

Mycobiota and Toxigenicity Profile of *Aspergillus flavus* Recovered from Food and Poultry Feed Mixtures in Cameroon

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Abstract

A total of 202 poultry feed and its raw ingredients collected from different agroecological zones of Cameroon were examined for total mycoflora and the ability of *A. flavus* isolates to produce aflatoxin B₁. Dilution plating was used for fungal isolation. The mean fungal contamination levels were significantly higher in maize and peanut meal as compared with broiler and layer feeds. In peanut meal and poultry feed, the most representative fungi were *A. flavus*, *A. niger*, *A. oryzae*, *F. solani*, *F. verticilloides*, *Penicillium spp*, and *Rhizopus spp*. Of all the fungi encountered, *A. flavus* was encountered in 90% of white maize and 28.5% of yellow maize samples. The frequency of isolation of the most representative fungi in peanut meal, broiler and layer feed was 100, 94, and 76.5% for *A. flavus* and 70.6, 82.3, and 76.5% for *Penicillium spp*, respectively. Molecular identification using the Intergenic Spacer Gene (IGS) for aflatoxin biosynthesis confirmed all fungi identified morphologically as *A. flavus*. Aflatoxin B₁ analysis showed that all the *A. flavus* isolates encountered were aflatoxin B₁ producers. Conclusion from this study indicates that the use of peanut meal in poultry feed is risky, and can impact poultry health and economic benefits.

Key words: aflatoxin, animal feed, fungal contamination, toxigenic

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Introduction

Fungal and mycotoxins contamination of food and poultry feeds can occur at each step along the chain from grain production, storage and processing. Mycotoxin producing fungi such as *Aspergillus*, *Fusarium* and *Penicillium* are of concern because they pose a relatively greater toxic threat to animals and humans (Sweany et al., 2011; Oguz, 2012). Labuda and Tančinová (2006) and González Pereyra et al. (2008) recovered several mycotoxigenic fungi from poultry feed and pig feed belonging to 3 and 22 genera respectively in Slovakia and Argentina. In Cameroon, Ngoko et al. (2001) isolated eight mycotoxin producer fungal species belonging to six fungal genera from maize in Western highland and Rain forest region. Most of these species have been reported to infect grain and poultry feed in several parts of the world (Somashekar et al., 2004; Fandohan et al., 2005; Akande et al., 2006; Rodrigues et al., 2011; Rodrigues and Naehrer, 2012). Fungi require nutrients for growth and their presence reduce the nutrient content of the grain and feed, affect palatability, animal performance and flock uniformity (Khlanguiset et al., 2011). Contamination with *Aspergillus flavus* and subsequent production of aflatoxin in grain or poultry feed mixtures is considered as one of the most serious safety problems in poultry industry throughout the world (Labuda and Tančinová, 2006; Shuaib et al., 2010; Rawal et al., 2010).

Poultry are the most sensitive of all farm animals to the toxic effects of even small amounts of aflatoxin B₁ (Mabett, 2005; Shuaib et al., 2010). Although, poultry do not generally have enough life span to develop cancer, aflatoxin B₁-related disorders adversely affect their health. Depending on the dose and exposure time, it can cause anorexia with poor growth rate, reduced feed utilization, decreased egg production and increase mortality (Akande et al., 2006).

Several physical factors including moisture, humidity, ambient temperature, storage time, pH and oxygen affect fungal growth and mycotoxins production (Kaaya and Kyamuhangire, 2006). In Cameroon, more than 60% of poultry farms are located in Western highland where temperature ranging from 18 to 35°C and high humidity generally over 80% ensures continuous presence of moisture in the air. These environmental factors amongst others are very suitable for fungi growth and mycotoxins production. In this area, mycotoxigenic seed-borne *Aspergillus* and *Fusarium* strains have been isolated from maize, ground nuts, and other common feed commodities by Ngoko et al. (2001 and 2008). There is no information about the levels of fungal and mycotoxins contamination of poultry feed in Cameroon.

The main objective of this study was to investigate the levels of mycobiota contamination and toxigenicity profile of *Aspergillus flavus* recovered from maize grain, peanut meal and poultry feed in Cameroon.

Materials and Methods

Location and sampling

A total of 202 maize grain, peanut meal, broiler and layer feeds samples were collected at poultry farms, poultry feed production sites or from poultry feed dealers between May and August 2012. The samples of peanut and feed mixtures were sourced from Bafoussam, Dschang and Bamenda in the Western highland region and from Yaoundé in Central region and from Douala in Littoral region of Cameroon. Maize was sourced from Western highland and in the Northern region. The samples were stored at room temperature (18–25°C) in plastics sacks until analysis in September 2012. All samples were sealed under vacuum to prevent air exchanges between the samples and the storage environment. There was no available information concerning the individual farms and conditions under which the commodities concerned were stored and handled.

Isolation and enumeration of fungi

Fine particles of samples were subjected to dilution plate technique to enumerate total fungi flora on a non-selective PDA (Potato extract 4.0 g, Dextrose 20.0 g, Agar 15.0 g) as described by Somashekar et al. (2004). Five grams of each sample was mixed with 45 mL of sterile distilled water on horizontal shaker (New Brunswick CO. INC, EDISON, N.J., USA) at 220 rpm at 25°C for 20 min. From the mixture, 1 mL of appropriate dilutions made up to 10^{-3} was applied in duplicates on a solid medium using surface-spread method. After 7 days of incubation at 25°C with a photoperiodicity of 12 hours, resulting colonies of fungal propagules referring to cfu (colony forming units) per gram of sample was counted. Macroscopic and microscopic identification of fungal species were carried out according to Samson et al. (2010). *Aspergillus* isolates from PDA were sub cultured on malt extract agar (MEA) for purification and DNA extraction.

Biochemical analysis and screening of *Aspergillus flavus* for aflatoxin B₁ production

All *Aspergillus* isolates identified on PDA were subcultured in duplicate on aflatoxin producing media, YESA (yeast extract 20.0 g, sucrose 150.0 g, MgSO₄ 7H₂O 0.5 g, and agar 20.0 g, Oxoid Ltd, Basingstoke, Hampshire, England) and incubated at 25°C for 7 days to evaluate aflatoxin B₁ production. Aflatoxin B₁ was extracted from approximately 5 g of the YESA with fungi colonies in 10 mL of 80% methanol using the standard ELISA extraction protocol essentially as described by the kit manufacturer (New Brunswick CO. INC, EDISON, N.J., USA). Aflatoxin B₁ was quantified using ELISA kit (aflatoxin B₁ ELISA Quantitative, Helica Biosystems, Inc, Catalog no. 941BAFLO1B1).

Aflatoxin extraction and quantification from maize and feed mixture samples

All the samples were grounded into fine particles using the Romer mill (Romer series II[®] MILL) and from each sample 5 g was weighed, mixed with 0.5 g sodium chloride and introduced in 50 ml falcon tube. A

volume of 10 ml of 80% methanol solution (methanol:water, 80:20, v/v) was added in the tube and blended at 225 rpm for 4 min at 25°C in a control environment shaker (New Brunswick CO. INC, EDISON, N.J., USA). The blended mixture was filtered using fluted filter paper and 2 ml of filtrate was diluted with 8 ml of distilled water into a clean tube, and mixed for 2 min with a linear mixer (DENLEY Spiramix 5, Sussex, England). From the diluted filtrate, 2 ml (0.2 g sample equivalent) was passed through Aflatest®-P affinity column at a rate of 1 to 2 drops/second and the column was rinsed twice with 5 ml of distilled water at the same rate. The affinity column was eluted with 1 ml HPLC grade methanol at the rate of 1 to 2 drops/second and elute collected in a glass tube. One ml of Aflatest® developer solution was added to the elute in the tube, mixed, and concentrations of the total aflatoxin ($\mu\text{g}/\text{kg}$) were detected after 60 second using calibrated Vicam fluorometer (Series-4EX, Source Scientific LLC, Watertown, MA, USA) with blank containing 1 ml methanol. The detection limit of this method was 2 $\mu\text{g}/\text{kg}$.

DNA extraction from fungi

In order to differentiate *Aspergillus flavus* from *Aspergillus parasiticus*, DNA was extracted from fungal mycelia for molecular identification. Young mycelia from pure colony was grown on MEA (Malt Extract 30.0 g, Mycological peptone 5.0 g, Agar 15.0 g) for 3 days at 25°C and DNA was extracted in duplicate following the modified cetyltrimethylammonium bromide (CTAB) method according to Somashekar et al. (2004). This method consists of double phenol-chloroform extraction followed by isopropanol precipitation and resuspension of DNA in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR amplification and primer design

PCR was used to amplify the Intergenic Spacer Gene (IGS) for aflatoxin biosynthesis on *Aspergillus* isolates in 20 μL reaction mixture containing: 2 μL of DNA (20 ng/ μL), 1 μL (5 pmoles/ μL) of each primer (IGS-F and IGS-R) and 16 μL of molecular water. The PCR reactions were run on Gene Amp PCR system 9700 (Perkin-Elmer, USA) under the following conditions: 94°C for 5 min followed by 40 amplifications cycles at 94°C for 20 seconds, 55°C for 20 seconds, 72°C for 1 minute and a final extension at 72°C for 10 min. The sequences of the primers used are as follows: IGS-F, 5'-AAGGAATTCAGGAATTCTCAATTG-3'; IGS-R, 5'-GTCCACCGGCAAATCGCCGTGCG-3'. This primer pair IGS-F/IGS-R was designed to amplify the target regions from isolates that correspond to a PCR product of 674 bp (El Khoury et al., 2011). The amplified products were examined by 0.8% w/v agarose gel electrophoresis.

The PCR products were subjected to endonuclease restriction enzyme digestion using BglII (FERMENTAS GMBH, Opel-strasse 9, D-68789 ST. Leon-Rot, Germany). The reaction was performed in a total volume of 20 μL containing 0.5 units of enzyme, 2 μL of buffer, 4 μL of PCR product, and 13.5 μL of ultrapure water. The reaction mixture was incubated at 37°C for 1 h. The resulted fragments were separated by electrophoresis on a 2% w/v agarose gel for 35 min at 100 V.

Statistical analyses

Fungal counts were transformed to $\log_{10}(x+1)$ to obtain homogeneity of variance. The results were analyzed by SPSS version 12.0 program for Windows. Analyses of variance (ANOVA) and Duncan's multiple range test at 5% significance level were used to compare the means moisture content of samples, fungal isolation frequency and aflatoxin B₁ production.

Results

Fungal survey

The five commodities had different levels of mycobiota contamination, ranging from 3.2 to 4.5×10^{-2} cfu g⁻¹ for yellow maize, 3.5 to 4.3×10^{-2} cfu g⁻¹ for white maize, 2.8 to 4.9×10^{-2} cfu g⁻¹ for peanut meal, 3.1 to 4.1×10^{-2} cfu g⁻¹ for broiler feed and 2.5 to 4.1×10^{-2} cfu g⁻¹ for layer feed (Table 1). The highest mean count of mycobiota was recorded in yellow maize (4.1×10^{-2} cfu g⁻¹) and the lowest in layer feed (3.3×10^{-2} cfu g⁻¹). The mean contamination levels were significantly different ($P < 0.05$) between analyzed commodities.

A total of 4 fungi genera (*Penicillium spp*, *Fusarium spp*, *Aspergillus spp* and *Rhizopus spp*) were detected in yellow and white maize. The fungal contamination frequency was higher in white maize as compared to yellow maize (Figure 1). The most frequent fungi in white maize were those from *Penicillium spp* (100%), *Fusarium verticilloides* (90%), and *Aspergillus flavus* (90%), while in yellow maize the most frequent were *Fusarium verticilloides* (86%) and *Aspergillus niger* (79%). Of all the fungi encountered, *Aspergillus flavus* which is the potential aflatoxins producer was present in 90% of white maize samples and only in 28.5% of yellow maize samples.

In peanut meal and poultry feed mixtures, the most representative fungi isolated were *A. flavus*, *A. niger*, *A. oryzae*, *Fusarium solani*, *F. verticilloides*, *Penicillium spp* and *Rhizopus spp* (Figure 2). *F. graminearium*, *F. culmorum* and *F. poae* were present in very small amount in peanut meal while *F. equiseti* was found only in broiler feed. *A. flavus* and *Penicillium spp* were most frequent amongst these three commodities with the frequency of isolation rates of 100, 94, and 76.5% for *A. flavus* and 70.6, 82.3, and 76.5% for *Penicillium spp* in peanut meal, broiler and layer feed, respectively.

Differentiation between *A. flavus* and *A. parasiticus*

The DNA of *Aspergillus* isolates was subjected to PCR. Differentiation between *A. flavus* and *A. parasiticus* was done using IGS specific primers and subsequent restriction digestion using the BglIII enzyme. From all the isolates, three distinct amplicons were observed on 2% electrophoresis gel at 362, 210 and 102 bp indicative of *A. flavus* (Figure 3). This indicates that all *Aspergillus* recovered from all the commodities was *A. flavus* which may be a potential risk factor to aflatoxin B₁ production in these commodities during processing and storage. Of all the fungi species encountered, *A. flavus* was the most frequently found in peanut meal and broiler feed as compared to maize and layer feed.

Table 1. Enumeration of total mycobiota and their colony forming unit from food and poultry feeds samples

Commodities	N	Range viable counts,	Mean viable counts	SEM
Yellow maize	14	3.2 - 4.5	4.1 ^c	0.11
White maize	10	3.5 - 4.3	4.0 ^{bc}	0.07
Peanut meal	17	2.8 - 4.9	3.7 ^{bc}	0.12
Broiler feeds	17	3.1 - 4.1	3.7 ^b	0.06
Layer feeds	16	2.5 - 4.1	3.3 ^a	0.12

^{a-c} Means within a column with different superscripts are different ($P < 0.05$)

Table 2. Production pattern of Aflatoxin B₁ by *Aspergillus flavus* isolated from peanut meal, maize and poultry feed mixtures in Cameroon

Commodities	Aflatoxin B ₁ production on YESA (<i>in vitro</i> test)			
	N	Range (µg/kg)	Mean (µg/kg)	Aflatoxin B ₁ production frequency
Yellow maize	14	1.5 – 2.2	2.0	<5 µg/kg 3/3 (100%)
White maize	10	2.9 – 368.1	148.1	<5 µg/kg 7/11 (63.6%)
				>100 µg/kg 4/11 (36.3%)
Peanut meal	17	1.2 – 234.2	178.8	<5 µg/kg 2/14 (14.2%)
				>100 µg/kg 12/14 (85.7%)
Broiler feeds	17	1 – 383.2	171.3	<5 µg/kg 8/16 (50%)
				>100 µg/kg 8/16 (50%)
Layer feeds	16	1.3 – 375.5	234.5	<5 µg/kg 3/11(27.2%)
				>100 µg/kg 8/11(72.7%)

***In vitro* toxigenicity profile and relationship between aflatoxin B₁ and *A. flavus* occurrence in contaminated food and feed**

All the *A. flavus* isolates were found to be aflatoxin B₁ producers, and a part for the strains recovered from yellow maize which produced very small amount of aflatoxin B₁ (1.5 to 2.2 µg/kg), all other *A. flavus* strains recovered from white maize, peanut meal, broiler and layer feed were highly toxigenic with mean aflatoxin B₁ production of 148.1, 178.8, 171.3, and 234.5 µg/kg, respectively (Table 2). Approximately 36.36, 50, 72.72, and 85.71% of isolates from white maize, broiler feed, layer feed and peanut meal, produced more than 100 µg/kg of aflatoxin B₁, respectively.

The *in vitro* aflatoxin B₁ content followed the same trend of *A. flavus* infection. It tends to be proportionally related to *A. flavus* infection range. However, this varies from one commodity to another with peanut meal having the highest aflatoxin B₁ concentration (Figure 4).

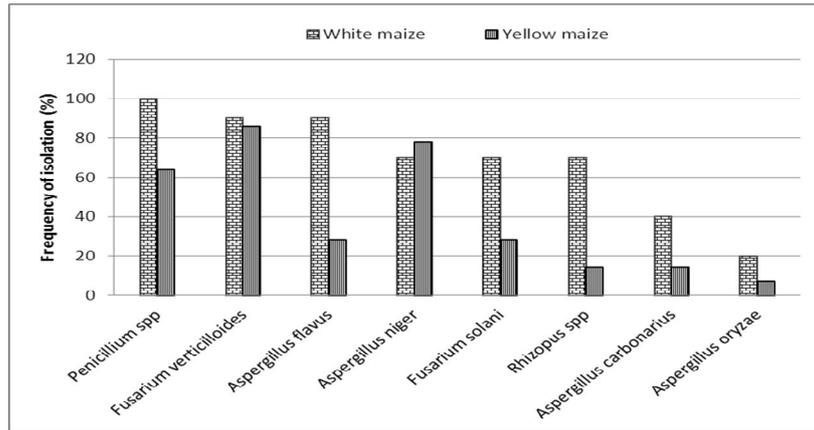


Figure 1. Mycobiota recovered from white and yellow maize in Cameroon.

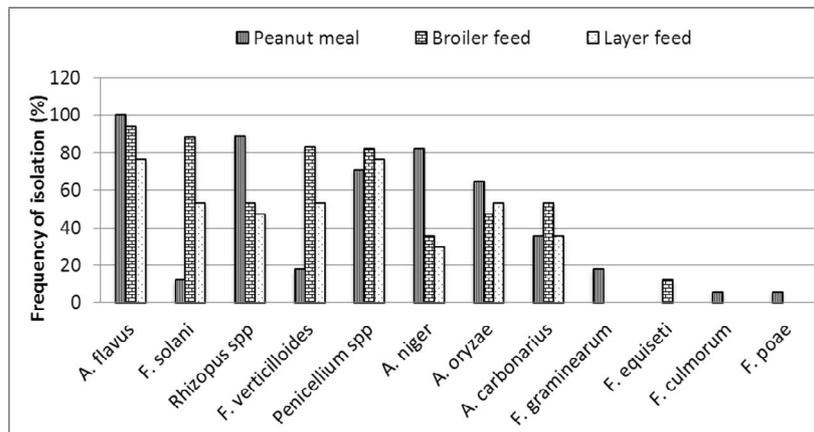


Figure 2. Mycobiota recovered from peanut meal, broiler and layer feeds in Cameroon.

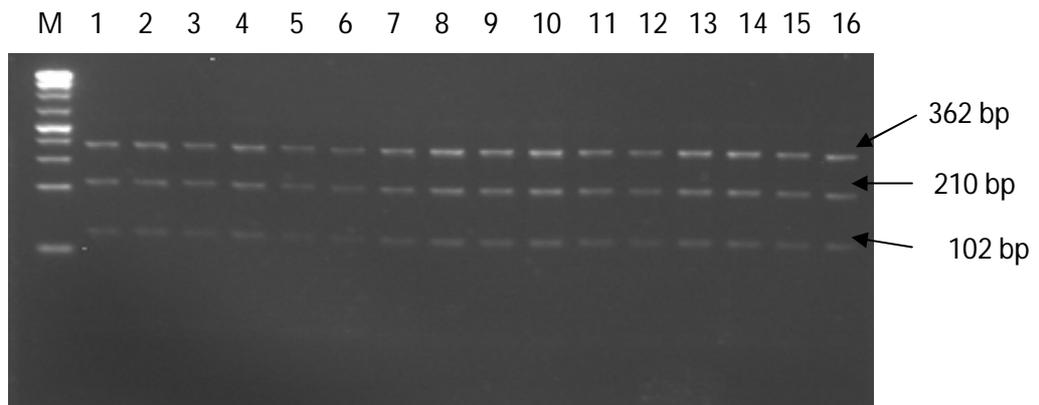


Figure 3. Representative 2% agarose gel showing PCR product amplified with IGSF/IGSR primers and digested with BglII restriction enzyme. M_1 kb⁺ DNA marker (GeneRuler, Fermentas), Lane 1 to 16_ *A. flavus*.

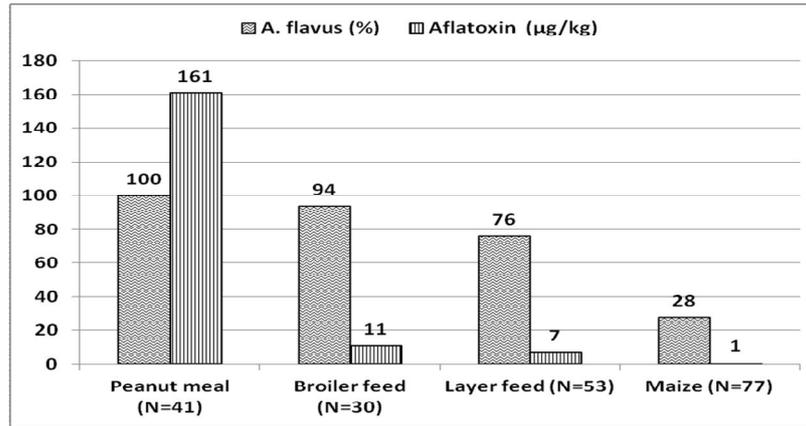


Figure 4. Relationship between *Aspergillus flavus* occurrence (%) and aflatoxin B₁ content ($\mu\text{g/kg}$) from the *in vitro* test.

Discussion

Mycobiota survey of samples from different areas of Cameroon showed that the total recorded counts in maize and peanut meal was significantly ($P < 0.05$) higher in comparison with feed mixtures. This result is in accordance with earlier studies showing contamination of feedstuffs and animal feed all over the world (Somahsekar et al., 2004; Gonzalez Pereyra et al., 2008). *A. flavus* which is the most important risk factor of aflatoxin production was present in 90% of white maize and in 28.5% of yellow maize samples. Thus, white maize could be more prone to aflatoxin contamination. The presence of β -caroten in yellow maize was reported to modulate fungal growth and the aflatoxin synthesis in plant (Norton, 1997) with *A. flavus* strains being significantly more sensitive than *A. parasiticus* strains.

Of all the fungi encountered in peanut meal, the most representative was *A. flavus* (100%), followed by *Rhizopus spp* (88.5%), *A. niger* (82.4%), *Penicillium spp* (70.6%) and *A. oryzae* (64.7%). The present results differ with the findings of Rostami et al. (2009) who reported that *A. flavus* (39.1%), *Penicillium* (9.2%), *Rhizopus* (7.2%), *Mucor* (2.5%), *Alternaria* (1.03%) and *Nigrospora* (0.5%) are dominant mold fungi contaminant of peanut meal in Iran. In this study, very large number of fungi was found to co-occur with very high frequency in peanut meal probably because this commodity content nutrients suitable for the growth of a variety of fungi population or they have similar growth conditions and they do not show antagonism when growing together in this substrate. Many of those fungi were reported to be high mycotoxins producers in peanut and many other commodities in Cameroon (Ngoko et al., 2008, Njobeh et al., 2009). The high aflatoxin content of peanut meal recorded in this study can be attributed to the high prevalence of *A. flavus* which is known to be the most important risk factor of production of this toxin in groundnuts and its sub products (Elshafie et al., 2011).

In poultry feed mixtures, *Penicillium spp* and *A. flavus* which is an important aflatoxin producer fungus were most frequent. Broiler feeds showed higher levels of aflatoxin. This can be explained by the fact that

proteins requirements of broilers chickens are higher than the needs of layers, and to meet the requirements, farmers used large quantities of peanut meal as cheapest proteins sources ingredient which is more susceptible to *Aspergillus* and aflatoxins contamination. This toxin level was found to be above the permissible limit (20 µg/kg) in poultry feed in many countries in the world (FDA, 2004). In poultry feed, *A. flavus* showed the highest frequency as compared to all other fungi which obtained moderate levels. Highest isolation frequency of *A. flavus* in farm-mixed and commercially prepared poultry feeds have also been reported in Nigeria which borders Cameroon (Adeniran and Makun, 2013). This result is different from the report of Muhammad et al. (2010) describing *A. niger* aggregates as predominant species followed by *A. flavus* in poultry feeds in Pakistan. The present results also contradict the findings of Labuda and Tančinová (2006) who reported *Penicillium spp* and *Fusarium spp* to be the typical fungal genera inhabiting Slovakian poultry feed mixtures.

All the IGS-PCR products subjected to restriction endonuclease analysis using BglII cleaved into three fragments of 362, 210 and 102 bp, suggesting that all *Aspergillus* strains recovered from all the commodities were *A. flavus* (El Khoury et al., 2011). This enzyme is known to cleave *A. parasiticus* sequence into 2 fragments of 363 and 311 bp (El Khoury et al., 2011). All the *A. flavus* isolates (100%) recovered in this study were aflatoxin B₁ producers. These strains were highly toxic as compared to the previous reports (Melki Ben Fredj et al., 2009).

Conclusion

This study showed that food and poultry feed from Cameroon is heavily contaminated by toxigenic *A. flavus* with significant level of aflatoxin B₁ contamination. The progressive use of peanut meal in poultry feed is an inherent risk factor for aflatoxigenic fungi and aflatoxin contamination. There is a great need to develop practical and cost-effective methods of preventing mould growth and detoxifying poultry feed; such as mixing aflatoxin binders in feed before consumption by poultry and other animals.

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