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Original Article

Incidence of *Aspergillus* Section *Flavi* and concentration of aflatoxin in feed concentrates for Cattle in Jos, Nigeria

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Abstract

Samples of two types of cattle feed concentrates compounded at the feed mill section of the National Veterinary Research Institute (NVRI), Vom, Nigeria were evaluated for incidence of toxigenic *Aspergillus* section *Flavi*. Aflatoxins in the feed were quantified by Thin-layer chromatography with fluorescent detection in order to determine the risk of aflatoxicosis to the cattle. Isolates of *Aspergillus* section *Flavi* were recovered from all analyzed feed samples at varying levels. *A. flavus* was predominant (>70%) in the feed samples and occurred as the only *Aspergillus* section *Flavi* member in the dairy feed type. The incidence of atoxigenic strains was significantly (P<0.05) higher than that of toxigenic strains in both feed types and exceeded 75% in each case. Aflatoxin B1 (AFB1), B2 and G1 were detected in the samples at varying concentrations. Aflatoxins contaminated more samples and in higher concentrations in the dry season than in wet season. The mean concentration of aflatoxins was significantly (P<0.05) higher in dairy concentrates than maintenance concentrates. About 92% of the concentrate feed samples for dairy cattle had AFB1 concentrations exceeding the stipulated 5 µg/kg maximum limit set by the European Union (EU) for dairy cattle. AFB1 concentrations in all samples of the maintenance feed concentrate were within the EU maximum acceptable limits of 20 µg/kg. This study has shown that the risk of aflatoxicosis is high in dairy cattle due to the high levels of AFB1 in the feed concentrates. This may affect the milk products obtained from the cattle due to biotransformation of AFB1 into AFM1.

Key words: Aflatoxins, mycology, *Aspergillus*, cattle, feed safety

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Introduction

Livestock production is a vital part of Nigeria's economy because of its key role in providing high quality food. The dairy industry in Nigeria contributes up to 38% to agriculture in form of milk, milk products and bone meal, while cattle meant for beef provides meat, hides, skin and bone meal. The annual growth rate of the livestock sub-sector is 4.3% (Uko, 2004). However, there is an assumption that naturally occurring toxigenic fungi, especially *Aspergillus*, and its toxic metabolites such as aflatoxin B1 (AFB1) in the feed and ingredients may pose a big threat to this industry in Nigeria. This is gotten from reports of other countries (Fink-Gremmels, 2008; Sultana and Hanif, 2009). There has been no report of the incidence of toxigenic *Aspergillus* and aflatoxins in cattle feeds from Nigeria.

In Nigeria, the two categories of feed concentrates for ranched cattle are dairy and maintenance feeds. Both contain similar ingredients which differ in proportion. For the dairy cattle, milk production is the focus; and as such, the dairy feed has up to 70% energy inclusive of 18% crude protein. The maintenance feed on the other hand has 50–55% energy (15% crude protein inclusive). The general ingredients are cereals, grains (maize, guinea corn and cassava), oil seeds (soybean, ground nut), fibre sources (rice bran, wheat offal), bone, blood and fish meals, limestone, and a vitamin-mineral premix in the case of dairy cattle.

Majority of the studies on the health and safety of cattle have focused on bacterial or viral diseases. Mycotoxin contamination of ruminant feed has been under studied, thus, creating paucity of data in this regard. This may have been due to the relatively tolerant nature of the ruminants to adverse effects of mycotoxins, presumably due to ability of rumen microflora to detoxify the mycotoxins (Kiessling et al., 1984). However, it is noted that the rumen metabolites are more than or equally as toxic as parent mycotoxins e.g. conversion of zearalenone to α-zearanol (Kiessling et al., 1984) and AFB1 to AFM1 (Henry et al., 2001). Due to these toxicological findings and the need to maintain a vibrant livestock industry, this study aimed at investigating the incidence of toxigenic *Aspergillus* section *Flavi* and aflatoxins in the feeds meant for ranched cattle in one of Nigeria's Veterinary Institute.

Materials and Methods

Sample collection site

The sampling site was the National Veterinary Research Institute (NVRI), Vom, located in Jos South Area of Plateau State. Vom, with subtropical climate, falls within the Northern Guinea Savannah (NGS) agro-ecological zone of Nigeria. It lies within Latitude 9^{o} 44' N and Longitude 8^{o} 47'E, and has average height, annual temperature, rainfall and humidity of 4015 ft (1223 m) above sea level, 20^{o}C, 1450 mm and 60%, respectively.

NVRI is a research institute known for its expertise in raising livestock for meat, milk and milk products. Annually, an average of 150 tonnes of concentrate feed is produced at NVRI, Vom. The meat and milk obtained from NVRI serve several neighboring communities and states in northern Nigeria and beyond. However, these products are not exported outside Nigeria.

Sampling

Two categories of cattle feed concentrates, maintenance and dairy rations, were used for this study. The feed types were randomly sampled by seasons (dry and wet) from the feed mill section of the NVRI, Vom. Dry season samples were collected in March while wet season samples were collected in June 2011. A 200g sub-sample was collected from each of the five randomly selected bags per 20 sampled bags. The weight of each randomly sampled bag was 30kg. The five collected sub-samples were mixed to give a 1kg bulk sample which was designated as Lot 1. This same sampling pattern was performed for 40 other bags to obtain lots 2 and 3, all for the dairy feed type in one season. The process was repeated for the wet season.

To determine the influence of storage on moldiness and mycotoxin accumulation, each lot was divided into 2 equal parts and labeled as 'fresh' and 'stored'. Each fresh sample was put in a *Ziploc* bag and stored at 4^{o}C to prevent further fungal proliferation and subsequent toxin production. The stored sample was put in a feed sack and kept at the feed house for 21days to mimic the storage

condition at the feed mill. At the expiration of the 21-day storage period, the stored sample was retrieved from the feed house and stored at 4°C prior to subsequent mycological and toxin analyses. The whole sampling process was performed for the maintenance ration.

Isolation and identification of Aspergillus section Flavi from cattle feeds

A total of 24 feed samples (12 each of maintenance and dairy rations) were examined by the dilution plating technique for *Aspergillus* section *Flavi*. A 20g representative sample per bulk sample was collected, comminuted and placed on freshly prepared modified Rose Bengal Agar (MRBA) (Cotty et al., 1994) after appropriate dilution in 10ml sterile water. Plates were incubated in darkness at 31°C for 3days. Each colony resembling *Aspergillus* section *Flavi* (typical green colonies) was transferred from plates with fewer than 8 colonies to the central point of 5/2 agar (5% V-8 juice and 2% agar, pH 5.2) for further colonial differentiation and identification. Only 10 isolates per sample were transferred. The inoculated 5/2 agar plates (Ø 5.5cm) were incubated at 31°C, unilluminated for 5days. The identification of the isolates was based on colony features and conidial morphology (x400) as described by (Klich and Pitt, 1988; Cotty, 1989; Cotty et al., 1999; Ehrlich et al., 2007). The presence and size of sclerotia on 5/2 agar was used to further characterize the *A. flavus* into L-strains. Isolates with green colored colonies and producing smooth conidia and large sclerotia (>400 µm in diameter) or no sclerotia were grouped as L-type *A. flavus*. Brown or yellow-brown colonies with rough conidia (x400) were identified as *A. tamarii*.

Screening of isolates for aflatoxin production

The aflatoxigenicity potential of 240 isolates of *Aspergillus* section *Flavi* obtained from the feeds was tested on neutral red desiccated coconut agar (NRDCA). This medium, which enhances the rapid production and visualization of aflatoxins based on UV fluorescence, was prepared as described by Atanda et al. (2011). Each isolate was inoculated at the central point of duplicate Petri dishes containing freshly prepared NRDCA and incubated unilluminated at 31°C for 3-7days. The ability of the isolates to liberate aflatoxin was determined under UV at 365nm after 12h of incubating the plates until the seventh day.

Aflatoxin estimation in cattle feeds

The total aflatoxin content in the feed samples was determined according to the method of AOAC International (2000) with slight modifications. Total aflatoxin standard and reagents (analytical grade methanol, chloroform, n-hexane and acetone) were purchased from Sigma-Aldrich (Steinheim, Germany).

25g of a communited feed sample was weighed into a 250ml Erlenmeyer flask and extracted for 30min on a wrist-shaker using 125ml of methanol/water (55:45 v/v) and 1g of NaCl. The mixture was filtered through two folds of Whatman No. 1 filter paper and the filtrate was transferred into a 250ml separatory funnel. The mixture was cleaned up with two changes of 20ml n-hexane followed by the collection of the bottom layer into another 250ml separatory funnel. Chloroform (25ml) was added to the filtrate and shaken vigorously. Following separation, the lower chloroform layer was passed through a bed of anhydrous sodium sulphate into a polypropylene cup to remove residual water. The collected extract was evaporated to dryness in a fume hood and the residue was reconstituted in 500µl chloroform. The extracts (40µl) and 50µl aliquots of 0.50µg/ml total aflatoxin standards were separated on pre-coated TLC plates (silica gel 60 F_{254}; 20 × 10cm; Merck, Germany) using chloroform-acetone-water (88+12+1.5) as development solvent. Plates were dried in a laminar flow hood and visualized under long wavelength UV light (365nm). Aflatoxin bands in sample spots were identified on the basis of co-migration and characteristic fluorescence with aflatoxin standard. For quantification of aflatoxin B1 (AFB1), the sample and standard spots on dried TLC plates were sprayed with trifluoroacetic acid and tetra oxo sulphate (VI) acid according to Bankole et al. (2006). The concentration of aflatoxins (µg/kg) in the samples was estimated using the formula described in Atanda et al. (2011).

Statistical Analysis

The data obtained in this study were analyzed using SAS version 9.1. ANOVA was carried out and treatment means were separated by Duncan's

multiple range tests. The experiment was set up in a factorial (2 × 2 × 2) completely randomized design. The effect of feed type, season and storage condition, and their interaction towards the biological variable (CFU, incidence of *A. flavus* and *A. tamarii*) was analyzed and reported as mean squares.

Results

Incidence of Aspergillus section Flavi in the feed types

There were no statistical differences (P>0.05) in Log_{10}CFU/g values in the dairy type between the stored samples and those that were analyzed fresh regardless the season, although the stored samples had higher CFU values. For the maintenance feed concentrates, stored samples of the wet season had the most fungal count (4.1 Log_{10}CFU/g) which was more significant (P<0.05) than the freshly analyzed samples of the same season (3.4 Log_{10}CFU/g). On the overall, CFU values were significantly (P<0.05) higher in concentrates meant for maintenance cattle (3.81 Log_{10}CFU/g) than in those for dairy cattle (3.16 Log_{10}CFU/g) (Table 1).

Amongst the members of the section *Flavi* group, only *Aspergillus flavus* and *A. tamarii* were isolated from the feed samples. *A. flavus* was predominant (>70%) in the feed concentrates. Only *A. flavus* was isolated from the dairy type while for the maintenance type, the incidence of *A. flavus* was highest (100%) and most significant (P<0.05) in stored samples collected in the wet season than the dry season samples. *A. tamarii* was not isolated from the stored maintenance samples collected during wet season.

Table 1: Incidence of *Aspergillus* section *Flavi* in fresh and stored cattle feed types collected from National Veterinary Research Institute, Vom, Nigeria, in two seasons

Feed type	Season*	Condition	$Log_{10}CFU^{\#}$	N^{+}	% *A. flavus*	% *A. tamarii*
Dairy	Dry	Fresh	3.2^{a}	30	100.0^{a}	0.0
		Stored	3.4^{a}	30	100.0^{a}	0.0
	Wet	Fresh	3.1^{a}	30	100.0^{a}	0.0
		Stored	3.4^{a}	30	100.0^{a}	0.0
	Mean	-	3.2	-	100.0	0.0
Maintenance	Dry	Fresh	4.0^{a}	30	73.3^{b}	26.7^{a}
		Stored	4.0^{a}	30	73.3^{b}	26.7^{a}
	Wet	Fresh	3.4^{b}	30	93.3^{ab}	6.7^{b}
		Stored	4.1^{a}	30	100.0^{a}	0.0
	Mean	-	3.8	-	85.0	15.0

*Seasons when samples were collected: dry season (March) and wet season (June).
#Logarithmic value for colony forming unit per gram of analyzed samples. Value not in percentage.
+Number of *Aspergillus* section *Flavi* isolated.
Values with different superscripts within a feed type in a column are significantly different by DMRT (P<0.05).

The independent effect of feed type, season and storage condition on the biological variables showed that only feed type and season significantly (P<0.01) influenced the incidence values of *A. flavus* and *A. tamarii* while feed type (P<0.01) and storage condition (P<0.05) significantly affected the obtained CFU. The effect of the interaction between feed type, season and storage condition showed no significance (P>0.05) for all biological variables. However, the interaction between feed type and season influenced the incidences of the *Aspergillus* species obtained (Table 2).

Incidence of atoxigenic and toxigenic A. flavus

The aflatoxigenicity potential of 240 isolates determined in this study is given in figures 1 and 2. The incidence of atoxigenic strains was significantly (P<0.05) higher than that of toxigenic strains in both feed types and exceeded 75% in each case. We observed that there was no significant (P>0.05) difference in the overall incidence of toxigenic strains in the feed types collected across seasons. The incidence of toxigenic strains

increased in stored samples of the maintenance concentrates than in fresh samples of same feed type regardless of season (Fig. 2). The pattern was not regular in the dairy type (Fig. 1).

Table 2: ANOVA mean squares showing the independent effect of feed types, season and storage condition, and their interaction towards CFU, incidence of *A. flavus* and *A. tamarii*.

Source	df[+]	% *A. flavus*	% *A. tamarii*	Log_{10}CFU
Feed type (F)	1	270.00**	27.00**	5.0926**
Season (S)	1	1633.33**	1633.33**	0.0002ns
Storage condition (C)	1	33.33ns	33.33ns	0.3115
F × S	1	1633.33**	16.33**	0.2357*
F × C	1	33.33ns	33.33ns	0.0593ns
S × C	1	33.33ns	33.33ns	1.3084**
F × S × C	1	33.33ns	33.33ns	0.0704ns
Rep	1	0.00ns	0.00ns	0.0000ns
Error	39	71.80	71.80	0.05
Total	47	-	-	-
CV[#] (%)	-	9.16	113.00	6.40

[+]degree of freedom; [#]Coefficient of variation; *significant at 5%, ** significant at 1%, ns= not significant

Aflatoxins in cattle feed samples

Table 3 presents the number of analyzed samples, percentage contaminated samples with aflatoxin, and levels of contamination (µg/kg) in the feed types across seasons. Only AFB1, B2 and G1 were detected in the samples. AFG1 was not detected in maintenance feeds collected in the dry season. B-aflatoxins contaminated all dairy samples in the dry season. The aflatoxins contaminated more samples in higher concentrations in the dry season (>83%) than the wet season (<67%). On the overall, the mean concentration of aflatoxins were significantly (P<0.05) higher in dairy concentrates (20.6µg/kg) than maintenance types (10.1µg/kg). In summary (Table 4), 91.7% of the concentrate samples for dairy cattle had AFB1 in concentrations above the EU regulated limits for dairy cattle (5µg/kg). The AFB1 concentration in all concentrate samples for maintenance cattle did not exceed the EU maximum acceptable limits (MAL) of 20µg/kg.

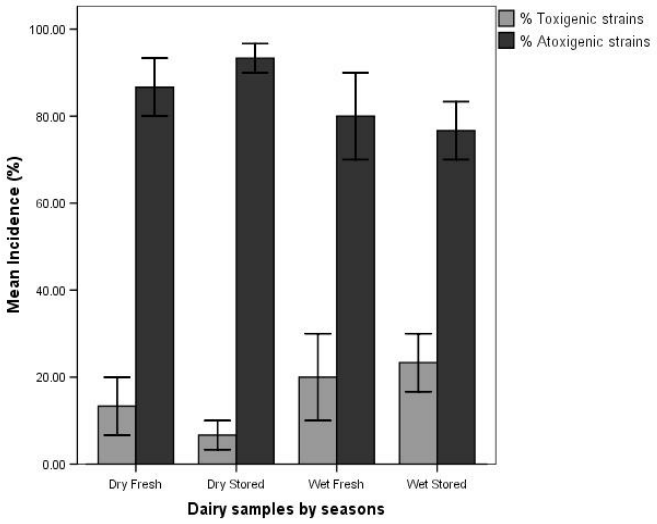

Fig. 1: Incidence of toxigenic and atoxigenic *Aspergillus* section *Flavi* in fresh and stored dairy concentrates collected in two seasons.

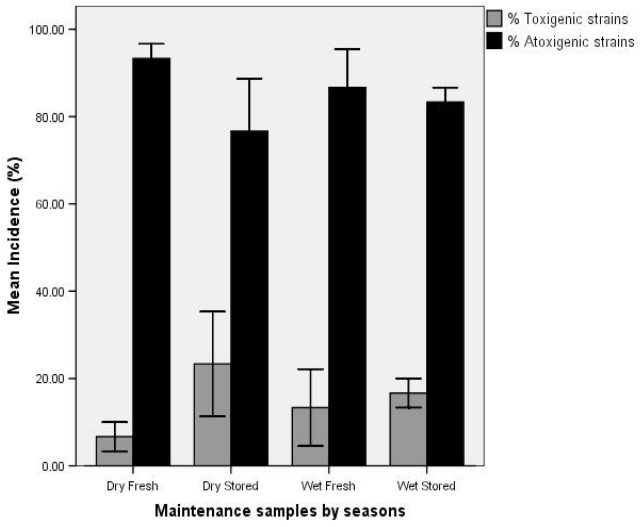

Fig. 2: Incidence of toxigenic and atoxigenic *Aspergillus* section *Flavi* in fresh and stored maintenance concentrates collected in two seasons.

Table 3: Distribution (%) and concentrations of aflatoxins (µg/kg) in cattle feed from Jos, Nigeria

Feed type	*Season	Aflatoxin B_1			Aflatoxin B_2			Aflatoxin G_1			Total aflatoxin[#]		
		Range	Mean	%	Range	Mean	%	Range	Mean	%	Range	Mean	%
Dairy	Dry (N^+=6)	7 – 18	9.83	100.00	7 – 11	9.17	100.00	0 – 10	6.50	66.67	0 – 39	25.50	100.00
	Wet (*N*=6)	0 – 13	7.67	83.33	0 – 12	3.67	33.33	0 – 15	4.33	33.33	0 – 28	15.67	83.33
	Overall (*N*=12)	0 – 18	8.75[a]	91.67	0 – 12	6.42[a]	66.67	0 – 15	5.42[a]	50.00	0 – 39	20.58[a]	91.67
Maintenance	Dry (*N*=6)	0 – 9	5.00	66.67	0 – 11	6.00	66.67	nd	nd	nd	0 – 18	11.00	66.67
	Wet (*N*=6)	0 – 10	4.00	50.00	nd	nd	nd	0 – 16	5.17	33.33	0 – 26	9.17	50.00
	Overall (*N*=12)	0 – 10	4.50[b]	58.33	0 – 11	3.00[b]	33.33	0 – 16	2.58[a]	16.67	0 – 26	10.08[b]	58.33

*Season of sample collection: dry season (March) and wet season (June).
[+]Number of samples analyzed for aflatoxins.
[#]Total aflatoxin= aflatoxin B_1+B_2+G_1. Aflatoxin G_2 not detected in any sample.
nd= not detected.
Mean aflatoxin values with different superscripts in a column are significantly different by DMRT (P<0.05).

Table 4: Summary of aflatoxin B1 (AFB1) concentrations (µg/kg) in cattle feed types from Jos, Nigeria

AFB1 concentrations (µg/kg)	Percentage samples per feed types	
	Dairy (N^+=12)	Maintenance (N^+=12)
<5	8.3	50.0
<10	66.7	91.7
<20	100.0	100.0

[+]Number of samples analyzed for aflatoxin B1.

Discussion

The presence and high incidence of *Aspergillus flavus* (>70%) isolates in all the analyzed feed samples indicate the suitability of the diverse feed ingredients and storage conditions for the proliferation of the mould (Fink-Gremmels, 2008). However, it was observed that the incidence of *A. tamarii* was very low in the dairy feeds while in the maintenance type, no occurrence was recorded. This is in accordance with the reports of Accensi et al. (2004) who made it clear that *A. flavus* was the most predominant member of *Aspergillus* in mixed feeds. They also reported a zero incidence for *A. tamarii* in 147 mixed feed samples. The total *Aspergillus* counts taken in this study are below the recommended maximum level for poor feed quality (5.0 Log_{10}CFU/g) (Chelkowski, 1991). Although the counts did not exceed the limit in any case, it should be noted that the counts were high enough (up to 4.1 Log_{10}CFU/g) to arouse safety questions about the feed quality. The presence of *A. flavus* in all the feed samples poses a threat because of the aflatoxigenic strains that were detected, albeit at very low levels.

Mycotoxin contamination of feeds accounts for significant economic losses in animal husbandry and undesirable trade barriers for raw materials and consumable products (Wu, 2006). Aflatoxins have been reported to occur in ruminant diet through the concentrates such as maize, ground nut cake and cassava (Frink-Gremmels, 2008). According to the diet recipe given earlier for the types of cattle (dairy and maintenance), it is observed that the dairy feed has more concentrates than the maintenance feed. This may have contributed to the significant (P<0.05) higher aflatoxin levels we obtained in the dairy samples than the maintenance feed. Specifically, the high incidence (91.7%) of dairy sample with AFB1 concentration exceeding the EU stipulated 5µg/kg maximum limit in contrast to the low incidence of toxigenic isolates is of great importance.

AFB1 has been noted to exert carcinogenic, teratogenic, hepatotoxic, mutagenic and immunosuppressive effects in cattle. In dairy cattle, the effects may lead to a serious drop in milk production and anemia. The milk can be further contaminated with AFM1 at concentrations estimated to represent 1–2% of the ingested AFB1 (Frink-Gremmels, 2008). Furthermore, AFB1 ingestion by dairy cattle is associated with reduced feed intake and overall retarded growth and development (Akande et al., 2006). Therefore the high incidence of AFB1 contaminated samples above 5µg/kg recorded in this study should not be played down. This is because AFB1, when ingested by cattle, is excreted into milk within 12 hours as AFM1. The carcinogenic potency of AFM1 is almost as high as that of AFB1 (Henry et al., 2001). The FDA limits for aflatoxin M1 in milk is 0.5µg/kg (Diaz et al., 2004), a concentration that is 10-fold lower than 5µg/kg. Therefore if 91.7% of the samples we tested far exceeded this maximum concentration of 5µg/kg, there is a tendency that a metabolite concentration higher than 0.5µg/kg required for milk will be excreted during production. This is a threat to the meat and milk industry especially to the unaware consumers.

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