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Prevalence and Mycotoxigenic Potential of Fungi in Fish Feed Collected from Fish Farms in Egypt with a Particular Reference to Aflatoxins Contamination

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Abstract

The current study is aimed to investigate the fungal contaminants in fish feed. Isolation of fungi was conducted on modified Dichloran 18% glycerol agar (DG18) and potato dextrose agar (PDA). Feed samples were assayed for aflatoxins using HPLC. A total of 43 species belonging to 19 fungal genera recovered from 45 fish feed samples. *Aspergillus* and *Penicillium* were the most predominant genera with isolation frequency values indicated the retrieval capability of DG18 over PDA medium. For instance, *Aspergillus* spp. recorded 60%, 53.3% while *Penicillium* spp. were 33.3%, 17.8% on DG18 and PDA respectively via direct plating. 41.4% of the tested isolates were mycotoxin producers. Aflatoxins B1, B2, G1 and G2 were detected by 6 out of 10 screened *Aspergillus* isolates. Fumitremorgins, Gliotoxin, Ochratoxin A and B, and Zearalenone were also detected. The feed samples of zero total count percentages (TC%) of *A. flavus* recorded the highest incidence of aflatoxins B2, G1 and G2 (2.3, 35.3 and 7.8 ng/g respectively). Meanwhile, the highest B1 concentration (3.7 ng/g) was recorded for the highest TC% interval studied (1:9 cfu/g). Thus, it is important to monitor the fungal load and mycotoxins in fish feed periodically using proper practical approaches.

KEYWORDS
Aflatoxins, *Aspergillus flavus*, Contamination, Fish feed, HPLC

INTRODUCTION

Egyptian aquaculture has witnessed a dramatic development over the past ten years, that drives the country to the sixth place worldwide in aquaculture production (Mehrim and Refaey, 2023). However, aquaculture sustainability is hindered by feed cost which accounts for 85% of production operating expenses (Adeleke *et al*., 2021). Diminishing this cost resolved when increased plant ingredients were introduced over the costly fishmeal. Nonetheless, agricultural crops are vulnerable to pre- and post harvest fungal invasion particularly when the moisture content of some sources is relatively high (>14%) (Neme and Mohammed, 2017).

Moreover, the climate change events as flooding, extreme heat, and water scarcity episodes, particularly in the Mediterranean basin, aggravate the challenges that agricultural crops facing (Mehrim and Refaey, 2023). High moisture levels combined with high temperatures (>25°C), prompt the growth of most fungi (El-sayed *et al*., 2015), whereas water depletion flourishes xerophilic molds which encounter low availability of substrate water (aw<0.85). These xerophiles play a key role in food and feed spoilage (Pitt and Hocking, 2022).

Some of these spoilage molds have the potential to produce mycotoxins. *Aspergillus*, *Penicillium*, and *Fusarium* are the most important fungal genera producing mycotoxins in food and feed. Aflatoxins include the four major types (B1, B2, G1 and G2) are the most ubiquitous mycotoxins that contaminate crops and most plant-based commodities and mainly produced by *A. flavus* and *A. Parasiticus*. Aflatoxin B1 is considered the most hazardous (Moretti and Susca, 2017). Depending on the exposure, aflatoxin-contaminated feed can adversely impact fish health. Highly contaminated feed caused acute aflatoxicosis in fish that could lead to raise mortality (Mahfouz and Sherif, 2015), while the prolonged feeding with lower aflatoxin concentrations caused tumors in livers and kidneys of fish (Agag, 2004).

Early investigations recorded mycotoxins contamination in Egyptian feed ingredients such as maize and rice germ (Abdelhamid, 1990). Later trails determined mycobiota and mycotoxins in the finished fish feed (El-Shanawany *et al*., 2005; Embaby *et al*., 2015; Khalifa *et al*., 2022). However, previous attempts overlooked elaborating xerophiles through fungal profiling via adopting specific culture media. Additionally, no trials related the natural occurrence of aflatoxins in fish feed to the detectable presence/absence of the main fungal producers in the same feed substrate.

Thus, the present study aimed to survey fungal species that contaminated finished fish feed collected from diverse fish farms distributed in Egypt. The influence of culture media and isolation techniques on the recovered fungi was also investigated and the natural co-occurrence of aflatoxins in some feed samples as well.

MATERIALS AND METHODS

Isolation and identification of fungi from feed samples

A total of 45 finished fish feed samples were collected from

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different fish farms in Egypt. The collected samples were placed in sealed sterile polyethylene bags and kept at 4oC until analysis. Ten grams of each sample were ground individually by an electric grinder. Dilution and direct plating techniques were used to isolate fungi from the feed samples. For dilution plates, isolation technique described by Johnson and Curl (1972) were followed. Meanwhile feed particles were inserted onto solidified agar for direct plating. Potato dextrose agar (PDA) was used as a routine isolation medium while, modified Dichloran 18% glycerol agar (DG18) as described by Hocking and Pitt (1980) was adopted to favor isolation and enumeration of xerophilic fungi. The inoculated plates were incubated at 25±2o C and checked up to 10 days. The identification of fungi was carried out according to Raper and Fennell (1965); Moubasher. (1993) and Pitt and Hocking (2009). For dilution method, the total count (TC) of each species per sample was calculated. Meanwhile, isolation frequency (percentage of samples in which each species was recorded/total examined samples) was considered for both isolation methods.

TLC screening of mycotoxigenic fungal species

Twenty-nine fungal isolates were assayed for mycotoxin production. One ml of spore suspension (5x106 spore/ml) of the each isolate was inoculated into 50 ml yeast extract sucrose broth medium (YES; 2 % yeast extract and 20% sucrose) and incubated at 25±2°C for 10 days as static culture (El-Shanawany *et al*., 2005). After incubation, each individual culture was mixed with chloroform (1:1 v/v), vortexed and then filtrated through Whatman No. 1 paper. The filtrate was separated using a separatory funnel and chloroform layer was passed over 2 g anhydrous sodium sulphate and evaporated in rotatory vacuum evaporator at 40°C (Khaddor *et al*., 2007). The clean extract was applied on silica gel Plate for detecting mycotoxins according to procedures described by Maenetje and Dutton (2007). TLC-plates were examined in UV-light (366 and 254 nm) after elution in chloroform/ methanol (97:3).

Fish feed screening for natural co-occurrence of aflatoxins (AFs) by high-performance liquid chromatography (HPLC)

Fifteen out of 45 fish feed samples were investigated for aflatoxins contamination by using HPLC method. The tested samples were nominated based on the data of total count percentage (TC%) of aflatoxin producers (*A. flavus* and *A. Parasiticus*). Twenty five grams of each sample were grinded then shaken individually for 30 min in 100 ml of methanol/water (55:45 v/v). Extraction procedure performed as previously described and analyzed using high-performance liquid chromatography (HPLC) according to the official AOAC method validated by GazioĞlu and Kolak (2015). Aflatoxins detection and quantification were achieved by Waters 2475 Multi λ Fluorescence Detector (Waters, Milford, MA, USA).

RESULTS AND DISCUSSION

Mycoflora in fish feed

The current investigated feed samples revealed high infestation with diverse fungal contaminants. A total of 43 fungal species belonging to 19 genera were recovered from the feed on both DG18 and PDA media by using dilution and direct plating techniques. Data presented in Table 1 showed that *Aspergillus* and *Penicillium* were the most dominant genera. *Aspergillus* represented by 11 species and two varieties of which *A. flavus*, *A. niger*, *A. glaucus* and *A. Parasiticus* were the most dominant species with isolation frequency of 31.1%, 24.4%, 20.0%, 11.1% on DG18 medium respectively. These findings are in accordance with the results found by Almeida *et al*. (2011) where *A. flavus*, *A. niger*, *A. glaucus* were the most frequently isolated mold from the tested fish feed as 40.2%, 39.1%, 29.9% respectively. *Penicillium* came as the second predominant genus represented by five species of which *P. chrysogenum* recorded the highest frequency (almost around 22%) over the other species. This is quite in agreement with Mohamed *et al*. (2017), where they documented *Penicillium* spp. as the second most abundant genera of which *P. brevicompactum* was dominant in Nile tilapia feed.

It is noteworthy that both former fungi revealed much more preference for DG18 medium over PDA. It was elaborated by their total count (cfu x 10/ g sample), where *Aspergillus* spp. recorded 212.7 on DG18 compared to 135.7 on PDA, while *Penicillium* spp. encountered 48.7 on DG18 compared to 31 on PDA. In addition, marginal xerophilic species in both genera revealed higher total count (cfu x 10/ g sample) on DG18 medium than on PDA such as *A. flavus* (64.7, 38.7), *A. glaucus* (117.7, 38.3), *P. chrysogenum* (40.7, 21.7) and *P. brevicompactum* (7.3, 1.3) respectively as visualized in Table 1. These observations are in accordance with results achieved by Copetti *et al*. (2009). They observed the highest incidence values of *Aspergillus* spp. and its teleomorph *Eurotium* spp. and then *Penicillium* spp. in commercial dry pet food, when using DG18 compared to other adopted media. They attributed this remark to the reduced water activity of DG18 media (aw = 0.955) that prompted such slow growing xerophiles to compete efficiently with the other osmophiles.

Regarding the influence of isolation technique on fungal diversity it was observed that some of *Hyphomycetes* such as *Biopolaris tripogenis*, *Stemphyllum vesicarium*, and two *Trichoderma* spp., despite they detected only once in this study, showed reluctance to be recovered with dilution technique on both media. With similar low incidence, other workers recorded one or more of the former species (Almeida *et al*., 2011; Barbosa *et al*., 2013). Nevertheless, with careful examination of relevant literature, a lack of knowledge considering the correlation between isolation techniques and fungal diversity was found.

Screening of Toxigenic Fungal species

Twenty-nine fungal isolates belonging to *Aspergillus*, *Penicillium* and *Fusarium* were assayed for toxigenic potentiality. Table 2 showed that out of the five *A. flavus* isolates tested: four were found to be aflatoxigenic (one isolate produced aflatoxins B1, B2, G1 and G2, two isolates produced only aflatoxins B1 and one isolate was G1 producer). Meanwhile, two out of five *A. Parasiticus* isolates produced both aflatoxins B1 and G1. Similarly, many researchers detected the four types of aflatoxins in *A. flavus* culture filtrates. El-Shanawany *et al*. (2005) detected different mycotoxins in silage samples as well as fungal isolates recovered thereof. Further, the toxigenic potential of some *A. flavus* and *A. niger* isolates recovered from stored seeds were tested by Al-Abdalall (2009), finding the four previously mentioned aflatoxin types.

Three isolates of *A. ochraceous* were screened for ochratoxin production, one produced Ochra A and another was Ochra B producer. These toxins are accused of liver and kidney cancer through prolonged exposure (Reddy and Bhoola, 2010). Gliotoxin was also determined by two *A. fumigatus* isolates out of five tested, while fumitremorgins produced by another third one. Land *et al*. (1987) reported that persons who have worked with moldy feed containing *A. fumigatus* were in serious neurological syndromes risk. Only one *Fusarium solani* extract out of the three

screened was able to produce Zearalenone. Nonetheless, none among the tested *Penicillium* extracts exerted any specific toxins.

Detection of aflatoxins in feed samples

Total count percentage (TC%) for *A. flavus* and *A. Parasiticus* was the criteria of choice in nominating feed samples for aflatoxins screening by HPLC. Five groups of total count group intervals (0.0, 0.1:0.9, 1:9, 10:20, 21:30 cfu/g sample) were allocated. After that, cases numbers of *A. flavus* total count that fit into each interval were expressed in parentheses. The former values calculated in relation to the total feed samples, where n=45, to

Table 1. Frequency percentages of fungi recovered from 45 fish feed samples and total count (TC) expressed as colony forming unit (CFU) per gram on Potato Dextrose Agar (PDA) and modified Dichloran 18% Glycerol Agar (DG18) media. Values in parentheses represent number of fungi in all examined samples; – = Not detected.

Isolated Fungi	Direct plating		Dilution plate			
	DG18	PDA	DG18		PDA	
	Frequency%	Frequency%	Frequency%	TC x 10 CFU/ g	Frequency%	TC x 10 CFU/ g
Absidia corymbifera	(2) 4.4	(3) 6.7	(2) 4.4	2	(3) 6.7	5.7
Alternaria alternata	(2) 4.4	(1) 2.2	(1) 2.2	0.3	(1) 2.2	0.3
Alternaria chlamydospora	-	-	-	-	(1) 2.2	0.3
Aspergillus spp.	(27) 60	(24) 53.3	(25) 55.6	212.7	(23) 51.1	135.7
A. candidus	-	-	(1) 2.2	4.7	(1) 2.2	0.3
A. egyptiacus	-	-	(1) 2.2	1.7	-	-
A. flavus	(9) 20.0	(6) 13.3	(14) 31.1	64.7	(12) 26.7	38.7
A. fumigatus	(2) 4.4	(2) 4.4	(1) 2.2	0.7	(3) 6.7	13
A. fumigatus var. albus	-	-	(1) 2.2	-	-	-
A. glaucus	(5) 11.1	(4) 8.9	(9) 20.0	117.7	(6) 13.3	38.3
A. niger	(13) 28.9	(12) 26.7	(11) 24.4	10.7	(5) 11.1	3.7
A. ochraceous	(1) 2.2	(2) 4.4	(5) 11.1	5.7	(2) 4.4	3.7
A. Parasiticus	(2) 4.4	-	(5) 11.1	6.7	(7) 15.6	34.3
A. sydowii	(1) 2.2	(1) 2.2	-	-	(1) 2.2	0.33
A. terreus var. aureus	(1) 2.2	(1) 2.2	(1) 2.2	0.3	-	-
A. ustus	(1) 2.2	-	-	-	(1) 2.2	2.3
A. versicolor	-	-	-	-	(2) 4.4	1
Biopolaris tripogenis	-	(1) 2.2	-	-	-	-
Chaetomium sp.	(1) 2.2	-	-	-	-	-
Cladosporium herbarum	(3) 6.7	(1) 2.2	(1) 2.2	0.7	(1) 2.2	0.3
Cladosporium cladosporiodes	(2) 4.4	-	(1) 2.2	2.3	-	-
Eurotium sp.	(5) 11.1	(3) 6.7	(2) 4.4	2.3	(1) 2.2	4
Eurotium repens	-	-	(1) 2.2	0.3	-	-
Emercilla nidulance	(2) 4.4	(1) 2.2	-	-	(2) 4.4	0.7
Fusarium solani	(1) 2.2	(1) 2.2	(3) 6.7	26.7	(3) 6.7	6.3
Fusarium monilliform	-	-	-	-	(1) 2.2	0.7
Fusarium verticillioides	-	-	-	-	(1) 2.2	16.3
Monoascus sp. 1	(1) 2.2	-	-	-	(1) 2.2	0.7
Monoascus sp. 2	-	-	(1) 2.2	3	(1) 2.2	5.3
Mucor hiemalis	-	-	(4)8.9	1.7	(1) 2.2	1
Mucor racemosus	(1) 2.2	(2) 4.4	-	-	-	-
Nigrospora oryzae	-	(3) 6.7	-	-	(1) 2.2	0.3
Paecilomyces variotii	(3) 6.7	(3) 6.7	(2) 4.4	10.7	(1) 2.2	0.7
Penicillium spp.	(15) 33.3	(9) 17.8	(15) 33.3	48.7	(14) 31.1	31
P. chrysogenum	(10) 22.2	(7) 15.6	(11) 24.4	40.7	(10) 22.2	21.7
P. brevicombactum	(3) 6.7	(1) 2.2	(3) 6.7	7.3	(3) 6.7	1.3
P. waxmanii (monoverticillata)	(1) 2.2	-	-	-	-	-
P. puberulum	(1) 2.2	-	(1) 2.2	0.7	-	-
P. oxalicum	(1) 2.2	-	-	-	(1) 2.2	8
Stemphyllum vesicarium	(1) 2.2	(4) 8.9	-	-	-	-
Rhizomucor sp.	-	(1) 2.2	-	-	(1) 2.2	-
Rhizopus stolonifer	(4) 8.9	(5) 11.1	(1) 2.2	0.3	(3) 6.7	1.7
Trichoderma harzianum	-	(1) 2.2	-	-	-	-
Trichoderma koningii	-	(1) 2.2	-	-	-	-
Ulocladium utrum	-	-	(2) 4.4	1.3	(2) 4.4	0.7
Yeast	(1) 2.2	(4) 8.9	(1) 2.2	0.3	(9) 20.0	110.3
Total species average count/medium				313.3		322

Table 2. Thin Layer Chromatograph Screening for Mycotoxins produced by selected isolates (29) of fungal isolates.

Species	No of isolates tested	Characteristic mycotoxins	No. of producing isolates
Aspergillus flavus	5	AFB1, AFG1	1
		AFB1	2
		AFG1	1
A. fumigatus	5	Gliotoxin	2
		Fumitremorgens	1
A. ochraceous	3	Ochra B	1
		Ochra A	1
A. Parasiticus	5	AFB1, AFG1	2
Fusarium solani	3	Zeralenone	1
Penicillium chrysogenum	5	Roquefortine C	-
P. brevicompactum	3	Mycophenolic acid	-

Table 3. Total count percent (TC%) of *Aspergillus flavus* per each sample out of the forty-five Feed Samples Total count (TC) expressed as colony forming unit (CFU) per gram on Potato Dextrose Agar (PDA) and modified Dichloran 18% Glycerol Agar (DG18) media at 25±2°C, and the detected aflatoxins (B1, B2, G1 and G2) in 15 feed samples 5 per each TC range category.

Group no.	TC range / sample (cfu/g)	TC% *A. Parasiticus*		TC% of *A. flavus*		Aflatoxin Fraction (ng/g)			
		PDA	DG18	PDA	DG18	B1	B2	G1	G2
Group 1	0	(39) 86.7	(40) 88.9	(30) 66.7	(30) 66.7	2.3±1.7	2.3±1.9	35.3±27.3	7.8±9.9
Group 2	0.1: 0.9	(4) 8.9	(2) 4.4	(8) 17.8	(6) 13.3	1.2±1.2	0.3±0.3	13.0±26.2	4.0±6.5
Group 3	1:09	(2) 4.4	(3) 6.7	(5) 11.1	(7) 15.6	3.7±5.1	1.6±1.2	18.2±20.6	2.4±4.2
Group 4	10:20	-	-	(1) 2.2	(1) 2.2	ND	ND	ND	ND
Group 5	21:30	-	-	(1) 2.2	(1) 2.2	ND	ND	ND	ND

determine TC%. Similirly, *A. Parasiticus* TC% was calculated. The first three groups were only assigned for aflatoxin screening. The means and standard deviation of aflatoxins (B1, B2, G1 and G2) were calculated for each group interval.

The results illustrated in Table 3 revealed that the first tested group that recorded no culturable aflatoxin producers (neither *A. flavus* nor *A. Parasiticus*) was surprisingly experienced the highest incidence of aflatoxin B2, G1 and G2 (2.3, 35.3 and 7.8 ng/g respectively) compared to the other tested groups. Meanwhile, the highest B1 concentration (3.7 ng/g) was recorded for the highest count range group studied (1:9 cfu/g). These data are in accordance with many studies that reported aflatoxin contamination in fish feed. Mohamed *et al*. (2017) determined Aflatoxin B1 in 21 out of 25 fish feed samples with concentrations ranging between 0.07 and 20.0 µg/kg. Further, a total of 15 fish feed samples were analyzed for aflatoxins through an experiment adopted by Victoria *et al*. (2020) finding that 87.5% of samples were contaminated with AFB1 ranging between 0.1 to 16.5 µg/kg.

However, few attempts related the existence of *A. flavus* and/or *A. Parasiticus*, as main aflatoxin producers, to the natural occurrence of aflatoxin in certain substrate. Bullerman *et al*. (1984) clarified that the presence of mycotoxins in certain substrate does not necessarily indicate the existence of producer molds. Additionally, if they are produced, they may remain in the substrate for a while longer the survival of the mold itself. Jaimez *et al*. (2003) detected aflatoxins in 21% of the analyzed feed samples and in 23% of feed raw material, with maximal amounts of 2.8 and 6.0 µg/kg of aflatoxin B1 respectively. They also notified that there is no direct link between the evident presence of *A. flavus* strains with demonstrated aflatoxin production capacity and the existence of aflatoxins in the same substrate. Moreover, Victoria *et al*. (2020) attributed the mycotoxin contamination of feed despite the absence of potential fungal producer to the limitation of culturing technique that might omit some molds due to the unsuitability of *in vitro* culture conditions.

CONCLUSION

The current study revealed that *Aspergillus* spp. were the dominant genera in the studied fish feed and some of which exerted toxigenic potentiality. Thus, proper and periodical monitoring of fish feed microbiological quality is of great importance. In this concern, adopting specific culture media plays a key role in reliable assessment of fungal load.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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