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Research Article

Pathological effects of *Aspergillus* toxicity on gill structure of *Litopenaeus vannamei* in Iran by two different toxicological investigations

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Abstract

This histological study was conducted to discover the response of *Litopenaeus vannamei* to various levels of aflatoxigenic fungi (*Aspergillus*) toxicity. For this purpose, 400 specimens of live shrimp were gathered from a shrimp culture site in Iran. According to the results of the high-pressure liquid chromatography (HPLC) analysis, *Aspergillus parasiticus* had a higher production capability of total aflatoxin (TAF) (1073.804 ng g^{-1}) compared to *Aspergillus flavus* (292.349 ng g^{-1}). Two experiments with different toxicities of *Aspergillus* were assayed. In both experiments, the shrimps in 6 experimental groups were exposed to 0, 1, 5, 10, 15, and 20 ml of fungal spore suspension (FSS) in the feed (E1) and culture medium water (E2) in triplicate in each group for 4 weeks. The histopathology results of the gill tissue in the control group were normal. However, the inflammation, hemocytic infiltration, melanization, edema, and necrosis (as the main histopathological changes in the gill tissue) were observed after 28 days of the experiment when the toxicity of both experiments reached 18 µg kg^{-1} total aflatoxin and 1 ml FSS in E1 and E2, respectively. Furthermore, it was recognized that the histological alterations index (HAI) of gill was higher in E2 (0-130) than in E1 (0-74).

Keywords: *Aspergillus parasiticus, Aspergillus flavus,* Aflatoxin, Gill, *Litopenaeus vannamei*

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Introduction

In recent decades, with the expansion of the shrimp farming trade, there has been an urge to observe the physiological conditions of farm-raised penaeids regarding the incidence of many issues through this trade (Deng *et al*., 2020; Fang *et al*., 2020; Jannathulla and Dayal., 2023). Many researchers have reported the fungal contamination in the shrimp propagation process that caused economic losses for the global aquaculture industry (Zeng *et al*., 2016; Deng *et al*., 2020; Kracizy *et al*., 2021; Su *et al*., 2023). Also, various studies have shown that mycotoxins as a secondary metabolite of *Aspergillus* fungi have become a serious issue in the aquafeed and have also affected the health of aquatic animals especially aquatic invertebrates such as *Fenneropenaeus indicus* (Ghaednia *et al*., 2013), Common carp (*Cyprinus carpio*) (Al-Rubaiy *et al*., 2018) and *Litopenaeus vannamei* (Zhao *et al*., 2017; Jamshidizadeh *et al*., 2019; Wang *et al*., 2019; Kracizy *et al*., 2021).

Aflatoxin is an aromatic hydrocarbon that belongs to an oversized cluster of mycotoxins created by fungal species such as the *Aspergillus* genus, notably *A. flavus* and *A. parasiticus*, and species of the *Penicillium* and *Rhizopus* genera (Albero *et al*., 2022; Mirza Alizadeh *et al*., 2022; Smaoui *et al*., 2023). These Aspergillus spp. create four aflatoxin varieties such as aflatoxins B1, B2, G1 and G2, which the symbols B and G represent abbreviations for Blue and Green fluorescent colors produced under UV irradiation (Al-Ghouti *et al*., 2022; Pisoschi *et al*., 2023). Among these varieties, AFB1 is known as the most potent teratogenic and is considered a carcinogen, hepatotoxic, and immunosuppressant with the greatest toxicity for aquatic animals and humans (Alam *et al*., 2022; Cao *et al*., 2022; Gao *et al*., 2023; Mohamed *et al*., 2023).

In marine shrimp, the toxicity of AF can lead to abnormalities, such as physiological disorders, digestive function problems, poor growth, and histological changes, which decrease aquatic animal production (Zeng *et al*., 2016; Jamshidizadeh *et al*., 2019; Deng *et al*., 2020; Kracizy *et al*., 2021; Su *et al*., 2023). To ensure the health of farm-raised shrimps and increase the efficiency of the aquaculture industry, the monitoring of aquatic feed and culture ponds for *Aspergillus* fungal contamination is an essential factor that is commonly used in most countries. Since fungi invasion is related to environmental conditions such as temperature and humidity and improper feed storage methods to the growth of toxigenic fungi, the probability of aflatoxicosis in an area with a humid tropical climate like the Hormozgan region in Iran is common. In this regard, preventing aflatoxicosis and minimizing the economic failure caused by aquatic diseases, depends on the monitoring of pathogenic microorganisms in aquaculture ponds.

In recent years, *Litopenaeus vannamei* (Boone, 1931) has become the most widespread crustacean species cultured worldwide, especially in Asian countries (Abdirad *et al*., 2022;

Hembrom *et al*., 2023; Mondal *et al*., 2023). Although many researchers have studied the effects of various toxic substances on *Litopenaeus vannamei*, the effect of total aflatoxin (TAF) toxicity as a result of fungal contamination in shrimp farms has not been considered yet. Since the gills are closely related to both the hemolymph or blood system and external environment of aquatic animals which are generally multifunctional for acid-base balance, osmoregulation, respiration, and excretion of nitrogenous waste in shrimp, the tissue of this organ acts as a host of a secondary metabolite of toxigenic fungus (Callaghan *et al*., 2016; Allen and Weihrauch, 2021). However, there is rare information about the effect of aflatoxins on the structures of the gill. Therefore, in the present research, it was tried to determine the pathogenicity of *Aspergillus* spp. For this purpose, histopathology of the gill of *Litopenaeus vannamei* was assayed as a biomarker for monitoring the physiological conditions or controlling these economically important pathogens in shrimp farms.

Materials and methods

Research design

In this research, toxic substances were tested for the histopathological study of the gill by employing two following experiments for 4 weeks (Fig. 1): 1) E1 (experimental shrimps exposed to aflatoxin in feed) and 2) E2 (experimental shrimps exposed to aflatoxin in culture medium water).

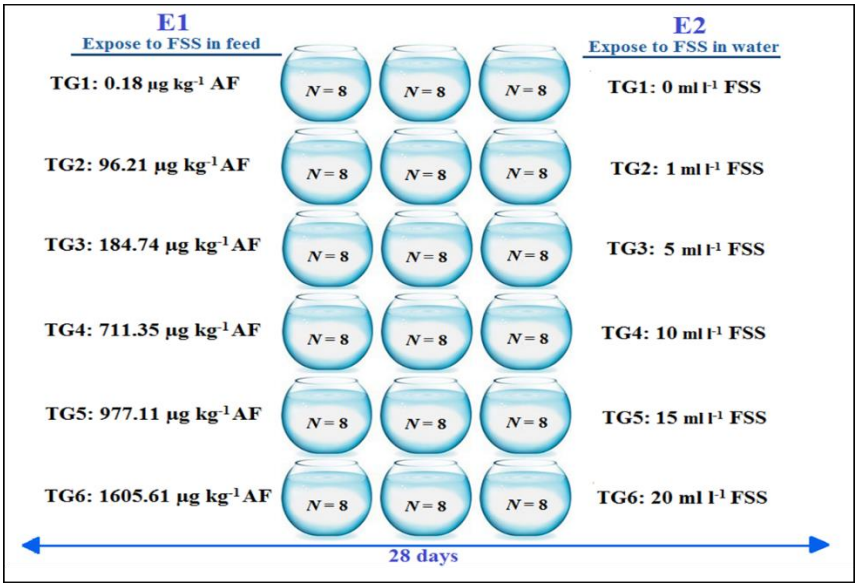

Figure 1: Design of experiments (E1 and E2). FSS: fungal spore suspension, AF: total aflatoxin detected in experimental diets.

In these experiments, several groups of 8 shrimps were introduced into glass containers with 20 l-adaptation culture medium water. In the first experiment (E1), shrimps in six testable groups were fed with aflatoxin-contaminated diets for 28 days as follows: diet 1 (control) without FSS and diets 2-6 (with 1, 5, 10, 15, and 20 ml of FSS per kg). Aflatoxin concentrations of the experimental diets

were found to be 0.18, 96.21, 184.74, 711.35, 977.11, and 1605.61 µg kg^{-1}, respectively. In the second experiment (E2), various volumes of fungal spore suspension (FSS) were transferred to 6 groups of 20 l-culture medium water as follows: group 1 (control) without FSS and treatment groups 2-6 with 1, 5, 10, 15, and 20 (ml) of FSS per l, respectively.

Toxic substances

Fungal strains, growth medium, and growth conditions

In this study, two species of aflatoxigenic *Aspergillus* including *A. flavus* (PTCC 5018), and *A. parasiticus* (NRRL 5286) were used to compare the TAF production. The growth method of mentioned strains was modified as follows: Briefly, fungi were cultured on sabouraud dextrose agar (SDA) in Petri dishes containing medium and were allowed to grow in the dark and sterile environment in an incubator at 27℃ for 7 days for the analysis of TAF production.

Quantification of total aflatoxin (TAF)

After 7 days of incubation in the dark at 27℃ on SDA medium, samples were prepared and extracted for aflatoxin quantitation by HPLC according to a previous study (Abbas *et al*., 2004). TAF (ng g^{-1}) in the samples was quantified to introduce a suitable species of *Aspergillus* with a high capacity of aflatoxin production for further process.

Experimental toxic substances

In the current study, the experimental protocol was modified and designed according to the previous studies (Casado *et al*., 2001; Lim *et al*., 2001; Bintvihok and Kositcharoenkul, 2006; Deng *et al*., 2010). After comparing the TAF production capacity between experimental fungi, *A. parasiticus* was cultured on SDA as explained in the previous section. To track of research process, the conidiospore from the culture was collected in 1 ml of sterile water. The spore concentration of the suspension samples was determined using a hemocytometer as described in (Nesci *et al*., 2003).

In the first experiment (E1), shrimps in six testable groups were fed with aflatoxin-contaminated diets for 28 days. The aflatoxin-contaminated shrimp meal was prepared using six group of commercial shrimp meal (100 gr) with the same basal combination (Table 1). Next, different volumes of FSS (with a concentration of 10^6 spore mL^{-1}) were placed on the diets with a moisture ratio of 20% separately, as follows: diet 1 without FSS (control) and diets 2-6 with 1, 5, 10, 15, and 20 (ml) of FSS per kg, respectively. To allow aflatoxin production, the mix is placed in an orbital shaker at 150 rpm and 27℃ for 7 days. Then, the meal contaminated with aflatoxin was oven-dried at 90℃ such that its moisture content was decreased to 10%. Finally, the TAF was subjected to feed analysis by the HPLC technique. The meal contaminated with aflatoxin was kept at -20℃ until use in the feeding experiment. In the second

experiment (E2), various volumes of fungal spore suspension (FSS, with a concentration of 10^6 spore mL^{-1}) were transferred to 6 groups of 20 l-culture medium water as follows: group 1 (control) without FSS and treatment groups 2-6 with 1, 5, 10, 15, and 20 (mL) of FSS per l, respectively.

Table 1: Basal components of the shrimp feed (g kg^{-1}diet).

Ingredients	Content
Fish meal	200
Soybean meal	300
Wheat flour	245
Soy protein concentrate	70
Beer yeast	40
Chicken meal	30
Shrimp meal	30
Soy oil	20
Fish oil	10
Soy lecithin	10
Vitamin premix1	10
Mineral premix2	10
Monocalcium phosphate	20
Choline chloride	5
Total	1000

1 Vitamin premix (kg 1 diet): vitamin A, 250 000 IU; riboflavin, 750 mg; pyridoxine HCl, 400 mg; cyanocobalamin 1 mg; thiamine, 250 mg; menadione, 250 mg; folic acid, 125 mg; biotin, 10 mg; a-tocopherol, 2.5 g; myo-inositol, 8000 mg; calcium pantothenate, 1250 mg; nicotinic acid, 2000 mg; vitamin D3, 45 000 IU; vitamin C, 7000 mg.
2 Mineral premix (kg 1 diet): ZnSO4 7H 2O, 0.04 g; CaCO3, 37.9 g; KCl, 5.3 g; KI, 0.04 g; NaCl, 2.6 g; CuSO4 5H2O, 0.02 g; CoSO4 7H 2O, 0.02 g; FeSO4 7H2O, 0.9 g; MnSO4 H2O, 0.03 g; MgSO4 7H 2O, 3.5 g; Ca(HPO4)2 2H2O, 9.8 g.

Shrimp and experimental conditions

In the present study, live and healthy samples (Juvenile Pacific white shrimps, *Litopenaeus vannamei*), with an average weight of 9.74±1.52 (g), were caught from the Tiab located in the east of Bandar Abbas, Hormozgan province (56°51′ E and 27°06′ N) in Iran and were transferred to the laboratory of Hormozgan University. The location of the sampling site is shown in Figure 2. Then, for the aim of adaptation, 8 shrimps were put into 18 fiberglass containers (DO 7.71±0.74 mg/L, 30.09±2.84 ppt, pH 8.23±0.32, and 26.06±2.80°C) for two weeks. Shrimp feed pellets were used for feeding shrimps four times a day during the adaptation period. The water was replaced every 24 h and the experiment was carried out for 28 days. Dead samples were eliminated and recorded during the experiment.

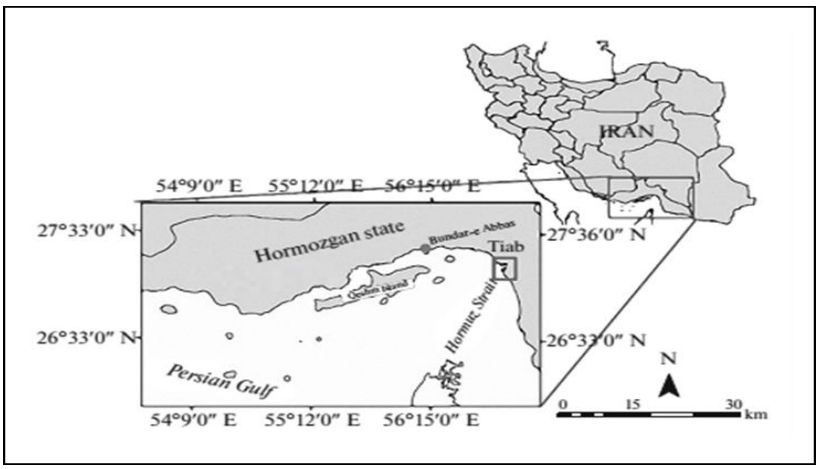

Figure 2: Location of sample collection.

Collection of sample

Shrimps were deprived of feed for 24 h before the sample collection process. Next, three shrimps from each aquarium were caught and their gill was separated for histological studies.

Histopathological process

Preparation of tissue slides

After 4 weeks of exposing the experiment, the gill of shrimps was evaluated using a light microscope (OLYMPUS) through routine histological methods. The gills were immersed in the Bouin's solution fixative for 24 to 72 h to prevent autolysis of this organ before further processing and were exposed to ethyl alcohol 70% for long-term maintenance. Next, following the routine H&E (Harri's hematoxylin and eosin phloxine) stain protocol, tissues were prepared for microscopic evaluation. Briefly, gills were dehydrated via a series of enhancing ethanol concentrations cleared in xylene and infiltrated with liquid paraffin at 57ºC using an automatic processor of tissue (DS 2080/H). Afterward, they were placed in paraffin as a tissue paraffin block. After trimming of tissue blocks, they were cut into 5-µm thick slides using a semiautomatic-rotary microtome. Finally, the samples were dyed with H&E stain protocol. The tissue slides were examined through an optical microscope and photomicrographs were performed by a digital microscope camera (Dino Eye AM423X, Taiwan).

Histological alterations index (HAI)

After investigating the photomicrographs of tissue slides, the histological damage intensity of the gill in experimental shrimps was measured as an HAI. Tissue injuries were categorized into three progressive stages during this method. Tissue damage levels are denoted as stage I, stage II, and stage III (Table 2) with "no effect on organ operation", "more intense injury and impaired organ operation", and "very intense and irretrievable changes to operation and structure", respectively (da Silva Montes *et al*., 2011).

Table 2: Classification of the histological alterations found in gill of *Litopenaeus vannamei* examined during experiments (da Silva Montes *et al*., 2011).

Stage I	Stage II	Stage III
Curling and clubbing of the lamellae	Inflammation of lamella	Necrosis
Lamellar epithelial hyperplasia	Hemocytic infiltration	
Atrophy of lamellae	Melanization	
Oedema	Pillar cell disruption	
Desquamation of epithelial lamellae		

Values of HAI were determined according to the following formula (Poleksić and Mitrović-Tutundžić, 1994):

$$HAI = [1 \times \Sigma \text{ (stage I)}] + [10 \times \Sigma \text{ (stage II)}] + [100 \times \Sigma \text{ (stage III)}]$$

In this histologic analysis, the average HAI value calculated using this method was classified into five categories: 0-10 (normal tissue operation), 11-20 (mild to moderate change), 21-50 (moderate to severe change), 51-100 (severe change), and >100 (irreparable change).

Statistical analysis

Statistical analysis of this research and plotting the graphs were carried out using SPSS 22 and Excel software packages, respectively. HAI variables were compared utilizing an independent Student's t-test (p<0.05). Other experimental parameters were compared by a One-way and Two-way ANOVA test (p<0.05). Eventually, the comparison between experimental groups was made by Duncan's multiple range test (p<0.05).

Results

Aflatoxin levels produced by experimental fungi

After incubation of *A. flavus* and *A. parasiticus* in the dark at 27℃ for 7 days, TAF was extracted from each sample. Table 3 shows levels of aflatoxins (ng g^{-1}) as mean±standard division of three independent plates for each fungal strain. Compared with *A. flavus*, aflatoxin production by *A. parasiticus* was found to be significant (p<0.05) after 7 days. A higher level of aflatoxin with significantly different compared with *A. flavus* (p<0.05) associated with AFB1 by 86.2% TAF (925.895 ng g^{-1}) in the *A. parasiticus* strain. In contrast, AFG1 and AFG2 production by *A. flavus* was not detected. Estimating the aflatoxin by HPLC method revealed the significant production of TAF in *A. parasiticus* by 1073.84 ng g^{-1}, indicating a 27.2% increase from 292.349 ng g^{-1} by *A. flavus*.

Table 3: Production of aflatoxins by experimental *Aspergillus* spp.

Aspergillus spp.	Aflatoxin type				Total aflatoxin
	B1	B2	G1	G2	
A. flavus (ng g-1)	5.528±0.603[b]	286.821±23.690[a]	NO	NO	292.349
A. parasiticus (ng g-1)	6.415±1.127[c]	925.895±24.241[a]	4.477±1.205[c]	137.017±39.012[b]	1073.804

Note: Data represent mean±*SD* of three replicates. Values with different superscripts in the same column indicate statistical difference at $p<0.05$

Aflatoxin levels in the experimental diets

In current study, aflatoxin contamination (0.18 µg kg^{-1}) was detected in diet without FSS (control group). However, aflatoxin concentrations of diets 2-6 (with 1, 5, 10, 15, and 20 ml of FSS per kg) were found to be 96.21, 184.74, 711.35, 977.11, and 1605.61 µg kg^{-1}, respectively (Table 4).

Table 4: Aflatoxin levels of the experimental diets.

Experimental diets	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fungi spore suspension (mL kg^{-1})	0	1	5	10	15	20
Detected Aflatoxin (µg kg^{-1})	0.18±0.02[f]	96.21±3.80[e]	184.74±25.33[d]	711.35±30.12[c]	977.11±35.07[b]	1605.61±44.18[a]

Note: Data represent mean±*SD* of three replicates. Values with different superscripts in the same column indicate statistical difference at $p<0.05$.

Gill histopathology and histological alterations index

In the present research, histological evaluation of the gill of shrimps indicated that the gill tissue of the control group was normal; like the typical histologic structure previously introduced for Penaeidae shrimps (Fig. 3).

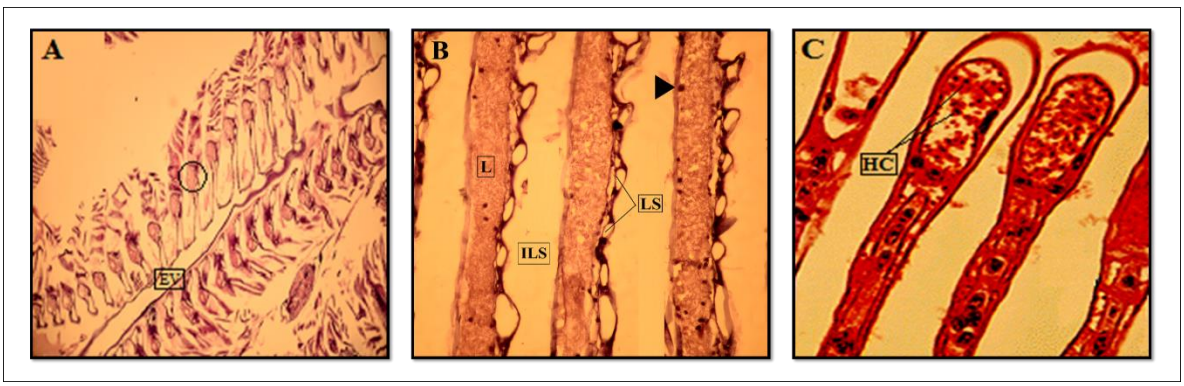

Figure 3: Normal structure of gill of *Litopenaeus vannamei*. Circle: each tip of filament is occupied by a hemolymphatic lacuna, EV: efferent vessel (a); L: normal lamellae structure, ILS: uniform interlamellar spaces, LS: lamellar sinus, Triangle: pillar cells (b); HC: hemocytes (c). Scale bars: a and b: 20µm; c: 10µm.

However, according to the study of tissue slides photomicrographs, when the feed and culture medium water was contaminated for experimental shrimps, notable histological damage was observed in shrimps from both experiments (Figs. 4 and 5). In E1, the gills indicated hemocytic infiltration and epithelial inflammation at a concentration of 711.35 µg kg^{-1} AF. Pathology of melanization was presented when the concentration of AF exceeded 977.11 µg kg^{-1} AF. Furthermore, a maximum HAI value of 48.3 and 74.3 was observed in the treatment groups of 5 and 6, suggesting moderate to severe and severe changes to the gills (Fig. 6). The fusion of lamella and hyperplasia of the basal epithelial cells was observed at a concentration of 711.35 until 1605.61 µg kg^{-1} AF (with different severities). (In E2, similar pathologies were detected coupled with necrosis, atrophied hemolymphatic lacuna, curling, and clubbing of the lamellae. This result is associated with the level of aflatoxin on the branchial lamellae (Fig. 5). These observations were reflected in the enhanced HAI values in treatments 4, 5, and 6 with means of 85.3, 117.6, and 130.3, respectively, indicating damage shift from severe to irreparable alteration in gill structure.

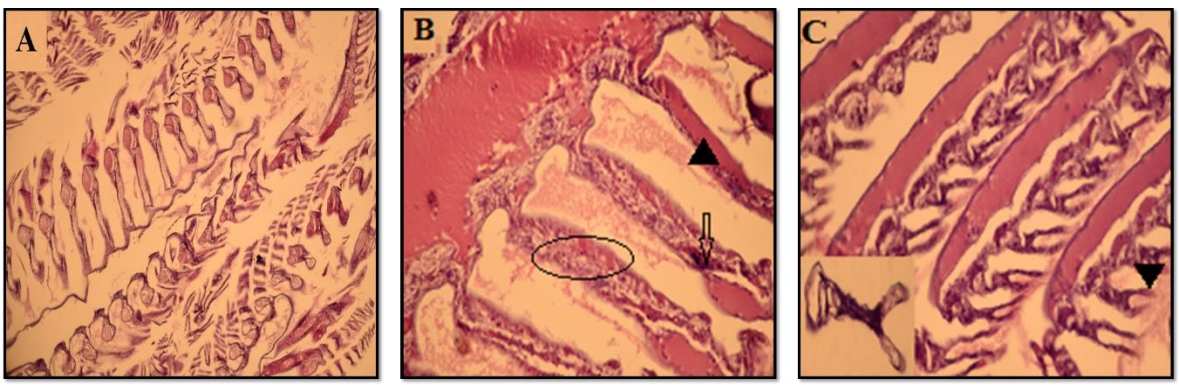

Figure 4: Gill structure of *Litopenaeus vannamei* exposed to aflatoxin in E1. Abnormal lamella (a); Arrow: melanisation, Circle: oedema, Triangle: haemocytic infiltration (b); Triangle: clubbing of the lamellae (c). Scale bars: a: 20µm; b and c: 10µm.

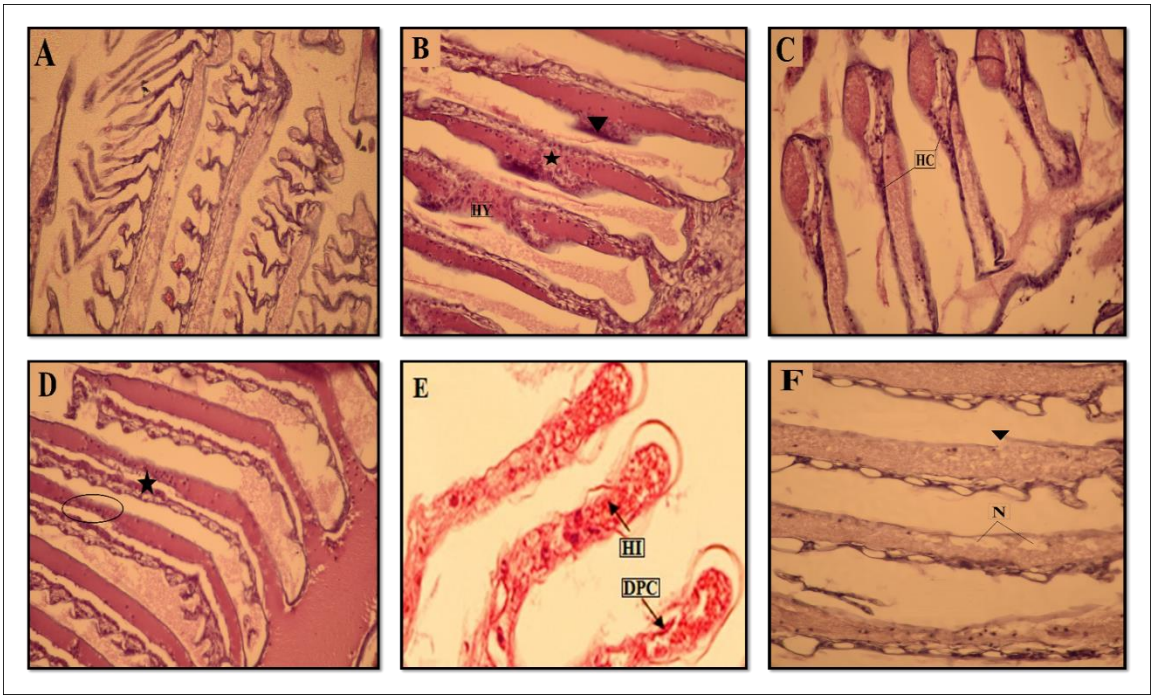

Figure 5: Gill structure of *Litopenaeus vannamei* exposed to aflatoxin in E2. Dissemination of gill lamellas coupled with curling and clubbing of the lamellae (a). HY: hyperplasia, Star: inflammation with accumulation of hemocytes, Triangle: melanization (b); HC: accumulation of hemocytes (c); Circle: atrophy of lamellae, Star: accumulation of hemocytes (d); DPC: disrupted pillar cells, HI: haemocytic infiltration (e); N: Necrosis in gill lamellae, Triangle: desquamation of the cuticle (f). Staining: H&E. Scale bars: a: 20µm; b-f: 10µm.

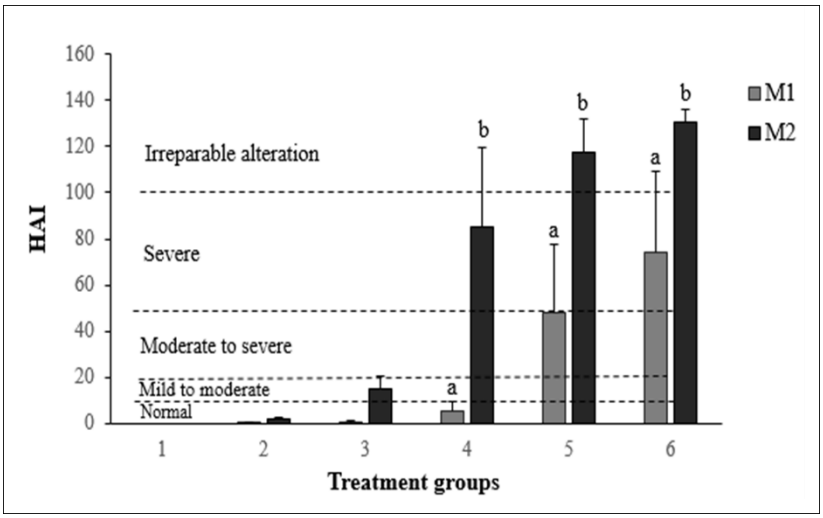

Figure 6: Histological alteration index (HAI±SD) values of gills in E1 (gray bars) and E2 (black bars). Different letters indicate significant differences (p<0.05) between experiments in the same treatment groups.

Figure 6 presents the values of HAI in both experiments in the experimental shrimps, which are significantly different between experiments in treatment groups of 4-6 (p<0.05). According to HAI values, the observed alterations in the gill of treatment shrimps were relevant to the concentration of dietary aflatoxin. Therefore, by increasing the content of

aflatoxin in the diet, the HAI value was also increased significantly (p<0.05). Evaluating the tissue photomicrographs indicated a maximum of HAI with a mean of 74.3 and 130.3 in a sixth treatment group in E1 and E2, respectively, indicating severe and irreparable alteration. Small values of HAI were detected to be equal to 0, 0.3, 0.6, 5 and 48 in E1 and 2, 15, 85, 117 and 130 in E2.

Discussion

All infective microorganisms possess some attributes that permit them to invade and cause harm to organisms that are exposed to their toxic substances. Toxicity is a term that refers to the flexibility of a being to provide toxins capable of reworking the conventional operation of cells or tissues and destroy them. Since some toxins are secreted outside the producer microorganism and cause severe harm after they penetrate the host organism (Atlas, 1995), the toxicant substances of *Aspergillus* spp toward shrimp were very high, showing their important histopathological effects.

Many researchers have reported that some strains of fungi are isolated from aquaculture ponds, aquafeed, and even the body of shrimp. A study of Ochoa *et al.* (2015) recognized that strains of *Geotrichum and Fusarium* isolated from the walls and bottoms of the culture ponds of *L. vannamei*. Also, aquaculture center in Asian countries like southern provinces of Iran is thought to be potential locations for fungal contamination (Gonçalves *et al.*, 2017; Gonçalves *et al*., 2018a, b; Jamshidizadeh *et al.*, 2019). This fungal contaminant might have occurred due to factors such as the low quality of aquafeed and pond water caused by inappropriate management of shrimp farms in a wet tropical climate. Therefore, such places provide a suitable spot for mold growth, followed by contamination of shrimp feed and water with fungal substances. Lahouar *et al.* (2016) have shown that temperature and water activity affect *Aspergillus* spp growth, leading to aflatoxin production. Among the isolated fungal strains such as *Penicillium*, *Fusarium*, *Cladosporium*, *Candida*, and different genera, *Aspergillus* spp is known to be the most important and powerful fungal species to produce aflatoxins, which are mycotoxins known as toxigenic potential (Guo *et al*., 2018; Yu *et al*., 2020; Navale *et al*., 2021; Wei *et al*., 2023).

Production of aflatoxin is reported to occur by 3 major species of *Aspergillus*; *A. flavus*, *A. parasiticus*, and less frequently by another *Aspergillus* species (Ahmed *et al*., 2020; Navale *et al*., 2021). Many researchers have recognized variations within the substance production of *A. flavus* and *A. parasticus*, although they are closely connected species (D'Mello and Macdonald, 1998; Klich and Pitt, 1988; Do and Choi, 2007; Nikolic *et al*., 2018; Rushing and Selim, 2019; Ráduly *et al*., 2020). In the current experiment, the aflatoxin production capability of important *Aspergillus* spp was analyzed with HPLC. As shown in Table 3, *A. parasiticus* synthesizes AFB and AFG

types, whereas *A. flavus* creates only AFB. Therefore, *A. parasiticus* produces a high concentration of TAF (1073.804), suggesting a better production capability of TAF than *A. flavus*. Many researchers have reported that over 90% of all *A. parasticus* produces high concentrations of aflatoxin, as well as B and G types. On the opposite side, it has been reported that no more than 50% of *A. flavus* can synthesize toxins and if they produce simply B aflatoxins (Rushing and Selim, 2019; Priesterjahn *et al*., 2020). In another study (Priesterjahn *et al*., 2020), *A. flavus* produced aflatoxins B1 and B2, whereas *A. parasiticus* generated aflatoxins B1, B2, G1, and G2. According to these results, in the current study, *A. parasiticus* was chosen for analysis of the toxicity of Aflatoxigenic *Aspergillus* and utilized for the preparation of experimental diets, water, and tracking the further process.

Nowadays, increasing amounts of plant proteins such as soybean, peanut, corn, cottonseed, or wheat are being combined into aquatic animal feeds to avoid the heavy prices of animal-based resources (Hua *et al*., 2019; Dawood and Koshio, 2020; Salehpour *et al*., 2022). A high amount of these plant components are polluted with mycotoxins over time (Dawood and Koshio, 2020; Jia *et al*., 2022). The most mycotoxins typically detected in aquaculture feed are different types of aflatoxin (AFB1, AFB2, AFG1, and AFG2) (Ottinger and Kaattari, 1998; Huang *et al*., 2011). In current research, it was discovered that although FSS was not added to the control shrimp diet, 0.18 µg kg^{-1} of TAF was detected. This result seems to be due to a suitable climate for fungus growth, the low quality of shrimp feed components, and improper strategies of shrimp processing and aquafeed storage in Hormozgan, Iran. Many studies have shown the prevalence of aflatoxin in industrial aqua feeds (Gonçalves *et al.*, 2017; Gonçalves *et al*., 2018a; Gonçalves *et al*., 2018b; Hassaan *et al*., 2020). The analysis of the mycotoxin prevalence in Asian countries (Gonçalves *et al.*, 2018b) indicates the common aflatoxin contamination of commercial aquafeed in this region, which is due to the climate conditions of these countries. This researcher has reported that aflatoxin contamination in 69% of the aqua feed samples caused a high level of aflatoxin with a quantity of 51.82 µg kg^{-1}. Bautista *et al.* (1994) examined industrial shrimp feeds in the Philippines and reported different levels of AFB1 contamination from non-found to 120 µg kg^{-1}. Deng *et al.* (2010) reported AFB1 contamination of 62.6, 13.3, and 1.6 µg kg^{-1} in peanut meal, soybean meal, and fish meal, respectively. Bintvihok *et al.* (2003) indicated the presence of aflatoxin in shrimp feeds utilized in aquaculture industry. Zeng *et al.* (2016) found 16.9 µg kg^{-1} of aflatoxin B1 within the control shrimp diet with no additional aflatoxin. Other studies also detected aflatoxin in the control aquatic diet (Han *et al*., 2010; Rajeev Raghavan *et al.*, 2011; Huang *et al*., 2014).

In aquatic animals, the gills are multi-functional organs that operate between the animal and the ambient environment. Gills are referred to as the foremost

susceptible organ because they are subjected to all or any toxicant pathogens within the environment (Salehpour *et al*., 2021; Lavanya and Dayakar, 2022). Also, Randall *et al.* (1998) incontestable that the gills are often a significant route of uptake even for chemical contamination. Histopathological changes in respiratory organ structure by any annoyance materials like poison dissolved or suspended within the water directly affect the mechanisms of respiration and osmoregulation. They eventually might lead to hypoxia, impaired gas exchange, metabolism failure, and issues with the ionic and acid-base balance of aquatic animals. (Callaghan *et al*., 2016; Allen and Weihrauch, 2021). Hence, aflatoxicosis may have an economically important impact on the production level of aquaculture farms. According to these issues, in the current study, histopathological damages of this substance in the gill of *l. vannamei* exposed to *A. parasiticus* metabolite were evaluated.

The histologic survey of the control gills within the present research and other scientists (de Araujo and Valenti, 2018), demonstrates that the gill structures of shrimps are shaped primarily by normal lamellae structure, efferent vessel, pillar cells, lamellar epithelium, hemocytes, and hemolymphatic lacuna. The role of hemocytes as a part of the cellular psychoanalytic process of crustaceans has been proved. Also, their larger presence is regarded as a symptom of inflammation (Frischer *et al*., 2022). Despite, increased level of hemocytes in the gill layer of *L. vannamei* can be a sign of blood circulation damage caused by inflammation. Shahafve *et al.* (2017) evaluated histopathology of gills in the fish treated with sublethal levels of aflatoxin (0.5, 0.7, and 1.4 mg kg^{-1} feed) for 21 days. Necrosis of gills, curling, and clubbing of the lamellae, inflammation of cells, and gill epithelium necrosis are the main histopathological alterations reported by this scientist within the gill of experimental fish. Necrosis is a lesion in which the cell cytoplasm is uniformly stained in all parts of the cell. In fact, necrosis is a sign of a decrease in cell activity and cell death. Eventually, these cells are cytolysis or phagocytes by lymphocytes (Gente *et al*., 1999; Xu *et al*., 2022).

Necrosis in the gill indicates aflatoxin effects on the endothelial cells of the vascular system and most pathologic damages are determined in these cells due to their sensitivity to aflatoxin (Zeng *et al*., 2019). Similar changes were determined in rohu fish (*Labeo rohita*) exposed to AFB1 (Sahoo *et al*., 2001). This researcher reported epithelium hyperplasia and cell necrosis in gill lamellae of rohu fed with aflatoxin-contaminated. Similar changes are determined in grass carp (*Ctenopharyngodon idella*), rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), juvenile maroon clownfish (*Premnas biaculeatus*), and crab (*Portunus pelagicus*) (Romano and Zeng, 2007; Rodrigues *et al*., 2014; Abdel-Daim *et*

al., 2020; Imani *et al*., 2020; He *et al*., 2023). Al-Azri *et al.* (2015) found the hyperplasia of the primary lamellae's epithelial layer, desquamation of epithelial cells, and cellular degeneration at aflatoxin concentrations more than 100 µg, which led to necrosis of epithelial tissues of gill by day thirty of the test. Hyperplasia of gill filaments is a long-term response of squamous cells that often occurs in response to low levels of harmful agents. Epithelial cell hyperplasia is a chronic response to microbial and parasitic infections or chemical agents. The destruction of pillar cells is due to the intense flow of blood into the gill blades or even the direct effect of pollutants on these cells. Swelling of the lamellae is the reason for chronic changes in the gill structure. By microscopic examination, it is clear that the epithelial cells accumulate at the upper layer of lamella, so they are club-shaped. (Sharifpour *et al*., 2011).

According to the literature, several studies report that toxic substances caused changes in the structure of the gill and the histological damage was enhanced by raising the concentration of toxic substances in gills. Nevertheless, the effects of fungal toxic metabolite in medium water of aquatic animals have received less attention. Soegianto *et al.* (2013) discovered that copper caused changes in the structure of the gill, where the histologic injury deteriorated with rising copper concentration, leading to gill hyperplasia and necrosis. Usman *et al.* (2013) stated that the gill structure of *L. vannamei* juveniles considerably altered with a sublethal exposure of lead for 15 days. Such exposure affects its organs including gill hyperplasia, hemocytic accumulation in lamellae, and inflammation. Moreover, these serious structural injuries suggest that copper may prevent the physiological functions of the gill. This researcher also showed that histologic injury will increase with increasing lead concentration. Similar microscopic anatomy alterations are discovered in different crustaceans subjected to heavy metals (Frías-Espericueta *et al*., 2008; Shukla *et al*., 2019; de Jesus *et al*., 2021) According to this study, once crustaceans are subjected to various levels of metals, they show changes including a blackened look of the gills, necrosis, and hyperplasia of gill cells, abnormal structure of the epithelium, and desquamation of cuticle. Furtado *et al.* (2015) found hyperplasia in the gills of *L. vannamei* subjected to concentrations of nitrate for a long period of the experiment. Lamela *et al.* (2005) identified edema within the gills after they cultured *L. schmitti* below low salinity and attributed it to a discount in osmoregulatory capability that influenced vascular permeableness.

According to the results of the present study, water and feed quality in deterioration aquaculture centers triggered changes to the structure of the gill and also the microscopic anatomy injury with an increasing quantity of FSS in water. Similarly, TAF in feed resulted in abnormal lamella (clubbing of the lamellae), edema, inflammation, hemocytic infiltration, and melanization (Figs. 4 and 5). In the current study, to

match the intensity of lesions in shrimps subjected to various experiments of aflatoxin contamination, histopathological study results of every experimental group were quantified semi quantitativly as histological alternation index (HAI) of gill (Table 2).

Pathologies that were maintained at stage I and were thought to be normal structures, after 4 weeks of both experiment, have turned into stage II tissue damage, actually changed from moderate to irreparable alteration. Within the treatment groups, HAI value in E1 grows at a lower rate than E2 reaching a severe level. However, injuries in E2 converted significantly to irreparable alteration that caused by water pollution with aflatoxin. In treatment group 4, once a normal structure of the gill was maintained in E1, injury in E2 accrued to severe alteration significantly. Also, when toxicity increased in treatment groups 5 and 6, the tissue injury accrued from moderate and severe in E1 severally to irreparable alteration in E2. Moreover, analysis of HAI indicated that the type and magnitude of histopathological alterations in gill tissue vary based on aflatoxin level in both experiments. So, once the largest amounts of toxicity were detected in treatment groups 5 and 6, the injury to the gills became irreparable and could have induced the high toxicity in experiment E2 (Fig. 6). Moreover, concerning the values of HAI (>100) in treatment groups exposed to FSS≥15 mL, we discovered cellular degeneration caused by changes from stage-3 lesions, like necrosis of the gill epithelial tissues of these experimental groups with a completely various severity in E2.

A Histological study by Ferguson (1989) recognized that the hyperplasia of gill lamellae could also be elicited by the impact of the poisonous substance that changes glycoprotein within the mucous secretion covering the cells. Therefore, it influences the negative bars of the epithelium and inflics interpolation to the adjacent lamellae. It seems that exposure to aflatoxin also enhanced the secretion of mucus by the gills, probably resulting in disabled gas exchange. Thus, they enhance the thickness of the epithelial cells that are in contact with the external environment. These alterations within the epithelial cells are examples of protection mechanisms against solid or microbial agents in suspension. Accordingly, they enhance the gap between the blood and the external environment. Eventually, they serve as an obstacle to the importation of contaminants (Hadi *et al*., 2009), due to the impaired oxygen uptake (Figueiredo-Fernandes *et al*., 2007).

The mentioned damage due to the presence of *Aspergillus* spp in the feed and water used in the aquaculture industry indicates the disability of gill function. In this regard, its potential to affect the respiratory/osmoregulatory capacity of *L. vannamei* can cause an economically significant effect on the production of farm-raised shrimp. Large amounts of toxicants, which contaminate shrimp farms through feed and water, are accumulated in gill filaments, covering their surfaces and

blocking the chambers of gill. Metabolic acidosis and tissue hypoxia and damage to immune system , as well as the flexibility of shrimp to infection are caused and as a result the adverse effects of poisons (Bauer, 1999; Scholnick *et al.*, 2006; Martin and Hose, 2010). Shrimp subjected to contaminant stress have low inviolability and reduced resistance against numerous toxicant substances (Liao *et al*., 2012; Li *et al*., 2017). In our experiment, contaminated feed and water confirm the histologic response in *L. vannamei*.

In the present study, more damage within the gills was determined in shrimp that were exposed to greater toxicity by the rise within the *A. parasiticus* spore suspension (up to 15 and 20 mL L^{-1}) in water (E2). The main damages related to the gills were hemocytic infiltration, lamellae inflammation, melanization, and necrosis. In this research, three phases of histological changes were found in gills under enhancing toxicity in feed and water: (i) inflammation in gill lamellae and edema; (ii) hemocytic infiltration by toxic detection; and (iii) melanization and necrosis. These pathologies can cause moderate to irreversible changes resulting in respiratory failure and a high risk of gathering the cultured shrimp. Besides, this study discovered that once toxicity increased in both experiments, a greater histological alteration index (HAI) is recorded in gills. This response reveals the sensibility of the gills to the existence of aflatoxigenic *Aspergillus* in feed and water. Impairment of gill function can cause respiratory loss of *L. vannamei*, leading to an economically significant effect on aquaculture. Since these fungi are capable of producing mycotoxins, further studies are necessary to regulate and interfere with these economically important pathogens in aquaculture. Thus, this study suggests monitoring the physiological conditions as a biomarker to control and prevent these economically important pathogens. Further research works should also be conducted to assess the impact of the toxic secondary metabolite of fungus at the histological levels in other organs of shrimp.

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