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Mycoflora, aflatoxigenic strains of *Aspergillus* section *Flavi* and aflatoxins in fish feed

A.A. Fallah[1,2*], E. Pirali-Kheirabadi[3], M. Rahnama[4], S.S. Saei-Dehkordi[1,2] and K. Pirali-Kheirabadi[2,5]

[1]Shahrekord University, Faculty of Veterinary Medicine, Department of Food Hygiene and Quality Control, P.O. Box 115, Shahrekord 34141, Iran; [2]Shahrekord University, Research Institute of Zoonotic Diseases, P.O. Box 115, Shahrekord 34141, Iran; [3]Fisheries Organization of Iran, Shahrekord branch, P.O. Box 214, Shahrekord 34141, Iran; [4]University of Zabol, Faculty of Veterinary Medicine, Department of Food Hygiene and Quality Control, P.O. Box 483, Zabol 98615, Iran; [5]Shahrekord University, Faculty of Veterinary Medicine, Department of Pathobiology, P.O. Box 115, Shahrekord 34141, Iran; fallah.aziz55@yahoo.com, fallah-az@vet.sku.ac.ir

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Abstract

Commercial feedstuffs are an important component in a modern aquaculture industry. Mycotoxin contamination of fish feeds represents a hazard to aquatic animals and human health. This study aimed to determine the mycoflora, aflatoxigenic strains of *Aspergillus* section *Flavi*, and occurrence of aflatoxins in fish feed samples. A total of 86 fish feed samples consisting of pellet, extruder, and scramble samples were collected from feed processing industries and fish farms in Iran. Identification of fungi, *in vitro* production of aflatoxins by *Aspergillus* section *Flavi* strains, and analysis of aflatoxins were done according to standard and established methods. The mean total fungal counts ranged between 2.16 and 4.74 log cfu/g in samples; and 2 samples (2.33%) exceeded the hygienic quality limit, i.e. 4 log cfu/g. *Aspergillus* was the predominant detected genera followed by *Penicillium* and *Fusarium*. Among the *Aspergillus* species, *Aspergillus flavus* was the most prevalent species, followed by *Aspergillus parasiticus*, *Aspergillus niger*, and *Aspergillus ochraceus*. All the *A. parasiticus* isolates and 48.1% of the *A. flavus* isolates produced aflatoxins *in vitro*. Contamination with aflatoxins were detected in 58 feed samples (67.5%), ranging between 0.46 and 68.5 µg/kg. The results revealed that the frequency of contamination with aflatoxins and prevalence of aflatoxigenic fungi in such a level can be a potential hazard for the aquaculture industry and human health. Regular monitoring of mycotoxins specially aflatoxins in feed supply chain can improve the situation through reducing economic losses and minimizing hazards to the public health.

Keywords: aflatoxins, *Aspergillus* section *Flavi*, aflatoxigenic fungi, fish feed

1. Introduction

Mycotoxins are secondary toxic metabolites of fungi, produced during the end period of the exponential growth phase and have no apparent function in fungi growth and metabolism. They are mainly produced by specific competent moulds genera like *Aspergillus*, *Penicillium* and *Fusarium* under favourable conditions of temperature and humidity. Contamination of feedstuffs with mycotoxins may lead to nutrient losses and pose a threat to animal and also human health due to the toxin transmission to humans through the food chain (Alldrick *et al.*, 2009; Fallah, 2010; Martins *et al.*, 2008; Razavilar, 1998; Thomson *et al.*, 2012).

Aflatoxins, the most studied group of mycotoxins, are produced by toxigenic strains of *Aspergillus* section *Flavi* species, particularly *Aspergillus flavus*, *Aspergillus parasiticus*, and to a lesser extent by *Aspergillus nomius*. The four major aflatoxins of concern are designated as B_1, B_2, G_1, and G_2, based on their fluorescence under UV light. Among them, aflatoxin B_1 (AFB_1) is the most frequent and toxic with a high toxicity for a large number of animal species (Cole and Cox, 1981; Creppy, 2002; Fallah *et al.*, 2011; Paramithiotis and Drosinos, 2010). Exposure of aquatic animals to aflatoxins resulted in reduced growth rate, hematological abnormalities, immunity impairments, necrotic hepatocytes, and hepatocellular carcinoma. Moreover, transmission of aflatoxins through contaminated

feed in seafood products may be a potential hazard for public health because of their mutagenic, carcinogenic, teratogenic, immunosuppressive, and other adverse effects (El-Sayed and Khalil, 2009; Royes and Yanong, 2010).

Aquaculture is one of the most important husbandry systems around the world, with an average annual growth rate of 8.8% since 1980 (FAO, 2012). Hence, the increasing demand of finished feed for farmed aquatic animals is evident. The safety and quality of feed are the factors affecting the development and productivity of aquatic husbandry. The increasing use of agricultural ingredients in aquatic feed formula enhances the risk of fungi and mycotoxins contamination. Regular monitoring of toxigenic fungi along the feed supply chain is required for developing strategies to prevent or control mycotoxin production.

Several studies in the world have been undertaken to determine the mycoflora and natural occurrence of mycotoxins in animal feedstuffs (Diaz *et al.*, 2009; Paramithiotis *et al.*, 2009; Rosa *et al.*, 2008; Saleemi *et al.*, 2010). However, referring to the existing scientific literature, no survey has been conducted in this field in Iran. Therefore, this study aimed to determine the mycobiota in different kinds of fish feed (pellet, extruder and scramble) and ability of *Aspergillus* section *Flavi* isolates to produce aflatoxins. The natural occurrence of aflatoxins in feed samples was also studied.

2. Materials and methods

Sample collection and culture conditions

A total of 86 fish feed samples (10 kg each) consisting of pellet (n=35), extruder (n=29), and scramble (n=22) were obtained from feed processing industries and fish farms in Chaharmahal-va-Baghtiari province, the first rank farmed fish production province in Iran (Annual Fishery Statistics, 2010), during April to December 2010. Each primary sample was finely ground and homogenised in a laboratory mill (Pars Khazar model BG-300P; Pars Khazar Industrial Co., Rasht, Iran). Laboratory samples (1.2-1.5 kg each) were obtained from primary homogenised samples. The analyses for mould counts were performed immediately. Then, the remaining of each sample was stored at 2 °C prior to aflatoxin analysis.

Ten gram of each sample was placed aseptically into a sterile stomacher bag (Seward Medical, UK) and homogenised with 90 ml of 0.1% (w/v) peptone water using a Stomacher® 400 laboratory blender (Seward Medical, Worthing, UK) at a medium speed for 3 min. From the resulting homogenate, 10-fold appropriate serial dilutions were prepared and aliquots consisting of 0.1 ml of each dilution (in duplicate) were spread on the surface of two media. The general culture medium used for enumeration of total mycoflora was dichloran rose bengal chloramphenicol agar (DRBC), with the composition of 10 g glucose, 5 g meat peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 25 mg rose bengal, 2 mg dichloran, 100 mg chloramphenicol, and 15 g agar per 1000 ml of distilled water. The low water activity medium used for development of xerophilic fungi was dichloran 18% glycerol agar (DG18), with the composition of 10 g glucose, 5 g meat peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 2 mg dichloran, 100 mg chloramphenicol, 220 g glycerol, and 15 g agar per 1000 ml of distilled water (Pitt and Hocking, 2009). All plates were incubated for 7-10 days at 25 °C in the dark. Only plates containing 10-100 colonies were used for enumeration and the results were expressed as log colony-forming unit per gram (log cfu/g) of sample. The colonies of fungi were transferred to malt extract agar slants and incubated for 7 days at 25 °C. Fungal genera were identified according to microscopic criteria in accordance with appropriate keys (Karim, 2006; Pitt and Hocking, 2009). Taxonomic identification of *Aspergillus* species was performed through microscopic and macroscopic criteria following the schemes suggested by Klich (2002) and Pitt and Hocking (2009). The results were expressed as isolation frequency for each genus (% of samples in which each genus was present), and relative density for each *Aspergillus* species (% of isolation of each *Aspergillus* species among total number of *Aspergillus* strains).

Aflatoxigenic ability of *Aspergillus* section *Flavi*

The capacity for producing aflatoxins was determined for all strains of *Aspergillus* section *Flavi* isolated from fish feed samples according to the method described by Bragulat *et al.* (2001) and Riba *et al.* (2010). The isolates were cultured on czapek yeast extract agar and incubated at 25 °C for 7 days. Afterwards, three agar plugs (10 mm in diameter) were removed from the central area of each colony using a cork borer. The plugs were weighed, transferred into small tubes and extracted with 1 ml of methanol for 60 min. The extracts were centrifuged at 13,000 rpm for 20 min and then filtered through a 0.45 µm Milipore filter (Merck Millipore, Darmstadt, Germany). The aflatoxins were determined by high performance liquid chromatography (HPLC) with fluorescence detection following the methodology subsequently described.

Aflatoxin analysis

Analysis of aflatoxins in fish feed samples was performed by the HPLC method of Institute of Standards and Industrial Research of Iran (ISIRI, 2003) with some minor modifications. A homogenised sample (50 g) was extracted with 300 ml of methanol:water (8:2, v/v) in a blender at high speed for 5 min. Five grams of sodium chloride and 100 ml of n-hexane were added prior to extraction. The extract was filtered through no. 4 filter paper (Whatman International Ltd., Maidstone, UK) and the hexane phase was discarded. Then, the methanolic extract (20 ml) was

diluted with phosphate-buffered saline (PBS) solution (120 ml). This diluted extract was further filtered through glass microfiber filter. After conditioning the Aflatest immunoaffinity column (Vicam, Watertown, MA, USA) with 10 ml of PBS, 70 ml of the filtrate was passed through it at a flow rate of 1 drop/s. The column was washed with 15 ml of water and dried by passing air. The aflatoxins were eluted with methanol using a two-step procedure. First, 500 µl of methanol was passed through the column by gravity and collected. After 1 min, the second portion of methanol (750 µl) was applied and collected. The eluate was diluted with water and analysed by HPLC.

The HPLC device used for determination of aflatoxins was Waters 2695 chromatograph (Waters Corporation, Milford, MA, USA). The mobile phase was water:methanol:acetonitrile (54:29:17, v/v/v) with a flow rate of 1.0 ml/min. Post column derivatisation was achieved with a Kobra cell and addition of potassium bromide to the mobile phase. The analytical column was Chromolith® performance RP-18E column (200 mm × 4.6 mm i.d.; Merck, Darmstadt, Germany). The fluorescence detector (Waters 474; Waters Corporation) was operated at extinction and emission wavelengths of 365 and 535 nm, respectively. The limit of detection was about 0.1 µg/kg for each of the aflatoxins (B_1, B_2, G_1 and G_2).

To validate our method, fish feed samples (50 g each) were spiked with AFB_1, AFB_2, AFG_1 and AFG_2 at levels of 1 and 5 µg/kg for each aflatoxins. Spiking was carried out in triplicate. The concentrations were determined using the previously described procedure. The recovery scores (mean±standard deviation) were as follows:

- 1 µg/kg spiking level: 86.3±2.35% for AFB_1, 84.7±4.06% for AFB_2, 79.6±2.91% for AFG_1 and 75.0±3.15% for AFG_2.
- 5 µg/kg spiking level: 85.6±3.41% for AFB_1, 82.2±2.99% for AFB_2, 75.1±3.39% for AFG_1 and 72.6±1.90% for AFG_2.

Statistical analysis

The data of fungal counts were transformed to log cfu/g of sample. To compare fungal counts on different culture media, statistical analysis was performed using t-test of GraphPad Prism software, version 3 (www.graphpad.com). The results were considered significantly different at $P<0.05$.

3. Results and discussion

Analysis of the mycoflora

The results of total mould counts in fish feed samples are depicted in Table 1. Statistical analysis revealed no significant differences ($P>0.05$) in counts between two culture media (DRBC and DG18). The mould counts of only 2 samples (2.33%), consisting of one pellet sample (2.86%) and one extruder sample (3.45%), exceeded the limit of 4 log cfu/g suggested by PDV (2009). It has been determined that the pelleting process can reduce fungal counts (Magnoli *et al.*, 2010).

Among the 8 fungal genera isolated from fish feed samples, *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. were the three most prevalent genera, with the isolation frequency of 63.5, 41.5 and 26.2%, respectively (Figure 1). The high prevalence of these genera which contain most of toxigenic fungi, could be a potential hazard for farmed fish species. Several previous studies revealed that *Aspergillus* and *Penicillium* were the predominant fungal genera in equine, cattle, and poultry feeds (Keller *et al.*, 2007; Rosa *et al.*, 2008; Saleemi *et al.*, 2010). In a recent study, Almeida *et al.* (2011) found that *Aspergillus*, *Penicillium*, *Cladosporium* and *Fusarium* species were present in feed for farmed sea bass.

The relative densities of *Aspergillus* spp. isolated from fish feed samples are shown in Figure 2. *A. flavus* was the predominant species (47.3%) followed by *A. parasiticus* (16.1%) and *Aspergillus niger* aggregate (13.9%). *Aspergillus* section *Flavi* species (*A. flavus*, *A. parasiticus*, *A. nomius*, and *Aspergillus tamarii*) which are the potential aflatoxigenic fungi, dedicated 65.8% of the total *Aspergillus* isolates. Moreover, the presence of *A. niger* aggregate (13.9%) and *Aspergillus ochraceus* (10.2%) as the potential ochratoxigenic fungi in such a level should not

Table 1. Total fungal counts (log cfu/g) of fish feed samples in different culture media.

Fish feed	No. of samples	DRBC		DG18		No. that exceed hygienic limit[a] (%)
		Mean±sem	Min.-max.	Mean±sem	Min.-max.	
Pellet	35	3.11±0.09	2.23-4.74	2.98±0.11	2.16-4.52	1 (2.86)
Extruder	29	2.80±0.19	ND[b]-4.68	2.63±0.22	ND-4.57	1 (3.45)
Scramble	22	2.55±0.20	ND-3.85	2.29±0.26	ND-3.68	0

[a] The hygienic limit for total fungal counts is 4 log cfu/g of feed sample (PDV, 2009).
[b] Not detected (detection limit: 2 log cfu/g).

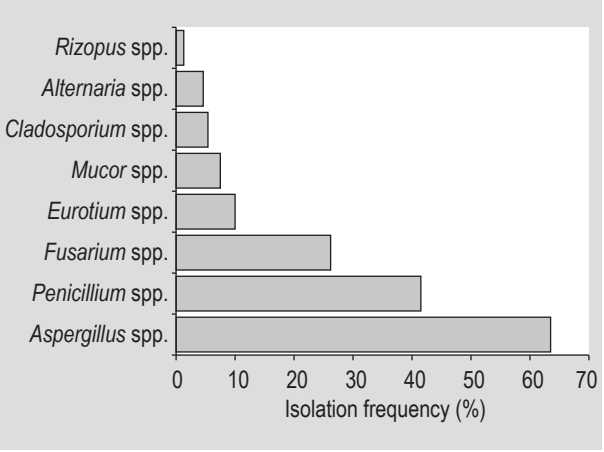

Figure 1. Isolation frequency (%) of different fungal genera isolated from fish feed.

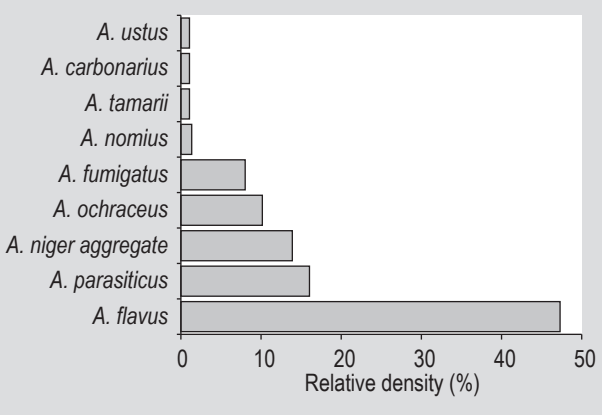

Figure 2. Relative density (%) of *Aspergillus* species isolated from fish feed.

be overlooked. Although, presence of mycotoxigenic fungi does not absolutely indicate mycotoxin production, survival of these strains in fish feed samples could be a potential risk for aquaculture industry. Almeida *et al.* (2011) found that *A. flavus* and *A. niger* were frequently isolated from farmed sea bass feed samples.

In vitro aflatoxin production

The ability to produce aflatoxins was determined for 246 isolates of *Aspergillus* section *Flavi* from fish feed samples. The results showed that all *A. parasiticus* isolates, 85 *A. flavus* isolates (48.1%), and only one isolate of *A. nomius* (20%) were aflatoxigenic. The isolates of *A. tamarii* did not produce aflatoxins (Table 2). According to Pitt (2006) about 50% of naturally occurring *A. flavus* and all of the *A. parasiticus* strains can be aflatoxigenic, which is in good agreement with the results of the present study. Saleemi *et al.* (2010) found that 83.3% of *A. flavus* and 85.7% of *A. parasiticus* strains isolated from Pakistani poultry feed were aflatoxigenic. In a previous study conducted in Colombia, all of the *A. parasiticus*, 76.5% of *A. flavus* strains, and none of the *A. tamarii* and *Aspergillus oryzee* strains isolated from animal feedstuffs produced aflatoxins (Diaz *et al.*, 2009). In another study, all the strains of *A. flavus* and *A. parasiticus* isolated from super premium pet food in Brazil were able to produce aflatoxins (Campos *et al.*, 2009). The variations in aflatoxigenic potential in isolates from different parts of the world might be due to differences in climatic conditions, the media and analytical method used, soil composition, and competition with other mycoflora (Diaz *et al.*, 2009).

As can be seen in Table 2, *A. flavus* isolates produced only B aflatoxins (AFB_1 and AFB_2), while *A. parasiticus* produced all four major types of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2). Also, *A. nomius* isolate produced AFB_1 and AFG_1. The present study confirm the observations of Pitt (2006) who reported that *A. flavus* produced only B aflatoxins, while the others produced B and G types.

Aflatoxin analysis in fish feed

The occurrence and levels of aflatoxin B_1 and total aflatoxins in fish feed samples are presented in Table 3. Aflatoxin B_1 was detected in 74.3% of pellet (mean 9.87 µg/kg), 65.5% of extruder (mean 7.96 µg/kg), and 45.4% of scramble samples (mean 5.97 µg/kg). The levels of the toxin in 22.9% of pellet and 20.7% of extruder samples exceeded the limit of Institute of Standards and Industrial Research of Iran (ISIRI, 2002) for aflatoxin B_1 in animal feedstuffs

Table 2. Production of aflatoxins in czapek yeast extract agar (CYA) by strains of *Aspergillus* section *Flavi* isolated from fish feed.

Species	No. of strains	No. of aflatoxigenic strains (%)	Mean±sem (range) (µg/g of CYA)			
			Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
A. flavus	177	85 (48.1)	48.4±3.49 (0.03-91.8)	9.23±0.87 (0.01-23.5)	0	0
A. parasiticus	60	60 (100.0)	50.7±4.51 (0.45-110.3)	10.3±1.99 (0.07-31.8)	7.78±1.29 (0.05-19.4)	0.71±0.08 (0.02-1.09)
A. nomius	5	1 (20.0)	0.12	0	0.05	0
A. tamarii	4	0	0	0	0	0

Table 3. Occurrence of aflatoxin B_1 and total aflatoxins in fish feed.

Fish feed	No. of samples	Aflatoxin B_1			Total aflatoxins		
		No. of positive samples (%)	Mean±sem (min.-max.)[a]	No. that exceed regulation[b] (%)	No. of positive samples (%)	Mean±sem (min.-max.)[a]	No. that exceed regulation[b] (%)
Pellet	35	26 (74.3)	9.87±2.90 (0.54-59.7)	8 (22.9)	28 (80.0)	12.1±3.54 (0.95-68.5)	6 (17.1)
Extruder	29	19 (65.5)	7.96±2.47 (0.46-38.7)	6 (20.7)	20 (68.9)	9.71±2.93 (0.46-41.4)	3 (10.3)
Scramble	22	10 (45.4)	5.97±1.13 (0.55-9.13)	0 (0.0)	10 (45.4)	7.09±1.40 (0.55-11.2)	0 (0.0)

[a] Mean±sem and range given between brackets (µg/kg) in positive samples.
[b] The ISIRI (2002) limit for aflatoxin B_1 and total aflatoxins in animal feedstuffs are 10 and 20 µg/kg, respectively.

i.e. 10 µg/kg. The frequency of total aflatoxins in pellet, extruder and scramble samples was 80, 68.9 and 45.4%, with mean values of 12.1, 9.71, and 7.09 µg/kg, respectively. Considering the ISIRI (2002) limit for total aflatoxins in animal feedstuffs (20 µg/kg), 17.1% of pellet, and 10.3% of extruder samples had the toxin in concentrations in excess of the limit. In Turkey, Altuğ and Beklevik (2003) verified the presence of aflatoixins in 49.5% of fish feed samples, and 23.5% represented levels higher than 20 µg/kg. In contrast, Almeida *et al.* (2011) reported that aflatoxins were not present in any of the feed samples for farmed sea bass in Portugal.

4. Conclusions

The results of the present study indicated that the incidence of the aflatoxigenic fungi and aflatoxins in fish feed were relatively high, and it can provide a potential hazard for aquaculture industry and human health. The best way to deal with this problem is to reduce aflatoxins contamination in feedstuffs by improving processing and storage practices. At the same time, attention should be given to regular monitoring of aflatoxins in finished fish feed and feed ingredients. Finally, feed samples with high levels of aflatoxins must be prohibited for animal consumption by the governmental authorities.

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