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Mycological Evaluation and Occurrence of Aflatoxins and Ochratoxin A in *Tilapia Oreochromis niloticus* Fish and Fish Products

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Abstract

A total of 100 random samples of whole Tilapia (*Oreochromis niloticus*) and fillet of Tilapia (50 of each) were collected from different fish markets in Gharbia Governorate to evaluate pH, mycological contamination, and their total aflatoxin and ochratoxin A residues. The obtained results revealed that the mean±SE pH values were 6.1±0.05 and 6.3±0.03 and the mean total mould count values±SE were $3.63\times10^{2}\pm8.75\times10$ and $1.65\times10^{2}\pm4.78\times10$ cfu/g of whole Tilapia and fillet of Tilapia, respectively. It was found that the mean value of the total aflatoxin (µg/kg) in the examined samples were 0.55±0.2 µg/kg for whole Tilapia and 0.68±0.06 µg/kg for the fillet samples. Whereas the mean values of ochratoxin A were 2.79±0.6 µg/kg for and 0.12±0.01 µg/kg for the whole tilapia and fillet, respectively. Six fungal species were identified in the current study. The most prevalent fungal species were *Aspergillus niger*, followed by *A. flavus*. However, the least dominant were *A. terreu*, *A. westerdijkiae*, and *A. pseudocaelatus*, *Alternaria alternate*, *Cladosporium cladosporidiae*, *Mucor* species and *Penicillium* species. In conclusion, the investigated whole tilapia and fish fillet is contaminated by mould. Aflatoxin and ochratoxin were detected in the examined samples. Therefore, continuous monitoring of the occurrence of mycotoxins in the retailed fish and fish products are highly recommended to ensure product safety.

KEYWORDS
Tilapia Oreochromis niloticus, Filet fish, Mould, Aflatoxin, Ochratoxin A

INTRODUCTION

Animal proteins are produced in large part from fish. Fish farming is spreading quickly over the world to meet the rising demand for fish for human use and to make up for the lack of animal protein. Due to its low cost and delicious flavour of Nile Tilapia, *Oreochromis niloticus* is regarded as one of the most popular fish in Egypt. It is commonly cultivated due to its quick growth, tolerance to disease and stress, ease of reproduction, and low energy and management needs (Shaltout *et al*., 2001; Shaltout, 2003; Nandlal and Pickering, 2004; Hassan *et al*., 2014; Shaltout *et al*., 2014; Shaltout *et al*., 2015; Shaltout *et al*., 2016; Edris *et al*., 2017a; El-khamisy, 2020).

Fish products may cause the environment to reabsorb moisture during storage, which accelerates microbial development and increases the presence of *Aspergillus* spp., *Rhizopus* spp., and *Penicillium* (Shaltout and Salem, 2000; Shaltout, and Hashim, 2002; El-Diasty and Salem, 2007; Edris *et al*., 2017b; Saad *et al*., 2020). Due to poor hygiene, cleaning, or maintenance in open and unprotected shells, the market may be a source of seafood contamination, allowing dust and fungal spores to accumulate on the product, resulting in fungal growth, toxin generation, and product spoiling (El-diasty 2004; Fredrick *et al*., 2016; Hassan *et al*., 2020).

According to Ali (2022), one of the main reasons for fish deterioration, which can endanger public health and generate financial losses, is microbial contamination of fish. *Aspergillus*, *Penicillium*, and slime, which are widespread and predominantly involved in saprophytic putrefaction processes, are examples of common fungal pollutants of concern. Because they can serve as secondary invaders on small, superficial lesions, saprophytic oomycetes are thought of as opportunistic pathogens.

Aspergillus flavus and *Aspergillus parasiticus* both have the ability to produce aflatoxins, a particular class of harmful secondary metabolite. They are crucial for both economic and health reasons. The International Agency for Research on Cancer has classed the known hepatocarcinogen aflatoxin B1 as a Class I human carcinogen. To preserve human health, its bioavailability must be decreased (Amnah, 2013). Ochratoxins (OTA) are a major problem. Fish may contain contaminants that are harmful to both human and animal health. They can have sub-chronic and chronic effects on people, although OTAs' acute toxicity is rarely documented in people (Hassan and Shaltout, 2004; Hassan *et al*., 2019; Saad *et al*., 2020).

As a result, the current study was conducted for the isolation and identification of fungi, as well as the detection of total Aflatoxin and Ochratoxin A in whole Tilapia fish and fillet, and to discuss their public health impact.

MATERIALS AND METHODS

Collection of samples

In the Gharbia Governorate, 100 samples of whole tilapia

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(*Oreochromis niloticus*) and 50 samples of tilapia fillet were gathered from various fish markets. Each sample was labeled, stored separately in a sterile plastic bag, and kept in the freezer. All of the gathered samples were quickly sent to the lab, where they underwent ELISA immunoaffinity analysis to determine whether they were contaminated with mould and how much total aflatoxins and ochratoxin A they contained.

Detection of pH in fish flesh (Pearson, 2006)

Preparation of samples (ISO 6887/3/2007)

Twenty five grams of fish product samples were blended and stirred for two minutes in a stomacher bag after being aseptically combined with 225 ml of water containing 0.1% peptone. To achieve a dilution of 10^{-1}. Shaking was used to combine the food homogenate, and then 1.0 ml was pipetted into a tube containing 9.0 ml of 0.85% saline and gently mixed. 1.0 ml of the first dilution was pipetted into the tube for the second dilution, which contained 0.85% saline. The mixture was allowed to settle for 5 minutes at room temperature after this stage, which was repeated until a 10^{-6} dilution. These microbiological tests were performed on the produced homogenate.

Total Mould Count (ISO 21527/2/2008)

One mL of the previously made serial dilutions was used to inoculate duplicate plates of Dichloran Rose Bengal agar plates. The inoculation plates were incubated for five days at 25ºC, and on the fifth day, the count was reported.

Identification of isolated mould (Pitt and Hocking, 2009)

The rate and pattern of growth as well as colour, texture, basal and surface mycelia, colony reversal, rate of colony expansion, and diameter might all be used to macroscopically identify an isolated mould. The produced slides were further examined microscopically to characterize the morphological structures of the mould growth. Low power and oil immersion lenses were used.

Detection of total aflatoxin (Sahar et al., 2013)

A competitive direct enzyme linked immunosorbent assay (CD-ELISA) method was used to evaluate the quantitative analysis of total aflatoxins. The technique is based on precise mycotoxin monitoring. The Veratox test kits (Neogen Crop, Lansing, MI. USK. Approved by the USDA-GIPSA (2008-011) and the AOAC research institute (certificate No. 950702) were utilized. According to the manufacturer's instructions, the analysis was completed. Awareness Technology Inc.'s log/log it program was used to calculate the concentration of aflatoxins (Anonymous, 2000; Stoloff *et al*., 1999). The concentration of total AF Levels in the tested samples were estimated from the standard curve relation optical density versus total AF standards. The detection of total aflatoxin was determined in Animal health Research, Giza, Egypt.

Detection of total aflatoxin and ochratoxin A (Baydar et al., 2007)

The procedure was carried out in accordance with the instructions provided by the manufacturer of the quantitative test kit for OTA, R- Biopharm, RIDASCREEN*Ocratoxin A (R- Biopharm, Darmstadt, Alernania), which has a detection limit of 1.25 g kg, a recovery percentage of approximately 100%, and a specificity for OAT of 100% (Biopharm 2021). The concentration of OTA in the sample was quantified by photometric measurement at 450 nm using an absorbance microplate reader, model number EL800 (Bio tech Instruments INC®, Winooski, VT, EE.UU). Six standard OTA solutions (0, 50, 100, 300, 900, and 800 ng/kg) were used to produce the calibration curve, and the OTA-specific RIDA® SOFT Win programme (Art, Z 9999, R- Biopharm®, Darmstadt, Germany) was used to handle all the experiment's data. The detection of ochratoxin A was determined in Animal health Research, Giza, Egypt.

Statistical Analysis

According to Feldman *et al*. (2003), the analysis of variance (ANOVA) test was used to statistically assess the results.

RESULTS

Tilapia is a popular fish due to its low to moderate fat level and excellent protein quality. As a result, this fish has a wide range of health benefits, including weight loss, increased metabolism, bone strengthening, lowering the risk of numerous chronic diseases, and preventing arthritis and cancer. Tilapia species are among the most frequently grown freshwater fish, distinguished by their high development rate and ability to co-culture with prawns in a polyculture system. Although *Oreochromis niloticus* has a quick growth rate, it does not withstand high salinity, and the demand for tilapia has increased exponentially in recent years, particularly in warm countries such as Egypt and the Middle East (Richardson, 2017).

Maize bran, wheat bran, rice bran, soy meal, cotton seed cake, fish meal, bone meal, and termites are used to make fish feeds (Aanyu *et al*. 2018). Unfortunately, some of these substances can be contaminated and colonized by fungus that create hazardous secondary metabolites known as mycotoxins (Rutaisire, 2007; Udomkun *et al*., 2017). These mycotoxins, if consumed, can harm the health of both fish and humans (Matejova *et al*., 2016). Fish contamination can also be linked to raw materials, workers, and processing instruments like forklifts via leaks, building openings, and vermin. Some diseases may even establish themselves in processing facilities by surviving in niches for extended periods of time. The quality of fish is a major concern for food processors, consumers, and public health authorities, and the provision of safe, healthy, and acceptable fish and its products as food to customers, as well as microorganism control, is critical

Table 1. Statistical analytical results of pH in the examined samples of examined samples and Acceptability of the examined samples according to their pH values (n=50).

Type of samples	No. of examined samples	Min	Max	Mean±S.E	Max. permissible limit			
					Accepted		Unaccepted	
					No.	%	No.	%
Tilapia Oreochromis niloticus fish	50	5.6	6.61	6.1±0.05	46	92	4	8
fillet of *Tilapia Oreochromis niloticus* Fish	50	6	6.72	6.3±0.03	45	90	5	10

to meeting these objectives (Adebayo- Tayo *et al*., 2012). Table 1 revealed that the pH values in the examined fish samples were varied from 5.60 to 6.61 with an average of 6.1±0.05 in Tilapia fish, 6.00 to 6.72, and an average of 6.30±0.03 in fillet of Tilapia. The observed results agreed to those of Zaky and Ibrahim (2017); Elsherief (2019). The Egyptian Organization for Standardization (EOS) reported that the crucial limits of pH for cold fish portions should not exceed 6.5. According to Daramola *et al*. (2007), pH is a measure of the extent of microbial spoilage in fish, and some proteolytic bacteria create acid after carbohydrate degradation, increasing the acid level of the medium. The pH value is an accurate predictor of freshness or deterioration. The decrease in pH is caused by the fish's carbohydrate being fermented to acids. Ozyurt *et al*. (2007) discovered that a pH range of 6.8-7 was indicated as an acceptable limit for fish, with values above 7 being spoilt.

Moulds in fish are caused by inadequate hygiene during the catching, handling, processing, salting, storage, transport, distribution, and marketing of fish. Mould contamination has created undesirable alterations in the fish, rendering it unsellable. Furthermore, the danger of consumers acquiring the related disease has increased, presumably due to the synthesis of aflatoxins by particular strains of fungi. Figure 1 stated that the mean±SE (log_{10} cfu/g) values of mould were 5.03±3.4 (log_{10} cfu/g) in Tilapia fish samples, while they were 3.08±2.6 (log_{10} cfu/g) in fillet of Tilapia fish samples. There was a significance difference ($P<0.05$) between the mean log counts of both fish and fillet samples. The results of total mould count of examined fish samples were nearly similar to those obtained (Barbosa *et al.,* 2013) who reported that mean mould counts were $2.9\times10^3\pm0.6\times10^3$. El-khamisy (2020) recorded that Nile tilapia fish was the highest one as mean mould count/g in ranged from $1.1\times10^5\pm3.4\times10^4$ cfu/g. *Tilapia nilotica* showed a higher total mould count than *Mugil cephalus*, according to Nader *et al*. (2019). In the tested *Tilapia nilotica* and *Mugil cephalus*, the mean total mould counts were 3.63 $x10^2$±8.75x10 and 1.65 $x10^2$±4.78x10 CFU/g, respectively. This could be linked to *Tilapia nilotica*'s higher moisture content than *Mugil cephalus*, which could lead to a higher mould contamination.

This fungal infection may suggest a lack of hygiene at the time of capture. Mould spores can grow in refrigerators, chillers, Fisher Hands, and garments due to environmental conditions (Mizakova *et al*., 2002; Reij and Den Aantrekker, 2004). Fungal contamination of fish can cause spoiling and the creation of mycotoxins, which are carcinogenic and can cause liver illness and organ damage in humans (Darwish *et al*., 2014).

Results in Table 2 showed six fungal species were isolated from Tilapia fish and fillet of Tilapia. Table 2 showed that *Tilapia nilotica* fish had the highest value of isolation percentage of *As-*

Table 2. Incidence of moulds species isolated from the examined fish samples.

Mould species	*Tilapia Oreochromis niloticus* fish		Fillet of *Tilapia Oreochromis niloticus fish*	
	No.	%	No.	%
Alternaria alternate	5	4.59	1	2.44
Aspergillus species				
A. flavus	25	22.94	10	24.4
A. niger	30	27.52	9	21.95
A. penicilloides	0	0	2	4.88
A. pseudocaelatus	2	1.83	0	0
A. terreus	3	2.75	0	0
A. westerdijkiae	3	2.75	0	0
Cladosporium cladosporidiae	13	11.93	2	4.88
EuPenicillium spp.	2	1.83	3	7.32
Mucor species	3	2.75	10	24.4
Penicillium species				
P. citrinum	10	9.17	2	4.88
P. corylophilium	5	4.59	1	2.44
P. griseofulvum	3	2.75	1	2.44
P. simplicissimum	5	4.59	0	0
Total	109	100	41	100

Table 3. Determination of total aflatoxin in fish flesh samples.

		Total aflatoxin positive samples		Amount of total aflatoxin (ppb)		
Type of fish product	No. of samples	No.	%	Min.	Max.	Mean±SE.
Tilapia Oreochromis niloticus fish	50	5	10	0.06	0.96	0.55±0.2
Fillet of *Tilapia Oreochromis niloticus*	50	4	8	0.71	0.83	0.68±0.06

Table 4. Determination of ochratoxin A in fish flesh samples.

		Ochratoxin A positive samples		Amount of Ochratoxin A (ppb)		
Type of fish product	No. of samples	No.	%	Min.	Max.	Mean±SE.
Tilapia Oreochromis niloticus fish	50	6	12	0.05	3.2	2.79±0.6
Fillet of *Tilapia Oreochromis niloticus*	50	5	10	0.09	0.15	0.12±0.01

pergillus niger (27.52%) followed by *A. flavus* (22.94 %), however, the lowest values were obtained for *A. terreus*, *A. westerdijkiae*, and *A. pseudocaelatus* 2.75, 2.7 5 and 1.83, respectively. While the values were obtained for *Cladosporium cladosporidiae* and *Alternaria alternate* 11.93%, and 4.59%, respectively. Fillet of Tilapia had *A. flavus* as the highest value of isolation percentage (24.4%) followed by *A. niger* (21.95%) while the lowest values were obtained for *P. citrinum* (4.88%), *P. corylophilium* (2.44%) and *P. griseofulvum* (2.44%), respectively.

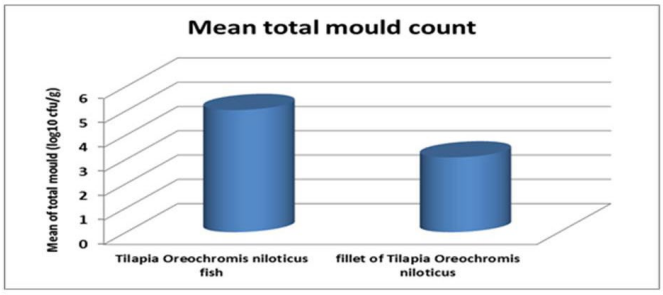

Fig. 1. Mean value of mould count (log_{10} cfu/g) in examined samples (n=50).

Contamination of fish feed with *Aspergillus* species, particularly *A. flavus* and *A. niger* at high humidity levels leads to increased fungal growth during feed storage at high humidity (Almeida *et al*., 2011; Kumar *et al*., 2017). Contaminated water, worker hands and food play a very important role in fish health (Nader *et al*., 2019).

In agreement with our findings, Junaid *et al*. (2010) reported that all stockfish samples were contaminated with fungi. Seven different fungi were found to be associated with the stockfish samples sold in the four different markets: *Mucor* Spp, *Asergillus flavus*, *Trichophyton verrucosum*, *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium* spp., and *Rhizopus* spp., among the isolated fungi, *Mucor* Spp was found to occur the most frequently. *A. niger*, *A. flavus*, *A. versicolor*, *A. parasiticus*, *Rhizopus* spp., *Mucor* spp., *Phoma herbarum*, and *Trichoderma hamatum* were isolated from *Tilapha nilotica*, according to Ali *et al*. (2011). According to Nader *et al*. (2019), *Aspergillus niger* (73.91%) and *A. flavus* (86.95%) were the most dominant mould species in tilapia fish, whereas *A. ochraceus*, *A. parasiticus*, and *Alternaria* species had the lowest rates. Therefore, improper sanitation during fish handling, catching, storage, marketing, and production may be the cause of the fungal contamination of fish (Shaltout and Hashim, 2002). By displaying a spectrum of four species, Zaky and Ibrahim (2017) noted that the dominant genus is *Aspergillus*; *Penicillium* follows with three species. From the studied fresh fish samples, the remaining taxa were only represented by two or one species.

According to information in Table 3, total aflatoxins residues were found in 5 (10%) of the whole Tilapia fish and 4 (8%) of the fillet samples. Total aflatoxin residues' concentrations (µg/Kg) ranged from 0.061 to 0.96 with a mean value of 0.55 to 0.20 in the whole tilapia and from 0.71 to 0.83 with a mean value of 0.68 to 0.06 in the fillet samples. These results were less than what Saad *et al*. (2020) reported. Due to the generation of mycotoxins, mould contamination poses a significant concern (El-Diasty and Salem, 2007; Saad *et al*., 2020). While modest amounts result in liver disorders and organ damage, high levels of these mycotoxins induce liver cancer.

The residual concentrations of total aflatoxin in the whole tilapia and the fillet were accepted according to FAO (2004) and European Commission (2006) and (2010) who reported that total aflatoxin should not exceed 4-15 µg/kg.

Ochratoxin A residues were found in 6 (12%) of the whole fish and 5 (10%) of the fillet samples, according to data shown in Table 4. Total ochratoxin A residue concentrations (µg/Kg) ranged from 0.05 to 3.20 in the analyzed samples, with a mean value of 2.79±0.6 in the whole tilapia and from 0.071 to 0.15 with a mean value of 0.12±0.01 in the fillet samples. Because the incidence of ochratoxin A does not exceed the international permissible limits (5 µg/Kg) previously recorded by FAO (2004), the examined Tilapia fish samples and fillet of Tilapia are considered acceptable. The health of both people and animals is seriously endangered by ochratoxin (OTA). They can have subchronic and chronic effects on people, but OTAs' acute toxicity is rarely documented in people. According to research conducted on animals, OTA is teratogenic, immunotoxic, hepatotoxic, and nephrotoxic (Richard, 2007).

According to Saad *et al*. (2020), ochratoxin A was present in 16.67% of smoked herring, 6.67% of canned sardines, and 6.67% of frozen fish fillets. Ochratoxin A was completely detected in 10% of the samples of fish products analyzed. Whereas the mean concentrations of ochratoxin A (±g/kg) were 3.24±0.39 µg/kg in the frozen fish fillets, 5.60±0.61 g/kg in canned sardines, and 6.52±0.74 g/kg in smoked herring samples. All the examined frozen fish fillets were accepted but 6.67% of the examined smoked herring and 3.33% of the examined canned sardines were rejected due to their high level of OTA.

Aflatoxin B1 was found in the feed components in Brazil at a mean value of 3.8 µg/kg (ranging from 1.6 to 9.8 g/kg). Additionally, it was found in commercial diets at mean concentrations of 1.40 µg/g together with fumonisins (mean, 1.60 µg/g). Aflatoxin B1 was also detected at 92.0% of locally produced fish feed in Nigeria, with concentrations as high as 550.8 µg/g. Ochratoxin A was also detected at 23.3% of the fish feed samples examined in Egypt (Oliveira and Vasconcelos, 2020).

CONCLUSION

Due to increasing mycotoxin contamination, the use of plant-based feed ingredient in fish farms can seriously jeopardize aquaculture productivity. Mycotoxins cause large economic losses in fish because they increase sickness, mortality, and the prevalence of reproductive issues while decreasing weight gain. Additionally, even trace amounts of mycotoxins can seriously endanger the health of fish consumers if they are consumed. On the other hand, chronic effects like cancer or immunodeficiency might result from prolonged human exposure to modest amounts of mycotoxins. Therefore, methods for reducing exposure and preventing contamination before and after harvest are of utmost relevance. To safeguard aquaculture as it develops, monitoring of raw materials and finished feed should become standard procedure.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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