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Research Article

The Improvement of Microbiological and Histopathological Findings of Fish Intoxicated with Aflatoxins and Ochartoxins by Adding Yeast In vitro

Heidy Abo El-Yazeed[1], Mai El- Dosouki[2], Nouran Kenawy and Mai H. Hanafy[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[2]Department of Fish diseases, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
***Corresponding author:** maihamed_vet@hotmail.com

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ABSTRACT

The present study was conducted to detect the prevalence of mycotoxins in fish feeds and to assess their effect on fish health and growth as well as to monitor the effect of adding yeast on the improvement of different parameters of intoxicated fish invitro. The aflatoxins and ochratoxins were measured in ration samples using Fluorometeric method and he effects of experimental feeding of ochratoxins (OA) and aflatoxins (AFs) contaminated ration on *Tilapia niloticus* was studied. The body weight gain in fish fed on ration contaminated with AFs and OA was significantly improved after addition of yeast to the mycotoxicated feed. On the other hand, the hepatosomatic index calculated in the present experiment was increased after administration of yeast to fish with the mycotoxicated feed. Currently, no aflatoxins and ochratoxins residues levels in flesh of *Tilapia niloticus* were detected at the end of experimental period. The histopathological examination of liver and kidney tissues of fish in treated groups was carried out as well. The beneficial effect of yeast was detected as a marked improvement of hepatic features.

Key words: Aflatoxins, Ochartoxins, Fish, Yeast, Fluorometer

INTRODUCTION

Aquaculture contributes greatly in global fish production as the world-wide consumption of fish is increasing. Fish meat represents one of the most vital sources of animal protein for human. Therefore, intensive rising of great numbers of fish has a great economic importance (Alceste and Jory, 2000).

Aquatic products constitute an important food source for both animal and human consumption, the increasing demand of which, makes the development of aquaculture mandatory **(**Myhr and Dalmo, 2005).

Aqua feeds basically depend on fishmeal as a protein source, but the trend in recent years has been shifted towards replacing fish meal with less expensive sources of protein of plant origin. Consequently, aquaculture feeds develop a higher risk of being contaminated by one or more types of mycotoxins (Klyszejko *et al.,* 2005)

Adding of plant based ingredients, such as corn, in aqua feeds promotes both the risk of introducing mycotoxins into the feed at the point of feed manufacturing and mycotoxins production during storage of processed feed. (Spring and Fegan, 2005). Contamination of aqua feed by mycotoxins occurs usually in countries with humid tropical climates due to many factors, among which are climatic conditions which enhance mold growth and inappropriate methods of both feed processing and storage. So far, more than 400 different mycotoxins have been reported that can be grouped into five main classes: aflatoxins, ochratoxins, fumonisins, zearalenone and trichothecenes (Cast, 2003).

One mould species can produce many types of mycotoxin. Consequently, any moldy sample may contain several mould species; because the sample may be contaminated with different mycotoxins. Thus, when a mycotoxin is detected, the examiner should suspect that other types are suspected to be present in a contaminated feed. (Abdelhamid *et al.,* 2004). In addition to the deleterious effects recorded for mycotoxins on both the health and growth of fish, also immunodulatory effects was detected. (Chavez-Sanchez, *et al.,* 1994). Moreover, many toxic effects were recorded for mycotoxins such as carcinogenicity, genotoxicity, oestrogenicity, hepatotoxicity, reproductive disorders, Nephrotoxicity, immunosuppression or dermal effects. (Bryden, 2012).

Aflatoxin is the metabolic by product of Aspergillus *flavus* and *Aspergillus parasiticus.* It is a toxic compound and the cause of high mortality in livestock, fishes and in

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some cases of human beings (Reed and Casali, 1987). Ochratoxins, the group of secondary metabolites produced by fungi belonging to *Aspergillus* and *Penicillium* genera, in which ochratoxin A (OTA) is the most toxic and abundant mycotoxin of these groups which is generally accompanied with contamination of corn, cereal grains and oil seeds and can affect animal performance by damaging of the kidney function**.** Ochratoxin A is an important mycotoxin not only because of its deleterious effects on fish production, but also because it contaminates the edible tissues of fish and other animals that consume it; therefore, it can reach the human food chain and affect the renal systems of those who eat contaminated products. Owing to the prevalence of OA in cereal grains involved in feed livestock in specific regions of the world, OA is recorded to be the cause of Balkan endemic nephropathy in humans who have eaten foods contaminated with OA (Bruce, 2010**).** The negative effects of mycotoxins include inhibition of DNA, RNA and protein synthesis. **(**Ottinger and Kaattari, 2000).

Mycotoxins are incriminated to cause a wide variety of adverse clinical signs among fishes depending on the type and dose of the mycotoxin, duration of exposure, the fish species/age, nutritional and health conditions at the time of exposure to contaminated feed (Horvath, 1998). Many researchers have reported that mycotoxins can act synergistically so that the deleterious effects of two mycotoxins will exceed the effects of each alone. According to FAO (food and agriculture organization) 25% of the world's crop harvests are contaminated by mycotoxins. The environment in Egypt (high temperature in summer and high humidity) exhibits suitable condition for the presence of mycotoxins.

MATERIALS AND METHODS

Ration preparation

Fifty samples of fish feed were subjected to mycological examination and measurement of aflatoxin B_1 and ochratoxin A using fluorometeric method and the samples which yielded higher levels were selected for further studies.

Detection of ochratoxins and aflatoxins using fluorometric assay

This was carried out through: Ochratest Fluorometer procedure & Aflatest Fluorometer procedure overview: according to VICAM manual.

Holding and management of fish throughout the experiment

Fish used in this study were kept in full glass aquaria supplied with chlorine free tap water with continuous aeration and filtration. The fish were fed pelleted, locally prepared diet **(**Jauncey and Ross, 1982).

Experimental feeding of fish with ochratoxicated and aflatoxicated ration with or without yeast

A total of 60 fish were divided into 3 groups (20 fish per group). The selected ration samples were divided into two portions; one portion was used for feeding the first group of fish (positive – Toxicated group). And fresh yeast was added to the other portion of the sample, which was used for feeding the second group of fish. While third group was given a ration free from mycotoxins and kept as negative control group. The experiment period extended for two months, in which fish were fed twice/day (about 5% of the weight of fish, 2 grams at the morning and 2 grams afternoon).

Clinical examination

The living fish during experiment were clinically examined for the general behavior, changes in color, respiratory manifestations, feeding and any clinical abnormalities on external body surface (skin, gills, eyes and mouth) according to the methods described by **(**Noga, 1996).

Post-mortem examination

The internal organs were exposed and examined macroscopically for any gross abnormalities in musculature. The collected fish were examined macroscopically using the methods described by (Amlacher, 1970).

Measurement of lipid peroxidase enzyme (n.mol/g tissue) in the liver of *Tilapia niloticus*

Lipid peroxidase levels were determined by measuring the product formed from the combination malondialdehyde and thiobarbituric acid (TBA) through High Performance Liquid Chromatography (HPLC) and UV/Vis detection, and using a modified version of the method used by (Almeida *et al.,* 2004).

Calculation of the Hepatosomatic index of *Tilapia niloticus* groups (test and control) during whole experimental period (8 weeks)

During the 8-week period of the experiment, 3 samples from each group were taken each at 2 weeks interval-to calculate the hepatosomatic index.

HIS= (Liver weight (g) /Fish weight (g)) x100
cited by Parameswaran and Liese, 1974.

Histopathological examination

Autopsy samples were taken from liver, gills, spleen and skeletal muscles of fish in different groups and fixed in 10% Formaldehyde saline for twenty four hours. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain for routine examination through the light electric microscope (Banchroft, *et al.,* 1996).

Detection of ochratoxins and aflatoxins residues in fish muscles using thin layer chromatography (TLC)

The flesh of previously fish samples was homogenized thoroughly in an electrical meat grinder. One hundred grams of the homogenized flesh was mixed with 10 ml of citric acid solution (20%) using of 30x1 cm stirring glass rod for five minutes, then mixture was re-stirred with 20 g of diatomaceous earth.

Samples of soft tissues (20 g of muscles) were taken from each experimental fish and stored at 4˚C and kept frozen until used. The residues of mycotoxins in fish muscles were detected and measured by TLC method.

RESULTS

Prevalence of different mould species isolated from examined ration samples

Moulds which were isolated from examined ration samples: *Penicillium* spp, *Aspergillus (flavus, niger)* and *Mucor* spp as 80, 60,40 and 20% respectively.

Measurements of ochratoxin A and aflatoxin B_1 in examined ration samples using Fluorometer

Aflatoxin B1 ranged from 5 to 13 ppb while Ocharatoxin a ranged from 7.6 to 38 ppb.

Results of experimental feeding of ochratoxins and aflatoxins contaminated ration on *Tilapia niloticus* different parameters

Body weight of *Tilapia niloticus* Groups (A, B, C) during whole experimental period (8 weeks) as shown in Table 1.

Table 1: Body weight of *Tilapia niloticus* Groups (A, B, C) during whole experimental period (8 weeks)

Time of assessment	Body weight in grams		
	Group (A) Contaminated ration with toxins	Group (B) Contaminated ration with toxins + yeast	Group (C) control group (Toxin free ration)
Day 0 (Arrival day)	200	200	200
2nd week	210	210	210
4th week	215	217	217
6th week	215	217	217
8th week	220	219	227
Mean	215	215.75	217.75
SE	3.4	3.5	4.5
P value	0.916 (Non-significant)		

Whereas, P value=ANOVA test, SE=standard error

Mortality rate of *Tilapia niloticus*

The mortality rate of *Tilapia niloticus* during the whole experimental period (8 weeks). % was calculated according to the total No. of examined samples in Table 2.

Hepatosomatic index of *Tilapia niloticus*

Results of the hepatosomatic index of *Tilapia niloticus* groups (test and control) during whole experimental period (8 weeks) in Table 3.

Lipid peroxidase levels in liver of *Tilapia niloticus*

Concentrations of lipid peroxidase enzyme (n. mol/g tissue) in the liver of *Tilapia niloticus.* Residual levels of ochratoxins and aflatoxins in flesh of *Tilapia niloticus*. Neither aflatoxins nor ochratoxins residues were detected in flesh of *Tilapia niloticus* in either tested or control groups in Table 4.

Histopathological findings of gills, liver, spleen and muscles of *Tilapia niloticus*

Control group: There was no histopathological alteration and normal histological structure of gills, liver, spleen, and skeletal muscles was observed.

DISCUSSION

Many toxic effects were recorded for mycotoxins including: carcinogenicity, nephrotoxicity, hepatotoxicity, genotoxicity, oestrogenicity, immunosuppression, reproductive disorders or dermal effects. (Bryden, 2012)

In the present work, the most predominant isolates were *Aspergillus flavus* with a percentage of 80%, followed by *Pencilluim* spp. with a percentage of 60%, *Aspergillus niger* with a percentage of 40% and *Mucor* spp. with a percentage of 20%. Nearly similar results were recorded by Barbosa *et al.,* 2013 who reported that total mould count of examined fish feeds ranged from $<1x10^2$ to $4.7x10^4$ CFU/g. In the same study, the most prevalent isolated fungal spp. was *Aspergillus* spp. followed by *Pencilluim* spp.

Fungal growth leads to reduction in the nutritional quality of fish feed also it could affect the palatability of feed and reduce the absorption of nutrients. (Barbosa *et al*., 2013).

Several strains of moulds particularly *Aspergillus flavus and A. ochraceus* were isolated from different types of fish and fish-feed were able to produce aflatoxins and ochratoxins. The produced aflatoxin B_1 and ochratocxin A were measured in the five ration groups by fluorometer and the results detected that the highest concentration of ochratoxin was in the fourth group at a level of 38 ppb, while the highest concentration of aflatoxins was in the second group at the level of 13 ppb. Whereas, the lowest concentration of ochratoxins was detected in the second group at the level of 7.6 ppb. While the lowest concentration of aflatoxins was observed in the third group at the level of 5 ppb. The total concentrations of aflatoxins ranged from 5 to 13 ppb and concentrations of ochratoxins ranged from 7.6 to 38 ppb.

Table 2: The mortality rate of *Tilapia niloticus* during the whole experimental period (8 weeks)

Experimental period	Group A Tested			Group B Tested			Group C Control		
	Total No.	No. of mortalities	%	Total No.	No. of mortalities	%	Total Nº	No. of mortalities	%
Week 1	20	-	-	20	-	-	20	-	-
Week 2	20	-	-	20	-	-	20	-	-
Week 3	20	-	-	20	-	-	20	-	-
Week 4	20	1	5	20	-	-	20	-	-
Week 5	19	3	15.7	20	1	5	20	-	-
Week 6	16	3	18.7	19	2	10.5	20	-	-
Week 7	13	1	8.3	17	1	5.8	20	-	-
Week 8	12	-	-	16	-	-	20	-	-

Table 3: Results of the hepatosomatic index of *Tilapia niloticus* groups (test and control) during whole experimental period (8 weeks)

Experimental period	Group A (tested) (Toxins)	Group B (tested) (Toxins + Yeast)	Group C (control) (Toxin free ration)
3rd week	1.8	1.7	2.3
	2.04	2.03	2.4
	2.3	1.77	1.85
Mean	2.04	1.83	2.18
SE	0.14	0.10	0.17
P value	0.283 (Non-significant)		
5th week	2.23	2.9	2.3
	2.9	1.3	1.4
	3.5	3.4	1.4
Mean	2.87	2.53	1.7
SE	0.37	0.63	0.3
P value	0.251 (Non-significant)		
8th week	1.93	1.84	2.8
	1.82	1.92	2.6
	1.29	2.24	2.2
Mean	1.68 b	3.0 a	2.5 a
SE	0.19	0.12	0.18
P value	0.031 (Significant)		

Whereas, P value=ANOVA test, SE=standard error, Significant=means with different letters (a, b) within the same row are significantly different at P value ≤0.05.

Table 4: Concentrations of lipid peroxidase enzyme (n. mol/g tissue) in the liver of *Tilapia niloticus*

Group	Group A	Group B	Group C
Concentration (n. mol/g tissue)	4.13	3.01	3.06

Aflatoxins were on the top of the list of mycotoxins to be investigated in aquaculture. As in other animal species, aflatoxin exerts carcinogenic effects in fish (Reed and Casali, 1987) Different researchers proved the presence of aflatoxin B_1 (AFB_1) in shrimp and fish feed (Abdelhamid, *et al.,* 1998 and Bautista, *et al.,* 1994). Although ochratoxin A has not been studied to the same extent as AFB_1 in aquaculture, there are several studies declaring the toxic effects of this toxin in different fish species (Manning *et al.,* 2003; Moussa and Khattab, 2003; Shalaby, 2004).

Most of the recorded levels of aflatoxins and ochratoxins were exceeding the permissible limits in food as recorded by WHO, 1979 who recommended that the aflatoxins must not be more than 15 ppb, while FAO (1995) presented that the levels of aflatoxins must be not exceeding 20 ppb in food. Hence, most of detected levels were health hazard for consumers where, cases of carcinogenic effects for internal vital organs are resulted specifically from liver and kidney. Moreover, the consumption of food contaminated with mould and their related toxins induced food poisoning, hemorrhages, hepatotoxicity, nephrotoxicity, neurotoxicity, dermatitis, carcinogenic, hormonal and immunosuppression effects (Hassan *et al.,* 2016).

As observed in the obtained data, all samples were subjected to analysis for the presence of mycotoxins showed co-occurrence of both AFB_1 and OTA. The present work has shown the simultaneous occurrence of two carcinogenic mycotoxins (AFB_1 and OTA) in feed intended for fish aquaculture. There are limited studies published on feed contamination with these two toxins intended for fish feed in Egypt.

The biological effects of mycotoxins depend mainly on the ingested concentration of existing mycotoxins, time of exposure and animal sensitivity. In addition, the mycotoxin affects are not only amplified by stress production but also increased in animals reared intensively (Binder, 2006*).*

In the present work, the effects of experimental feeding of ochratoxin A and aflatoxin B_1 contaminated ration on *Tilapia niloticus* were undertaken. The diminished body weight gain in fish fed on ration contaminated with AFs and OTA was significantly improved after addition of yeast to the mycotoxicated feed. Whereas, the mortality rates which was observed in the mycotoxicated fish group were completely absent by adding yeast to the toxicated group. On the other hand, the hepatospmatic index calculated in the present experiment increased after administration of yeast to fish with the mycotoxicated feed. Currently, no aflatoxins and ochratoxins residues levels in flesh of *Tilapia niloticus* were detected at the end of experimental period.

Several authors illustrated the effects of addition of antitoxins to animal feeds to ameliorate the toxic effects of mycotoxins as using of yeast (Hassan *et al.,* 2012), probiotic preparation (Nabawy *et al.,* 2014) and N. acetylcysteine and probiotic (Hassan, *et al.,* 2012).

Regarding the histopathological findings of gills, liver, spleen and muscles of *Tilapia niloticus*: in the present study, the histopathological examination of liver and kidney tissues of fish in the treated groups was investigated. The sensitivity of different tissues to the toxic effect of mycotoxins depends mainly on the bio-distribution of the certain enzymes, which are responsible for the metabolic transformation of AFs into toxic active intermediate metabolites (Singh and Clausen, 1980).

The present study recorded criteria, which are related to the toxic changes of aflatoxicosis and ochratoxicosis, in addition to the degree of their mitigation using different investigated therapeutic agents. They lead to damage of all component of the cell that includes lipids, proteins and DNA through induction state of oxidative stress (Chandra *et al*., 2015). Additionally, when aflatoxins and OTA enter the cell, they bind with DNA and thereby inhibits the action of RNA polymerase, followed by inhibition of mRNA which is reflected by marked reduction in the protein synthesis, so aflatoxins act as an inhibitor of protein synthesis at some specific stages. The cytopathic effect of mycotoxicosis occurs in 2 forms, both of necrosis and apoptosis. Apoptosis could be attributed to the ability of AFB1 to increase the expression of pro apoptotic proteins p53 and bax and decreased the expression of bcl2 (Brahmi, *et al.,* 2011), while necrosis begins when the glutathione stores are almost depleted as a result of aflatoxin metabolism (Abdel-Wahhab, *et al.,* 2010)**.** In the present study, AFB1 attacks the soluble cell compounds as well as membranes, eventually leading to the impairment of cell functioning and cytolysis (Berg, *et al.,* 2004**),** that appeared in the form of severe vacuolar degeneration, and impairment of cellular membranes.

Regarding the effect of addition of yeast to the mycotoxicated feed to fish, it promoted the intracellular

GSH which restored its levels following depletion through elevation of the glutathione peroxidase and glutathione reductase activities, total glutathione, and total reactive antioxidant potential levels and caused a reduction in the thiobarbituric acid reactive substances, lipid hydroperoxides, carbonyl protein and hydrogen peroxide concentrations (Hassan, *et al*., 2016). This beneficial effect of yeast was detected as a marked improvement of hepatic features manifested by improvement of the vacuolar degenerative changes at both low and high doses and nuclear membrane integrity. In addition, the ability of yeast, as a natural antioxidant, to compete the aflatoxin and ochratoxin pathogenic effects through the immune strengthening effect and protection of lipid and protein from oxidative damage was proved (Ouwehand, *et al.*, 2002).

Moreover, the known immune effect of probiotics was detected in the form of marked increase in the number of mutli-nucleated giant cells, which are the end result of macrophage activation and fusion (Saleh, 2010). The obtained results were confirmed by previous investigators as (El-Mahalaway, 2015).

The role of biological compounds of *Streptomyces* spp. and many natural herbal- based oils in control of renal medulla pathogenic effect caused by fungal pathogens had been detected in form of hyaline cast formation and coagulative necrosis of collecting tubules lining epithelia, while, Stocker *et al.*, 2012**,** reported sloughing of epithelial lining of the tubules which resulted in cellular casts. However, no improvement of the renal pathological features in yeast treated group could be recorded. On the other hand, evidence indicating that GSH conjugation plays an important role in the formation of toxic metabolites from a variety of chemicals that have accumulated. Thus, several classes of compounds are converted, via conjugation with GSH, into either cytotoxic, genotoxic, or mutagenic metabolites. The specificity and rate of mercapturic acid de-acetylation determine its toxicity; hence the mercapturate metabolites of aflatoxin and ochratoxins may be in relation with their cyto- and nephrotoxicity (Stocker, *et al.*, 2012). Therefore, the use of feed additives of natural origins to avoid the toxic effect of mycotoxins and certain biological preparation as probiotic and N. acetylcysteine and yeast have been successfully proved to inhibit mold growth and to reduce the incidence of aflatoxicosis and ochratoxicosis in fish.

Conclusions

In general, mycotoxicosis can be controlled by improving the methods of harvesting management, storing and transportation of feed in good environmental conditions. The frequent testing program of feed for mycotoxins contamination is mandatory and the addition of yeast to fish ration has been proved to alleviate the deleterious effects of mycotoxins on fish health and flesh quality.

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