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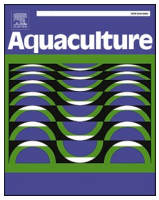

Fungal contamination and mycotoxins in aquafeed and tissues of aquaculture fishes and their biological control

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ABSTRACT

The presence of mycotoxins in food and feed is a significant issue, especially in fish farming where it can negatively impact farmed fish species. In this study, samples of aquaculture feed and fishes tissues were collected for fungal isolation and mycotoxins analysis. Levels of aflatoxins (AFs) and ochratoxin A (OTA) in the muscles and organ (liver and kidneys) were compared in three fish species. Furthermore, the volatile organic compounds (VOCs) emitted from a novel *Bacillus cereus* BC344–2 strain were tested against the growth and mycotoxin production of *Penicillium* spp., *Aspergillus* spp., and *Fusarium* species. There was a moderate fungal load in the aquafeed with $1.30 \times 10^2 \pm 2.6 \times 10^2$ (mean ± SD) CFU/g with high contribution of *Aspergillus* and *Penicillium* fungi. OTA and AFs were detected in 95% and 66.7% of the tested aquafeed samples, respectively, with 66.7% of the samples co-contaminated with both mycotoxins. Although AFs contents were within permissible limits, 4.8% of samples showed OTA contamination exceeding the permissible limits (5 μg/kg). Both mycotoxins were found in the liver samples, but none of the fish meat (muscle) samples were found to be contaminated with OTA. The in vitro biocontrol co-incubation assay showed that BC344–2 VOCs had a significant inhibitory effect on the growth and sporulation of the three exposed fungi. *P. verrucosum* showed the highest sensitivity with a 42.4% inhibition ratio, followed by *F. solani* (17.5%) and *A. flavus* (11.5%). Additionally, BC344–2 VOCs suppressed OTA and AFs synthesis by *P. verrucosum* and *A. flavus*, respectively. Gas chromatography-based analysis of headspace volatiles in BC344–2 volatilome revealed five bioactive compounds with BTH aldehyde and 1-Heptadecanol being the most probable antifungal compounds responsible for the inhibitions. Given the high detection rate of OTA and AFs in the fish tissue and feed samples, along with regular monitoring of mycotoxins, the biocontrol approaches using bacterial volatiles such as BC344–2 VOCs could be useful to ensure feed and food safety.

1. Introduction

Fish, a vital component of food in almost all countries across the globe, has been an important dietary source of protein throughout human history. Apart from being protein source, fish meat compared to terrestrial animal meat is rich in essential micronutrients including polyunsaturated omega-3 fatty acids, vital minerals, water-and fat-soluble vitamins and trace elements (Tacon et al., 2010; Tacon and Metian, 2013). Given the high nutritional value of fish, the consumption of fish meats has been associated with several health benefits including a reduction in the risk of death from cardiovascular and coronary heart diseases, stroke and cancer (Gunge et al., 2017; Hengeveld et al., 2018). More recently, in the European Environment Agency (EEA, 2016) report, global fish consumption is reported to have almost doubled from an average consumption of 9.9 kg/capita to 19.7 kg/capita and countries in Europe and Asia among other continents are estimated to have consumed an average of 22.6 kg/capita and 21.3 kg/capita of fish, respectively per year between 1960 and 2013 (Belchior et al., 2016).

According to Food and Agriculture Organization (FAO, 2018), aquaculture is growing more rapidly (at the pace of 5.8% annual growth in 2000–2016) than any other food production system. Moreover, a considerable proportion of the fish entering global markets for consumption today is sourced from fish farming contributing to 68% of global aquaculture output (Nogueira et al., 2020). In the Middle East, where aquaculture is mainly used for domestic production, has increased from 21.4% in 2001 to 44% in 2011, with Egypt, Saudi Arabia and Iran as the main producers in the region (Towers, 2014). More specifically, in Qatar, aquaculture production centers producing tilapia

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and shrimps have currently been established as alternative to the weakened captured fisheries (Abusin and Mandikiana, 2020). The productivity of these aquaculture production systems especially intensive aquaculture systems rests on the provision of protein-rich feed which is estimated to account approximately fifty-percent of total production cost (Marijani et al., 2019). This high cost of fish feed, in addition to the environmental (Tacon and Metian, 2008) and sustainability concerns (Boyd, 2015) associated to its production, has, in the past decade, resulted in a global shift from the use of fishmeal as the sole protein-ingredient to the partial (65–75% of fishmeal) and complete replacement (0–10% fishmeal) with plant-based ingredients (Tacon et al., 2010). Soybean meal for instance is reported to be the most promising substitute of the plant-based protein because of their substantial protein content and their amino-acid composition similarity to fishmeal (Chakraborty et al., 2019). There are also emerging trends of non-plant-based ingredients such as microalgae as partial protein source in fish feed composition (Nagappan et al., 2021). Nevertheless, majority of fish feed marketed and supplied in the world today including in Qatar, contains ingredients from plant origin which are an ideal substrate for the development of toxigenic fungi and under favorable conditions, their respective synthesis of mycotoxins.

Mycotoxins, known poisons are secondary metabolites produced by toxigenic fungi mainly from genera *Aspergillus, Penicillium and Fusarium* and present on nearly all agricultural products and by-products worldwide causing huge economic and health impacts (Magnoli et al., 2019). Over 400 mycotoxins that have been identified, the significant ones include aflatoxins (AFs), ochratoxins (OTA), fumonisins (FBs) deoxynivalenol (DON) and zearalenone (ZEA) (Mostrom, 2015). In aquafeed of plant-based ingredients such as wheat, corn and soybean meal, the risk of mycotoxin production especially AFs) and OTA are enhanced during prolonged storage in hot and humid environments which facilitate active fungal colonization of mostly *Aspergillus and Penicillium* spp.

Mounting evidence has also been established on co-contamination of fish feed and feed ingredients with mycotoxins (Smith et al., 2016). Co-contamination can occur when feed or feed ingredient is infested by a fungus capable of producing multiple toxins simultaneously or when a single ingredient is inhabited by a number of different fungi resulting in the production of different multiple toxins. It can also occur due to mixing of separate feed ingredients consisting of one or more toxins during feed formulation and manufacturing. In the study of (Gonçalves et al., 2018) where 41 samples were tested, level of co-contamination was found to be 76% with only 7% containing no quantifiable amount of the evaluated mycotoxins. Taking into account the toxicities of mycotoxins and their almost unpreventable invasion of the food chain, stringent regulation has been established by many countries especially developing countries to control mycotoxins in food. The European Commission (EC, 2006) has set maximum aflatoxin levels in cereal-based foods at 2 μg/kg (AFB1) and 4 μg/kg (AF total), respectively, while the maximum AFs levels in feed have been set at 20 μg/kg. Similarly, with the exception of pigs and poultry, the OTA levels for feed material and food are 250 μg/kg for feed material, 5 μg/kg for fish feed and 3 μg/kg for food. Like other Gulf Cooperation Council (GCC) countries, in Qatar, mycotoxins levels in food and feed are regulated in accordance with GCC Standardization Organization (GSO) guidelines. There are no specific set regulatory mycotoxins limits for aquaculture feed, however depending on the nature and composition of animal feed, AFs total at the levels of 20–100 μg/kg are fixed permissible in GCC countries (GSO, 2019).

Application of chemical pesticides in crops is often regarded as the suitable control strategy of toxigenic fungi and mycotoxins. But the residual transmission of pesticides to the human food chain through agricultural products, and the development of fungal strains that are resistant to pesticides is a major issue associated with the application of these chemicals for the prevention of fungal infection (Mehta et al., 2018). In recent decade, however, the replacement of these synthetic fungicides with environmentally friendly and economically efficient safer for non-target biological species as prototype/strategies has witnessed tremendous development (Tilocca et al., 2020). In particular, numerous bacterial and yeast strains as biocontrol strategies have been investigated for their inhibitory activity against mycotoxin-producing toxigenic fungi (Köhl et al., 2019; Tilocca et al., 2020). More specifically, the volatiles and diffusible compounds of generally regarded as safe (GRAS) yeasts and bacteria have been widely explored for their antagonistic activity against mycotoxin-producing toxigenic fungi (Abdallah et al., 2022; Alkuwari et al., 2022; Ul Hassan et al., 2019, 2021). In this regard, the volatile organic compounds (VOCs) produced by several *Bacillus* spp., have been succesfully tested both in vitro and in vivo for their antagonatic potential against toxigenic fungi (Ul Hassan et al., 2023).

The objective of this study is to examine the occurrence of fungal communities and mycotoxins in fish feed that is distributed and sold in Qatar, as well as to investigate the presence of mycotoxins in the liver and muscle tissues of fish that have been naturally exposed to contaminated feed. Additionally, the study explores the biocontrol effects of a *Bacillus cereus* strain that acts as an antagonist against fungal growth and mycotoxin synthesis in fish feed.

2. Materials and methods

2.1. Aquafeed and fish sampling

A total of 21 fish feed samples were collected from two different source in Qatar. Following standard sampling technique, four samples ($n = 4$) were collected from a major feed market, located in Doha and the remaining 17 fish feed samples ($n = 17$) were procured from the Aquatic Fisheries Research Center (ARC) in Ras Matbekh, Al khor, Qatar. The details of samples including their brand names, composition, origin and site of collection has been mentioned in Supplementary Table 1. The sample's origin was regionally categorized into Europe ($n = 11$) and Asia ($n = 10$) because none of the sample were locally prepared. Samples were grounded to powder and preserved at room temperature in 50 mL sterile airtight tubes and subsequently used for the determination of mycobiota and selected mycotoxins. Similarly, for the aquaculture fish tissues, a total of fifteen muscle samples ($n = 15$) and five liver samples ($n = 5$) of three commonly consumed farmed fished species (sooly, kurkufan and rohu) in Qatar were procured from Qatar fish market. Sooly (*Lethrinus microdon*) and Kurkufan (*Rhabdosargus haffara*) are reared locally in Qatar, while rohu (*Labeo rohita*) is an imported (India) farmed fish. The liver and muscle samples were aseptically extracted from the farmed fish samples, labelled, and preserved at −21 °C in sterile airtight tubes for further preparation and subsequent extraction for the selected mycotoxin content.

2.2. Determination of toxigenic mycobiota in fish feed

To determine the toxigenic mycobiota, aquafeed samples were processed as outlined by (Hassan et al., 2018) and morphologically identified following (Pitt and Hocking, 2009) identification keys. For isolation, 100 mg of grounded feed sample were mixed in 1.5 mL tube containing 1 mL of sterile distilled water. After thorough mixing, 100 μL of suspended samples were plated on Dichloran-Rose Bengal Chloramphenicol agar (DRCB) agar (31.7 g DRB agar and 100 mg chloramphenicol (Liofilchem – Zona Ind.le – Roseto d. Abruzzi (TE) - ITALY) in 1 L of water) and spread using sterile spreader. To estimate total culturable fungi in the feed, DRBC medium was used due to its selectivity for mold, ease in colony counting and effectivity in inhibiting bacterial growth (Pitt and Hocking, 2009). Plates were incubated at 28 °C for 5 days. Fungal contamination levels were determined afterwards by calculating the colony forming units (CFU/g) of each sample as below.

$$\text{Colony forming unit (cfu/g)} = \frac{No.of\ colonies\ X\ Dilution\ factor}{Weight\ of\ feed\ (g)}$$

For the morphological identification, a needle pick spore from isolated colonies were suspended in 1.5 mL tube containing melted agar (0.2%) and Tween 80 (0.05%). Spores from the suspension were transferred with a sterile needle and trigonally point-inoculated on the identification medias; Czapek yeast extract agar (CYA, Sigma-Aldrich, Steinheim, Germany) and malt extract agar (MEA, Scharlau, Barcelona, Spain). Fungal colony characters (reverse and observe color, size, shape) along with microscopic morphologies were recorded after 7 days of incubation and compared with Pitt and Hocking (2009) identification keys. The identified fungi were preserved for future use in a 25% glycerol prepared in a broth of Potato-Dextrose (PDB) and stored at −80 °C freezer (SANYO, Osaka, Japan).

2.3. Determination of OTA and AFs in feed and tissue samples

The presence of the two mycotoxin, total aflatoxin (AFs) and ochratoxin A (OTA) in the feed and tissues were analyzed through ELISA assay. ELISA test manual of R-Biopharm (Germany) were followed for mycotoxin extraction in the feed and the ELISA test manual of Bio-Scientific (Austin, TX USA) was followed for the tissue (liver and muscle) samples. To prepare the feed for mycotoxin quantification, total AFs (including AFB1, B2, G1 and G2) in the feed (2 g) were extracted with 10 mL of 70% methanol and shaken for 20 min at 200 RPM with an incubating shaker (BINDER, Germany). The resulting mixture was filtered using Whatman No. 4 (Whatman, Inc., Clifton, NJ, USA) filter paper as described by (Ul Hassan et al., 2019). In total, 100 μL of the filtrates were diluted with 600 μL of distilled water and 50 μL of diluted filtrates were used per well in the ELISA test. For OTA extraction, 5 g of the grounded feed were mixed with the diluted (25 mL) ECO extractor, vortexed for few seconds with the vortex (Vortex-Genie2, USA), shaken for few minutes with incubator shaker and centrifuged with the Thermo Scientific Centrifuge (Am Kalkberg, Germany). The 50 μL diluted extract pipetted into the ELISA wells were obtained from the mixture (500 μL of centrifuged supernatant and 500 μL of wash buffer solution).

To prepare the fish tissues for mycotoxin testing, sample paste (2.5 g of liver or 5 g of muscle) was mixed in 25 mL of 70% methanol for AFs extraction and 25 mL of diluted ECO extractor for OTA extraction. After mechanical shaking in the incubated shaker, the tubes were centrifuged for 10 min at 1500 RPM. To a 500 μL of supernatant, 500 μL of sterile distilled water were added. Similarly, to a 500 μL of supernatant OTA extract, a 500 μL buffer solution were added. In all cases, ELISA wells were loaded with a 50 μL of the diluted extract. Levels of AFs and OTA in both feed and tissue samples were quantitatively determined with RIDASCREEN® Aflatoxin total and RIDASCREEN® Ochratoxin A for AFs and OTA respectively. The absorbance of microplates at 450 nm was measured using an ELISA plate reader, TECAN Sunrise™ (Männedrof, Switzerland), and the data was processed using RIDASOFT® Win (Z9996), a data reduction software also from R-Biopharm, Germany.

2.4. Antagonistic activities of bacterial volatiles on toxigenic aquafeed isolates

To investigate the in vitro antifungal activity of bacteria on fungal growth and mycotoxin synthesis, a co-incubation assay was performed as described by (Zeidan et al., 2018). *Bacillus cereus* (BS344–2) used in this assay was isolated from apricot jam, imported from Lebanon, and sold in Qatar market. Matrix Assisted Laser Time of Flight (MALDI-TOF) mass spectrophotometry (MS) were used for the identification of the bacteria isolates (Ul Hassan et al., 2021). For the co-incubation assay, an aliquot (10 μL) of fungal spores suspension was centrally point-inoculated on PDA plates and immediately plates cover was substituted with base plate of bacterial culture on TSA. The resulting inversion was tightly sealed and secured with several layers of Parafilm® and two layers of transparent duct tape to prevent the escape of BC344–2 volatiles. In case of control fungal plates, sterile TSA plates were used for sealing against fungal spores inoculated on PDA. All treated and control plates were incubated at 28 °C. Colony characteristics including sporulation and size were observed and measured at the 3rd, 5th and 7th days of incubation. At each timepoint at least 6 replicates were studied. Fungal growth inhibition ratio was calculated based on the colony size of control (C) and treated (T) fungi as described by (Saleh et al., 2021).

$$\textit{Fungal growth inhibition ratio}\ (\%) = \frac{C - T}{C} X\ 100$$

The effect of the bacteria volatiles on the mycotoxin synthesis potential of the *Aspergillus* spp., and *Penicillium* spp., were determined. For this purpose, a cork borer was used to remove three plugs (6 mm each) from the colonized media. The plugged media was weighed and recorded following the protocol of Zeidan et al. (2018) for AFs extraction from *Aspergillus* spp. However, for OTA extraction from the *Penicillium* spp., similar protocol was followed except that $NaHCO_3$ was used for the resuspension of the extracts. After extraction, ELISA test was performed on all treated and control samples to determine and compare the concentration of mycotoxin.

2.5. Analysis of bacterial volatiles using gas chromatography mass spectrophotometry

To identify active bacterial volatile compounds liable for the inhibition of fungal growth and mycotoxin, gas chromatography mass spectrometry (GC–MS) based analysis were performed. For this purpose, BC344–2 cells were inoculated in a broth of tryptic soy (TS) in an Erlenmeyer flask (250 mL) and subsequently fitted with a cork. As described by (Saleh et al., 2021) two glass tubes were passed through the cork, one just above the TSB level (~1 cm) and other near the neck of the flask. As part of the setup, a glass pasture pipette loaded with activated charcoal were connected and sealed with a layer of Parafilm® to the other end of later tube for the collection of bacterial volatiles. Nitrogen gas was gently introduced in the first tube after 48 h of incubation at 30 °C under continuous shaking, in order to push the head space bacterial volatiles in tube having activated charcoal. Methylene chloride was used for the elution of the captured volatiles on the activated charcoal. The resulting solution were submitted to GC–MS/MS (Agilent 7890 A, CA, U.S.A.) analysis as detailed in the work of (UI Hassan et al., 2019). In the control flasks, volatiles emitted from TSB without bacterial inoculation were also submitted for GC–MS/MS analysis.

2.6. Statistical analysis

The number of colony forming units (CFU/g) was determined by analyzing each feed sample in triplicate. The aggregate mean and standard deviation (SD) were computed by combining the mean values of all samples in Microsoft Excel 2016. RIDA®SOFT Win program was used to determine the average of mycotoxins levels in feed samples, and MS Excel 2016 was used to calculate the overall average of each commodity. The variance test, ANOVA were used for the analysis of biocontrol experimental data. Statistical Package for Social Sciences (SPSS) was used as post-doc testing to compare the means of the data. p-values of ≤ 0.05 were kept for any significant difference at all times.

3. Results and discussion

3.1. 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 in this study ranged from 0 to 1.23×10^3 CFU/g with 80.9% of the count $<1 \times 10^2$

and 19.1% > 1×10^2 CFU/g (Supplementary Fig. 1). This demonstrates that majority of the sample had low levels of colony count which might be attributed to good handling and storage practices. This became more evident in the obtained mean fungal total count of $1.30 \times 10^2 \pm 2.6 \times 10^2$ (mean ± SD). None of 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.

On the basis of morphological features, eight (08) *Aspergillus* spp., seven (07) *Penicillium* spp., one (1) *Fusarium* sp., were identified. Four (04) fungal species were not identified. Many studies have confirmed similar predominance of *Aspergillus* and *Penicillium* species in the fish feed pellets (Pereyra et al., 2011). The isolation of a *Fusarium* spp. in this study corresponds to the results obtained by (Cardoso et al., 2013.) in fish feed. In the present study, *A. flavus* was the most occurring fungi followed by *P. verrucosum* and *P. islandicum*. High percentage of *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. flavus* is one the major producer of AFs and *P. verrucosum* is 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 *Fusarium* specie was morphologically identified as *F. solani*. In Qatar, *Aspergillus* and/or its mycotoxins have been found to be the most prevalent among the feed and food products (Hammami et al., 2014; Hassan et al., 2018).

3.2. Occurrence of AFs and OTA in aquafeed samples

The levels of AFs and OTA in the tested aquafeed samples are summarized in Table 1. OTA was the most prevalent mycotoxin, with 95% of the samples tested positive, followed by AFs (66.7% samples positive). The frequency of AFs and OTA prevalence in this work is higher than that reported by Buck (2005), which was 17% and 4%, respectively in commercial aquafeed. In a similar work on commercial feed intended for fish (Hashimoto et al., 2003), 28.5% of the samples were contaminated with AFs. In contrary to the prevalence noted in this work, it is quite infrequent for OTA to be more prevalent than AFs in fish feed samples. Many studies have reported higher prevalence of AFs compared to OTA (Barbosa et al., 2013; Marijani et al., 2017, 2019). In at least one study, Marijani et al. (2017) found only OTA in the cottonseed cake intended for fish feed formulation. With regards to the contamination levels, none of the AFs contaminated samples showed levels higher than EU permissible limits of 20 μg/kg. However, for OTA, 4.8% of the samples was above permissible limit of 5 μg/kg. Unlike the finding of this study, Kholife et al. (2019) reported levels of AFs and OTA higher than EU permissible limits in 42.86% and 66.7% of aquafeed samples, respectively. In the present study, AFs were in higher concentration, with a range of 1.83–15.94 μg/kg and an average (mean ± SD) concentration 6.85 ± 5.23 μg/kg (Table 1) compared to OTA (range 1.49–7.63 μg/kg and an average 2.27 ± 1.30 μg/kg). In line with the present study AFs were found in 84% of sampled fish feed in Kenya with an average concentration of 7.0 μg/kg and at a range of 1.8 to 39.7 μg/kg (Mwihia et al., 2018). Meanwhile, in Argentina, (Greco et al., 2015) detected OTA in 25% of the rainbow trout feed samples at an average concentration of 5.26 μg/kg.

A significant proportion of aquafeed samples showed co-occurrence of AFs and OTA as it has been observed in other studies (Barbosa et al., 2013). In total, 66.7% of the feed samples in this study were contaminated with AFs + OTA, and only 28.6% of the feed were contaminated with single mycotoxin and 4.7% did not contain measurable levels of any of the two tested mycotoxins (Table 1). This level of co-occurrence (AFs + OTA) reported in this study was higher than those reported by Smith et al. (2016) as 35.7%. Our findings also revealed a distinct pattern of distribution of mycotoxins in aquafeed samples imported from European and Asian countries, probably associated with climate differences such as temperature, humidity and annual rainfall between the two regions. AF and OTA were found to be present in 55% and 100% of the samples imported from Europe (Table 1) but had relatively low average (mean ± SD) concentrations 3.20 ± 1.15 μg/kg and 2.07 ± 0.37 μg/kg, respectively. The occurrence of the same mycotoxins in samples imported from Asia was relatively higher in percentage (AF = 80% and OTA = 90%) and at contamination level (AFs: 9.60 ± 5.47 μg/kg and OTA: 2.52 ± 1.93 μg/kg). In addition, the percent of negative samples for AFs (20%) and OTA (10%) in samples imported from Asia is lesser than those imported from Europe (AFs = 45% and OTA = 0%). An interesting observation in this regional comparison was the level of mycotoxin co-occurrence. Mycotoxins co-occurrence for samples imported from Europe and Asia was 54.5% and 80%, respectively. These observations are in line with the findings of Gonçalves et al. (2018) with mycotoxin co-occurrence of 50% and 84% for Europe and Asia, respectively.

3.3. Detection of AFs and OTA in aquaculture fish tissues

AFs and OTA were detected in 40% and 20% of the tested liver samples at mean concentrations of 2.89 ± 1.37 μg/kg and 1.30 μg/kg, respectively (Table 2). In case of fish muscles, OTA was not detected in any sample, however, AFs (20%) were detected at an average level of

Table 2
Levels of AFs and OTA in the aquaculture fish tissues.

Parameters	Tissue			
	Liver ($n = 5$)		Meat ($n = 15$)	
	Mycotoxin			
	AFs	OTA	AFs	OTA
No. of positive Sample (%)	2 (40)	1 (20)	3 (20)	0 (0)
Mean ± SD (μg/kg)	2.89 ± 1.37	1.30	1.38 ± 0.28	nd
Min-Max (μg/kg)	1.93–3.86	≤ 1.30	1.08–1.62	<dl
Samples > EU permissible limits	0	0	0	0
Co-occurrence of AFs + OTA (%)	20		0	

Table 1
Mycotoxins contamination in aquafeed on total samples and the basis of their origin.

Parameters	Aquafeed (total samples)		Europe*		Asia**	
	AFs	OTA	AFs	OTA	AFs	OTA
No. of positive sample (%)	14 (66.7)	20 (95)	6 (55)	11 (100)	8 (80)	9 (90)
Levels of mycotoxins; Mean ± SD (μg/kg)	6.85 ± 5.23	2.27 ± 1.30	3.20 ± 1.15	2.07 ± 0.37	9.60 ± 5.47	2.52 ± 1.93
Min-Max levels (μg/kg)	1.83–15.94	1.49–7.63	2.01–4.61	1.49–2.89	1.83–15.94	1.56–7.63
Samples > EU permissible limits (%)	0	4.8	0	0	0	4.8
Co-occurrence of AFs + OTA (%)	66.7		54.5		80	

* Samples originated from Germany, Greece and Turkey. **Sample originated from Japan, Thailand and India.

1.38 ± 0.28 μg/kg. In contrast to these findings, OTA was found in muscle of 60 farmed gilthead seabream and European seabass sold in the Italian market (Meucci et al., 2021). In the same study, Meucci et al. (2021) reported highest levels of OTA in kidney, followed by liver and muscles.

In this study, the presence of mycotoxins in fish tissue samples showed species wise variation (Table 3). For instance, in the liver samples of rohu (*Labeo rohita*), AFs and OTA were detected at an average concentration of 3.86 μg/kg and 1.30 μg/kg, respectively. Although, OTA were not detected in rohu muscle samples, 33.3% of the sample were contaminated with AFs with a mean concentration of 1.54 μg/kg. This level of AFs in muscle of rohu is lesser than those found in the muscle of seabass at 5 μg/kg in the study of El-Sayed and Khalil (2009). Kurkufan (*Rhabdosargus haffara*) on the other hand, tested positive only for AFs in both liver and muscle samples at an average concentration of 1.93 μg/kg (50%) and 1.08 μg/kg (16.7%), respectively. Neither AFs nor OTA was detected in Sooly (*Lethrinus microdon*) tissues (liver and muscle) samples. Kurkufan and Sooly are marine and locally farmed fish in Qatar and this is the first study reporting mycotoxins accumulation patterns on marketed farmed fish in the country. Our results suggest that the locally farmed fish species seem to pose little to no risk to public health compared to imported farmed fishes. However, a more robust study involving big sample size needs to be conducted to make definitive conclusion.

3.4. Biocontrol activity of BC344–2 VOCs against mycotoxigenic fungi of aquafeed

In the present work, antagonistic activity of bioactive volatile organic compounds of a novel *Bacillus cereus* strain (BC344–2) against the growth and mycotoxin synthesis of toxigenic fungi isolates of *P. verrucosum, A. flavus* and *F. solani* was studied. The exposure of the three toxigenic isolates resulted in fungal growth inhibition in terms of decreased growth rate and a significant alteration of their sporulating capacity as measured at the 3rd, 5th and 7th of co-incubation experiment.

In case of *A. flavus*, VOCs exposed fungal colonies at day 3rd, 5th and 7th showed an average colony diameter of 3.3 mm, 4.5 mm and 5.5 mm which were lower compared to the (unexposed) control fungi with these values of 3.6 mm, 4.7 mm and 5.8 mm. In addition to the reduction in colony size, a significant reduction in sporulation was observed (Fig. 2). In the work of Ul Hassan et al. (2019), *B. licheniformis* volatiles resulted in 49% reduction in colony diameter of *A. flavus* due to the presence of 3 methyl butanol as the major bioactive compound in the headspace volatiles. Likewise, on exposure to *B. megaterium* volatiles composed of mainly palmitic acid and tetracosane, Saleh et al. (2021) observed 29.4% reduction in *A. flavus* growth. Similarly, in a study of Mannaa and Kim (2018), bioactive volatiles of three bacteria strains including *B. megaterium* were reported to have significant reduction in the mycelia growth of *A. flavus* due to the presence of 5-methyl-2-phenyl-1H-indole as the main antifungal compound in the headspace. In a peanut preservation study, *A. flavus* was significantly inhibited by *B. subtilis* volatiles mix mainly 2,3-butanedione (Ling et al., 2022). The major bioactive compound in this study were BTH aldehyde, 2-Nitrocyclododecane and 1-heptadecanol. Josselin et al. (2022) reviewed the effects of bioactive volatilome on fungal growth and observed a significant sensitivity of *A. flavus* to aldehydes, alcohols and terpenes.

Table 3
Specie-wise mycotoxin contamination on the farmed fish samples.

Parameters	Rohu (*Labeo rohita*)		Kurkufan (*Rhabdosargus haffara*)		Sooly (*Lethrinus microdon*)	
	Mycotoxin					
	AFs	OTA	AFs	OTA	AFs	OTA
Liver						
No of samples tested	2	2	2	2	1	1
Positive samples (n)	1	1	1	0	0	0
% Positive	50	50	50	0	0	0
*Mean ± SD (μg/kg)	3.86	1.3	1.93	0	0	0
Muscle						
No of samples tested	6	6	6	6	3	3
Positive samples (n)	2	0	1	0	0	0
% Positive	33.3	0	16.7	0	0	0
*Mean ± SD (μg/kg)	1.54 ± 0.12	0	1.08	0	0	0

* Means were calculated on the basis of positive samples only.

The exposure of *P. verrucosum* to BC344–2 VOCs resulted in a significant decrement in colony size compared to VOCs unexposed fungi. The average colony diameters of *P. verrucosum* were 0.97 mm, 1.1 mm and 1.27 mm on days 3, 5 and 7 of VOCs exposure, respectively. These values were significantly lower than control fungi which showed diameter of 1.37 mm, 1.83 mm and 2.20 mm on day 3, 5 and 7, respectively. With an increasing duration of exposure, the rate of inhibition also increased starting at 29.3% at day 3 to 40.6% at day 7. The high growth inhibition ratio and sensitivity of *P verrucosum* to bacteria and yeast VOCs is not uncommon as it has been reported in the previous studies (Saleh et al., 2021; Ul Hassan et al., 2019, 2021; Zeidan et al., 2018). The VOCs produced by BC344–2 also significantly inhibited the sporulation of *P. verrucosum* (Fig. 2). This alteration in the development of fungal spores (sporulation) noted in the present study is most likely be affiliated with BC344–2 VOCs having 1-Heptadecanol. 1-Heptadecanol is functional group of alcohol and it has been reported that inhibition of spore germination can occurs as a result of alcohols, such as isoamyl alcohol, becoming adsorbent on the spore surface and adhering there for an extended period of time thus inhibiting sporulation (Ando et al., 2012). In addition, the restriction of fungal spore germination by *Bacillus* spp., and yeast VOCs against *A. flavus, P. verrucosum* and *Fusarium* spp., have also been reported in early studies of Ul Hassan et al. (2021); Zeidan et al. (2019).

The response of *F. solani* to *B344–2* VOCs also revealed a decrease in colony size in comparison to the control (unexposed fungi) as shown in Figs. 1 and 2. On exposure to BC344–2 VOCs at day 3, 5, and 7, the average colony diameter of *F. solani* was 1.97 mm, 2.0 mm and 4.23 mm, respectively compared to the control fungi where colony diameters were 2.3 mm, 3.37 mm and 5.13 mm. Fungi showed maximum FGI ratio at day 5 (40.6%) before declining to 17.5% at day 7. This growth inhibition activity is most likely attributed to the production of BTH aldehyde and 1-Heptadecanol or a combination of the hydrocarbons by BC344–2. *F. solani* and *P. verrucosum* tested in this study, appear to be most sensitive to these compounds due to the high rate of fungal inhibition 40.6% and 40.0%, respectively recorded during the fifth days of exposure. In the work of Guevara-Avendaño et al. (2019) VOCs emitted by seven *Bacillus* isolates shown to antagonistically reduce mycelia growth of *F. solani* with inhibition percentage higher than 20%. Although the volatile profiles constituted mainly ketones, pyrazines and sulfur-containing compound. Volatiles of *Bacillus* spp. have been shown to demonstrate an effective antagonism against mycotoxigenic *Fusarium* spp., (Lee et al., 2017). Moreover, alcoholic antifungal compounds extracted from seaweed have been found to be very effective against *F. solani* (El-Din and Mohamed, 2018).

3.5. Effect of BC344–2 VOCs on mycotoxin synthesis by fungi

Exposure to BC344–2 VOCs resulted in reduction of OTA synthesis by *P. verrucosum* and AFs by *A flavus*. In the colonized media plugs, acquired from the BC344–2 volatiles exposed *P. verrucosum*, OTA synthesis was completely blocked in contrast to those obtained from unexposed fungi with a mean OTA concentration of 1.94 μg/kg (Fig. 3). The high sensitivity of *P. verrucosum* to BC344–2 VOCs as explained in previous section may have altered the enzymatic activity of the fungi and thus curtailing its ability to synthesize OTA (Wheatley, 2002). Reduction in OTA synthesis by VOCs of *Bacillus* sp., has been reported in other studies

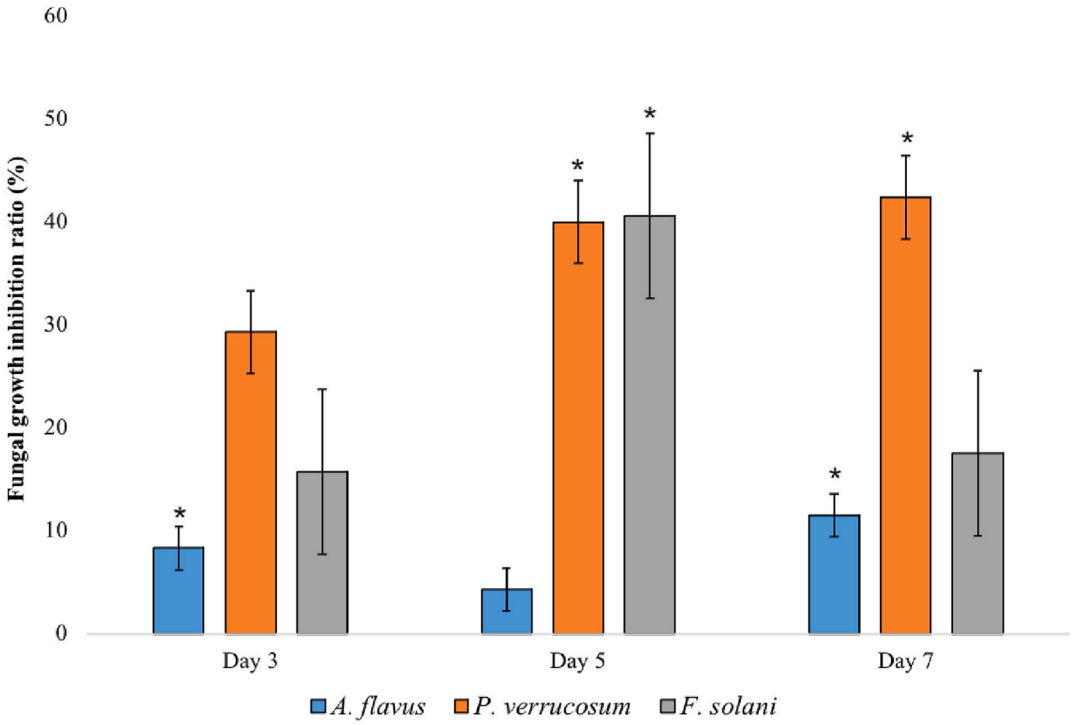

Fig. 1. Fungal growth inhibition (%) was measured as reduction in colony size relative to the control on days 3, 5, and 7 after *B. cereus* BC344–2 exposure. PDA plates were point-inoculated with spores of selected fungi and sealed against colonies of BC344–2 on TSA. Fungal growth inhibition was calculated compared at different timepoints and was considered as significant at $p \leq 0.05$.

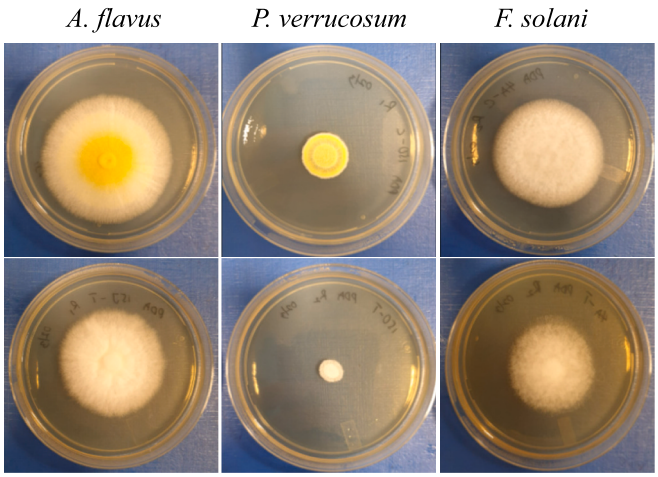

Fig. 2. Biocontrol activity of BC344–2 against toxigenic fungi at day 7 of coincubation. *B. cereus* BC344–2 antifungal activity was tested against *Penicillium*, *Fusarium* and *Aspergillus* fungi. Fungi in 2nd row are control (not exposed to bacterial VOCs), while those in 3rd row were exposed to bacterial volatiles.

(Higazy et al., 2021; Zeidan et al., 2019). In the case of *A. flavus*, the AFs content in the colonized media plugs exposed to BC344–2 VOCs were 0.092 μg/kg compared to the control (unexposed) with mean concentration of 0.13 μg/kg. In other words, the exposed fungi were able to synthesize 29.2% less AFs than un-exposed fungi. This is almost in line with the moderate effect of the VOCs on the growth of *A flavus* (least sensitivity) compared to *P. verrucosum* and *F. solani* tested in this study. In contrast to these findings, exposure *to B. licheniformis and B. megaterium* volatile showed complete and significant AFs reduction by *A. flavus* in the study of Ul Hassan et al. (2019) and (Saleh et al. (2021), respectively.

3.6. Analysis of bioactive bacterial volatile molecules

B. cereus BC344–2 emitted volatiles were subjected to GC–MS/MS, which revealed the presence of BHT-aldehyde, 1-Heptadecanol, 2-Nitrocyclododecane, Diethylmethyle Borane, and 2-Methyl-7-nonadecene. These molecules were lacking in the volatile profile of control flask containing only TSB, thus signifying that the antifungal activity of *B. cereus* (BC344–2) was either as a result of one of these five compounds or their combined effects. The antifungal activities of the families of these compounds identified in B344–2 have been reported to be present in the headspace of several *Bacillus spp* (Choub et al., 2022; He et al., 2020; Zhang et al., 2013). For instance, aldehydes like *O*-anisaldehyde were found to be most abundant volatiles in the headspace of *B. atrophaeus* responsible for the highest inhibition on the mycelial growth of *B. cinerea* (Zhang et al., 2013). BTH aldehyde were also found as the major compound in the headspace of a *B. cereus* strain (BC344–2) responsible for the complete inhibition of *A. niger* and *A. carbonarius* (Abdullah et al., 2022). Also, benzaldehyde in addition with other three VOCs were found in the headspace of *B. subtilis* as main antibacterial control agent (Rajer et al., 2017). Furthermore, several alcohols like ethanol, 1-octanol, 1-heptanol, cyclohexanol and 2-ethyl 1-hexanol, 1-decanol and phenol have been shown to have strong antifungal activity (Abdallah et al., 2022; Chaves-López et al., 2015; Wang et al., 2022). In the work of (Rajaofera et al., 2019), the hydrocarbon (octadecane) in addition with organic acids (chloroacetic acid, and hexadecanoic acid) were identified as key inhibitory compounds produced by *B. atrophaeus* against *C. gloeosporioides*. These findings highlight the potential use of *B. cereus* 344–2 volatiles in the preservation and storage of food and agricultural products.

3.7. Conclusion

Our findings demonstrate that aquafeed marketed in Qatar from some Asian and European countries are generally contaminated with moderate levels of mycotoxigenic fungi and mycotoxins. Although the

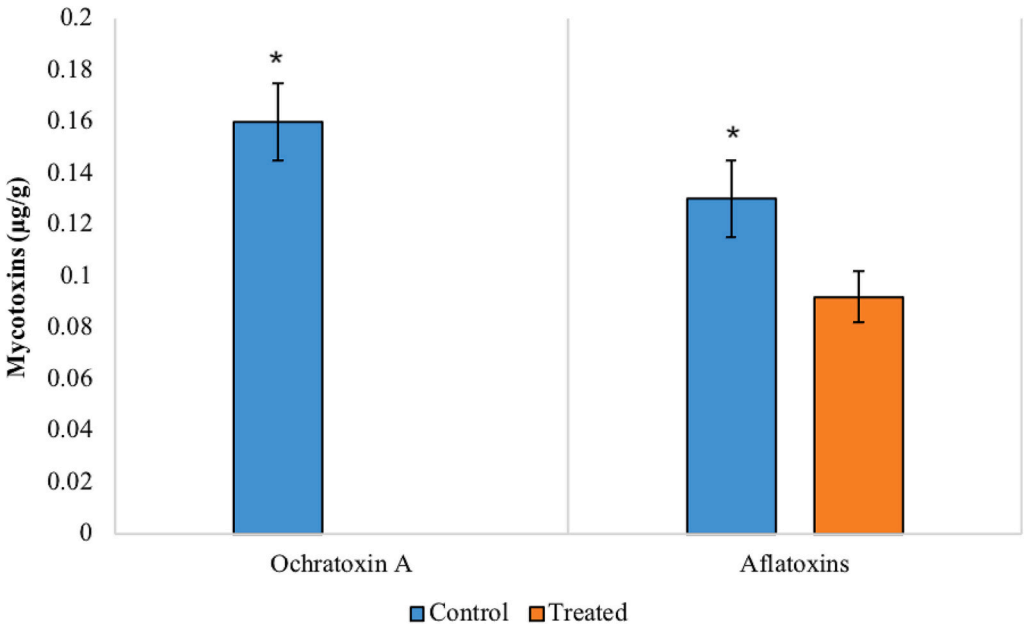

Fig. 3. Inhibition of OTA synthesis by *P. verrucosum* and AFs by *A. flavus* exposed to B344–2 VOCs. Values were considered as significant at $p \leq 0.05$.

levels of individual mycotoxins are generally within the permissible limits, however, the simultaneous occurrence of the two toxins, AFs and OTA in the feed samples might possibility lead to synergistic effects on aquaculture fish and subsequently on consumer health. This necessity to regularly monitor the levels is reinforced considering the detection of AFs and OTA in the liver of marketed fish. Although studies in this field (Bernhoft et al., 2017) has suggested that human exposure to these toxins through the consumption of toxins-fed fish is unlikely. The findings of the current study, in which AFs were detected in the muscle of rohu and kurkufan, suggest the need for additional research into the safety of these types of commercially consumed fish products (both local and imported) given an understandable potential risk to public health. On a positive note, exploration of in vitro biocontrol co-incubation assay indicated *P. verrucosum* as the most sensitive with 42.4% inhibition rate followed by *F. solani* (17.5%) and *A. flavus* (11.5%). On protein-rich TSA media, exposure to *P. verrucosum* and *A. flavus* to *B344–2* VOCs suppressed their respective synthesis of AFs (AFB1, AFG1, and AFG2) and OTA. The headspace bacterial volatiles analysis indicated BTH aldehyde and 1-Heptadecanol as the most probable antifungal compounds responsible for the inhibitions. Considering the inhibitory effect of *B. cereus* (B344–2) antifungal volatile compounds produced on the growth and mycotoxin production of representative fish feed isolates, it is possible that bacterial isolate can be applied in the food preservation against fungal attacks as well as mycotoxins accumulation.

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Credit author statement

S.J., and Z.U.H.: Conceptualization; A.B., Z.U.H., M.A.A. and S.J.: Methodology; A.B., Z.U.H., and S.J.: Validation and analysis of results; S. J: Resources provided; A.B., Z.U.H., and S.J.: Writing and reviewing; S.J. and Z.U.H.: Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

No data was used for the research described in the article.

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