Malaysian Journal of Microbiology, Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Bioburden, phenotypic and spectroscopic characterisation of toxigenic and atoxigenic *Aspergillus* section *Flavi* from poultry feeds in Kelantan, Malaysia and Katsina, Nigeria

Baha'uddeen Salisu[1,2], Siti Marwanis Anua[1]*, Wan Ishak Wan Rosli[1] and Nurzafirah Mazlan[3]

[1]School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[2]Department of Microbiology, Umaru Musa Yar'adua University, PMB 2218, Katsina, Nigeria.
[3]Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
Email: smarwanis@usm.my

Received 4 July 2022; Received in revised form 4 December 2023; Accepted 26 December 2023

ABSTRACT

Aims: The natural coexistence of high humidity and warm temperatures in Malaysia and Nigeria and poor storage facilities used by most poultry feed vendors provide suitable conditions for the proliferation of aflatoxigenic fungi and aflatoxigenesis. This study aims to characterise and evaluate the toxigenicity of *Aspergillus* section *Flavi* (ASF) from Malaysian and Nigerian poultry feeds.
Methodology and results: This study utilised standard mycological techniques to determine the bioburden and distribution of mycoflora in 132 and 144 Malaysian and Nigerian poultry feeds, respectively. The ASF isolated from the samples were tested for aflatoxigenicity by thin-layer chromatography (TLC) and then characterised by multivariate using attenuated total reflectance fourier transformed infrared spectroscopy (ATR-FTIR). A total of 128 and 75 mould fungal isolates belonging to 12 and 11 species were obtained from the Malaysian and Nigerian samples with a bioburden ranging from 2.0 to 6.97 log CFU/g and the highest overall mean count of 5.66 ± 4.51 log CFU/g and 5.6 ± 4.76 log CFU/g, respectively. *Aspergillus fumigatus*, *Aspergillus felis*, *Aspergillus flavus/parasiticus* and *Fusarium graminearum* were predominant in poultry feeds from both countries. Overall, 16 ASF were isolated (Malaysia = 7, Nigeria = 9), of which only three produce aflatoxins. The multivariate cluster analysis of ATR-FTIR spectra showed 97.78% similarity between the toxigenic and atoxigenic ASF with primary differences at 600 to 800 cm^{-1} and 2927 to 4000 cm^{-1} only.
Conclusion, significance and impact of study: The bioburden of fungal flora in the samples was higher than the ICMSF's acceptable range of 2.0 to 5.0 log CFU/g, indicating that they could be hazardous to poultry and necessitate stricter control measures. Irrespective of the country/source of samples, the ATR-FTIR has discriminated the toxigenic from atoxigenic ASF, implying its promising prospects for rapid identification of toxigenic ASF.

Keywords: Aflatoxigenicity, *Aspergillus* section *Flavi*, chromatography, poultry feed, spectroscopy

INTRODUCTION

Aflatoxins are a category of hazardous proteinous polyketide fungal exometabolites/secondary metabolites with a low molecular weight that typically contaminate preserved staples in warm, humid environments. They are cardiotoxic, nephrotoxic, neurotoxic, immunotoxic, enterotoxic, genotoxic, hepatotoxic and highly carcinogenic to animals and humans. They are the most abundant, toxic, carcinogenic mycotoxins (Cary *et al.*, 2018) and the most significant threat to food security because they contaminate a large fraction of agricultural products worldwide, resulting in severe economic loss, reduced farm/domestic animal productivity and a manifestation of aflatoxicosis in both humans and animals, particularly in less developed countries where the consumption of susceptible crops (cereals and nuts) is high (Benkerroum, 2020).

Aflatoxins are mainly produced by the typical mycoflora of food grains, including 18 species of *Aspergillus* section *Flavi* (*Aspergillus flavus*, *Aspergillus aflatoxiformans*, *Aspergillus parasiticus*, *Aspergillus novoparasiticus*, *Aspergillus nomius*, *Aspergillus pseudonomius*, *Aspergillus pseudotamarii*, *Aspergillus arachidicola*, *Aspergillus transmontanensis*, *Aspergillus cerealis*, *Aspergillus mottae*, *Aspergillus pseudocaelatus*, *Aspergillus luteovirescens*, *Aspergillus pipericola*, *Aspergillus minisclerotigenes*, *Aspergillus togoensis*,

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

Aspergillus sergii and *Aspergillus austwickii*) (Gonçalves e*t al.*, 2012; Frisvad *et al.*, 2019), three *Emericella* species from *Nidulantes* section (*Emericella venezuelensis*, *Emericella olivicola* and *Emericella astellata*) (Frisvad *et al.*, 2004), two *Aspergillus* species from *Ochraceorosei* section (*Aspergillus rambellii* and *Aspergillus ochraceoroseus*) (Lizárraga-Paulín *et al.*, 2012) and two *Aschersonia* species (*Aschersonia marganita* and *Aschersonia coffeae*) (Kornsakulkarn *et al.*, 2012). These aflatoxin-producing fungi, generally known as aflatoxigenic fungi (ATF), constantly contaminate several agricultural products before and after harvesting (Hendrich, 2017).

Livestock feeds are significantly more prone to a high incidence of contamination by ATF and mycotoxins owing to their multicomponent raw materials from various agricultural commodities originating from varied sources with varying fungal loads (Xu *et al.*, 2018). Aflatoxins have been found in animal diets in excess of the minimal allowable levels in a number of studies (Greco *et al.*, 2014; Habib *et al.*, 2015; Ibrahim *et al.*, 2018; Salisu and Almajir, 2020). This high rate of feed contamination is especially typical in nations with hot and humid climates (Mgbeahuruike, 2016), such as Nigeria and Malaysia. Thus, the need for continuous screening of commodities for ATF and aflatoxin in the mentioned countries is imperative. Furthermore, as many new potentially toxic ATF are being discovered (Frisvad *et al.*, 2019), knowing the local ATF and their toxigenicity is essential for effective aflatoxin control.

Therefore, the present study aims to (i) determine the bioburden of filamentous fungi in poultry feeds from Katsina state (Nigeria) and Kelantan state (Malaysia); (ii) phenotypically/morphologically identify the species of the isolates; (iii) determine the species distribution and frequency of isolation in each poultry feed category (starter feed, layer feed, grower feed and finisher feed) for each country; and (iv) to characterise the ATF isolates into toxigenic and atoxigenic strains by TLC and ATR-FTIR methods. It is hypothesised that a detailed comparison of the ATR-FTIR spectra of toxigenic and atoxigenic ASF from different regions could provide valuable diagnostic information for rapid spectroscopic differentiation of the two strains without necessarily performing the tedious cultural and biochemical characterisation approach: hence the significance of the present study.

MATERIALS AND METHODS

Study location

In this study, samples were collected from two different countries, Malaysia and Nigeria. Both countries have hot and humid environmental conditions favouring the growth and development of aflatoxin-producing fungi and aflatoxigenesis. A stratified research design was utilised to select one state from each country (Kelantan state in Malaysia and Katsina state in Nigeria) for the sampling. During the sampling period, the average humidity and temperature were respectively 71% and 31.6 °C in Kelantan (Malaysia) and 74% and 33.2 °C in Katsina (Nigeria).

Sample collection and preparation of composite samples

The poultry feed samples in the chosen study locations were selected from various vendor shops using a purposive-convenience sampling strategy. The condition of the poultry feed shops/storage facilities used by the poultry feed sellers was noted during the sampling. Most of the storage facilities do not have air conditioning systems or good ventilation outlets and thus, harboured high humidity and temperature, which are ideal for fungal growth and aflatoxigenesis.

The poultry feed samples were collected according to the International Commission on Microbiological Specifications for Foods' approved procedure (ICMSF) as described previously (Salisu *et al.*, 2020; 2022). Approximately 1000 g of each sample was purchased from each vendor by combining three samples (250-300 g each) taken from three locations within the vendor's poultry feed bag or container. Based on the availability of the poultry feed samples in the chosen sampling points, a total of 276 samples were collected from the two countries: 132 samples from Malaysia comprising 33 samples each of layer, starter, grower, and finisher feeds; and 144 samples from Nigeria comprising 36 samples each of layer, starter, grower and finisher feeds. Next, 44 and 48 composite samples were made from the 132 and 144 samples by combining and mixing three samples of the same variety collected from different sellers in the same sampling location. All the samples were stored at 4 °C in the dark prior to the mycological analyses.

Determination of fungal bioburden in the samples

The fungal population (colony forming units per gram, CFU/g) in each sample was determined according to ICMSF's protocol (ICMSF, 2006; Pitt and Hocking, 2009). Each sample was grounded into crystalline powder and subsequently, 10 g of the powder was macerated in 90 mL of sterile peptone water for 30 min to allow sufficient time for the fungal spores and mycelia to separate from the fine powder of the samples and then homogenised by blending for 15 to 20 sec.

Subsequently, serial dilution of the homogenate using 4 test tubes containing sterile peptone water as a diluent to achieve a dilution set of 10^{-1} to 10^{-4} for each sample was carried out, after which an aliquot of 100 µL from each tube was inoculated, by spread-plating, in triplicate on sterile potato dextrose agar media plates (PDA) (HiMedia India, M096), prepared and dispensed according to the manufacturer's instruction (Salisu and Almajir, 2020) and incubated at 28 ± 2 °C for five days. The plates were observed on the 3rd and 5th days of incubation. The various filamentous fungal colonies

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

growing from each plate were counted according to their type and used to calculate each sample's bioburden (total colony forming units (CFU) per gram).

Phenotypic identification of the fungal isolates

The various fungal colony types originating from each sample were sub-cultured repeatedly on Sabroud dextrose agar (SDA) (HiMedia India, M063) to obtain pure colonies of each isolate (Salau *et al.*, 2017), after which the pure cultures were sub-cultured at 30 °C for seven days on Cyzepak's agar (CZA) medium (Oxoid, CM0097), SDA and PDA for macroscopic cultural observation. Furthermore, isolates presumed to be *Aspergillus* section *Flavi* (ASF) species were sub-cultured at 30 °C for two days on *Aspergillus flavus-Aspergillus parasiticus* agar (AFPA) differential medium (Oxoid UK, CM0731) to identify the two most notable aflatoxin producers (*A. flavus* and *A. parasiticus*) (Wan Syahidah *et al.*, 2017). Next, the isolates were prepared for microscopic examination using the slide culture technique, stained with LPCB (lactophenol cotton blue) on a clean glass slide and examined under a bright-field microscope with ×10 and then ×40 objective lenses (Salisu *et al.*, 2020).

Finally, each isolate was identified phenotypically based on its respective microscopic characteristic features (colour, shape, size, texture and septation of the hyphae; the arrangement of the mycelium, type of conidiophoric vesicles, spore heads, ornamental forms, phialides, annellides, conidial arrangements; the presence of macroconidia, chlamydoconidia and fruiting bodies) and macroscopic appearance/cultural characteristic features on AFPA, CZA, PDA and SDA (colony colour or surface pigmentation, topography, texture, reverse colour, mycelial arrangement, spore shape and nature of spores) (Samson *et al.*, 2014; Kidd *et al.*, 2016; Salau *et al.*, 2017; Gordon and Julie, 2018; Noman *et al.*, 2018). The identification of each isolate was double confirmed by the Microbiology Technologist in the Environmental and Occupational Health Laboratory of the School of Health Sciences, Universiti Sains Malaysia.

Screening of the *Aspergillus* section *Flavi* (ASF) species for aflatoxigenicity

The isolates were screened for aflatoxin production following the protocol of Salisu *et al.* (2020) with slight modification. Each isolate was sub-cultured on PDA, SDA and AFPA media by spread plating and incubated at 30 °C for 7 days. Plates were viewed under UV light at 365 nm to detect aflatoxin fluorescence (if any) around the colonies. Next, the whole agar culture from each plate was blended in 250 mL of methanol: water solvent (3:1 v/v) for 20 sec, filtered the supernatant through muslin cloth and then filter paper to obtain clear filtrate, and subjected to the filtrate for solvent evaporation in a vacuum evaporation oven at 30 °C (Memmert, Schwabach Germany). The resulting extract was resuspended in 2 mL of the extraction solvent and subsequently analysed for aflatoxins by TLC, according to Salisu *et al.* (2021). Ten µL of the various extracts were spotted separately on 20 × 20 cm TLC plates (Merck 60 F_{254}, Merck KGaA, Darmstadt, Germany) alongside the positive control (certified mix 4 aflatoxin standard supplied by Pribolab, Singapore) and negative control (methanol-water solvent). Plates were developed in a TLC tank using 200 mL of acetonitrile: dichloromethane (3:17 v/v) as mobile phase and subsequently visualised at 366 nm using a CAMAG TLC scanner to detect and photographed the aflatoxin bands in the positive samples. Aflatoxigenic isolates were confirmed as those whose extracts exhibit TLC bands with fluorescence under UV that matches any of the four aflatoxin standards (positive controls) (Salisu *et al.*, 2020).

ATR-FTIR spectroscopic characterisation of the ASF isolates

The ASF isolates were analysed by ATR-FTIR spectroscopy following the protocol of Atkinson *et al.* (2014) with slight modification. Fungal spores and mycelia were aseptically harvested from 7-day-old ASF culture on PDA by adding 5 mL of methanol + water, 3:1 (v/v), in the fungal culture and loosening the colonies using sterile forceps. The suspension of the colonies was centrifuged at 10,000 rpm for 1 min, then discarded the solvent, added another 5 mL of methanol + water, 3:1 (v/v), vortexed and centrifuged at 10,000 rpm for 1 min. The washing step was repeated two more times, after which a small portion of the growth was loaded on the ATR diamond crystal (Lumos Bruker, USA) of the FTIR spectrometer (Bruker Tensor27, USA) and scanned 32 times through 600 cm^{-1} to 4000 cm^{-1} at a resolution of 4 cm^{-1}, smoothed at polynomial order 3.0, and averaged as a single spectrum. Each sample was analysed in triplicate to minimise sampling error and averaged as a single spectrum. The ATR crystal interface was adequately cleaned (3 times with 75% methanol) and dried between successive sample applications. A background spectrum was also recorded prior to each sample measurement and subtracted from the sample's spectrum. After processing, the various absorbance responses for each spectrum were imported into Minitab 18 statistical software (Minitab LLC, USA) for multivariate analyses.

Statistical analysis

Version 24 of IBM SPSS statistical software was used to analyse the results. Descriptive statistics, mainly the measures of central tendency (frequencies, mean and standard deviation), were used to summarise the fungal bioburden in the various samples. On the other hand, a comparison of contamination levels between four sample categories from the two countries was performed using one-way ANOVA. Independent t-test was also used when comparing findings between the two countries.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

RESULTS

Bioburden of mycoflora in the poultry feed samples

The obtained fungal bioburden per gram of the composite samples showed that all the poultry feed samples analysed were contaminated by at least one or more filamentous fungi at variable contamination levels. Except for starter feeds and finisher feeds from Nigeria, which has significantly higher fungal contamination levels (p<0.05) compared to other feed categories in both countries, there was no statistically significant difference between the average fungal bioburden in the Malaysian and Nigerian samples (p>0.05) (Table 1).

Overall, 128 and 75 fungal isolates were obtained from the Malaysian and Nigerian samples, respectively. The average fungal count in the samples ranges between 2.0 to 6.97 log CFU/g in Malaysia and 2.0 to 6.18 log CFU/g in Nigeria, with the starter feed from Nigeria having the highest overall mean count (5.66 ± 5.51 log CFU/g). About 70% of the isolates obtained in the poultry feed from both countries have been reported from common food grains (maize, rice, wheat and peanuts) in the same sampling markets in Malaysia (Salisu *et al.*, 2022) and Nigeria (Salisu *et al.*, 2020), suggesting a possible common source of contamination since fungi are capable of disseminating from their primary source to distal areas in the environment through the air-borne spores (Paulussen *et al.*, 2017; Tsang *et al.*, 2018).

Phenotypic identification of the fungal isolates

The phenotypic identification result showed that the 128 and 75 fungal isolates obtained from the Malaysian and Nigerian poultry feed samples in this study belonged to 14 different species of fungi with multiple variable frequencies of isolation in the samples. The various phenotypic features of the isolates and the reference keys used to identify them are summarised in Table 2. Nine of the 14 species were common in the samples from both countries. Three species were only isolated from Malaysian poultry feeds, and the other two species were isolated only from the Nigerian samples. The distribution and frequency of occurrence of all the isolates in each poultry feed type from both countries are shown in Figures 1 and 2.

In general, the 128 isolates from Malaysia belonged to 12 species of filamentous fungi, in which *Neoscytalidium dimidiatum*, *A. fumigatus*, *F. graminearum* and *A. felis* were predominant with isolation frequencies of 23 (17.9%), 17 (13.3%), 17 (13.3%) and 14 (10.9%) out of 128, respectively (Figure 1). On the other hand, 11 species make up the 75 fungal isolates from Nigeria, of which 52% (39 isolates) were species of *A. fumigatus* (14.7%), *A. niger* (14.7%), *A. flavus/parasiticus* (12.0%) and *A. nidulans* (10.7%) (Figure 2).

Screening of the *Aspergillus* section *Flavi* (ASF) species for aflatoxigenicity

A total of 16 ASF species (collectively referred to herein as *A. flavus/A. parasiticus*) were isolated from the poultry feed samples analysed. Of the 16 ASF, seven were isolated from Malaysian samples (Figure 1) and the other nine were isolated from the Nigerian samples (Figure 2). The aflatoxigenicity screening showed that only one out of the seven ASF from Malaysia is aflatoxigenic, capable of producing both type B (AFB1 and AFB2) and type G (AFG1 and AFG2) aflatoxins. In contrast, two of the nine ASF from Nigeria had the type B aflatoxins. The typical aflatoxin bands discovered in the extracts of the aflatoxigenic species are shown in Figure 3 on the scanned TLC plate at 365 nm.

Spectroscopic characterisation of the ASF isolates

The 16 ASF isolates in this study were resolved into 3 groups by the multivariate cluster analysis of the Mid-Infrared (MIR) ATR-FTIR spectral data: two closely related clusters (99.3% similarity) containing the aflatoxigenic isolates and one cluster containing non-aflatoxigenic isolates. Figure 4 depicts typical ATR-FTIR MIR spectral fingerprint data of the clusters of the ASF isolates and the multivariate clusters based on the three-dimensional principal component's resolution and a dendrogram of the complete linkage correlation coefficient distance. The discriminating analysis revealed 97.8% similarity between toxigenic and atoxigenic species'

Table 1: Summary of bioburden of mycoflora in the poultry feed samples.

Country	Statistics	Composite poultry feed categories				p-value
		Finisher	Grower	Layer	Starter	
Malaysia	Sample size (n)	11	11	11	11	[a]0.9989
	N	33	19	40	36	
	Mean (log CFU/g)	5.32	5.28	5.15	5.08	
	Standard deviation	4.58	4.46	2.34	4.23	
Nigeria	Sample size (n)	12	12	12	12	[a]0.0268*
	N	33	19	40	36	
	Mean (log CFU/g)	5.51	5.20	5.11	5.60	
	Standard deviation	4.49	4.56	4.38	4.76	
Malaysia vs Nigeria	p-value	[b]0.2483	[b]0.8301	[b]0.9180	[b]0.8703	

Where N = Total number of fungal isolates, *p<0.05, which indicates significant variation among the feed categories, Statistical test - [a]One-way ANOVA; [b]Independent t-test.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

Table 2: Phenotypic characterisation of the mycoflora isolated from the Malaysian and Nigerian poultry feeds.

Isolate No	Source	Observed phenotypic characteristics of the isolate		Identified species name	Reference(s) applied to identify the species name of the isolates
		Microscopy	Cultural characteristics (Macroscopy)		
1	Malaysian samples only	The hyphae were hyaline, septate and branched with uniseriate phialides and columniform conidia. The phialides curved roughly parallel to each other and covered only the upper two-thirds of the vesicle.	The colonies appeared bluish-green with a milk-velvety surface and milk reverse side. Differentiated from *A. fumigatus* based on its inability to grow above 48 °C.	*A. felis*	(Kidd *et al.*, 2016; Gordon and Julie, 2018)
2	Both Malaysian and Nigerian samples	The hyphae were septate, hyaline with long rough conidiophores bearing globose vesicles containing uniseriate or biseriate phialides covering the entire vesicle.	Colonies were bright, yellow-green, umbonate with whitish areal hyphae and white border and a pale yellow to milk reverse side. All the isolates produce aspergillic acid on AFPA medium within 48 h of incubation.	*A. flavus*/ *A. parasiticus*	(Frändberg *et al.*, 2003; Samson *et al.*, 2014; Gordon and Julie, 2018)
3	Both Malaysian and Nigerian samples	Septate hyaline hyphae with long conidiophores bearing flask-shaped vesicles containing uniseriate or biseriate phialides covering the upper third of the vesicle.	Isolates possessed bluish-green umbonate colonies with whitish areal hyphae and a white-grey border, and a pale yellow to milk reverse side.	*A. fumigatus*	(Kidd *et al.*, 2016; Gordon and Julie, 2018)
4	Nigerian samples only	Septate hyaline hyphae with short conidiophores bearing small globose vesicles with biseriate phialides covering the upper half of the vesicles. Numerous Hulle cells were also seen.	Velvety colonies with dark green to olive colouration and a yellow reverse side.	*A. nidulans*	(Samson *et al.*, 2014; Kidd *et al.*, 2016; Gordon and Julie, 2018)
5	Both Malaysian and Nigerian samples	Branched septate hyaline hyphae with long smooth conidiophores bearing large globose vesicles surrounded by radiate biseriate phialides.	Downy white to yellow umbonate colonies with dark brown to black pinpoints and white border, with milk to a pale-yellow reverse side.	*A. niger*	(Samson *et al.*, 2007; Pitt and Hocking, 2009; Kidd *et al.*, 2016)
6	Both Malaysian and Nigerian samples	Dark brown septate hyphae with suberect, pale brown septate and highly branched conidiophores bearing brown and barrel-shaped conidia.	Isolates produced cottony colonies with brown-black colour at both front and reverse sides.	*Fonsecaea pedrosoi*	(Kidd *et al.*, 2016; Gordon and Julie, 2018)
7	Both Malaysian and Nigerian samples	Hyphae were branched and septate bearing multicellular spindle-like septate conidia.	Isolates appeared as cottony, velvety colonies with pink in the centre and a white border with a pale brown reverse side. Distinguished from *Acremonium*	*F. graminearum*	(Leslie and Summerell, 2006; Samson *et al.*, 2007; 2010; Kidd *et al.*,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

(Continue)

Isolate No	Source	Observed phenotypic characteristics of the isolate		Identified species name	Reference(s) applied to identify the species name of the isolates
		Microscopy	Cultural characteristics (Macroscopy)		
			spp. based on their fluffy/woolly texture.		2016; Gordon and Julie, 2018)
8	Nigerian samples only	The hyphae are branched, bearing short phialides with clusters of two-celled kidney-shaped microconidia. A moderate number of thick-walled curved macroconidia and some single chlamydospores were also seen.	The isolates appeared as cream velvety lavender colonies with cottony surfaces and milk reverse side.	*F. solani*	(Kidd *et al.*, 2016; Gordon and Julie, 2018)
9	Both Malaysian and Nigerian samples	Medium phialides with many pink septated microconidia and fewer straight macroconidia. Chlamydospores were absent.	Purple colonies with a cream surface, slightly raised centre and a purple reverse side.	*Fusarium verticilloides*	(Leslie and Summerell, 2006; Kidd *et al.*, 2016; Gordon and Julie, 2018)
10	Malaysian sample only	Branched and septate hyphae with no conidiophores. Arthroconidia are rectangular and flattened on the ends.	Cottony white growth with a pale yellow to a greyish reverse side.	*Neoscytalidium dimidiatum*	(Kidd *et al.*, 2016; Gordon and Julie, 2018)
11	Both Malaysian and Nigerian samples	Hyaline septate hyphae bearing simple conidiophores that support multiple chains of single-celled conidia.	Milk umbonate colonies with a cottony periphery and a pale-yellow reverse side.	*Penicillium cheresanum*	(Watanabe, 2002; Pitt and Hocking, 2009; Kidd *et al.*, 2016; Gordon and Julie, 2018)
12	Both Malaysian and Nigerian samples	Hyaline septate branched hyphae with numerous conidiophores bearing brush-like clusters of phialides that carry chains of conidia.	Blue-green umbonate colonies with yellowish pigment and milk border with a pale yellow reverse side.	*Penicillium chrysogenum*	(Watanabe, 2002; Pitt and Hocking, 2009; Kidd *et al.*, 2016; Gordon and Julie, 2018)
13	Malaysian sample only	Rhizoid-borne, long, unbranched brownish sporangiophores bearing round black sporangia with round columellae.	Brownish black and umbonate colonies with a wide milk border and a pale-yellow reverse side.	*Rhizopus nigricans*	(Gordon and Julie, 2018)
14	Both Malaysian and Nigerian samples	Rhizoid-borne, long, unbranched sporangiophores bearing round black sporangia with round columellae.	Cottony whitish, lid-lifting growth spotted with a black and greenish colour and a pale-yellow reverse side.	*Rhizopus stolonifer*	(Watanabe, 2002; Kidd *et al.*, 2016; Gordon and Julie, 2018)

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

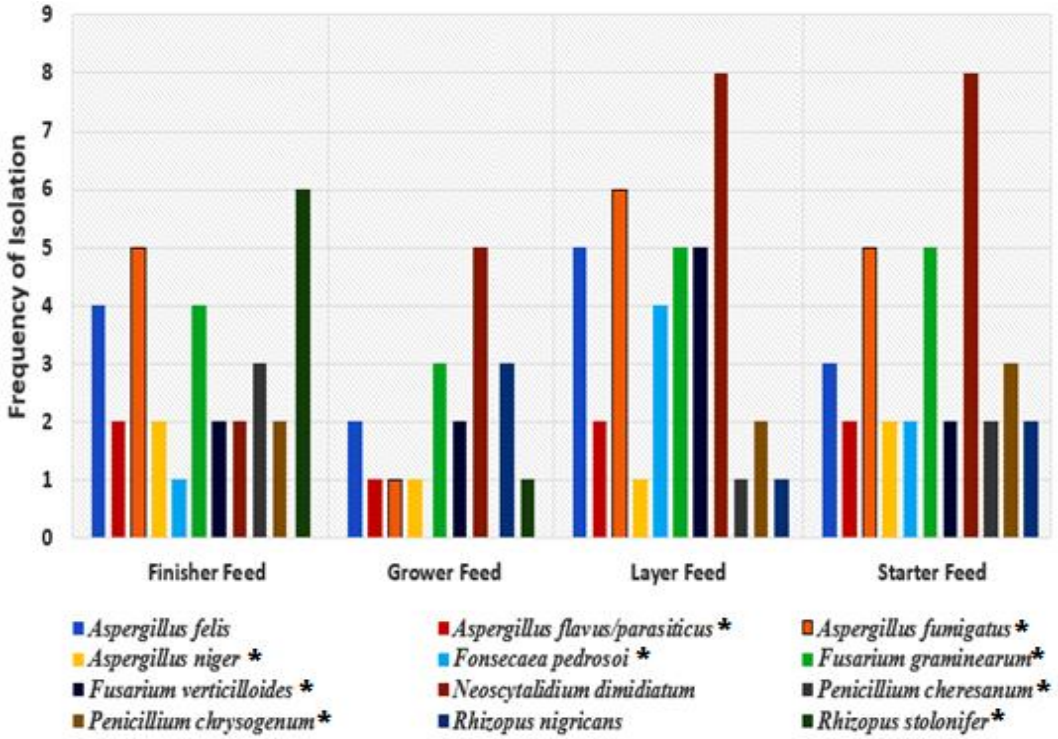

Figure 1: Frequency of occurrences and distribution of the fungal isolates in the poultry feed samples from Malaysia. Species with asterisk (*) are common in samples from both Malaysia and Nigeria.

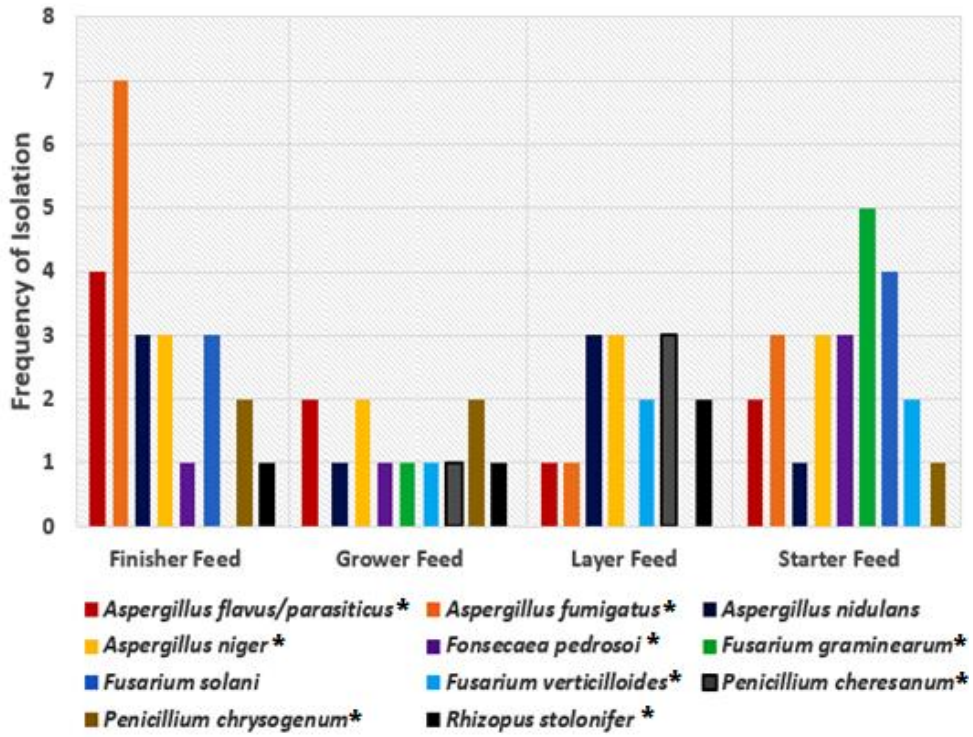

Figure 2: Frequency of occurrences and distribution of the fungal isolates in the poultry feed samples from Nigeria. Species with asterisk (*) are common in samples from both Malaysia and Nigeria.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

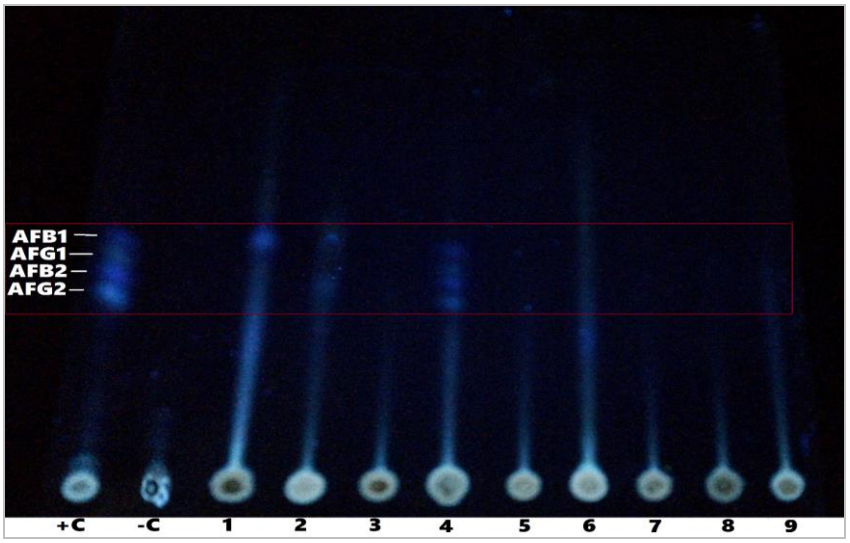

Figure 3: Aflatoxin bands in the agar extracts of the ASF species. The TLC plate was imaged in a UV chamber at 365 nm. Lanes 1 to 9 represent samples analysed. Lanes 1 and 2 produced only type B aflatoxins; Lanes 4 produced both type B and type G aflatoxins; Lanes 3, 5, 6, 7, 8 and 9 were for the non-aflatoxigenic ASF species. +C = Mixed aflatoxin standard extract (positive control), -C = Blank agar extract (negative control).

spectra, as would be predicted for phylogenetically related species. The spectra had several peaks that were identical to one another and which, among other things, stand for proteins, primary and secondary amides (Amide I and Amide II), lipids, phospholipids, polysaccharides and nucleic acids. Only a few wavenumbers (600 to 800 cm^{-1}, 2927 to 4000 cm^{-1}) showed differences.

DISCUSSION

Considering the significant economic contribution of poultry factories in terms of both meat and egg demands in nations such as Malaysia and Nigeria, contamination of poultry feeds by toxic fungi in these countries might have a significant negative influence on human health and the economy. These fungi can produce and accumulate mycotoxins in poultry feeds, which could be transferred to livestock and, subsequently, humans via eggs (Greco *et al.*, 2014; Alshannaq and Yu, 2017) and meat (Magnussen and Parsi, 2013).

However, as only a few studies are reporting consumer-level fungal contamination of chicken feed in Malaysia and Nigeria, it is clear that our research findings offer insight into the underlying mycoflora contamination of these items. The average contamination level across all sample categories was higher than the ICMSF's acceptable range of 2.0 to 5.0 log CFU/g (ICMSF, 2006) and it is consistent with other studies that document excessive fungal contamination in poultry feeds and food grains from Malaysia (Samsudin and Abdullah, 2013; Zulkifli and Zakaria, 2017; Norlia *et al.*, 2018; Yazid *et al.*, 2021) and Nigeria (Ezekiel *et al.*, 2014; Monday *et al.*, 2014; Egbuta *et al.*, 2015; Ibrahim *et al.*, 2018; Okafor and Eni, 2018; Salisu and Almajir, 2020; Salisu *et al.*, 2020). On the contrary, Reddy *et al.* (2011) and Roslan (2016) reported lower fungal bioburden in Malaysian poultry feeds and grain-based foods, respectively. However, it should be highlighted that their samples were from supermarkets rather than poultry shops, which typically lack the air conditioning equipment seen in supermarkets.

Another important observation in the present study is that the standard deviations for the fungal bioburden were very high in all the poultry feed categories. This implies higher heterogeneity in the samples' contamination levels because some of the samples had as low as 2.0 log CFU/mL fungal bioburden, whereas some other samples had as high as 6.76 log CFU/mL fungal bioburden. This further indicates that although the mean bioburden of fungi in each poultry feed category is higher than the permissible level recommended by the ICMSF, many of the samples are safe for poultry consumption because their contamination is within the acceptable limit set by the ICMSF.

In general, the high bioburden of fungi obtained in the present study may be due to long-time storage and poor storage conditions used by the poultry feed sellers, as opined by Habib *et al.* (2015), Ibrahim *et al.* (2018) and Salisu and Almajir (2020) who observed that majority of the Nigerian poultry sellers use insufficient and poor storage facilities (locked-up stores without air conditioning system or good ventilation). As a result, livestock feeds are frequently contaminated with mycotoxins and fungi in Nigeria.

Morphologically, the phenotypes of the fungi that were found in the poultry feed in the present study matched those that had been reported in the fungi identification literature (Samson *et al.*, 2014; Kidd *et al.*, 2016; Salau *et al.*, 2017; Gordon and Julie, 2018; Noman *et al.*, 2018). The most prevalent species (*Aspergillus*, *Penicillium* and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(2) 2024, pp. 120-131
DOI: http://dx.doi.org/10.21161/mjm.220010

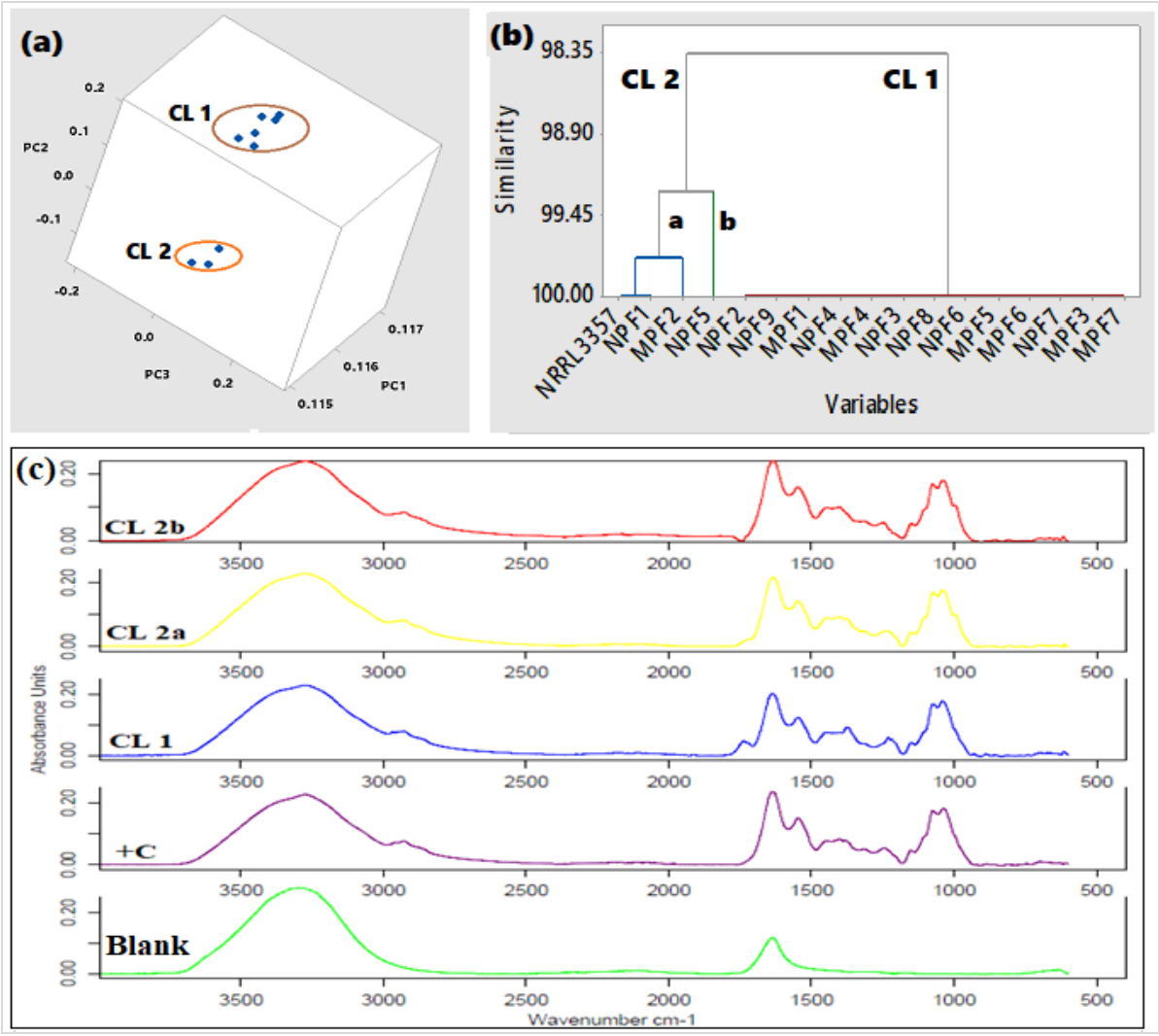

Figure 4: Clusters of the toxigenic and atoxigenic ASF isolates resolved by (a) principal components analysis and (b) dendrogram based on the complete linkage correlation coefficient distance and their (c) typical ATR-FTIR MIR spectral fingerprints. CL 1 is the cluster of the atoxigenic ASF isolates; CL 2 is the cluster of the toxigenic ASF isolates; +C is positive control (*A. flavus* NRRL 3357); the blank spectrum is for the solvent used in harvesting and washing of the fungal mycelium prior to spectral acquisition.

Rhizopus), identified in this study's samples agreed with earlier reports of fungal contamination of feeds and foods in Malaysia (Reddy and Salleh, 2011; Samsudin and Abdullah, 2013; Roslan, 2016) and Nigeria (Makun *et al.*, 2012; Nura *et al.*, 2016; Keta *et al.*, 2017) in which these species were listed alongside others that were not isolated in this study as significant fungal contaminants of feeds and foods in the two countries. However, the overall high prevalence of *A. flavus/A. parasiticus* and the extremely low prevalence of *A. fumigatus* identified in this study have supported Roslan's findings, where these species were reported as the most prevalent in some poultry feed samples from Malaysia (Roslan, 2016).

The identification and characterisation of mycoflora from environmental samples have been accomplished using ATR-FTIR spectroscopy and multivariate statistical analysis (Greene *et al.*, 1992; Bhat, 2013; Kaya-Celiker *et al.*, 2016). In this study, as well as in earlier studies by Fischer *et al.* (2006) and Bhat (2013), it was discovered that members of the *Aspergillus* spp. had distinct characteristic wavenumbers (1765-1590 cm^{-1}, 1470-1275 cm^{-1}, 930-715 cm^{-1}, 1170-1000 cm^{-1}) along their FTIR spectra that correlate to the fungal fingerprints, polysaccharides, Amide II and Amide I, respectively.

Results in the present study showed that the FTIR spectra of the two toxigenic clusters (cluster 1a and cluster 1b), depicted in Figure 4, differ mostly in the frequency ranges of 4000 to 2927 cm^{-1} and 1630 to 600 cm^{-1}. Most cluster 1a isolates displayed minor absorbance peaks at wavenumbers (3075 to 3061 cm^{-1}, 3720 to 3517

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

cm^{-1}, 3879 cm^{-1} and 3959 cm^{-1}) that indicate variable O-H stretching at the fingerprint region, whereas atoxigenic isolates (cluster 2) and cluster 1b (toxigenic isolates) did not. It also has minor absorbance peaks at 892 cm^{-1}, 931 cm^{-1}, 1376 cm^{-1}, 1395 cm^{-1}, 1429 cm^{-1}, 1589 cm^{-1} and 1627 cm^{-1}, which are all absent from the atoxigenic (cluster 2) isolate as well as the toxigenic cluster 1b isolates. Contrarily, several common absorbance peaks at 856-859 cm^{-1}, 3744-3755 cm^{-1}, 3800-3804 cm^{-1}, 3850-3859 cm^{-1}, 3888-3889 cm^{-1} and 3966-3981 cm^{-1} are shared by the toxic cluster 1b and the atoxigenic isolates (cluster 2) but not by the cluster 1a isolates. However, there were some absorbance maxima at wavenumbers between 1071.32 and 1074 in isolates from both toxigenic clusters (clusters 1a and 1b). 63 cm^{-1}, 1230.77-1233.8 cm^{-1}, 1315 cm^{-1}, 1401.79-1409.2 cm^{-1}, 1443.39-1445. 47 cm^{-1}, 1633.13-1637. 53 cm^{-1}, 3775.69-3776.63 cm^{-1} and 3939.46-3940 cm^{-1}. When the spectra of toxigenic and atoxigenic species were compared, all of them were absent in the atoxigenic isolates' spectra (cluster 2). It has been documented that, in contrast to *A. parasiticus*, toxigenic *A. flavus* showed a distinct peak at 1375.31 cm^{-1} (Bhat, 2013). On the other hand, the *A. parasiticus* displayed smaller peaks between 800 cm^{-1} and 500 cm^{-1}, which are absent in *A. flavus* (Bhat, 2013). The results from this investigation and comparable studies (Greene *et al.*, 1992; Bhat, 2013; Kaya-Celiker *et al.*, 2014; 2015; 2016) show that amides, carbonyls from esters and methylene groups are the predominant absorbance peaks that distinguish the toxigenic and atoxigenic *Aspergillus* spp.

CONCLUSION

The poultry feed samples tested in this study were highly contaminated by notable mycotoxigenic fungi, particularly members of the *Aspergillus* section *Flavi* and *Fusarium* spp., which are known to produce potent carcinogenic mycotoxins. There were no significant differences between the fungal bioburden from Malaysian poultry feed and those from Nigeria. Still, the mean bioburden in all the sample categories was above the countries' permissible limit and the ICMSF. As a result, more research is needed, particularly intervention studies that will examine the level of mycotoxins in poultry feeds and assess and improve stakeholders' knowledge, attitude and practice regarding fungal and mycotoxin contamination of poultry feeds in the study regions. The toxigenic and atoxigenic ASF spectral data/cluster obtained in this study might be utilised as reference spectra for rapid screening of these toxigenic fungal species in chicken feeds and foods in the study region.

ACKNOWLEDGEMENTS

The Ministry of Higher Education Malaysia's Fundamental Research Grant Scheme (FRGS/1/2019/SKK06/USM/03/1) funded this research. The authors are also thankful to Muhammad Syafiq Sidek Ahmad, Siti Kurunisa Mohd Hanafiah and the UPMS personnel of Universiti Sains Malaysia's School of Health Sciences for their assistance.

REFERENCES

Alshannaq, A. and Yu, J. (2017). Occurrence, toxicity, and analysis of major mycotoxins in food. *International Journal of Environmental Research and Public Health* **14(6), 632.**

Atkinson, C., Pechanova, O., Sparks, D. L., Brown, A. and Rodriguez, J. M. (2014). Differentiation of aflatoxigenic and non-aflatoxigenic strains of Aspergilli by FT-IR spectroscopy. *Applied Spectroscopy* **68(8), 920-924.**

Benkerroum, N. (2020). Aflatoxins: Producing-molds, structure, health issues and incidence in southeast Asian and sub-saharan African countries. *International Journal of Environmental Research and Public Health* **17(4), 1215.**

Bhat, R. (2013). Potential use of fourier transform infrared spectroscopy for identification of molds capable of producing mycotoxins. *International Journal of Food Properties* **16(8), 1819-1829.**

Cary, J. W., Gilbert, M. K., Lebar, M. D., Majumdar, R. and Calvo, A. (2018). *Aspergillus flavus* secondary metabolites: More than just aflatoxins. *Food Safety* **6(1), 7-32.**

Egbuta, M. A., Wanza, M. M. and Dutton, M. F. (2015). Evaluation of five major mycotoxins co-contaminating two cereal grains from Nigeria. *International Journal of Biochemistry Research and Review* **6(4), 160-169.**

Ezekiel, C. N., Atehnkeng, J., Odebode, A. C. and Bandyopadhyay, R. (2014). Distribution of aflatoxigenic *Aspergillus* section *Flavi* in commercial poultry feed in Nigeria. *International Journal of Food Microbiology* **189, 18-25.**

Fischer, G., Braun, S., Thissen, R. and Dott, W. (2006). FT-IR spectroscopy as a tool for rapid identification and intra-species characterization of air-borne filamentous fungi. *Journal of Microbiological Methods* **64(1), 63-77.**

Frändberg, E., Pitt, J. and Olsen, M. (2003). Quality control of *Aspergillus flavus* and *A. parasiticus* agar and comparison with dichloran 18% glycerol agar: A collaborative study. *International Journal of Food Microbiology* **89(1), 99-102.**

Frisvad, J. C., Hubka, V., Ezekiel, C. N., Hong, S. B., Nováková, A., Chen, A. J. *et al.* (2019). Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. *Studies in Mycology* **93, 1-63.**

Frisvad, J. C., Samson, R. A. and Smedsgaard, J. (2004). *Emericella astellata*, a new producer of aflatoxin B 1, B 2 and sterigmatocystin. *Letters in Applied Microbiology* **38(5), 440-445.**

Gonçalves, J. S., Ferracin, L. M., Vieira, M. L. C., Iamanaka, B. T., Taniwaki, M. H. and Fungaro, M. H. P. (2012). Molecular analysis of *Aspergillus* section *Flavi* isolated from Brazil nuts. *World Journal of Microbiology and Biotechnology* **28(4), 1817-1825.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Gordon, L. L. and Julie, A. R. (2018). Color Atlas of Mycology: An Illustrated Field Guide Based on Proficiency Testing. College of American Pathologists, Northfield, Illinois. **pp. 1-133.**

Greco, M. V., Franchi, M. L., Golba, S. L. R., Pardo, A. G. and Pose, G. N. (2014). Mycotoxins and mycotoxigenic fungi in poultry feed for food-producing animals. *The Scientific World Journal* **2014, Article ID 968215.**

Greene, R. V., Gordon, S. H., Jackson, M. A., Bennett, G. A., McClelland, J. F. and Jones, R. W. (1992). Detection of fungal contamination in corn: Potential of FTIR-PAS and -DRS. *Journal of Agricultural and Food Chemistry* **40(7), 1144-1149.**

Habib, M. A., Abdu, P., Kwanashie, C. N., Kabir, J. and Negedu, A. (2015). Isolation and identification of *Aspergillus* species from poultry feeds in Kaduna State, Nigeria. *Microbiology research International* **3(2), 27-32.**

Hendrich, S. (2017). Mycotoxins. *In*: Food Toxicology: Current Advances and Future Challenges. Sachan, A. and Hendrich, S. (eds.). Apple Academic Press, New York. **pp. 407-446.**

Ibrahim, R. A., Salisu, B., Anua, S. M., Abdulkadir, B., Yakubu, A., Adamu, U. *et al.* (2018). Cancer risk, fungal and mycotoxin contaminations of poultry feed products from some major processing companies in Nigeria. 37th National Annual Scientific Conference of the Nigerian Society of Biochemistry and Molecular Biology, Nigeria.

ICMSF, International Commission on Microbiological Specifications for Foods. (2006). Microorganisms in Foods 6: Microbial Ecology of Food Commodities. Springer, New York.

Kaya-Celiker, H., Mallikarjunan, P. K. and Kaaya, A. (2015). Mid-infrared spectroscopy for discrimination and classification of *Aspergillus* spp. contamination in peanuts. *Food Control* **52, 103-111.**

Kaya-Celiker, H., Mallikarjunan, P. K. and Kaaya, A. (2016). Characterization of invasion of genus *Aspergillus* on peanut seeds using FTIR-PAS. *Food Analytical Methods* **9(1), 105-113.**

Kaya-Celiker, H., Mallikarjunan, P. K., Schmale, D. and Christie, M. E. (2014). Discrimination of moldy peanuts with reference to aflatoxin using FTIR-ATR system. *Food Control* **44, 64-71.**

Keta, J. N., Shehu, K., Wagini, N. H., Yahayya, U., Mohammad, N. K., Gudu, G. B. *et al.* (2017). Fungi associated with the foliar diseases of cultivated rice (*Oryza sativa*) in birnin Kebbi, Kebbi State, North-Western of Nigeria. *Katsina Journal of Natural and Applied Sciences* **6(1), 107-113.**

Kidd, S., Halliday, C., Alexiou, H. and Ellis, D. (2016). Descriptions of Medical Fungi. Edn. 3rd. Mycology Unit, Women's and Children's Hospital, Australia.

Kornsakulkarn, J., Saepua, S., Srichomthong, K., Supothina, S. and Thongpanchang, C. (2012). New mycotoxins from the scale insect fungus *Aschersonia coffeae* Henn. BCC 28712. *Tetrahedron* **68(40), 8480-8486.**

Leslie, J. F. and Summerell, B. A. (2006). The *Fusarium* Laboratory Manual. Blackwell Publishing, USA.

Lizárraga-Paulín, E. G., Moreno-Martínez, E. and Miranda-Castro, S. P. (2012). Aflatoxins and their impact on human and animal health: An emerging problem. *In*: Aflatoxins – Biochemistry and Molecular Biology. Guevara-Gonzalez, R. G. (ed.). IntechOpen Limited, United Kingdom.

Magnussen, A. and Parsi, M. A. (2013). Aflatoxins, hepatocellular carcinoma and public health. *World Journal of Gastroenterology* **19(10), 1508-1512.**

Makun, H. A., Anjorin, S. T., Abidoye, A. S., Rufai, A. R. and Kabiru, Y. A. (2012). Incidence and botanical control of seed-borne fungi of cowpea in Niger State, Nigeria. *ARPN Journal of Agricultural and Biological Science* **7(8), 654-658.**

Mgbeahuruike, A. C. (2016). Aflatoxin contamination of poultry feeds in Nigerian feed mills and the effect on the performance of Abor acre broilers. *Animal Research International* **13(2), 2436-2445.**

Monday, I. E., Francis, J. I. and Mohammad, S. U. (2014). Microbiological quality of ready-to-eat foods (rice and moimoi) sold by food vendors in Federal Polytechnic Bali, Taraba State Nigeria. *IOSR Journal of Environmental Science, Toxicology and Food Technology* **8(2), 145-149.**

Noman, E. A., Al-Gheethi, A. A., Ab. Rahman, N. N. N., Hideyuki, N., Talip, B. B. H., Mohamed, R. M. S. R. *et al.* (2018). Phenotypic identification of *Penicillium* spp. isolated from clinical wastes based on microstructure characteristics. *Malaysian Journal of Microbiology* **14(2), 88-95.**

Norlia, M., Nor-Khaizura, M. A. R., Selamat, J., Bakar, F. A., Radu, S. and Chin, C. K. (2018). Evaluation of aflatoxin and *Aspergillus* sp. contamination in raw peanuts and peanut-based products along this supply chain in Malaysia. *Food Additives and Contaminants: Part A* **35(9), 1787-1802.**

Nura, M., Abubakar, A., Auyo, M, I., Sunday, E. and Kutama, A. S. (2016). Isolation and identification of fungi associated with tigernut milk drink (Kunun Aya) in Dutse, Jigawa State. *Global Advanced Research Journal of Agricultural Science* **5(7), 302-308.**

Okafor, S. E. and Eni, A. O. (2018). Microbial quality and the occurrence of aflatoxins in plantain/yam and wheat flours in Ado-Odo Ota. *IOP Conference Series: Earth and Environmental Science* **210, 012017.**

Paulussen, C., Hallsworth, J. E., Álvarez-Pérez, S., Nierman, W. C., Hamill, P. G., Blain, D. *et al.* (2017). Ecology of aspergillosis: Insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. *Microbial Biotechnology* **10(2), 296-322.**

Pitt, J. I. and Hocking, A. D. (2009). Fungi and Food Spoilage. Edn. 3rd. Springer, New York.

Reddy, K. R. N. and Salleh, B. (2011). Co-occurrence of moulds and mycotoxins in corn grains used for animal feeds in Malaysia. *Journal of Animal and Veterinary Advances* **10(5), 668-673.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Reddy, K. R. N., Farhana, N. I. and Salleh, B. (2011). Occurrence of *Aspergillus* spp. and aflatoxin B1 in Malaysian foods used for human consumption. *Journal of Food Science* **76(4), T99-T104.**

Roslan, Z. B. (2016). Prevalence of *Aspergillus* sp. contamination in commercial poultry feed and feed ingredients in Malaysia. Ph.D. Thesis. Universiti Putra Malaysia, Malaysia.

Salau, A. I., Shehu, K. and Kasarawa, A. B. (2017). Morphological characterization of mycotoxigenic fungi contaminating groundnut products in Sokoto state. International Journal of Research in Pharmacy and Biosciences **4(4), 1-7.**

Salisu, B., Anua, S. M., Wan Ishak, W. R. and Mazlan, N. (2021). Development and validation of quantitative thin layer chromatographic technique for determination of total aflatoxins in poultry feed and food grains without sample clean-up. *Journal of Advanced Veterinary and Animal Research* **8(4), 656-670.**

Salisu, B., Anua, S. M., Wan Ishak, W. R., Mazlan, N. and Lawal, U. (2020). Incidence, distribution and phenotypic characterisation of aflatoxigenic fungi contaminating commonly consumed food grains in Katsina State, Nigeria. *Malaysian Journal of Medicine and Health Sciences* **16(Suppl 11), 18-27.**

Salisu, B., Anua, S. M., Wan Ishak, W. R. and Mazlan, N. (2022). Mycotoxigenic fungi contamination of grains and peanuts from open markets in Kelantan, Malaysia. *Food Research* **6(1), 69-77.**

Salisu, B. D. and Almajir, I. R. (2020). Aflatoxins and aflatoxigenic fungal contamination of common poultry feed products in Katsina State, Nigeria. *Novel Research in Microbiology Journal* **4(1), 653-665.**

Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C. and Andersen, B. (2010). Food and Indoor Fungi. Edn. 2nd. Westerdijk Laboratory Manual Series. Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Samson, R. A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J. C. and Varga, J. (2007). Diagnostic tools to identify black aspergilli. *Studies in Mycology* **59, 129-145.**

Samson, R. A., Visagie, C. M., Houbraken, J., Hong, S., Hubka, V., Klaassen, C. H. W. *et al.* (2014). Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Studies in Mycology* **78(1), 141-173.**

Samsudin, N. I. P. and Abdullah, N. (2013). A preliminary survey on the occurrence of mycotoxigenic fungi and mycotoxins contaminating red rice at consumer level in Selangor, Malaysia. *Mycotoxin Research* **29(2), 89-96.**

Tsang, C. C., Tang, J. Y. M., Lau, S. K. P. and Woo, P. C. Y. (2018). Taxonomy and evolution of *Aspergillus*, *Penicillium* and *Talaromyces* in the omics era – Past, present and future. *Computational and Structural Biotechnology Journal* **16, 197-210.**

Wan Syahidah, H., Selvaneswary, K., Normah, M., Syafiq, M. I. and Suhaimi, D. (2017). Survey on occurrence of aflatoxins in chicken feeds from Peninsular Malaysia. *Malaysian Journal of Veterinary Research* **8(1), 103-112.**

Watanabe, T. (2002). Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species, Edn. 2nd. CRC Press, Boca Raton.

Xu, L., Zhang, Z., Zhang, Q., Zhang, W., Yu, L., Wang, D. *et al.* (2018). An on-site simultaneous semi-quantification of aflatoxin B1, zearalenone, and T-2 toxin in maize- and cereal-based feed via multicolor immunochromatographic assay. *Toxins* **10(2), 87.**

Yazid, S. N. E., Ng, W. J., Selamat, J., Ismail, S. I. and Samsudin, N. I. P. (2021). Diversity and toxigenicity of mycobiota in grain corn: A case study at pioneer grain corn plantations in Terengganu, Malaysia. *Agriculture* **11(3), 237.**

Zulkifli, N. A. and Zakaria, L. (2017). Morphological and molecular diversity of *Aspergillus* from corn grain used as livestock feed. *HAYATI Journal of Biosciences* **24(1), 26-34.**