B aflatoxins; and thus have been ascribed to the L-type, while *A. parasiticus* and S_{BG} isolates usually produce both B and G aflatoxins but are distinguished from each other by cultural characteristics (Cotty, 1989; Garber and Cotty, 1997; Cotty and Cardwell, 1999; Atehnkeng et al., 2008; Diedhiou et al., 2011).

The available reports on mycoflora in poultry feed focused more on *A. flavus* (Adebajo, 1992; Magnoli et al., 1998; Accensi et al., 2004; Osho et al., 2007; Saleemi et al., 2010; Azarakhsh et al., 2011; Astoreca et al., 2011) and only a few reported *A. parasiticus* in feed, albeit at very low occurrence levels (Saleemi et al., 2010; Astoreca et al., 2011; Monge et al., 2013). Previous studies showed that *A. flavus* was predominant (up to 40%) on chick, grower and layer mashes in Nigeria (Osho et al., 2007); however, the occurrence of *A. tamarii* and S_{BG} in poultry feed has not previously been reported.

Aflatoxin contamination in poultry feed alongside the presence of *Aspergillus* species has also been widely evaluated (Adebajo, 1992;

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Dalcero et al., 1998; Magnoli et al., 2002; Astoreca et al., 2011; Monge et al., 2013), but none of these study reported the correlation between incidence of toxigenic isolates of *Aspergillus* section *Flavi* with levels of aflatoxins in the feed. Aflatoxins especially aflatoxin B_1 (AFB_1) are regarded as potent hepatocarcinogens and immunosuppressants, and there are reports showing that this group of mycotoxins pose the biggest threat to the poultry industry through low productivity and death (van Egmond, 1989; Chukwuka et al., 2010; Pedrosa and Borutova, 2011). There is therefore the need to clearly evaluate the potential risks of aflatoxicosis in the Nigerian poultry industry for prompt legislative actions and mitigation of aflatoxin contamination in the feed. This study was designed to determine and evaluate the distribution and aflatoxin-producing potentials of *Aspergillus* section *Flavi* in five commercial poultry feed formulas sold across five agro-ecological zones (AEZs) in Nigeria. We also established the correlation between incidence of *Aspergillus* section *Flavi* and aflatoxin contamination in the feed samples.

2. Materials and methods

2.1. Survey and sampling of poultry feed samples

Surveys were conducted in 17 States representing five AEZs of Nigeria between October and November 2009 in order to determine the distribution of *Aspergillus* section *Flavi* in commercial poultry feed in Nigeria. The sampling points (States) and AEZs are given in Fig. 1. Ezekiel et al. (2012) describe other details of sampling. Briefly, 58 commercial poultry feed samples categorized as: chick mash ($n = 7$), grower mash ($n = 14$), layer mash ($n = 14$), broiler starter ($n = 11$) and broiler finisher ($n = 12$); were collected from the 17 states. The compositions of the various feed types were reported in Ezekiel et al. (2012). Each sample was collected from at least three points of the 50 kg bag of bulk feed into clean zip-lock bags. Samples were transported to the Mycotoxin Laboratory of International Institute of Tropical Agriculture (IITA), Ibadan for further analysis. About 50 g representative samples were randomly taken per feed sample and divided into two parts: part A, for moisture content determination, and part B for isolation of *Aspergillus* section *Flavi*. Part A samples were analyzed immediately to avoid change in moisture content, while part B samples were stored at 4 °C and analyzed within 48 h.

2.2. Determination of moisture content of feed

The method described by Dalcero et al. (2002) was adopted for the estimation of moisture content in the feed. Ten grams of each feed was dried for 5 h at 85 °C in an oven with forced air circulation. Triplicates of each sample were weighed and the initial moisture content (%) was calculated by subtracting the final weight from the initial weight and dividing by the initial weight then multiplied by 100.

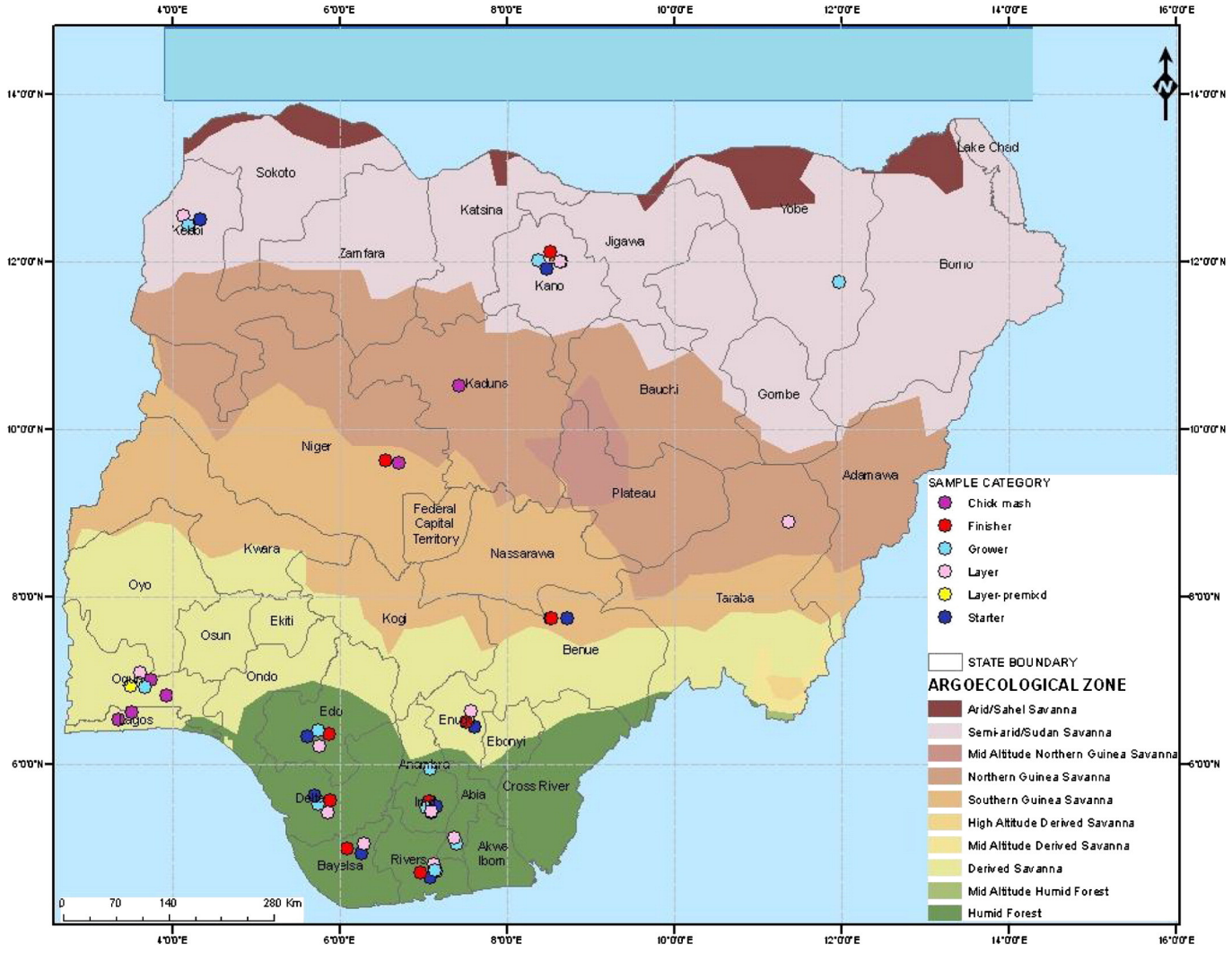

Fig. 1. Map of Nigeria showing the locations and feed types sampled in each of the states.

2.3. Assessment of poultry feed for Aspergillus section Flavi

2.3.1. Isolation of Aspergillus section Flavi

Ten grams of each part B sub-sample was appropriately diluted in 90 ml of sterile distilled water. The mixture was homogenized for 2 min and 0.05–0.1 ml aliquots were taken and surface inoculated in triplicate on modified Rose Bengal Agar (mRBA, Cotty, 1994; Probst et al., 2007). The inoculated plates were incubated without illumination at 31 °C for 3 days. Distinct colonies with a typical green appearance resembling members of *Aspergillus* section *Flavi* were transferred to 5/2 agar (5% V-8 juice and 2% agar, pH 5.2) for further characterization (Atehnkeng et al., 2008). The relative populations of *Aspergillus* section *Flavi* in each feed sample were calculated and expressed as logarithm of colony-forming units per gram (Log_{10}CFU/g).

2.3.2. Characterization of Aspergillus section Flavi isolates

Isolates were transferred from mRBA plates onto 5/2 agar for characterization of *Aspergillus* section *Flavi* colonies. Only mRBA plates with 8–10 colonies were used (Diedhiou et al., 2011) and 20 isolates from a set of triplicate mRBA isolation plates per feed sample were transferred to 5/2 agar plates. The inoculated 5/2 agar plates were incubated in the dark at 31 °C for 5 days. After 5 days, each single colony on 5/2 plate was tentatively identified. All identified isolates were maintained first as agar plugs at 31 °C in 4 ml vials containing 2 ml sterile distilled water, and thereafter, on silica granules (Grade H, Type II, 6–12 Mesh, Merck KGaA, Darmstadt, Germany) at 4 °C.

Identification of *Aspergillus* section *Flavi* was based on a combination of macroscopic and microscopic characters exhibited by each species on specific media and aflatoxin profile as reported by Cotty (1989), Cotty and Cardwell (1999), Atehnkeng et al. (2008) and Diedhiou et al. (2011). Isolates with greenish-yellow colonies that produced smooth conidia and large (>400 μm average diameter) or no sclerotia on 5/2 were identified as L-type *A. flavus*, while those with numerous and small sclerotia (<400 μm average diameter) were tentatively regarded as unnamed taxon S_{BG} (Cotty and Cardwell, 1999). Isolates appearing as dark green colonies on 5/2 agar and showing rough conidia (×400) were initially termed *A. parasiticus* and confirmed using their aflatoxin profile. Isolates with yellow–brown colonies and rough conidia (×400) were identified as *A. tamarii*, a putative non aflatoxin-producing species.

In order to confirm the identities of all the isolates of *A. flavus*, unnamed taxon S_{BG}, *A. parasiticus* and *A. tamarii*, each isolate was inoculated on freshly prepared *A. flavus* and *A. parasiticus* agar (AFPA; Pitt et al., 1983). The inoculated plates were incubated in the dark for 7 days at 31 °C and later observed for color reaction. Isolates that produced a bright orange reverse were confirmed as *A. flavus* L-strain, unnamed taxon S_{BG} and *A. parasiticus*; while *A. tamarii* produced brown spores and a brown to tan reverse (Cotty, 1997). A second confirmatory technique based on aflatoxigenicity screening of each isolate was adopted in the characterization of the isolates. All isolates were tested for aflatoxin production on healthy maize grains (details are described below in Section 2.4). Colonies with characteristic *A. flavus* appearance and that produced only B aflatoxins or no aflatoxins were confirmed as L-type *A. flavus* (toxigenic or atoxigenic isolates). Isolates producing both B and G aflatoxins were confirmed as either S_{BG} strain or *A. parasiticus* following previous colonial and microscopic descriptions.

2.4. Aflatoxigenicity testing of Aspergillus section Flavi isolates

A total of 1006 *Aspergillus* isolates were tested for B and G aflatoxin production according to the healthy maize grain colonization (MGC) test described by Probst et al. (2010) but with slight modifications.

2.4.1. Maize preparation and inoculation with test isolates

The healthy maize grains used for this study were collected from the maize breeding unit of IITA and were sorted to remove grains with insect infestation, physical damage and shriveling. Sorted grains were analyzed for aflatoxin using thin layer chromatography aided by densitometry (see Section 2.4.2.). Ten grams of aflatoxin-free maize grains was weighed into 40 ml vials and rinsed twice in sterile distilled water to wash off any surface contaminants. Two milliliters of distilled water was added to each vial prior to autoclaving at 121 °C for 10 min. The vials containing autoclaved maize were cooled overnight for moisture equilibration. One milliliter of sterile distilled water was added to each vial to provide sufficient moisture for fungal growth and subsequent toxin production. The sterilized grains in each vial were inoculated with 500 μl spore suspensions containing approximately 1×10^6 conidia/ml of an isolate and incubated without illumination at 31 °C for 7 days. Three vials were inoculated with spore suspension of each isolate and triplicate control vials for isolates from samples within same location were maintained. The control vials were treated in the same way as the test vials, except for inoculation.

2.4.2. Extraction and quantification of aflatoxins in inoculated maize grains

Aflatoxin extraction from the inoculated maize was performed according to Probst et al. (2010). The inoculated grains in each vial were comminuted in 50 ml of 80% methanol for 3 min using a high speed Waring blender (Waring Commercial, Springfield, MO). The mixture was shaken in an orbital shaker for 30 min and allowed to stand for 10 min. Aliquots (4 μl) of the supernatant were directly spotted on High Performance Thin Layer Chromatography (HPTLC) pre-coated plates (20 × 10 cm Silica gels 60, F_{254}; Merck, Germany). B and G aflatoxin standards were spotted alongside the samples and the plates were developed in ethyl ether–methanol–water (96:3:1). The dried plates were visualized under ultraviolet light (365 nm) and scored visually for the presence or absence of aflatoxin.

The 80% methanol extracts from vials with no detectable aflatoxins were partitioned twice using 20 ml sterile distilled water and 25 ml dichloromethane in each instance. The lower phase of the extract was collected into a polypropylene cup by filtration through a bed of anhydrous sodium sulfate. The extract was evaporated to dryness, then the residue was dissolved in 500 μl dichloromethane and spotted on pre-coated HPTLC plates alongside aflatoxin standards of known concentrations. The plates were developed, and spots were visualized and scored for the presence or absence of aflatoxin as described earlier.

Aflatoxins were quantified in the positive samples using a scanning densitometer (CAMAG TLC Scanner 3 with Win-CATS 1.4.2 software, Camag AG, Muttenz, Switzerland). Chromatograms were generated and interpreted by factoring in all the variables including sample size, volume of extraction solvent, volume of mixture spotted on the HPTLC plate, and concentration of standards applied to the TLC plate. The concentration of aflatoxins (B_1, B_2, G_1 and G_2) in each sample was estimated with a detection limit of 0.5 ng/g. Quantification of aflatoxins enabled the evaluation of the proportion of toxigenic to atoxigenic strains in each sample, feed type across the AEZ, as well as determining the contribution of each species to aflatoxin production. The in vivo AFB_1 producing potential of toxigenic isolates belonging to a species was calculated as follows:

Aflatoxin-producing potential attributed to species x = [(sum of aflatoxin B_1 produced by all isolates of the species x) / (sum of aflatoxin B_1 produced by all isolates of all species *in vivo*)] × 100.

2.4.3. Quantification of aflatoxin B_1 in poultry feed

The concentrations of AFB_1 in the feed were quantified using liquid chromatography tandem mass spectrometry and reported in Ezekiel et al. (2012). The data obtained from that study were used for correlation analysis in this study.

2.5. Data analysis

The SAS system (version 9.1, SAS Institute Inc., NC) was used for statistical analysis of data on the moisture content, propagule density of

Aspergillus section *Flavi*, strain distribution, proportion of toxigenic to atoxigenic strains in feed across agro-ecological zones, and quantities of types of aflatoxin produced by toxigenic isolates. Means were separated by Duncan's multiple range test and tested for significance by one-way analysis of variance at $\alpha = 0.05$. Pearson's correlation analysis was used to determine the relationship among incidence of species, aflatoxigenic isolates and aflatoxin concentration in the feed.

3. Results

3.1. Moisture content of poultry feed

The moisture content of the feed ranged from 9.8% to 12.4%. There were no significant ($p > 0.05$) differences in the moisture content of the feed across the five AEZs, even though feed samples from HF had the highest moisture content (11.2% ± 0.04), while those from NGS had the lowest (9.9% ± 0.01). The moisture content of the feed types were as follows: 9.9 ± 0.03% for chick mash, 10.0% ± 0.03 for broiler starter, 10.2% ± 0.01 for grower mash, 10.2% ± 0.02 for broiler finisher and 9.9% ± 0.01 for layer mash (data not shown).

3.2. Incidence of Aspergillus section Flavi species in the feed samples

A total of 1006 *Aspergillus* section *Flavi* isolates were obtained from 51 of the 58 (87.9%) feed samples. The other seven feed samples showed zero concentration of the section *Flavi* propagules. Only *A. flavus*, unnamed taxon S_{BG}, *A. parasiticus* and *A. tamarii* were isolated from the feed samples. The S-strain of *A. flavus* that produces only the B-type aflatoxin was not detected.

The percentage of contaminated feed samples, concentration of *Aspergillus* section *Flavi* propagules, incidence and distribution of species within *Aspergillus* section *Flavi* in the feed types across AEZs are shown in Fig. 2 and Table 1. All samples of chick mash, broiler starter, broiler finisher, 9 (64.3%) of grower mash and 12 (85.7%) of layer mash were contaminated with species of *Aspergillus* section *Flavi*. The mean population density of *Aspergillus* section *Flavi* in the feed samples across the five AEZs was 3.56 Log_{10}CFU/g. *A. flavus* was the most prevalent (overall mean: 91.8%) of the *Aspergillus* section *Flavi* isolates obtained from the feed in the AEZs. *Aspergillus parasiticus* had the least incidence (overall mean: 0.1%) and was isolated only from a layer mash sample collected from the DS zone (Table 1). The unnamed taxon S_{BG}, though lower (overall mean: 1.9%) in incidence than *A. flavus* and *A. tamarii* (overall mean: 6.2%), was also isolated from chick mash, broiler starter and finisher feed (Fig. 2) across all AEZs except the DS (Table 1). There was no significant difference ($p > 0.05$) in the incidence of *A. flavus* and *A. tamarii* in the feed across the AEZs; whereas, S_{BG} isolates were significantly ($p < 0.05$) higher in NGS than other zones (Table 1).

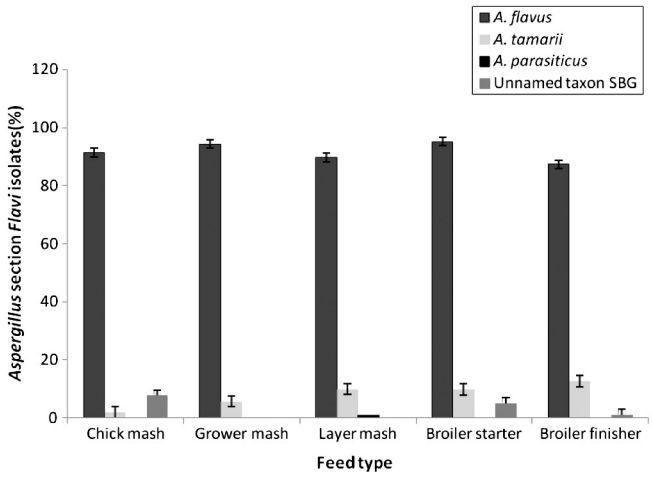

Fig. 2. Incidence (%) of *Aspergillus* section *Flavi* isolates in five major commercial poultry feed types in Nigeria. Vertical lines on bars indicate the standard error of mean ($\alpha = 0.05$).

3.3. Distribution of toxigenic and atoxigenic Aspergillus section Flavi in the feed

Only 293 isolates (29.1%) of the 1006 tested isolates produced aflatoxins. All S_{BG} (19) strains and the *A. parasiticus* isolate produced both B and G aflatoxins, whereas 273 (29.5%) of the 924 *A. flavus* isolates were toxigenic and produced only the B aflatoxins. In contrast and as expected, *A. tamarii* (62) strains did not produce aflatoxins on maize grains. There were significant ($p < 0.05$) differences in the incidence of toxigenic *Aspergillus* section *Flavi* isolates, whereas no significant ($p > 0.05$) differences were observed for the atoxigenic isolates (Table 2). Chick mash had the highest incidence (44.4%) of toxigenic isolates, whereas grower mash had the least incidence (19.9%) (Table 2). For comparison across AEZs, the highest incidence (43.2%) of toxigenic isolates was obtained from feed samples from the DS zone, with the least (19.2%) obtained from SGS (Fig. 3). Generally, the incidence of atoxigenic isolates was higher than that of toxigenic isolates in all feed types across the five AEZs.

3.4. Aflatoxins produced by toxigenic isolates

All toxigenic isolates of *Aspergillus* section *Flavi* obtained from the feed samples produced aflatoxins in varying concentrations in inoculated maize grains. The concentration of B aflatoxins produced by the toxigenic isolates ranged from 40 ng/g to 441,000 ng/g, and concentrations of G aflatoxins were between 2800 ng/g and 341,000 ng/g (Table 3). *A. flavus* L-strain found in grower mash feed from DS produced the highest B aflatoxin (AFB_1) (440,521 ng/g), whereas taxon S_{BG} in chick mash from NGS produced the highest quantity of G aflatoxin (AFG_1) (340,984 ng/g). Furthermore, toxigenic isolates from grower mash feed produced the highest mean AFB_1 concentration (55,637 ng/g), and this was significantly ($p < 0.05$) more than the mean AFB_1 concentration produced by toxigenic isolates in layer mash (Table 3).

Analysis of the quantities of AFB_1 produced in maize grains inoculated with toxigenic isolates of *Aspergillus* section *Flavi* showed that between 1.3 and 5.7% of the 293 toxigenic isolates produced AFB_1 in quantities exceeding 100,000 ng/g (Fig. 4). The highest contribution of AFB_1 was by toxigenic isolates obtained from the chick mash feeds. Majority (9.2–19.3%) of the toxigenic isolates produced between 10,001 ng/g and 100,000 ng/g AFB_1, and 2.2–4.2% of the isolates produced 1–1000 ng/g AFB_1 in maize grains (Fig. 4).

A. flavus isolates from all feed types, except the chick mash, had similarly high potential (87–100%) to produce AFB_1 in maize grains; however, S_{BG} from the chick mash feed showed more AFB_1 producing capacity (53.7%) than *A. flavus* (Fig. 5). S_{BG} obtained from other feed types had between 1.6 and 12.9% potential for AFB_1 production and only *A. flavus* in the grower mash feed produced AFB_1. *A. parasiticus* which was only isolated from layer mash had 0.4% potential to produce AFB_1.

3.5. Correlation studies between species of Aspergillus section Flavi and AFB_1 in feed

The relationships among incidence, proportion of aflatoxigenic *Aspergillus* section *Flavi* species and aflatoxin levels in the feed samples are given in Table 4. The population of *A. flavus* in the feed samples had a significant positive influence ($r = 0.90$, $p < 0.001$) on the overall population in contrast to the contributions of other species of *Aspergillus*. Although all isolates belonging to the unnamed taxon S_{BG} produced aflatoxins, the correlation between frequencies of S_{BG} strains and toxigenic strains in the samples, though high ($r = 0.50$), was not significant ($p > 0.05$). Only one *A. parasiticus* isolate was obtained from the feed samples; therefore,

Table 1
Distribution of 1006 isolates belonging to species within *Aspergillus* section *Flavi* isolated from feed samples across five agro-ecological zones in Nigeria.

AEZ[a]	Mean $Log_{10}CFU$[b,d]	% contamination[c]	Number isolated	Mean distribution of species (%)[d]			
				A. flavus	S_{BG}	*A. parasiticus*	*A. tamarii*
DS	4.11a	100.0a	226	96.9a	0.00	0.4a	2.7a
HF	3.06a	81.5a	440	89.2a	0.6b	0.00	10.2a
NGS	2.71a	100.0a	60	80.0a	16.7a	0.00	3.3a
SGS	2.84a	100.0a	120	98.4a	0.8b	0.00	0.8a
SS	3.26a	80.0a	160	86.4a	3.6b	0.00	10.0a
Mean[e]	3.56	87.9	–	91.8	1.9	0.1	6.2

[a] AEZ: DS, Derived Savannah; HF, Humid Forest; NGS, Northern Guinea Savannah; SGS, Southern Guinea Savannah; and SS, Sudan Savannah.
[b] Average logarithm of colony forming units per gram feed samples from the AEZs.
[c] Percentage of feed samples contaminated with *Aspergillus* section *Flavi* propagules.
[d] Means with same alphabet in a column are not significantly different by Duncan multiple range test at $\alpha = 0.05$.
[e] Average CFU and percentage distribution of species in *Aspergillus* section *Flavi*.

it is not a good representation in the correlation studies. Furthermore, there was a significant ($p < 0.01$) inverse ($r = -0.89$) relationship between *A. flavus* incidence and AFB_1 levels in the feed, while the populations of S_{BG} correlated significantly ($p < 0.001$) and positively ($r = 0.99$) with AFB_1 levels in the feed (Table 4). There was also an inverse significant correlation ($r = -0.99$, $p \leq 0.05$) between the incidence of *A. tamarii* and proportion of toxigenic strains in the feed, while the relationship was positively significant ($r = 0.62$, $p \leq 0.01$) for the incidence of *A. tamarii* and proportion of atoxigenic strains in the feed samples. As expected, *A. tamarii* had no relationship ($r = 0.00$) with AFB_1 levels in the feed.

4. Discussion

Mycotoxin (e.g. aflatoxin) contamination of poultry feed ingredients and finished feed are of high importance in the poultry industry due to the associated risks they pose. Animal mycotoxicoses, caused by toxins from toxigenic fungi in poultry feed formulations, are a leading cause of low productivity and mortality of poultry birds (Huff et al., 1988). Commercially available poultry feed in Nigeria are made from processed cereals and nuts and frequently include maize and groundnut. These crops are prone to toxigenic fungi and aflatoxin contamination (Bankole and Adebanjo, 2003; Kpodo and Bankole, 2008). Occasionally, bad quality grains that are unfit for human consumption are channeled into the poultry production line. This practice tends to increase the levels of mycotoxins such as aflatoxins in the finished feed as the grain quality is compromised. This may lead to further accumulation of mycotoxins above the acceptable limits as revealed by the results of this study. In addition, feed formulated using contaminated cereals affects cross-border trade where the concentration levels exceed the regulatory limits of the importing countries (Wu, 2006).

The high occurrence of *A. flavus* in the feed samples in the AEZs is in line with previous reports of Adebajo (1992), Magnoli et al. (1998), Accensi et al. (2004), Osho et al. (2007), Saleemi et al. (2010) and Azarakhsh et al. (2011). However, the incidence of *A. flavus* in feed samples in this study was much higher than those reported by Accensi et al. (2004) and Osho et al. (2007) who found 43.5% of *A. flavus* in mixed feed from Spain and 40%, in chick, layer and grower mashes from Nigeria. Only a few reports have indicated the presence of *A. parasiticus* in poultry feed (Saleemi et al., 2010; Astoreca et al., 2011). The recovery of only one *A. parasiticus* isolate in this study is similar to the work of Astoreca et al. (2011) who also recovered one *A. parasiticus* isolate out of 91 *Aspergillus* section *Flavi* isolates from poultry feed in Argentina. Furthermore, there is paucity of data in the literature on the occurrence of the unnamed taxon S_{BG} in poultry feed and in this study, S_{BG} isolates were found in higher proportions than *A. parasiticus* in the feed types. The occurrence of S_{BG} and *A. parasiticus* in chick mash, broiler starter and layer mash feed samples is of great concern. Both species of *Aspergillus* are known to produce the high concentrations of B and G aflatoxins (Atehnkeng et al., 2008; Diedhiou et al., 2011). The high distribution of S_{BG} isolates in the feed from NGS zone corroborates previous reports from Nigeria on the preference of this species for hot regions (Atehnkeng et al., 2008; Donner et al., 2009).

Within *Aspergillus* section *Flavi*, several new species with small sclerotia (S-morphotype) have been described recently using molecular and phylogenetic analysis. Some of these species are strain S_{BG},

Table 2
Mean incidence (%) of toxigenic and atoxigenic *Aspergillus* section *Flavi* isolates in five major commercial poultry feed types in Nigeria.

	Incidence (%)[a]					X[b] (%)	
	Chick mash	Grower mash	Layer mash	Broiler starter	Broiler finisher		
Toxigenic	44.4a	19.9b	23.7b	30.7ab	28.3ab	27.6	–
Atoxigenic	55.6a	49.4a	61.3a	69.3a	71.7a	–	63.3

[a] Mean values with same alphabet in a row are not significantly different by Duncan multiple range test at $\alpha = 0.05$.
[b] X: mean of means.

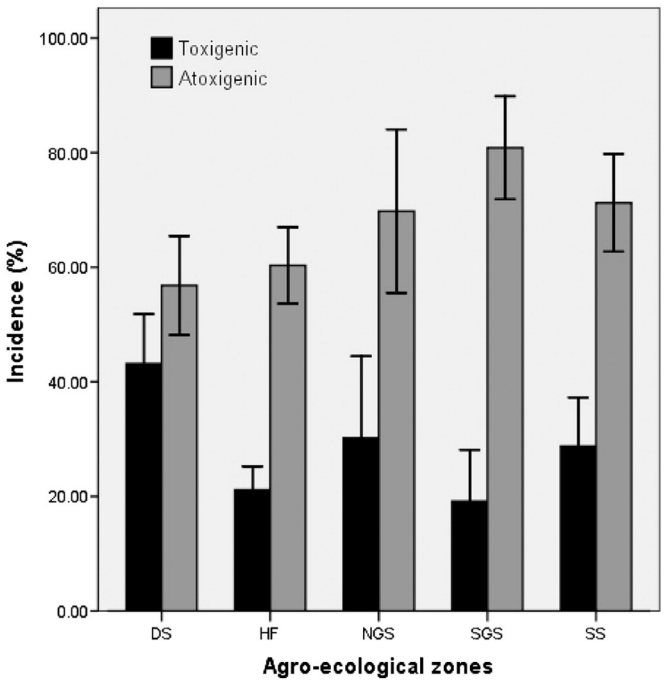

Fig. 3. Distribution of toxigenic and atoxigenic *Aspergillus* section *Flavi* isolates from commercial poultry feed in five agro-ecological zones of Nigeria. DS, Derived Savannah; HF, Humid Forest; NGS, Northern Guinea Savannah; SGS; Southern Guinea Savannah; and SS, Sudan Savannah. Vertical lines on bars indicate the standard error of mean ($\alpha = 0.05$).

Table 3
Concentration of aflatoxins produced in maize grains inoculated with aflatoxigenic isolates from five commercial poultry feed types in Nigeria.

Feed types		Aflatoxin concentration (ng/g)								
		A. flavus		Unnamed taxon S_{BG}			*A. parasiticus*			Average toxin[b]
		N^a	B_1	N^a	B_1	G_1	N^a	B_1	G_1	B_1
Chick mash	Mean	52	23,954	10	145,547	158,888	–	–	–	43,566ab
	Range		40–112,533		3757–355,543	5134–340,984		–	–	40–355,543
Grower mash	Mean	50	55,637	–	–	–	–	–	–	55,637a
	Range		420–440,521		–	–		–	–	420–440,521
Broiler starter	Mean	54	39,593	8	62,179	106,551	–	–	–	42,507ab
	Range		336–290,064		2536–161,680	2825–294,714		–	–	336–290,064
Broiler finisher	Mean	60	41,044	1	99,162	101,390	–	–	–	41,997ab
	Range		268–334,027		–	–		–	–	268–334,027
Layer mash	Mean	57	30,543	–	–	–	1	20,364	81,543	30,367b
	Range		137–271,855		–	–		–	–	137–271,855

[a] Number of toxigenic isolates.
[b] Mean aflatoxin of all three species. Average values followed by same alphabet in a column are not significantly different by Duncan multiple range test ($\alpha = 0.05$).

A. minisclerotigenes and *A. parvisclerotigenus* (Varga et al., 2011; Probst et al., 2014). These S-morphotype species are known to produce large amounts of aflatoxins. It has been shown that in West Africa, Strain S_{BG} is the predominant species with S-morphotype (Probst et al., 2014). Though the overall incidence of toxigenic isolates was lower than that of the atoxigenic isolates, the probable high risk of aflatoxicosis due to the presence of the toxigenic isolates should not be over looked. The toxigenic isolates were more prevalent in feed eaten in the early stage of the birds' development and in particular, the occurrence of unnamed taxon S_{BG} due to its prolific aflatoxigenic nature (Atehnkeng et al., 2008). The high quantity of B aflatoxins produced by the toxigenic isolates on inoculated maize implies the potential risk of aflatoxicosis posed to the birds especially at their early stages of growth. This also supports a recent report from Monge et al. (2013) on the occurrence of AFB_1 in starter feeds.

The positive correlations between the incidences of *A. flavus* and S_{BG} in the feed samples and proportion of toxigenic section *Flavi* isolates are in line with the report of Donner et al. (2009). The report suggested that both populations in Nigerian soils belong to the aflatoxin-producing members within the section *Flavi* community though the S_{BG} strain produces higher amounts of aflatoxins than *A. flavus*. The significant inverse correlation between incidence of *A. flavus* and AFB_1 levels in the feed as well as significant positive correlation between the incidence of S_{BG} and AFB_1 levels shows that the latter species contributed more to the high concentrations of AFB_1 in the feed than *A. flavus* regardless of their low occurrences in the samples. In addition, the inverse correlation between the occurrence of *A. tamarii* and proportion of toxigenic strains in the feed, and positive significant correlation between the incidence of *A. tamarii* and proportion of atoxigenic isolates confirm the fact that *A. tamarii* isolates do not produce aflatoxins. This is in line with the numerous reports that suggest *A. tamarii* as a putative atoxigenic member of the section *Flavi* (Cotty, 1997; Atehnkeng et al., 2008; Diedhiou et al., 2011).

Another interesting fact is the positive but non significant relationship between aflatoxin-producing ability of toxigenic isolates obtained from the MGC test and concentrations of AFB_1 detected in the 58 feed samples by LC-MS/MS. This is an indication that the toxigenic isolates obtained from the feed did not exhibit their full aflatoxigenic potential in the feed where they occurred as compared to the quantities of toxins produced on inoculated maize grains. This may have been due to any or all of the following: (1) the critical moisture levels in the feed samples, (2) competition arising from the co-occurrence and activities of other species of the section *Flavi* or other fungi in the feed, and (3) presence of aflatoxin inhibitors/binders in the feed. It has been reported that moisture levels are critical factors for aflatoxin biosynthesis in substrates (Adebajo, 1992; Chukwuka et al., 2010). Probst et al. (2011) also stated that competition by atoxigenic strains of *A. flavus* or other moulds was capable of affecting aflatoxin production by toxigenic isolates. Furthermore, aflatoxin binders or inhibitors such as clay, UT-Aflatrol, organic acids and yeast cell walls which are usually included during feed formulation in the poultry industry may interfere with aflatoxin production or accumulation in the feeds (Coelho, 1990; Kolossova et al., 2009).

In conclusion, this study has shown the widespread contamination of poultry feed in the Nigerian market by toxigenic *Aspergillus*. We have also established a correlation between incidence of *A. flavus* and aflatoxin-producing ability of isolates *in vitro*, incidences of S_{BG} and

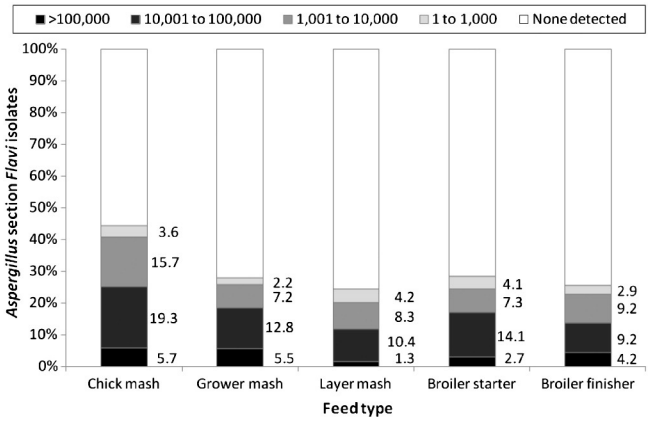

Fig. 4. Percent of *Aspergillus* section *Flavi* isolates that produced various quantities of aflatoxin B_1 (ng/g) in artificially inoculated maize grains in the laboratory.

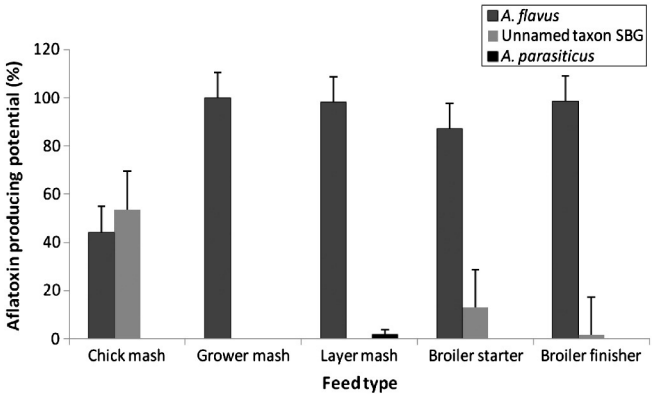

Fig. 5. Aflatoxin-producing potential of *Aspergillus* section *Flavi* isolates from five major commercial poultry feed types in Nigeria. Vertical lines on bars indicate the standard error of mean ($\alpha = 0.05$). Aflatoxin-producing potential attributed to a species was calculated by dividing the sum of aflatoxin B_1 produced by all isolates of a species by the sum of aflatoxin B_1 produced by all isolates of all species in vivo, multiplied by 100.

Table 4
Correlations[a] of relationships among the quantity (CFU/g)[b] of *Aspergillus* section *Flavi* in the feeds[c], the proportion of *Aspergillus* section *Flavi* species (*A. flavus*, unnamed taxon S_{BG} and *A. tamarii*), toxigenic or atoxigenic isolates and the mean aflatoxin B_1 (AFB_1) in the 58 feed samples.

	Cfu[c]	%*A. flavus*	%S_{BG} strain	%*A. tamarii*	%Toxigenic	%Atoxigenic	AFB_1 (μg/kg)
Cfu	1.00						
%*A. flavus*	0.90***	1.00					
%S_{BG} strain	−0.52	−0.84	1.00				
%*A. tamarii*	−0.81	−0.47	−0.08	1.00			
%Toxigenic	0.83*	0.05	0.50	−0.99*	1.00		
%Atoxigenic	−0.96*	−0.98*	−0.17	0.62**	−0.64	1.00	
AFB_1	−0.60	−0.89**	0.99***	0.00	−0.05	−0.18	1.00

[a] Correlation coefficients significant with $p < 0.001 = ***$, $0.01 \geq p > 0.001 = **$, $0.05 \geq p > 0.01 = *$.
[b] Colony forming unit per gram feed sample.
[c] Number of poultry feed types = 5.

A. parasiticus and AFB_1 concentrations in the feed, aflatoxin-producing potential of the *Aspergillus* section *Flavi* isolates from the feed and aflatoxin levels in the feeds. The high occurrence of *Aspergillus* section *Flavi* isolates in the poultry feed samples reported here is an indication of the poor quality of the feed commercially available to farms as majority of the species of *Aspergillus* section *Flavi* isolates are toxigenic (Varga et al., 2009). They are also capable of reducing the overall nutritional quality of the feed by their deteriorative activities (Adebajo, 1992). Interventions are therefore urgent in order to reduce toxigenic *Aspergillus* and aflatoxin contamination of poultry feed in Nigeria. Efforts should be collective – involving grain farmers, feed millers, poultry farmers and other stakeholders – towards adopting an integrated control approach. Suggested actions include sourcing of high quality grains/ingredients, scheduled testing and regulation of raw materials and compounded feeds for compliance of aflatoxin standards, and provision of good storage facilities for long term keeping of the feeds.

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