Isolation and Identification of fungi in Aquaculture from Zawiya and Zuwara, Libya

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Abstract

Fish feed is very important for aquaculture production because it usually constitutes over 50% of the production costs and it has a significant impact on the quality, safety and nutritional value of farmed fish. It seems to be particularly prone to deposition in several fish tissues representing a risk for human consumption. *Aspergillus* spp is the major producer of aflatoxin that commonly contaminate food and feed all over the world. A total of 30 finished fish feed samples were collected from major feed market and feed factories some Zawiya and Zuwara farms. Fungal counts were obtained on PDA culture media in the ranges of $< 10^2$ to 5.04×10^4 $CFUg^{-1}$. A total of five genera of moulds were identified with *Aspergillus spp* being the most prevalent 60%, followed by *Penicillium* spp.16.6 %. Other fungi from the genera *Fusarium* spp. 10%, *Mucor* sp, 3.3% and *Alternaria* sp. 3.3% were found in smaller amounts. Despite good screening programs, selection of high quality raw materials and feed ingredients and good storage conditions it is difficult to guarantee the absence of mycotoxins in aquaculture feeds. Therefore, it is essential to develop effective methods for managing the risks posed by mycotoxin contamination.

Keywords: **aquaculture; fish feed, *Aspergillus spp,* mycotoxins**

Introduction

Aquaculture is currently the fastest growing animal production sector in the world. It is developing, expanding and intensifying in almost all regions of the world (Subasinghe et al., 2009) With growing demand for aquaculture comes increasing concern about the reliable supply of raw materials needed to support this growth. Aqua feeds traditionally depend on fishmeal as a protein source, but the trend in recent years has moved towards replacing fish meal with less expensive sources of protein of plant origin (Nagappan et al., 2021). As a result of this trend, aquaculture feeds have a higher risk of contamination by one or more types of aflatoxins. (Alberts et al., 2006 ; Oliveira et al., 2013).

With increased demand for fish as human food, fish farming is expanding all over the world to overcome animal protein deficiency. Seabass, Seabream and Nile tilapia is considered one of the most popular and cheapest fish in Libya. Now it is widely cultured because of its high growth rate, ability to withstand diseases, ability to spawn easily, in addition to the minimal requirements regarding management and energy inputs (Hams *et al*., 2017). Contamination of feed and other products by yeast and mold varies according to several factors such as moisture, temperature, and hygienic conditions (Milićević *et al*., 2010).

Fish feed is a major cost item in aquaculture industry representing around 50% of the total production costs(Marijani et al., 2019).. Plant-based ingredients used are associated with fungal contaminants during the initial stages of crop production, in addition, there are economic losses that result from contamination of crops and animal feed with mycotoxin (Bandyopadhyay and Cotty, 2011; Nogueira et al., 2020).). Feed can be contaminated during processing with fungal spores, particularly in grain grounding and feed pelleting (Embaby *et al*., 2015). Moreover, feed storage in addition to environmental factors may increase fungal growth in feed, and this in turn results in mycotoxin production which have been identified as a worldwide food and feed safety issue (Mahfouz and Sherif, 2015). In aquaculture, aflatoxins are the most frequently recorded mycotoxins, it seems to be particularly prone to deposition in several fish tissues representing a risk for human consumption (Gonçalves *et al.,* 2018). Aflatoxin is a toxic compound produced by *Aspergillus flavus and A. parasiticus*. The molds can grow in improperly stored feeds and feeds with inferior quality of ingredients. (Smith et al., 2016).

Aflatoxins represent a serious source of contamination in foods and feeds in many parts of the world. These toxins have been incriminated as the cause of high mortality in livestock and in some cases of death in human beings (Murjani, 2003; Abdallah et al., 2022. Aflatoxin B1 is known to be the most significant form that causes serious risk to animals and human health. The carcinogenic effect of aflatoxin B1 has been studied in fishes such as salmonid, seabream, rainbow trout, channel catfish, tilapia, guppy and Indian major carps (Lovell, 1992; Wu, 1998; Murjani, 2003) and *Penaeus monodon* (Bautista *et al*., 1994). There are very few researches regarding the effect of aflatoxin on Nile tilapia *Oreochromis niloticus* (Frisvad et al., 2006; Ehrlich et al., 2007). These aflatoxin producers are frequently isolated from contaminated food and feed all over the world. Animals exposed to aflatoxins through their diet undergo acute or chronic intoxication caused by mycotoxin ingestion (Bandyopadhyay and Cotty, 2011). For example, fish exposed to such mycotoxins and/or their producing fungi would have reduced growth rate when Seabream were fed diets containing 1.8 milligrams (mg) of AFB1 per one kilogram (kg) of feed for 75 days., In addition, tissue abnormality or lesions in the livers of these Seabream showed the beginnings of cancer development, reduced immunity and increased mortality (Tuan et al., 2002; GSO, 2019).
This may progress to a gradual decline in quality of reared fish stock and pose serious challenges to aquaculture industry (Fallah *et al*., 2014). It is nearly impossible to practically control the proliferation of fungi and subsequent mycotoxin contamination in most agricultural systems (Barbosa et al., 2013; Marijani et al., 2019). Consequently, numerous countries have established or proposed regulations for controlling aflatoxin in food and feed. Seabass and Nile tilapia is the most commonly farmed fish species in East Africa (Greco et al., 2015). Some farms depend on locally-made commercial fish feed produced by using locally available ingredients, whereas other farms sometimes use imported feed. Although fish feed quality standards exist in Libya, standards for manufacture, distribution, storage and handling of ingredients are not continously regulated. Chromatographic techniques, in particular high-performance liquid chromatography (HPLC), is one of the most accurate methods used for quantifying mycotoxins and nowadays is the most commonly used for detecting and identifying a wide diversity of natural toxic compounds including aflatoxins (Scarlett *et al.,* 2012). Few articles on mycotoxin contamination in fish feed have been reported in Africa(Njobeh *et al*.,

2012). The objective of this study is to examine the occurrence of fungal communities and isolation and identification in fish feed that is distributed and sold in Libya,

Materials and methods

Sample collection

The research materials consisted of 30 representative fished samples which were collected from two different source in Libya, 14 samples (*n* = 14) were collected from a major feed market, located in Zawiya and Zuwara city and the remaining 16 fish feed samples (*n* = 16) were procured from the fish farms of Zawiya and Zuwara city during a 2-month period. The samples (each about 500g) were stored at -4°C and analyzed the day after collection. Samples were divided into two part: one for determining moisture content and mycological examination, which were investigated immediately whereas the other part was stored at -20 until it was used for isolated and identification. To determine moisture content of feed samples, 2 g of each sample were dried in an oven with forced air circulation for 16 hours at 80 ºC. The samples (three replicates each) were weighed and the initial water content was determined according to the method described by (Dalcero *et al.*, 1998). Only plates containing 10-100 CFU were used for counting and the results were expressed as CFU per gram of sample. (the feed hygienic quality limits is 1×10^4 CFU G^{-1}) Good Manufacturing Practices (GMP, 2008). Selected colonies were transferred for further sub-culturing on plates with fresh agar media for purification.

Fungal isolation and identification

Fungi were isolated using the dilution plate technique, 10 g of each sample were mixed with 90 mL of 0.1% peptone water solution on a horizontal shaker at 220 rpm for 20 min at 25 °C to homogenize. Ten-fold appropriate serial dilutions were prepared before aliquots (1.0 mL) from each dilution were inoculated in triplicates onto plates of Potato Dextrose agar (PDA; Difco, USA)) using surface-spread method, Plates were incubated at 25°C for 7 days (Pitt and Hocking 2009). Selected colonies were sub-cultured on plates with fresh Czapek yeast extract agar (CYA, Sigma-Aldrich, Steinheim, Germany) media for purification. Single spore technique was also used to purify the obtained isolates as described by Leslie and Summerell (2006). Each isolated mould colony (reverse and observe color, size, shape) was observed microscopically for morphological characterization and identification to genera/species level. This was done by their macro- and micro morphology features using appropriate identification keys Pitt and Hocking (2009) identification keys.

Results and Discussion

Mycological isolation and identification

The presence of filamentous fungi in aquafeed which may develop in pre-harvest field conditions and/or post-harvest storage and handling has been a subject of pertinent concern particularly on their role in fish and the subsequent risk to human health (Gonçalves et al., 2020).

In the present study the presence of fungal communities in marketed fish feeds was studied. The fungal total count on all tested samples on PDA media in this study ranged from $< 10^2$ to 5.04×10^4 CFUg^{-1} with 46.6 % of the count $<1 \times 10^2$ and 20 % $> 1 \times 10^2$ CFU/g. (Table 1, Figure 1,2).

This demonstrates that majority of the sample had high levels of colony count which might be attributed to bad handling and storage practices. the samples analyzed in this study were above the levels designated as hygienic feed quality limits of 1×10^4 CFU/g (GMP, 2008).

These results were in line with those obtained in finished feed collected from tilapia farms in Brazil (Barbosa et al., 2013) and rainbow trout hatcheries in Argentina (Greco et al., 2015). Although in both studies, 10% and 10.7% of their respective analyzed samples were above the levels recommended as hygienic feed quality limits. The presence of high fungal colony counts particularly above permissible limits in animal feed suggest a decline in the palatability, edibility and overall nutritional value of the feed for animal nutrient absorption.

The most frequently isolated fungus was *Aspergillus* spp (18) 60 %., Other frequently isolated moulds included *Penicillium* spp. (05) 16.6%, *Fusarium* spp. (03)10 %, *Mucor* sp. (01)3.3 % and *Alternaria* sp. (01) 3.3% were identified. (Table 2. Figure 3,4,5). Many studies have confirmed similar predominance of *Aspergillus* and *Penicillium* species in the fish feed pellets (Pereyra et al., 2011). in fish feed. In the present study, *A. parasiticus* was the most occurring fungi followed by *P.chrysoganum* and *P. citrinum*. High percentage of *A. parasiticus and A.flavus* had been reported in aquafeed samples in Brazil (Cardoso et al., 2013) and in finished fish feed samples in Kenya (Marijani et al., 2017). *A. parasiticus, A. flavus* is one the major producer of AFs and *P. chrysoganums* produces OTA (Oliveira and Vasconcelos, 2020). *A. parasiticus and A. niger* were also identified which are also producer of AFs and OTA, respectively (Kholife et al., 2019). The isolation of a *Fusarium* spp. in this study corresponds to the results obtained by (Tan et al., 2011.), the *Fusarium* specie was morphologically identified as *F. solani*. In Libya.

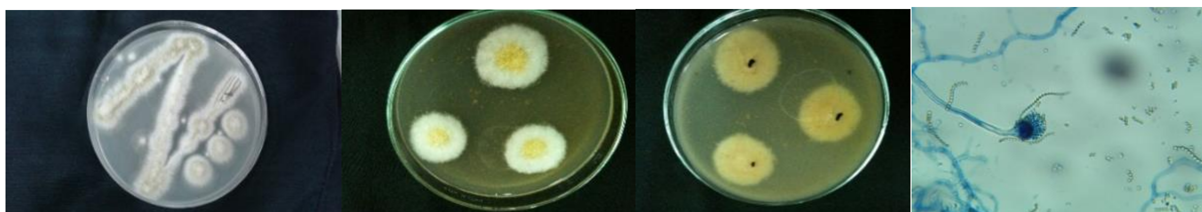

Fig .1: Morphological identification of *Aspergillus parasiticus* showing Colony on Czapek yeast extract agar (CYA), Colony reversed, vesicle (seen under light microscope at 40×).

Fig .2: Morphological identification of *Aspergillus flavus* showing, Colony on CYA, Colony reversed, and vesicle (seen under light microscope at 40×).

Table .1: Distribution of isolated fungi among fish feed samples

Samp .code	name of each sample	Content protein (%)	Moisture content	CFU/gm	Isolated fungi
1.	Floating feed	30%	0.019	< 10^2	*A,niger*
2.	sinking feed	25%	0.067	2.7 x 10^3	*F. solani*
.3	sinking feed	25%	0.053	< 10^2	*,A.parasiticus*
4.	sinking feed	25%	0.064	< 10^2	*A. parasiticus.*
5.	sinking feed	25%	0.053	3.4 x 10^3	*A.Flavus,*
6.	Sinking feed	25%	0.147	5.04 x 10^4	*A.niger, Mucor sp*
7.	Floating feed	32%	0.079	< 10^2	*P.chrysogenum*
8.	sinking feed	30%	0.072	4.2 x 10^4	*A. parasiticus,P.citrinum.*
9.	sinking feed	30%	0.063	3.2 x 10^2	*A.Flavus, P.sajarovii*
10.	sinking feed	30%	0.068	4.6 x 10^2	*A. parasiticus., A.Flavus*
11.	sinking feed	30%	0.071	< 10^2	*A. parasiticus,*
12.	sinking feed	30%	0.141	4 x 10^4	*A. parasiticus.*
13.	Floating feed	30%	0.065	5.01 x 10^4	*A.Flavus,*
14.	sinking feed	25%	0.079	< 10^2	*,P.chrysogenum,*
15.	sinking feed	28%	0.067	2 x 10^3	*A.terreus, Pinicillium.sp*
16.	sinking feed	28%	0.056	2.4 x 10^4	*A.parasiticus*
17.	Floating feed	35%	0.044	3.8 x 10^4	*F. oxysporum, A. parasiticus.*
18	sinking feed	25%	0.085	2.4 x 10^4	*F. oxysporum* *A. parasiticus.*
19.	Floating feed	30%	0.055	3.6 x 10^3	*A. parasiticus,*
20.	Floating feed	30%	0.042	5.4x 10^3	*A.niger. Alternaria sp.*

 Maximum recommended level (feed hygienic limit): 1 x 104 CFU-1

 Detection limit: 1 x 10^2**CFU**

The number of isolates, the isolation frequency and the relative density of the identified fungi are listed in Table (2).

Fungi species	No. of isolates	Fr[a] (%)	RD[b] (%)
Aspergillus spp	18	60	64.3
Penicillium spp	5	16.6	17.8
Fusarium spp	3	10	10.7
Mucor sp	1	3.3	3.5
Alternaria sp	1	3.3	3.5
Total No. of fungal isolates	28		
Total samples	30		

[a]: Frequency = $(\frac{ns}{N})$ × 100, ns: the number of samples whereas genus/species occurred, N: the total number of collected samples.

[b]: Relative density = $(\frac{ni}{Ni})$ × 100, ni: the number of isolates of a genus/species, Ni: the total number of fungal isolates

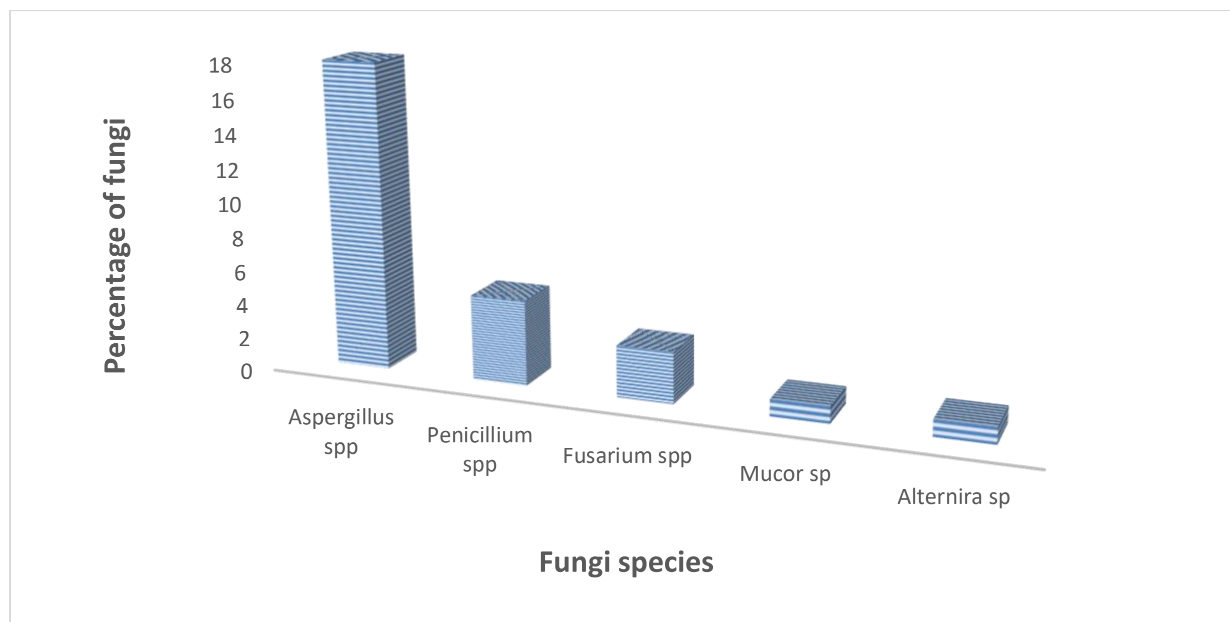

Fig. 3. Percentage of moulds contamination fish feed

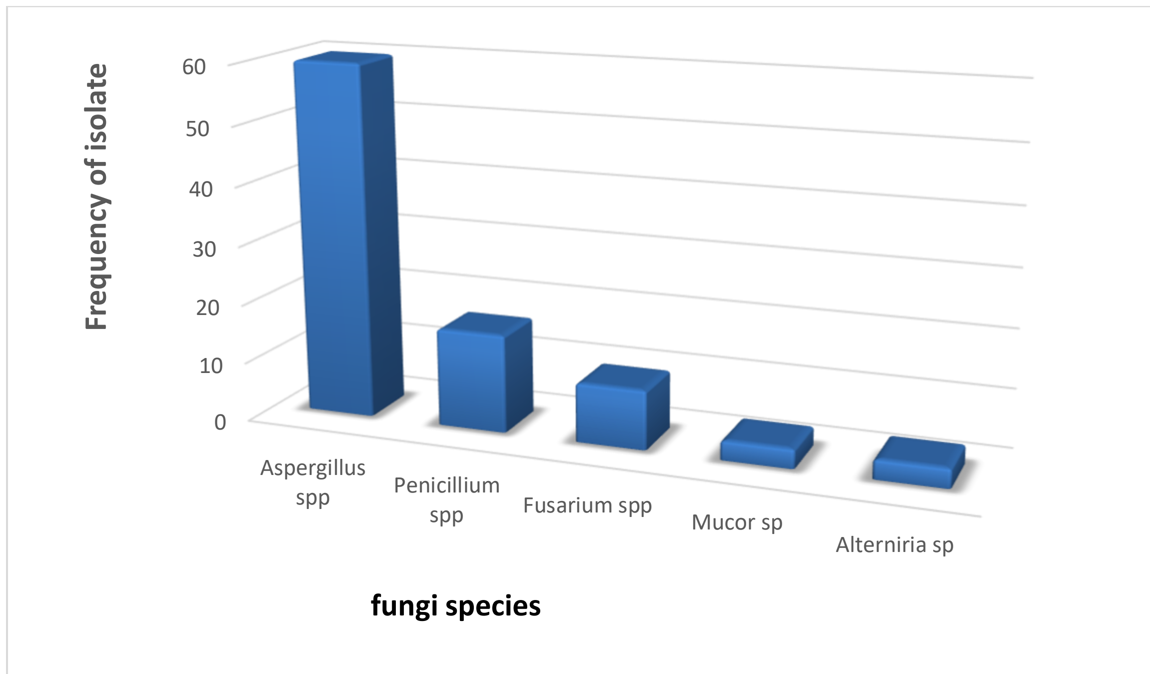

Fig. 4. Frequency of isolation of fungi species recovered from the examined fish feed samples

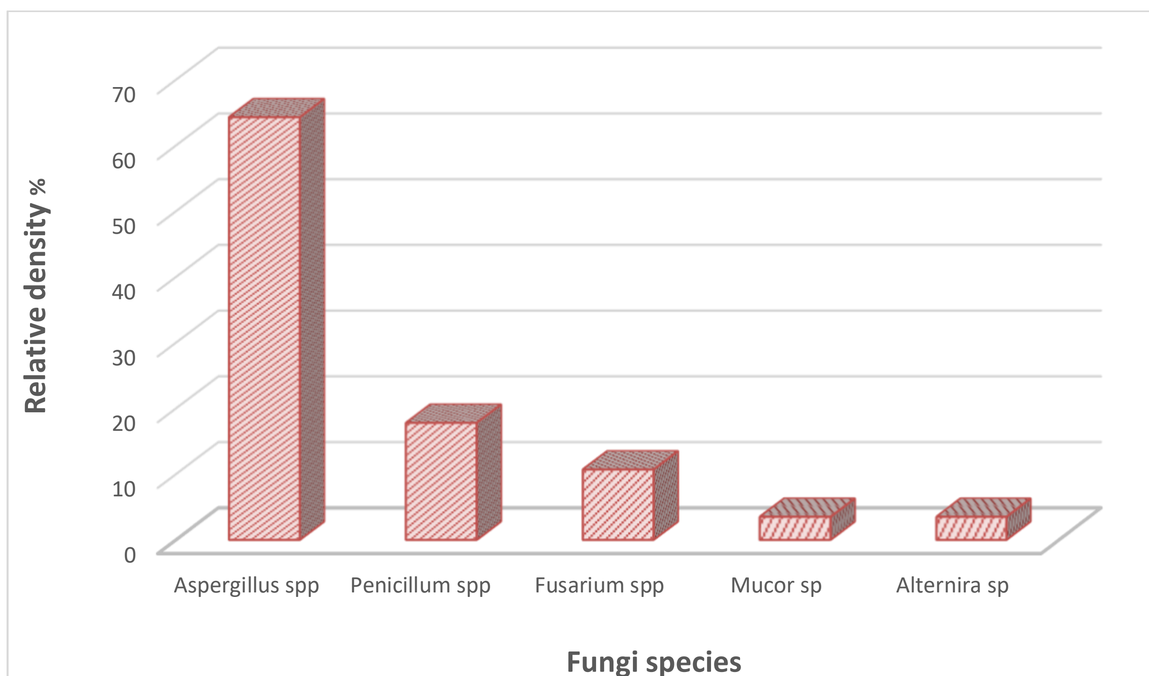

Fig. 5. Relative density of Fungi species recovered from the examined fish feed samples

Isolated species in our case are mostly storage contaminators, implicating that the high number of contaminated feed is most probably the result of manipulative mistakes (storage duration, temperature and humidity levels, etc.), during storage of feedstuffs or feed. Fungal colonization, growth and synthesis of toxins, results from the complex interaction of several factors (water availability, temperature and incubation time) and therefore, an understanding of each factor involved is essential for understanding the overall process and predicting fungal spoilage in agricultural and food products (Pardo *et al.,* 2005).
Improper storage accompanied by too high a temperature and elevated moisture content in the grain favour further mycotoxin production and lead to reduction in grain quality (Ramirez *et al.,* 2004). It is well known that cereal infection with moulds and toxin production depends strongly on environmental conditions (damp climate, cool temperatures). However, these data must be interpreted with caution, as they were calculated from a limited number of samples. The results of the mycoflora analysis carried out in this study are similar to previous results found by other authors (Milićević et al., 2010 and Gonçalves et al., 2020).

According to the regulation on maximal quantities of harmful components in feed, processed feeds and feed ingredients are not in compliance with standards of the hygiene quality if they contain above 1 X 10^4 CFU/g (GMP, 2005). By applying this principle, it was found that 23.3 % of the feed samples collected during this study didn't meet standards of mycological adequacy as they exceeded the feed hygienic quality limits. Fungal growth leads to the reduction of the nutritional quality of fish feed samples that could affect the palatability of feed and reduce the animal's nutrient absorption. Like our finding, Barbosa *et al*., (2013) and Ebeneezar, *et al*., (2018) obtained counts over the proposed limits in fish feed samples in Brazil and in India, respectively. Microbial contamination could be attributed to a low-quality substrate and/or an improper sanitation during handling, transportation (Grace, 2015). It is known that, microbe-free feed can't be produced without adversely affecting its nutritional value. Therefore, efforts should be made to reduce the number of microorganisms in feed as much as possible through appropriate quality control measures in different stages of feed processing and storage. So that the goal is not sterile feed but feed with "safe contamination level". Fungal contamination is very important for determining the probability that the feed contains mycotoxins, especially with improper storage conditions. If such contaminated feed is consumed by fish, it may cause acute deleterious effects leading to massive mortality. It has been estimated that near one-third of the world's crops are affected by mold growth, particularly aflatoxigenic fungi. Moreover, *A. flavus* , *A.parasiticus* and *A. niger* represent the most common *Aspergillus* spp. (Magnoli et al., 2019). In the present study, five mold species were identified in collected fish feed samples. The frequency of contamination of fish feed by *Aspergillus* spp., (60%) which was predominantly recovered from feed samples in accordance to the isolated species reported by Gerbaldo *et al*., (2012).

CONCLUSION

Aquaculture is very important natural sources both strategic and vital for all in the world. Aquaculture will continue to play an important role in the global supply of fish in the future. Negative effects of waste from mycotoxin to aquatic environment are increasingly recognized in Aquaculture. To minimize the risk of moulds exposure, close tripartite cooperation among the trade, the public and the government is essential. Properly planned use of aquaculture waste alleviates water pollution problems and not only conserves valuable water resources but also takes advantage of the nutrients contained in effluent. Aquaculture development must be sustained by basic and applied research and development in major fields such as nutrition, genetics, system management, product handling, and socioeconomics. One approach is closed systems that have no direct interaction with the local environment. The goal of aquaculture is grow in a manner that does not harm to aquatic ecosystems Therefore, monitoring of environmental effects of mycotoxin in aquaculture is very important for aquatic ecosystems conservation.

The present article indicates different fungal species of contamination of finished fish feed, but especially the highly frequency species *Aspergillus spp.* This study alarms us about the potential risks of moulds if contaminate raw materials such as fish feed and reflects that used ingredients are important vehicles for contaminating finished fish feed as they may be heavily contaminated by toxins.

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