Mycobiota and aflatoxin B_1 contamination of rainbow trout (*Oncorhinchus mykiss*) feed with emphasis to *Aspergillus* section *Flavi*

Alinezhad S.[1]; Tolouee M.[2]; Kamalzadeh A.[3,4]; Motalebi A. A.[5]; Nazeri M.[6]; Yasemi M.[3]; Shams-Ghahfarokhi M.[7]; Tolouei R.[8]; Razzaghi-Abyaneh M.[1, 8]*

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Abstract

In the present study, natural occurrence of fungi and aflatoxin B_1 (AFB_1) in trout pellet feed and feed ingredients randomly obtained from feed markets was investigated. The samples were cultured on the standard isolation media for 2 weeks at 28 ºC. Identification of fungal isolates was implemented based on the macro- and microscopic morphological criteria. AFB_1 was detected using high performance liquid chromatography (HPLC). Based on the results obtained, a total of 109 fungal isolates were identified of which *Aspergillus* was the prominent genus (57.0%), followed by *Penicillium* (12.84%), *Absidia* (11.01%) and *Pseudallscheria* (10.10%). The most frequent *Aspergillus* species was *A. flavus* (60.66%) isolated from all the feed ingredients as well as pellet feed. Among 37 *A. flavus* isolates, 19 (51.35%) were able to produce AFB_1 on YES broth in the range of 10.2 to 612.8 µg/g fungal dry weight. HPLC analyses of trout feed showed that pellet feed and all feed ingredients tested except gluten were contaminated with different levels of AFB_1 in the range of 1.83 to 67.35 µg/kg. Unacceptable levels of AFB_1 were reported for feed including soybean, fish meal and wheat. These results indicate the importance of AF contamination of trout feed in amounts higher than the acceptable level as a risk factor for fish farming production.

Keywords: Aflatoxin, *Oncorhynchus mykiss*, *Aspergillus flavus*, Mycobiota, Trout feed

1-Department of Aquatic Animal Disease, Faculty of Specialized Veterinary Science, Islamic Azad University, Science and Research Branch, Tehran, Iran.
2- Department of Horticulture, Faculty of Agriculture, Azad Karaj University, Karaj, Iran.
3- Institute of Scientific-Applied Higher Education of Jahad-E-Agriculture, Tehran 1457896681, Iran.
4- Animal Sciences Research Institute, Karaj 31585-1483, Iran.
5- Iranian Fisheries Research Organization, Tehran 14155-6116, Iran.
6- Chineh Feed Mill Co., Hashtgerd 14565-455, Iran.
7- Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran 14115-331, Iran.
8- Department of Mycology, Pasteur Institute of Iran, Tehran 13164, Iran.
*Corresponding author's email: mrab442@yahoo.com

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Introduction

Secondary metabolism is a hallmark of filamentous fungi and the diversity and complexity of secondary metabolites are astounding (Bennett and Klich, 2003). Various molds are capable of producing toxic secondary metabolites named "mycotoxins". A wide range of agriculture commodities, food and feedstuffs are susceptible to contamination with toxigenic moulds which are mainly belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* (Bennett and Klich, 2003). They invade crops in the field before and during harvest and may grow on food and feed during storage under suitable conditions of temperature and humidity (Smith et al., 1995; Berry, 1998). Aflatoxins (AFs) are important fungal toxic compounds, which are produced by an expanding list of closely related fungi mainly belong to *Aspergillus* section *Flavi* specially *Aspergillus flavus* and *A. parasiticus* (Eaton and Groopman, 1994). These fungi are widely distributed in soil, air, organic materials and plant parts all over the world (Bennett and Klich, 2003). Toxigenic *A. flavus* strains that possess all necessary genes for AF biosynthesis produce either AFB_1 and/or AFB_2. All the *A. parasiticus* strains produce AFG_1 and AFG_2, in addition to AFs B (Eaton and Groopman, 1994).

Among 20 known AFs, AFB_1 is the most important toxic compound that causes serious risk to human and animal health (Coulombe, 1993; Hussein and Brasel, 2001; Diaz et al., 2009; Sepahdari et al., 2010). It is a hepatotoxic, carcinogenic, mutagenic, teratogenic and immunosuppressive agent in various animal species from aquatic animals to terrestrial livestock. Due to extreme toxicity and wide spread occurrence in staple food and feed, AFB_1 currently is one of the most important mycotoxins that is under regulation by the Food and Drugs Administration (FDA) (Coulombe, 1993).

Despite the interesting data have now been exist about aflatoxicosis in animals, the presence and impact of AFs in farmed aquatic species are still underestimated. Very little has been documented on the toxicity of AFB_1 for cultured aquatic invertebrates fed by artificially contaminated diets. The history of aflatoxicosis in fish returns to nearly 50 years ago when an outbreak of hepatoma was reported in rainbow trout hatcheries in USA due to fed with AF contaminated cottonseed meal as a raw ingredient in trout meal (Ashley and Halver, 1963).

It seems that aflatoxicosis is a disease that can affect various freshwater aquatic species and arises when feed contaminated with AFs is eaten by the fish (Ashley, 1970; Bautista et al., 1994). AF contamination of feed and feed ingredients used in aquaculture has been reported for a wide range of substrates including rice, maize, peanuts, cottonseed, fish meal and dried fish (Ashley and Halver, 1963; Ashley, 1970). AFs exert a substantial impact on the fish farming production, causing anemia, hemorrhage, liver damage, weight loss, increased susceptibility to secondary infectious diseases, increased mortality and a gradual decline of reared fish stock quality, thus representing a significant problem in aquaculture systems (Santacroce et al., 2008). The carcinogenic effect of AFB_1 has been studied in fishes such as

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salmonid, rainbow, channel catfish, tilapia, guppy and Indian major carps and *Penaeus mondon* (Jantrarotai and Lovell, 1990; Tacon, 1992; Chavez et al., 1994; Lovell, 2001; Spring and Fegan, 2005).

It has been demonstrated that rainbow trout is extremely sensitive to AFB_1 (Cagauan, 2004; Eaton and Groopman, 2004). In general, trout are carnivorous in natural habitat, and therefore they are unlikely to be exposed to the toxin. However, in culture farms in which they are provided with commercial mixed feed comprising some different feedstuffs such as soybean meal, fish meal, wheat flour, starch, gluten as well as vegetable oil, vitamin, mineral and other additives, they are likely to be exposed to some different mycotoxins (Chelkowski, 1991; Yiannikouris and Jouany, 2002; Binder, 2006; González-Pereyra et al., 2008). Improper methods of feed processing and storage are among the most important factors favoring the growth of AF-producing molds and they are major elements that can be controlled by the fish producer (Payne et al., 1988; Fraga et al., 2007).

Cultured rainbow trout is one of the most important sources of sea foods in Iran. Based on the Food and Agricultural Organization (FAO) report, annual production of rainbow trout reached to around 618,000 tons in 2006 in the world of which 47,275 tons were produced in Iran as the forth producer of rainbow trout (FAO, 2008). With respect to the above-mentioned data and the significance of AFs in aquaculture, evaluation of the natural occurrence of AFs and aflatoxigenic fungi in trout feed is quit important. The present study was undertaken to determine the total mycobiota in trout feed ingredients and pellet feed with emphasis to AF-producing *Aspergillus* species and to investigate the natural occurrence of AFB_1 in feed ingredients and pellet feed used in trout nutrition.

Materials and methods

Chemicals

AFB_1 standard was the product of Sigma Chemical Co. St. Louis, Mo. USA. TLC silica gel 60 F_{254} plates were purchased from E. Merck, Germany. All other solvents and reagents were of analytical grade prepared from E. Merck, Germany unless otherwise specified.

Sample Preparation

Feed ingredients comprising soybean, wheat, wheat flour, fish meal, starch, gluten, as well as pellet feed were collected from a feed manufacturing plant for rainbow trout. One sample (10 kg each) was taken every month from March to July 2009 according to the approved method No. 7570, Institute of Standard and Industrial Research of Iran (ISIRI). The subsamples (1 kg each) were randomly selected for mycological and toxicological examination. All the samples were stored at 4 ºC before use.

Mycobiota Determination

A. parasiticus NRRL 2999, a known producer of AFs B and G series was used throughout the study as a control. To isolate the fungi, the subsamples of each feed were separately cultured on selective isolation media using spread plating method (Samson et al., 2000). At first, the samples were homogenized and then a part of each sample weighing 20 g mixed with

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180 ml of saline solution (0.85% sodium chloride) and 0.05% Tween was shaken for 30 minutes. Then, 0.5, 0.75 and 1 ml of dilution was transferred onto Dichloran Rosebengal Chloramphenicol agar (DRCA) and *Aspergillus flavus*/*parasiticus* Agar (AFPA) as appropriate isolation media. The cultures were incubated at 28 ºC for at least 2 weeks and they were checked daily for any visible fungal growth. In general, *Aspergillus* section *Flavi* colonies in AFPA turn out to be yellow-orange in reverse color as a specific identification criterion. Fungal genera and species were also isolated by transferring the colonies grown on AFPA and/or DRCA to Potato Dextrose Agar (PDA) plates. Czapek dox (CZ) agar was used for specific observation of the morphological traits of the *Aspergillus* colonies. After purification of all the fungi, slide cultures of fungal colonies grown on PDA were prepared to determine microscopic characteristics. Final identification of *Aspergillus* section *Flavi* was done based on the morphological criteria (Raper and Fennel, 1965; Samson et al., 2000).

Screening of Aspergillus flavus Isolates for AF Production

Initial screening process was carried out by observation of blue flourescence on the aflatoxin-producing ability (APA) medium supplemented with 0.3% *β*-cyclodextrin under UV light (365 nm) according to Fente et al. (2001). *A. flavus* isolates were cultured on APA by direct inoculation of fungal spores in the center of agar plates (8 cm Dia.). Flourescence production on the medium was assessed after 7 days incubated at 28 ºC.

Culture Conditions on YES Broth

AF-producing ability of positive samples were confirmed using thin layer chromatography (TLC). For assessment of AFB_1, all the isolates were cultured on yeast extract-sucrose (YES) broth (2% yeast extract and 18% sucrose) medium according to Razzaghi-Abyaneh et al. (2006). The medium was divided in 5 ml aliquots in 25 ml capacity Erlenmeyer flasks and sterilized by autoclaving at 121 ºC for 15 min. The cultures incubated at 28 ºC for 96 h in static condition after inoculating with 1×10^6 fungal conidia/ml.

Fungal Growth Determination

All contents of each flask including the culture media and fungal biomass were filtered through a thin layer of cheese cloth and were then thoroughly washed with distilled water. Total mycelia was placed in a stainless steel container and allowed to dry at 80 ºC till a constant weight was obtained. The net dry weight of mycelia was then determined.

Determination of AFB_1 in YES Broth and Trout Feed

AFB_1 was extracted from YES broth with chloroform using a separatory funnel. For the feed samples, a 25 g portion of each subsample was extracted with 100 ml of acetonitrile: water (90: 10, v/v) by a blend jar according to the procedure of González Pereyra et al. (2008). The chloroformic extracts were then concentrated by a rotary evaporator (EYELA N-1000, Japan) near to dryness and analyzed using TLC on 20 × 20 cm silica gel 60 F_{254} plates (E. Merck, Germany). TLC plates were developed using chloroform-methanol (98:2, vol/vol)

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as mobile phase. AFs B_1 and/or B_2 were observed under UV light (365 nm) as blue spots. AF concentration was measured using high performance liquid chromatography (HPLC; KNAUER D-14163 UV-VIS system, Berlin, Germany) (Razzaghi-Abyaneh et al., 2009). Fifty microliters of each sample (culture filtrate) were injected into the HPLC column (TSKgel ODS-80TS; 4.6 mm ID × 15.0 cm, TOSOH BIOSCIENCE, Japan) and eluted at a flow rate of 1 ml/min. by water/acetonitrile/methanol (60:25:15, v/v/v). The amount of AFB_1 was calculated at a wavelength of 365 nm by comparison of under-curved area of unknown samples with authentic standards treated in the same manner. The retention time of AFB_1 was 11.0 min.

Statistical Analysis

The quantitative data of AFB_1 production were subjected to the Analysis of Variance (One-way ANOVA) in Tukey range. Aflatoxin B_1 was the factor and AFB_1 amounts in feed ingredients and pellet feed samples taken every month from March to July 2009 (5 samples for each feed) were dependent variables. The differences with $P<0.05$ were considered significant.

Table 1: Distribution of fungal genera in trout feed

Fungal genera	Number of isolates in:							
	Soybean	Wheat	Wheat flour	Fish meal	Starch	Gluten	Pelletfeed	Total[a]
Aspergillus	5	11	11	10	4	11	9	61 (55.96)
Mucor	0	0	1	0	1	0	0	2 (1.83)
Penicillium	3	0	5	0	0	4	2	14 (12.84)
Alternaria	0	0	1	0	0	0	1	2 (1.83)
Scopulariopsis	2	0	0	0	0	0	0	2 (1.83)
Paecilomyces	1	0	0	0	0	1	0	2 (1.83)
Absidia	2	0	5	0	1	4	0	12 (11.01)
Cladosporium	0	0	0	0	0	1	0	1 (0.92)
Rhizopus	0	0	0	0	1	0	0	1 (0.92)
Pseudallscheria	0	0	0	0	0	0	11	11 (10.10)
Ulocladium	0	0	0	0	0	0	1	1 (0.92)
Total	13	11	23	10	7	21	22	109

The numbers in parentheses are frequencies (%) of the fungal genera isolated from feed samples.

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Results

Mycobiota of Trout Feed

As indicated in Table 1, a total of 109 fungal isolates belong to the eleven genera with different frequencies were isolated from trout feed. The highest fungal contamination was related to wheat flour (21.10%), followed by pellet feed (20.18%) and gluten (19.27%). Members of the genus *Aspergillus* were reported from all the feed samples, while the genera *Pseudallscheria* and *Ulocladium* were exclusively isolated from pellet feed. The other 8 genera were randomly distributed in feed samples. The genus *Aspergillus* was the most frequent fungal group (57.0%), followed by *Penicillium* (12.84%), *Absidia* (11.01%) and *Pseuallscheria* (10.10%). Table 2 shows the frequency and distribution of *Aspergillus* species in trout feed. A number of 53 *Aspergillus* isolates belonged to the 5 species including *A. flavus* (60.66%), *A. niger* (19.67%), *A. fumigatus* (3.28%), *A. clavatus* (1.64%) and *A. ochraceus* (1.64%) as well as 8 *Aspergillus* spp. were isolated from feed samples. *A. flavus* as the most prevalent species was isolated from all feed ingredients and pellet feed.

Table 2: Frequency and distribution of *Aspergillus* species in trout feed

Aspergillus species	Feed sample							Total[a]
	Soybean	Wheat	Wheat flour	Fish meal	Starch	Gluten	Pellet feed	
A. flavus	5	11	7	6	4	2	2	37 (60.66)
A. niger	0	0	4	1	0	2	5	12 (19.67)
A. clavatus	0	0	0	0	0	1	0	1 (1.64)
A. fumigatus	0	0	0	1	0	1	0	2 (3.28)
A. ochraceus	0	0	0	0	0	1	0	1 (1.64)
Aspergillus spp.	0	0	0	2	0	4	2	8 (13.11)
Total	5	11	11	10	4	11	9	61

[*] The numbers in parentheses are frequencies of the *Aspergillus* species isolated from

Aflatoxigenic and Non-Toxigenic Strains of A. flavus

Table 3 illustrates the results of AF producing ability of the *A. flavus* isolates. Initial screening of the *A. flavus* isolates on APA showed that only 12 out of 37 isolates produced blue fluorescence under UV light. Based on TLC results, besides of

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all 12 APA positive isolates, 7 of APA negative ones were aflatoxigenic. These results indicated that among a total of 19 (51.35%) aflatoxignic *A. flavus* isolates, 8 (42.10%) were able to produce AFB_1 and 11 (57.90%) produced both AFs B_1 and B_2 on yeast-extract sucrose (YES) broth. HPLC analyses of toxigenic *A. flavus* culture filtrates revealed that AFB_1 was produced in the range of 10.2 to 612.8 µg/g fungal dry weight (Table 4).

Table 3: The incidence and frequency of aflatoxigenic and non-toxigenic *A. flavus* strains in trout feed

Feed sample	*Aspergillus flavus* isolates (No.)	Aflatoxin-producing isolates[a] (No.)		Non-toxigenic isolates (No.)	Blue fluorescence on APA under UV light (365 nm)
		B_1	B_1& B_2		
Soybean	5	3	1	1	2
Wheat	11	2	3	6	3
Wheat flour	7	1	2	4	1
Fish meal	6	0	3	3	3
Starch	4	1	1	2	2
Gluten	2	0	0	2	0
Pellet feed	2	1	1	0	1
Total	37	19 (51.35%)[b]		18 (48.65%)[b]	12 (32.43%)[b]

[a] Aflatoxin production was noticed as blue fluorescence on silica gel 60_{F254} TLC plates under UV (365 nm) light.
[b] The numbers in parentheses are the frequencies of the *Aspergillus flavus* isolates regard to aflatoxin and blue fluorescence producing ability.

Natural Occurrence of AFB_1 in Trout Feed

in trout feed showed that pellet feed and all feed ingredients tested except gluten were contaminated with different levels of the toxin in the range of 0.06 to 212.18 µg/kg (Table 4). The range of AFB_1 produced in fish meal, soybean, wheat, pellet feed, wheat flour and starch were as 1.48-212.18, 7.33-65.56, 2.06-23.99, 0.12-20.09, 0.06-5.47 and 0.58-4.00 µg/kg, respectively. As indicated in Table 4, fish meal was the most contaminated feed with an average AFB_1 level of 67.35 µg/kg, followed by soybean (30.88 µg/kg), wheat (12.40 µg/kg) and pellet feed (8.56 µg/kg). Wheat flour and starch had a low level of AFB_1 contamination with the averages of 2.29 and 1.83 µg/kg, respectively. The differences between the mean level of AFB_1 in fish meal and soybean were significant with those of wheat, pellet feed, wheat flour and starch (P<0.05).

Discussion

It has been estimated that approximately 25% of crops are affected by mycotoxins in the world. AF contamination of feeds

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use in aquaculture is a common problem that poses both economic and health concerns in fishery production especially in developing countries (Spring and Fegan, 2005; Santacroce et al., 2008). Among farming fish, rainbow trout is considered to be the most sensitive one to AFB_1. In the present study, trout pellet feed and feed ingredients randomly obtained from feed markets were evaluated for mycobiota and natural contamination with AFB_1. *Aspergillus*, *Penicillium* and *Absidia* were the most prominent genera isolated. It is interesting to note that the genus *Pseudallscheria* was the forth prominent fungal genera exclusively isolated from the pellet feed. The significance of contamination and pathogenecity of this fungus need to be studied further.

More than half of all the fungi isolated from trout feed in our study belonged to the genus *Aspergillus* of which around 60.0% were identified as *A. flavus*. The results obtained from some similar studies carried out in other countries are in line with the outcome of this study (Keller et al., 2007; Diaz et al., 2009). For instance, based on a study conducted on animal feedstuffs in Colombia, 54.40% of samples were found to contain *Apergillus* spp., of which 56.0% belonged to the members of *Aspergillus* section *Flavi* (Diaz et al., 2009). *A. flavus* (40.54%) has also been identified as the most prevalent *Aspergillus* species according to a survey implemented on equine feeds study (Keller et al., 2007). Among other *Aspergillus* species isolated in the present study, *A. ochraceus* is mycotoxin producer (i.e. ochratoxin A), *A. clavatus* is involved in the etiology of mycoses and fungal allergies, *A. niger* is human pathogen and an environmental contaminant and *A. fumigatus* is one of the most important agents of nosocomial fungal infections in the world. So, they must be considered as potential public health hazards affecting both human and animals.

Approximately 50% of *A. flavus* strains isolated from trout feed were able to produce AFs. AF producers were isolated from pellet feed and all feed ingredients except gluten. The percentage of aflatoxigenic fungi among *A. flavus* isolated from animal feed is depend on several factors including the type of feed, environmental conditions, culture conditions, detection method, etc. AF-producing ability by *A. flavus* strains has been reported in a wide range from 1.60% for the strains isolated from poultry feed (Labuda and Tančinová, 2006) to 76.0% for those isolated from animal feed in India (Dutta and Das, 2001). Cutuli et al. (1991) reported that 75.0% of *A. flavus* strains isolated from trout feed were aflatoxigenic on natural media.

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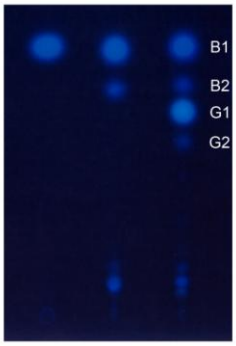


Figure 1: TLC profile of aflatoxin production on a silica gel 60 F_{254} plate. From left to right: AFB_1 standard, *A. flavus* A-11 (isolated from soybean) and *A. parasiticus* NRRL 2999.

Table 4: HPLC results of aflatoxin B_1 production by toxigenic *A. flavus* isolates on yeast extract sucrose (YES) broth and on naturally contaminated trout feed[*]

Feed source	Fungus	Fungal dry weight (mg)	AFB_1 concentration (µg/g dry weight)	AFB_1 occurrence in feed source (µg/kg)	
				Range	Mean
Soybean	*A. flavus* A-11	112.4 ± 18.0	10.2 ± 1.0	7.33-65.56	30.88
	A. flavus A-2	88.2 ± 12.1	329.7 ± 19.6		
	A. flavus A-23	85.2 ± 15.4	57.4 ± 6.6		
	A. flavus A-4	100.1 ± 9.8	612.8 ± 49.9		
Wheat	*A. flavus* A-15	103.7 ± 8.9	98.5 ± 5.4	2.06-23.99	12.40
	A. flavus A-12	78.9 ± 12.5	301.1 ± 20.9		
	A. flavus A-7	83.9 ± 9.7	143.8 ± 15.2		
	A. flavus A-18	109.2 ± 12.9	189.9 ± 15.7		
	A. flavus A-10	90.8 ± 13.6	183.0 ± 20.4		
Wheat flour	*A. flavus* A-1	95.1 ± 10.8	312.9 ± 23.5	0.06-5.47	2.29
	A. flavus A-19	115.3 ± 12.4	213.5 ± 39.1		
	A. flavus A-22	91.1 ± 11.7	118.7 ± 10.8		
Fish meal	*A. flavus* A-23	96.9 ± 8.7	412. 8 ± 22.6	1.48-212.18	67.35
	A. flavus A-17	106.3 ± 9.6	129.4 ± 18.7		
	A. flavus A-5	88.1 ± 10.6	511.7 ± 54.3		
Starch	*A. flavus* A-3	100.5 ± 12.9	88.2 ± 6.5	0.58-4.00	1.83
	A. flavus A-18	92.1 ± 7.3	286.4 ± 21.6		
Pellet feed	*A. flavus* A-37	93.4 ± 7.1	19.6 ± 2.3	0.12-20.09	8.56
	A. flavus A-21	80.8 ± 5.2	10.6 ± 1.9		

[a]No aflatoxin B_1 was detected in "gluten" which was contaminated with non-toxigenic *A. flavus* isolates.

Evaluation of natural occurrence of AFB_1 in trout feed in the present study revealed a relationship between the presence of AF-producing fungi and the occurrence of toxin in the feed samples. All feed samples contaminated with aflatoxigenic *A. flavus* strains were found to be contained different amounts of AFB_1. No AF was detected in gluten as a trout feed ingredient which it was not contaminated with AF-producing fungi. Although there is not an established guideline demonstrate the acceptable level of AFs for all the animal feed, Food and Drug Administration (FDA) approved a general action level of 20 ppb for AFB_1 in all feeds.

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In the present study, some feed ingredients including soybean, wheat and fish meal contaminated with aflatoxigenic fungi had unacceptable levels of AFB_1 (higher than 20 ppb). On the other hand, the level of AFB_1 in all wheat flour and starch samples was lower than the accepted level of 20 ppb. The amount of AFB_1 detected in pellet feed was in the acceptable level except for one sample which had a borderline level of 20.09 ppb. Unacceptable levels of AFB_1 in some animal feed samples have been reported by the other researchers as well (Charoenpornsook and Kavisarasai, 2006; Fraga et al., 2007; Keller et al., 2007; Motallebi et al., 2008).

On the basis of results obtained, different amounts of AFB_1 were produced by *A. flavus* strains isolated from each feed on YES broth. Likewise, a wide range of AFB_1 was detected in feed samples naturally contaminated with the AF-producing fungi. These results indicate that the ability of *A.* These results indicated that the ability of *A. flavus* strains isolated from each feed for AFB_1 production on YES broth was not necessarily correlated with the correspondence level of the toxin naturally produced in that feed. As a general fact, the presence of AF-producing fungi on food or feed does not necessarily mean the presence of AFs, vice versa. The condition for toxigenic fungal growth and AF production is different. Many factors such as substrate, pH, moisture and temperature affect the presence of AFs in a feed during storage. In fact, the *A. flavus* growth and AF production mostly occur in improperly stored feeds, feedstuff, and feed with inferior quality of ingredients in different seasons (Charoenpornsook and Kavisarasai, 2006). In fact, AF-producing fungi being killed or removed during processing, but the AFs remain in the final product.

Overall, results of the present study indicate that the high levels of AFB_1 in trout feed could be considered as a high risk for aquaculture as well as for the human health trough indirect exposure from fish meat consumption. Therefore, constant monitoring of trout feed for AF and AF-producing fungi is an urgent need which enable us to save the public health through omitting the potential sources of AF contamination from trout feed chain.

Conflict of interest statement

There is no conflict of interest for the authors.

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