Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (5): 473-481, 2023
DOI: 10.9775/kvfd.2023.29447

Research Article

Determination of the Mycotoxin Activity of Filamentous Fungi Isolated from the Intestinal Region of Adult Honey Bees by the PCR and UHPLC-Orbitrap-HRMS Methods

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How to cite this article?

Tarhane S, Dursun İ, Mor B, Kanıcı Tarhane A, Kızıltepe Ş, Filazi İ, Güven A: Determination of the mycotoxin activity of filamentous fungi isolated from the intestinal region of adult honey bees by the PCR and UHPLCOrbitrap-HRMS methods. *Kafkas Univ Vet Fak Derg,* 29 (5): 473-481, 2023.
DOI: 10.9775/kvfd.2023.29447

Article ID: KVFD-2023-29447
Received: 29.03.2023
Accepted: 23.08.2023
Published Online: 01.09.2023

ABSTRACT

Honey bees are threatened by many fungal, parasitic and bacterial diseases. This study was aimed at identifying filamentous fungi colonizing the intestinal region of dead adult honey bees and determining resultant mycotoxin activity and its potential adverse implications for bee and human health by the PCR and UHPLC-Orbitrap-HRMS methods. For this purpose, dead bees were collected from the ground in front of 95 hives displaying mass mortality in the Hatay, Iğdır and Bingöl provinces, and 22 filamentous and 6 yeast-like fungi were isolated and identified from the intestinal region of these bees. Of the filamentous fungal isolates, 8 were identified as *Aspergillus* spp., 3 as *Fusarium* spp., 2 as *Alternaria* spp. 5 as *Penicillium* spp., 4 as *Mucor* spp., and 2 as *Rhizopus* spp.. The PCR analysis of the filamentous fungi using primers targeting the aflatoxin and ochratoxin A genes revealed the presence of aflatoxin in only 1 out of the 22 samples. Aflatoxin and ochratoxin were not detected in any of the other samples. The UHPLC-Orbitrap-HRMS method revealed the presence of aflatoxin B1 in 17, both B1 and B2 in 6, B1, B2 and G1 in 5, and aflatoxin G2 in 1 of the filamentous fungal samples. The results obtained in the present study suggest that filamentous fungi may produce mycotoxins in the intestinal region of honey bees, and thereby, honey bees may distribute mycotoxins into the environment and cause indirect adverse effects on human and animal health.

Keywords: Filamentous fungus, Honey bee, Mycotoxin

Introduction

Honey bee diseases were first recognized by beekeepers and recently, have gained increased importance in the veterinary field [1]. Many parasites and pathogens threaten honey bees [2], and microbes associated with honey bees are either pathogenic or nonpathogenic. The common pathogenic bacteria of honey bees are *Escherichia coli* and species of the genera *Klebsiella, Proteus* and *Pseudomonas*. Fungal agents isolated from honey bees are mostly identified as *Penicillium* spp., *Aspergillus* and occasionally as *Torulopsis* spp. While fungal agents can colonize the intestinal system of honey bees without causing any harm, sometimes colonization may result in mass bee deaths [3].

Although fungal agents are generally known as plant and insect pathogens, they also cause diseases in vertebrates [4].

Fungal pathogens can be life threatening for both plants and animals, and their distribution has increased with climate change and rising temperatures [5].

Toxic substances known as mycotoxins are produced by mold fungi and can cause food poisoning and damage to organs such as the liver and kidney. Mycotoxins are produced primarily by species belonging to the genera *Aspergillus, Penicillium* and *Fusarium*, but other fungal species can also produce these toxic compounds [6].

Mycotoxins are defined as secondary metabolites of mold fungi that adversely affect human, animal and plant health. The contamination of food products with mycotoxins is a serious problem worldwide. Approximately 500 different mycotoxins have been identified to date, and new mycotoxins continue to be discovered [7].

Mold fungi are reproduced by spores, which are resistant to almost all kinds of environmental conditions, and their growth in crops may adversely affect plant products. Mycotoxins associated with filamentous fungi may harm animal and human health through the intake of contaminated feed and food products [8].

The toxic effects of mycotoxins are well-known in veterinary practice and are encountered in both developed and developing countries consequential to the growth of filamentous fungi under favourable social, economic and meteorological conditions (such as temperature and humidity) [9].

Filamentous fungi are widely distributed in nature and can survive adverse environmental conditions in the spore form, which is resistant to even very high temperatures. The growth of filamentous fungi is followed by the production of mycotoxins, which are toxic to animals and humans even at small concentrations [10].

Farm animals are frequently exposed to mycotoxins, and pigs are especially very sensitive to their adverse effects [11]. In the livestock industry, mycotoxins are common feed pollutants. Studies have shown that mycotoxins cause immunotoxicity and adversely affect the reproduction of animals [12].

Fungi, along with bacteria, are commonly observed in plants and animals, and although known as plant pathogens, can also colonize the intestinal region of honey bees, making these insects an important vector of these agents [13].

Honeybees and their hives host many saprophytic fungi and mycotoxins. When below a certain concentration, most mycotoxins are able to be neutralized by honey bees [14], yet even a dose of 5 ppm of some mycotoxins such as aflatoxin can cause the death of bee larvae [15]. The intestinal fungi of honey bees not only use the food stores in the hive, but also cause toxicity in bees by producing mycotoxins [16].

Due to the very low concentrations of mycotoxins, sensitive analytical procedures are required for their detection in biological samples. Most of the analytical methods used for mycotoxins are performed with liquid chromatographic (LC) quantification systems combined with other detection techniques such as spectrophotometry, fluorescence, mass spectrometry (MS) or MS/MS. Recently, liquid chromatography (LC) Orbitrap high resolution mass spectrometry (HRMS) has been effectively used for the identification and screening of non-target compounds in metabolomic strategies for the study of the bioaccumulation, toxicokinetics and excretion of mycotoxins and their metabolites as well as the target analysis of mycotoxins [17].

Maintaining the health of honey bee colonies is of great importance for the global agricultural sector, given the critical role of honey bees in crop pollination. Several factors including adverse environmental conditions, stress, pesticides and pathogens cause bee mortality [18,19].

In the present study, it was aimed to identify the fungal agents colonizing the intestinal region of dead honey bees and investigate whether they were related to mycotoxin activity, honey bee mortality, and indirectly human health.

Material and Methods

Ethical Statement

This study was performed with the permission of the Local Ethics Committee of Kafkas University (KAÜ-HADYEK, Decision number: 2022-213, Date: 27. 12. 2022).

Handling and Culture of Specimens

Dead bees were collected into ziplock bags from the ground in front of a total of 95 randomly-selected hives displaying mass mortality in three provinces, including Hatay (H) (17 hives), Bingöl (B) (54 hives), and Iğdır (I) (24 hives). The pile of dead bees in front of each hive was sampled from the top, middle and bottom layers to represent the whole population. The samples were delivered to the laboratory after each bag was numbered.

In the laboratory, the samples were sterilized first with 70% ethanol for 60 sec, then with 5% NaOCl for 60 sec to prevent external surface contamination, and washed in distilled water [20,21]. Next, the samples were transferred to 10 mL-cryotubes and chopped in a tissue slicer (Qiagen Tissue Lyser). After being inoculated onto Saburoud dextrose agar, the samples were incubated at 25°C under aerobic conditions. Fungal colonies that grew in the culture medium were conventionally identified under the light microscope as described by Navi et al.[22].

Table 1. *Primers targeting the aflatoxin and ochratoxin A genes*

Gene	Sequences	Length Base Pair (bp)	Reference
*Afl*D (Nor1)	F: 5'-GTCCAAGCAACAGGCCAAGT-3' R: 5'-TCGTGCATGTTGGTGATGGT-3'	66bp	[23]
Ochratoxin-A	F: 5'-AGCATCTATGCTGGCCAATC-3' R: 5'-AATGTACTCTCGCGGGCTAA-3'	187bp	[24]

Molecular Methods

DNA Isolation

For the molecular identification of mycotoxins, one piece of each of the identified filamentous fungi was transferred to a cryotube containing 500 µL of physiological saline and mechanically disrupted in a cell disintegrator.

DNA isolation from the disrupted fungi was performed with the chloroform isoamyl alcohol method. Primers targeting the aflatoxin and ochratoxin A genes were used for PCR *(Table 1)*.

PCR and PCR Conditions

For each sample, a PCR reaction mixture of 25 µL, which consisted of 2.5 µL DNA, 16.5 µL DNA-free water, 1 µL of each primer (total 2 µL), 0.5 µL dNTP mix, 0.5 µL Taq polymerase enzyme and 3 µL buffer, was prepared. The reaction products were incubated at 98°C for 30 sec, annealed at 94°C for 10 sec and amplified in a thermal cycler at 52°C for 15 sec, at 72°C for 15 sec and at 72°C for 1 min. The amplified products were run on 1.5% agarose gel. The bands that formed during electrophoresis were viewed under ultraviolet light. Bands of 66 bp corresponded to aflatoxin, and those of 186 bp corresponded to ochratoxin A [22-24].

Reagents and Chemicals for Mycotoxin Detection and Quantitation

Methanol (99.95%) of LC-MS grade and formic acid (99%) of LC-MS grade were purchased from CARLO ERBA (Val-de-Reuil Cedex, France), and ammonium formate of LC-MS grade was purchased from Honeywell™ Fluka™ (Seelze, Germany).

One-mL of an analytical standard mixture in acetonitrile (purity 99.9%) containing Aflatoxin B1 (1.2 µg/mL), Aflatoxin B2 (0.32 µg/mL), Aflatoxin G1 (0.88 µg/mL), and Aflatoxin G2 (0.28 µg/mL) was purchased from n'Tox (Bordeaux, France) (Certificate Number: 1622614458). Furthermore, 1.1 mL of Ochratoxin A in 100 µg/mL acetonitrile (purity 99.9%) was purchased from n'Tox (Bordeaux, France) (Certificate Number: 1604676695).

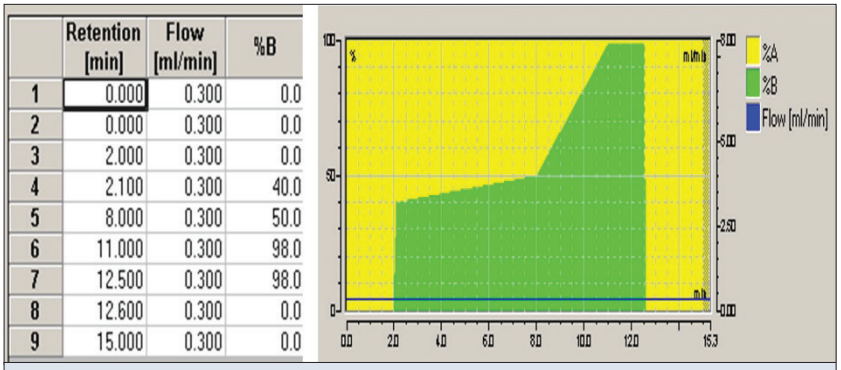
Ultra-High Performance Liquid Chromatography Coupled to Orbitrap High-Resolution Mass Spectrometry (UHPLC-Orbitrap-HRMS)

UHPLC-Orbitrap-HRMS analyses were conducted using an UHPLC system equipped with a DIONEX UltiMate 3000 RS pump, DIONEX UltiMate 3000 RS autosampler and DIONEX UltiMate 3000 RS column oven, and a high-resolution Orbitrap mass spectrometer (Orbitrap-HRMS, Exactive PlusTM, Thermo Fisher Scientific, Bremen, Germany) with a heated electrospray ionization interface (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA). The Orbitrap-HRMS instrument was calibrated with positive (Pierce™ LTQ Velos ESI Positive Ion Calibration Solution) and negative calibration solutions (Pierce™ Negative Ion Calibration Solution) using an automatic syringe injector (Thermo Fisher Scientific, USA). In the UHPLC-Orbitrap-HRMS analyses, UHPLC and part of MS were run simultaneously with the TraceFinder 3.2 program (Thermo Scientific) installed on the system computer, and the data were collected and recorded with the Xcalibur software version 2.1.0.1140 (Thermo Fisher Scientific).

Chromatography and High-Resolution MS Conditions

A Thermo SCIENTIFIC part no 17326-102130 (Dim. 100 mm × 2.1 mm, particle size: 2.6 µm) column was used for chromatographic analyses. The column oven was operated at a temperature of 30°C. The elution gradient was set as 1 mM ammonium formate (Fluka) prepared in ultrapure water obtained by the Ultrapure water system (GFL 2004/ Human power 1) for use as the mobile phase A, and methanol (Sigma) of 99.9% purity and LC-MS grade and 1mM ammonium formate (Fluka) for use as the mobile phase B. Separation was carried out applying the conditions presented in *Fig. 1*, with a sample injection volume of 20.0 µL and gradient elution conditions at a flow rate of 0.3 mL min-1. The analysis time was set to a total of 15 min.

The heated electrospray interface (HESI II, Thermo Fisher Scientific, San Jose, CA, USA) was set in only positive (ESI) mode using the following operating parameters: spray



	Retention [min]	Flow [ml/min]	%B
1	0.000	0.300	0.0
2	0.000	0.300	0.0
3	2.000	0.300	0.0
4	2.100	0.300	40.0
5	8.000	0.300	50.0
6	11.000	0.300	98.0
7	12.500	0.300	98.0
8	12.600	0.300	0.0
9	15.000	0.300	0.0

Fig 1. Subsequent gradient conditions in elution

voltage 3.5 kV; sheath gas (N2>99%) 35 (adimensional); auxiliary gas (N2>99%), 7 (adimensional); aux gas heater temperature 350°C; and capillary temperature 350 °C; S-lens RF level 50.

Mass spectra were obtained using two alternative acquisition functions: (1) Full MS, ESI+ (high collision dissociation (HCD) collision cell was closed without fragmentation), (2) All Ion Fragmentation (AIF), MS/MS, ESI+ operated fragmentally (HCD on, collision energy = 25 eV). The MS scan range was 200-450 m/z for the Full MS and AIF mode; the resolution was 17500; automatic gain control (AGC target) was $5x10^6$; and maximum IT was set to 2 ms. Mass accuracy was checked daily with multiple compound standards and calibrated weekly with mass accuracy standards.

Method Validation

UHPLC-Orbitrap-HRMS analysis was performed to determine the mycotoxin profiles of the samples. The stock standards of Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2 and Ochratoxin A were diluted to different concentrations as shown in *Table 2* with a 1:1 methanol-water solution. The external calibration curves given in *Fig. 2* were constructed and all measurement data were calculated using these calibration graphs.

Quan peak (Parent Ion) and confirming ions (Fragment Ions), retention time (RT), concentration ranges, ion mode (polarity), and the HCD collision energy of the mycotoxins are given in *Table 3*.

Table 2. *Calibration solutions of the Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2 and Ochratoxin A standards at different concentrations*

Mycotoxins	Level 1 (ng/mL)	Level 2 (ng/mL)	Level 3 (ng/mL)	Level 4 (ng/mL)	Level 5 (ng/mL)	Level 6 (ng/mL)
Aflatoxin B1	0.043	0.214	0.429	1.071	2.143	4.286
Aflatoxin B2	0.011	0.057	0.114	0.286	0.571	1.143
Aflatoxin G1	0.031	0.157	0.314	0.786	1.571	3.143
Aflatoxin G2	0.010	0.050	0.100	0.250	0.500	1.000
Ochratoxin A	-	0.100	0.250	0.500	1.000	2.500

The validation parameters of the UHPLC-Orbitrap-HRMS mycotoxin analysis method were used as performance criteria for method validation. The quan peak (parent ion), fragment ions (m/z) (confirming ion-1 and confirming ion-2), chromatograms and calibration curves used in UHPLC-Orbitrap-HRMS are shown in *Fig. 2*. The determination coefficient (R2), limit of detection (LOD) (ng/mL), limit of quantification (LOQ) (ng/mL), and recovery (%) (In the sample without analyte, 0.250 ng/mL for Ochratoxin, Level 2 concentrations in *Table 2* for Aflatoxin B1, Aflatoxin B2, Aflatoxin G1 and Aflatoxin G2 were spiked[17].) for the analysis of mycotoxins using the UHPLC-Orbitrap-HRMS method's validation parameters are provided in *Table 4*.

Table 3. *Optimized Orbitrap-HRMS parameters for the mycotoxins*

Mycotoxins	RT (n=9)	Parent Ion (m/z)[b]	Fragment Ions (m/z)		Concentration Range (ng/mL)	Adduct	Ion Mode	HCD CE
		Quan Peak (m/z)	Confirming Ion-1 (m/z)	Confirming Ion-2 (m/z)				(Fragmentation) (eV)
Aflatoxin B1	7.67±0.005	313.07066	285.07486	270.05148	0.043-4.286	M+H	Positive	25
Aflatoxin B2	7.14±0.004	315.08631	287.09082	259.05988	0.011-1.143	M+H	Positive	25
Aflatoxin G1	6.48±0.005	329.06558	311.05423	243.06474	0.031-3.143	M+H	Positive	25
Aflatoxin G2	6.07±0.005	331.08123	313.08123	303.08578	0.0100-1.000	M+H	Positive	25
Ochratoxin A	11.00±0.005	404.08954	257.02072	239.00957	0.100-2.500	M+H	Positive	25

RT: Retention time, HCD CE: Higher collisional dissociation collision energies.

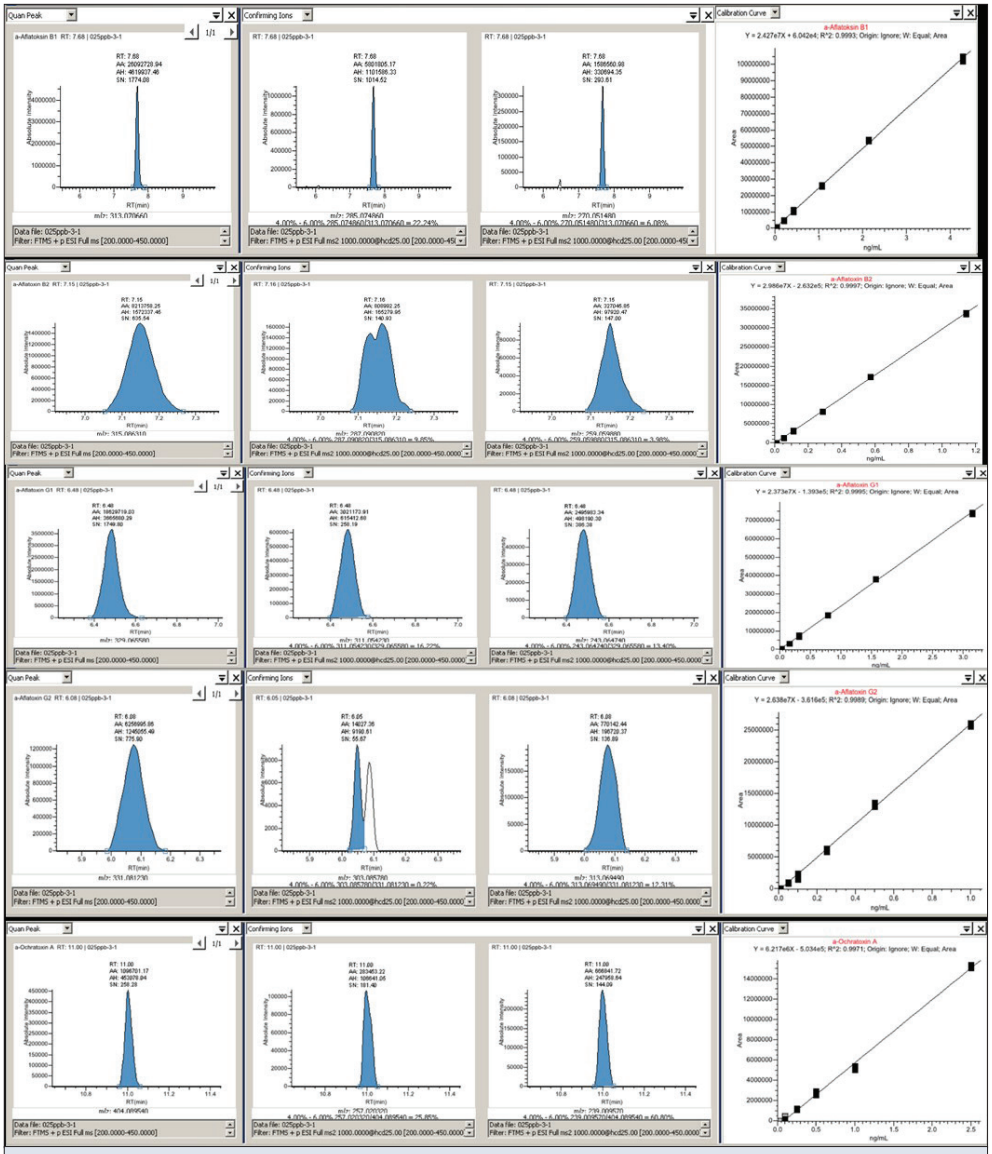


Fig 2. UHPLC-Orbitrap-HRMS Quan Peak (Parent Ion), Fragment Ions (m/z) (Confirming Ion-1 and Confirming Ion-2) chromatograms of mycotoxins, calibration curves

Table 4. Validation parameters of the UHPLC-Orbitrap-HRMS method used for mycotoxin analysis

Mycotoxins	% Recovery (n=7)	% RSD (n=7)	LOD (µg/kg) (n=7)	LOQ (µg/kg) (n=7)	R^2
Aflatoxin B1	96.19	0.88	0.005	0.018	0.9993
Aflatoxin B2	94.24	1.64	0.003	0.009	0.9997
Aflatoxin G1	93.99	1.81	0.008	0.027	0.9995
Aflatoxin G2	99.91	4.01	0.006	0.020	0.9989
Ochratoxin A	106.80	4.63	0.037	0.124	0.9971

% RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification, R^2: Correlation coefficient

Results

Microscopy

Fungi that grew in the culture medium were examined under a light microscope, and 22 out of the 95 samples were identified as filamentous fungi whilst 6 were identified as yeast-like fungi. Of the filamentous fungi, 8 were identified as *Aspergillus* spp., 3 as *Fusarium* spp., 2 as *Alternaria,* 5 as *Penicillium* spp., 4 as *Mucor* spp. and 2 as *Rhizopus* spp. *(Fig. 3) (Table 5).*

Table 5. *Fungi isolated from the intestinal tract of dead bees*

Provinces	Number of Hives	Culture-negative	*Aspergillus* spp.	*Alternaria* spp.	*Mucor* spp.	*Fussarium spp*	*Penicillium* Spp.	*Rhizopus* spp.	Yeast-like
Hatay	17	9	3	0	0	0	1	2	1
Iğdır	24	15	2	1	0	1	2	0	2
Bingöl	54	34	3	1	4	2	2	0	3
Total	95	57	8	2	4	3	5	2	6

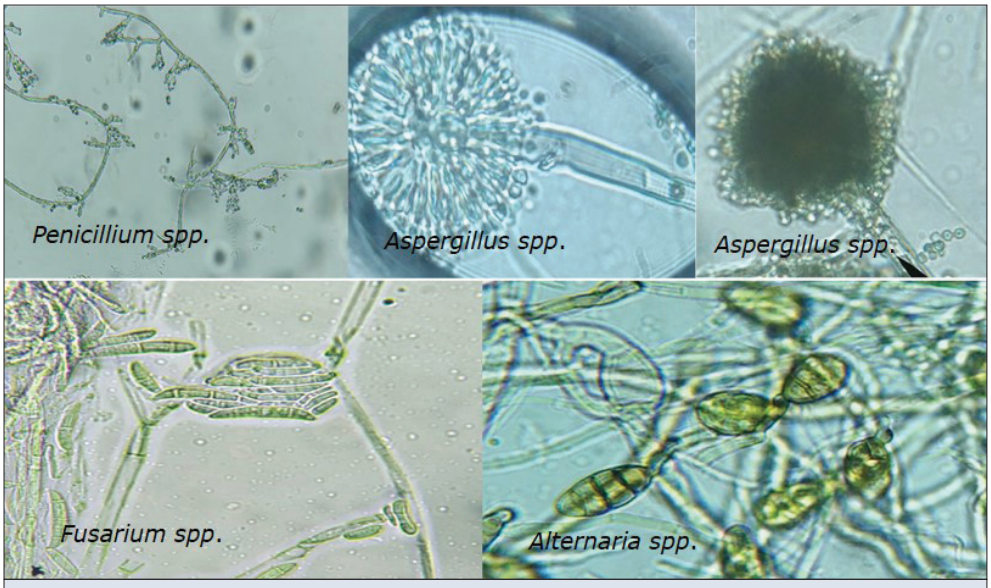


Fig 3. Micrographs obtained under the light microscope

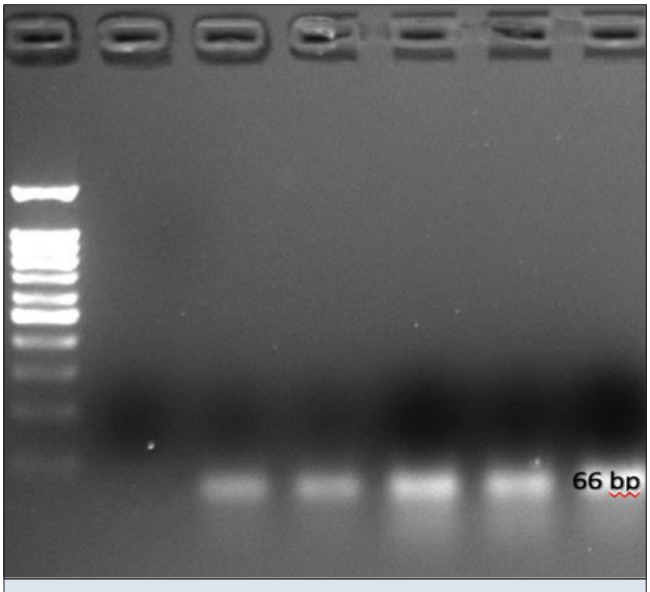


Fig 4. Electrophoresis image of the PCR products

According to PCR analyses performed with primers targeting the aflatoxin and ochratoxin A genes in filamentous fungi, aflatoxin was detected in only 1 out of the 22 samples, and neither aflatoxin nor ochratoxin were detected in the other samples.

Results of Mycotoxin Analysis by UHPLC-Orbitrap-HRMS

The qualitative and quantitative analyses of the mycotoxin content of the samples were determined using an UHPLC-HR-Orbitrap-MS device and a validated method *(Table 6)*.

Table 6. *Results of mycotoxins analysis by UHPLC-HR-Orbitrap-MS*

No	Sample Name	Aflatoxin B1 (µg/kg)	Aflatoxin B2 (µg/kg)	Aflatoxin G1 (µg/kg)	Aflatoxin G2 (µg/kg)	Ochratoxin A (µg/kg)
1	B8	nd	nd	nd	nd	nd
2	B8-1	nd	0.038	nd	nd	nd
3	B13	nd	nd	nd	nd	nd
4	B19	nd	nd	nd	nd	nd
5	B19-1	nd	0.034	nd	nd	nd
6	B22	nd	0.045	nd	nd	nd
7	B26	nd	0.074	nd	nd	nd
8	B31	nd	nd	nd	nd	nd
9	B33	nd	0.036	nd	nd	nd
10	B38	nd	nd	nd	nd	nd
11	B54	nd	0.037	nd	nd	nd
12	H1-6	0.076	0.037	0.035	nd	nd
13	H2-6	0.026	0.043	nd	nd	nd
14	H10-1	0.027	0.049	5.167	nd	nd
15	H10-2	0.081	0.038	6.284	nd	nd
16	H11	0.255	0.047	0.041	nd	nd
17	H11-2	0.078	0.058	0.170	nd	nd
18	H12	nd	0.460	nd	nd	nd
19	H20	nd	0.212	nd	nd	nd
20	H22	nd	0.133	nd	nd	nd
21	I10	nd	0.037	nd	nd	nd
22	I16	nd	0.036	nd	0.033	nd

nd: not detected

Ochratoxin A was not detected in any of the samples. Aflatoxin G2 was determined at a concentration of 0.033 µg/kg in only sample I16. Aflatoxin B2, which was the most common mycotoxin in the samples, was detected within a concentration range of 0.034-0.460 µg/kg. Aflatoxin B8, B13, B19, B31 and B38 were not detected in the samples. Aflatoxin B1 was detected in only 6 samples: H1-6 (0.076 µg/kg), H2-6 (0.026 µg/kg), H10-1 (0.027 µg/kg), H10-2 (0.081 µg/kg), H11 (0.255 µg) /kg), H11-2 (0.078 µg/kg)), and aflatoxin G1 was detected in only 5 samples: H1-6 (0.035 µg/kg), H10-1 (5.167 µg/kg), H10-2 (6.284 µg/kg), H11 (0.041 µg/kg), H11-2 (0.170 µg) /kg).

Discussion

Honey bees are exposed to the threat of multiple pathogenic microorganisms throughout their lives. Fungi can affect bee larvae without showing any symptoms, especially with an increase in air temperature. *Aspergillus flavus, A. niger* and *A. fumigatus* facilitate the exposure of bees to other disease-causing agents by suppressing their immune system [25].

Nearly a hundred species of mycotoxins produced as secondary metabolites by filamentous fungi have been identified, and most of them cause poisoning, organ damage, and carcinogenic effects in humans and animals [26]. In the present study, aflatoxin B2 was detected in 17 out of 22 filamentous fungi samples, aflatoxin B1 and B2 were detected in 6 out of 17 samples, and aflatoxin B1, B2 and G1 were detected in 5 samples. These results show that bees can be exposed to mycotoxins due to unhygienic conditions and environmental effects, spread them to plants, and infect bee larvae and other bee products. Thus, mycotoxins are likely to show adverse effects on the health of humans and animals directly or indirectly.

Aspergillus spp. and *Ascosphaera apis* are pathogenic to bees. *Aspergillus* spp. cause chalkbrood disease and *A. apis* causes stonebrood disease in bee larvae [27]. In the present study, *A. apis* was not isolated and identified from any of the samples, but *Aspergillus* spp. was identified in 8 out of 22 samples. *Aspergillus* species cause stonebrood disease in bee larvae and affect adult bees by suppressing their immune system [28]. In this study, the identification

of *Aspergillus* spp. suggests that these fungal agents could have caused the death of adult bees, and that they could have been transmitted from adult bees to larvae during feeding.

Isayeva et al.[29] examined the mycobiota of bees and isolated fungi from 130 out of 250 samples. These 130 fungal isolates were identified as belonging to 52 species, including among others *Alternaria alternata, Aspergillus flavus, Candida albicans,* and *Cladosporum herbarum*. These fungal species are reported to have toxigenic and allergenic effects on human and animal health. The identification of *Alternaria* spp., *Aspergillus* spp., and other filamentous fungi in honey bees in the present study shows similarity to the results reported by Isayeva et al.[29] and some differences are attributed to climatic and environmental conditions.

In the present study, while the aflatoxin gene was detected in only 1 sample by the PCR method, ochratoxin A was not detected in any of the samples. UHPLC-Orbitrap-HRMS results revealed the presence of aflatoxin B2 in 17 samples and aflatoxin B1 and aflatoxin B2 in 6 samples. In previous studies aimed at the investigation of the presence of mycotoxins by PCR, this method was proven to be useful in determining the presence of some mycotoxin genes [22,30]. Levin [31] reported that, unlike bacterial toxins encoded by a single gene, mycotoxins are encoded by multiple genes and cannot be detected using the PCR method. Furthermore, it has been reported that the presence of mycotoxin genes does not mean that the particular fungus species synthesize mycotoxins. Levin's findings explain the difference between the PCR and UHPLC-Orbitrap-HRMS results obtained in the present study.

Mycotoxin production by filamentous fungi has been demonstrated in several studies and aflatoxin B1 has been reported to be produced by *Aspergillus* spp. Honey bees are highly sensitive to aflatoxin B1, but an understanding of their sensitivity to other mycotoxins requires further research. It has been shown that 5 μg g^{-1} of dietary aflatoxin can cause high mortality in honey bees [32]. Aflatoxins reduce the resistance of honey bees to mycotic infections by directly affecting the central nervous and endocrine systems [33]. In the present study, aflatoxin B2 was detected in 17 of the filamentous fungi identified. Both B1 and B2 were detected in 6 samples, B1, B2 and G1 were detected in 5 samples, and aflatoxin G2 was detected in 1 sample. Aflatoxin was detected in 18 samples, corresponding to 82% of the 22 samples, and suggested that the cause of death of the collected adult bee samples could be mycotoxins produced by filamentous fungi.

In a study conducted by Decker et al.[13] on the intestines of 45 bees, it was reported that the fungal species identified could differ with the bacterial flora of the sampling site, and that bees could be the vector of fungi and transmit plant diseases. The same study pointed out to the need for further research to identify the species and potential roles of fungi in honey bees.

In a study conducted by Kis et al.[34] on 30 honey samples collected in Croatia between 2012 and 2017, *Penicillium, Alternaria* and *Cladosporium* were isolated and identified from 47.3% of the samples. It was also reported that *Mucor, Aureobasidium, Acremonium, Botrytis, Stachybotrys* and *Paecylomyces* were isolated and identified from 5.26% of the samples.

The present study suggests that fungal agents isolated and identified from adult honey bees can be transmitted to honey by adult bees, and that these fungal agents may threaten both human and animal health due to mycotoxin production.

The relationship between plant pathogens and honey bees has been demonstrated in many studies. Honey bees, which are important pollinators, also act as a vector of fungal, parasitic and viral plant pathogens. Honey bees can resist these agents up to certain limits [35]. In the present study, it was determined that the fungal agents isolated from the intestinal region of dead honey bees showed mycotoxin activity. AFB1, AFB2, AFG1 and AFG2 are toxic for both humans and animals. These aflatoxins are reported to have hepatotoxic, nephrotoxic and immunotoxic effects. Also, AFB1 is carcinogenic for humans. These mycotoxins may accumulate over time and cause mortality in honey bees. The destruction or treatment of plants infected with fungal diseases may reduce the spread of these mycotoxin-producing fungi into the environment by honey bees.

In conclusion, filamentous fungi can produce mycotoxins in the intestinal tract of honey bees. These mycotoxins can be spread to the environment by bees and adversely affect animal and human health. The mycotoxin activities of filamentous fungal agents, which are plant pathogens and for which pollinator honey bees may act as a vector, increase over time and cause bee mortality. Although reports are available on the isolation of filamentous fungi from the intestinal region of honey bees, further research is needed to determine the mycotoxin activity of these fungal agents in bees. This study is expected to contribute to future research in this field.

Availability of Data and Materials

Datasets analyzed during the current study are available in the author (S. Tarhane) on reasonable request.

Acknowledgements

We would like to express our endless thanks to our esteemed professors, who are always with us with their support, Prof. Dr. Salih OTLU, Prof. Dr. Bayram Cemil BİLGİLİ, Assoc., Lect. Bedri

EMİNSOY, Assoc. Prof. Dr. Melike Bilge KAMAÇ and the valuable personnel of Çankırı Karatekin University Central Laboratory.

Financial Support

No funding was received for conducting this study.

Conflict of Interest

The authors declare that there is no conflict of interest and they do not have any financial interests.

Author Contributions

All authors contributed to the understanding and design of the study. Sample collection was done by ST, İD, AKT, ŞK. The samples were prepared and analyzed by ST, İD, AKT, AG. The first draft of the article was written by ST and all authors commented on previous manuscripts of the article. All authors have read and approved the final article.

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