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Identification of Toxigenic Aspergillus Species in Rice Produced in Khuzestan and Mycotoxins in Iimported Cereals

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ABSTRACT

Background: Due to their high amount of carbohydrate and enough moisture, cereals are a good environment for the growth of toxigenic fungi. Because of the carcinogenicity and mutagenicity of mycotoxins, preventing them from entering the food chain is essential. Therefore, the present study was conducted to determine the amount and type of contaminated imported cereals and rice produced in Khuzestan province.

Materials & Methods: In October and November 2015, a total of 50 random samples of rice was collected from paddy fields. Aspergillus were identified based on available diagnostic criteria and PCR. The amount and type of aflatoxin in rice samples and mycotoxins in imported cereals (winter 2015 to autumn 2016) were evaluated by HPLC.

Results: Based on one sample t-test and comparing the mean of mycotoxins contaminating cereals in different seasons with national maximum standard, the amount of mycotoxins in barley and wheat were within the standard range but %8.4 of corn was higher than the permitted level (ppb5). Analysis of aflatoxins in rice also showed that 16 samples were contaminated with aflatoxin B1. *Aspergillus flavus* was the major pollutant (%42.1) isolated from rice.

Conclusion: *Aspergillus flavus* is the major producer of aflatoxin B1 in domestic rice. Examination of imported cereals also showed high rates of fungal growth and production of secondary metabolites, possibly due to inadequate storage conditions, high temperature and humidity. Therefore, it is recommended to strengthen the monitoring tools in the processing and storage of rice and cereals.

Keywords: Mycotoxin, HPLC, Carcinogenic, Aspergillus, Cereals

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Introduction

Mycotoxins are low-molecular-weight natural products that are produced by filamentous fungi as secondary metabolites **(1)**. There are five important mycotoxins that are naturally present in crops, including Aflatoxin produced by *Aspergillus flavus*, ochratoxin produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, Zearalenone and doxy-nivalenol produced by *Fusarium graminearum*, and Fumonisin produced by *Fusarium verticillioides* **(2)**. Six of the 18 different types of aflatoxin identified are of major importance, including B1, B2, G1, G2, M1, M2. Aflatoxin B1 has been classified as a Group A carcinogen by world health organization (WHO), for its constant contribution to the pathogenesis of liver cells **(1,5)**. Currently being used to detect a wide variety of mycotoxins, High Performance Liquid Chromatography (HPLC) leads to the production of

reliable data and accurate, precise and repeatable results, and will usually be of interest to researchers (6).

Although the weather in a particular country may not cause the development of a particular mycotoxin such as aflatoxin, the problem may come from another country in the form of crops such as peanuts and corn.

Materials and Methods

Isolation of Aspergillus Species

Collecting Rice Samples

In November 2015, 50 samples of rice husk from Baghmalek (23 samples of Champa), Shadegan (12 samples of Anbarboo) and Shooshtar (15 samples of Anbarboo) were collected through non-random convenience sampling. About 300 gr of rice husk was collected and transferred to the laboratory in sterile bags.

Isolation of Aspergillus Colonies from Rice Samples

One hundred rice seeds were randomly selected from each sample after washing and culturing on Sabouraud dextrose agar (SDA), based on colonial appearance and reproductive organs.

Identification of Aspergillus Strains Based on Morphological Traits

After 5 to 7 days of daily growth of the fungi and the type and shape of the colonies (Figure 1), Aspergillus fungi were identified by mycological and physiological methods according to John I. Pitt Diagnostic Key.

Classification of this group based on morphological identification is a traditional way that is very difficult and can lead to misdiagnosis, especially for *Aspergillus niger* species, which are a group of morphologically indistinguishable species (8-10).

Identification of Aspergillus Strains Based on ITS Sequencing

Fungal Mycelium Production and DNA Extraction

The grown mycelium mass was collected in SDB medium, washed with sterile distilled water and then dehydrated. The mycelium mass was powdered in liquid nitrogen. DNA isolation was performed with the modification of Raeder & Broda (1985) (with minor modification) (11,12).

Mycelium powder was purified and protein degraded in three successive stages using phenol, chloroform, and isoamyl alcohol. The DNA was finally collected by ethanol precipitation and washed with 70% ethanol.

ITS Gene Amplification

The ITS1-F and ITS4-R primer pairs were used to amplify around 600 bp of ITS regions. The reaction was performed in a thermocycler (Biorad) with the following temperature program: 3 min at 95°C and then 35 cycles, 95°C for 30 sec., 52°C for 40 sec., 72°C for 1 min, and a final extension step at 72°C for 5 min.

Measurement and Determination of Mycotoxins Level and Type by HPLC

Sample Collection

The analysis of mycotoxins in imported cereal was carried on 50 samples out of 2750 samples referred from Imam Khomeini Port Customs in winter, spring, summer and autumn 2016. It should be noted that the basis for the determination and detection of mycotoxins by the HPLC method was the same in all cases according to the standards of the National Iranian Standards Organization.

Sample Preparation

Fifty grams of samples were weighed for testing mycotoxins according to Iranian National Standard No. 6872 “Human-Animal Feed, Measurement of Group B and G Aflatoxins”, No. 9238 “Cereals and its Products - Measurement of Ochratoxin A”, No. 9239 “Cereals and Products” Its weights - the zearalenone measurement”, and No. 9240 “doxylamine content determination”

Measuring the Amount and Type of Mycotoxins

The toxin was extracted from the samples by solvent extraction (methanol 80%). The extract was diluted with Watman filter paper to a certain concentration after being filtered through a sinter filter. Extracts obtained from the immunoaffinity column containing specific antibodies (ZearalaTest™, AflaTest™, OchraTest ™, DONTest ™) were passed at a drop per second rate and the antigen present in the extract was bound to specific antibodies in the column. Injection, isolation, detection, and determination of Mycotoxins was calculated by reversed-phase HPLC columns and derivative and fluorescence detector, through comparison of the standard substrate surface with an unknown specimen, taking into account the dilution factor in ng/g (HPLC device, KNAUER, Germany). In order to calculate the recovery rate of mycotoxin toxins with a concentration of 1.6 ppb in healthy specimens, spiked specimens were read and extracted in a similar manner. Recovery rates of mycotoxins were in the range of 0-6%. This range is acceptable by national standards and indicates that the extraction operations are well performed.

Results

Investigation and Identification of Aspergillus Isolates from Rice

Of the 19 samples sequenced, 8 isolates (group I) were identified as *Aspergillus flavus*, 2 isolates (group II)

as *Aspergillus terreus*, 1 isolate as *Aspergillus nidulans* (Group III), 3 isolates as *Aspergillus tubingensis*, 3 isolates as *Aspergillus niger* and 1 isolate as *Aspergillus SP* (Figures 2 and 3).

The ITS region sequences in the studied samples showed 99-100% similarity to the type strains of any gene in the NCBI database (Table 1).

Table 1. Identification results of isolates based on blast morphology and search and their access number registered in the gene bank

Aspergillus has the most similarity	Isolate name (registered in GenBank)	Gathering location	Accession Number of Sequences Registered for this Study in GenBank
			ITS
Aspergillus flavus	*Sho_R1f*	Shooshtar	KY490723
	Sho_R2f	Shooshtar	KY490717
	Bag _R6f	Baghmalek	KY490710
	Bag _R7f	Baghmalek	KY490714
	Bag _R8f	Baghmalek	KY490709
	Bag_R10f	Baghmalek	KY490722
	Sha_R15f	Shadegan	KY490712
	Sha_R18f	Shadegan	KY490711
Aspergillus terreus	*Sha_R12t*	Shadegan	KY490721
	Sha_R14t	Shadegan	KY490708
Aspergillus tubingensis	*Sha_R11tu*	Shadegan	KY490713
	Sha_R13tu	Shadegan	KY490707
	Sha_R16tu	Shadegan	KY490706
Aspergillus nidulans	*Bag_R5ni*	Baghmalek	KY490724
Aspergillus niger	*Sho_R4n*	Shooshtar	KY490718
	Bag_R9n	Baghmalek	KY490715
	Sha_R17n	Shadegan	KY490720
	Sha_R19n	Shadegan	KY490719
Aspergillus SP	*Sho_R3a*	Shooshtar	KY490716

***All samples have 100-99% sequence similarity to standard samples in the NCBI database**.

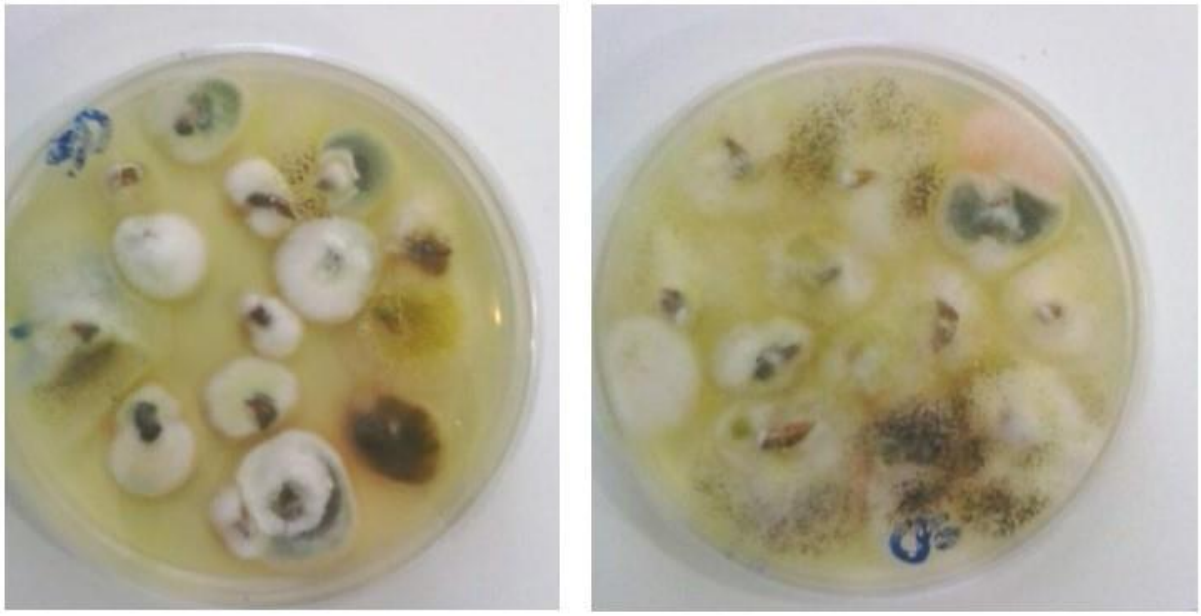

Figure 1. Fungal colonies appearing on the SDA medium at 28°C for five days.

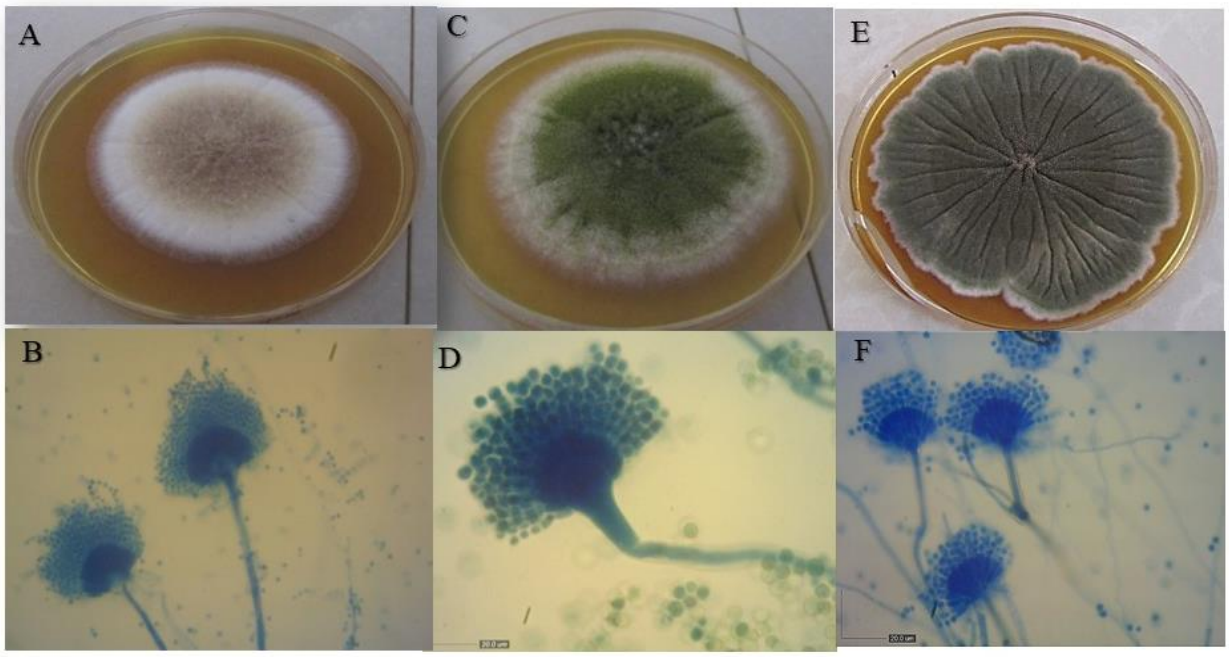

Figure 2. Microscopic image of Aspergillus terreus (A: conidiophore (x40); B: Isolated image on the plate, (Aspergillus flavus; C: conidiophore (x40) D: isolate image on the plate) and Aspergillus nidulans (E: conidiophore (x40) F: isolate image on the plate)

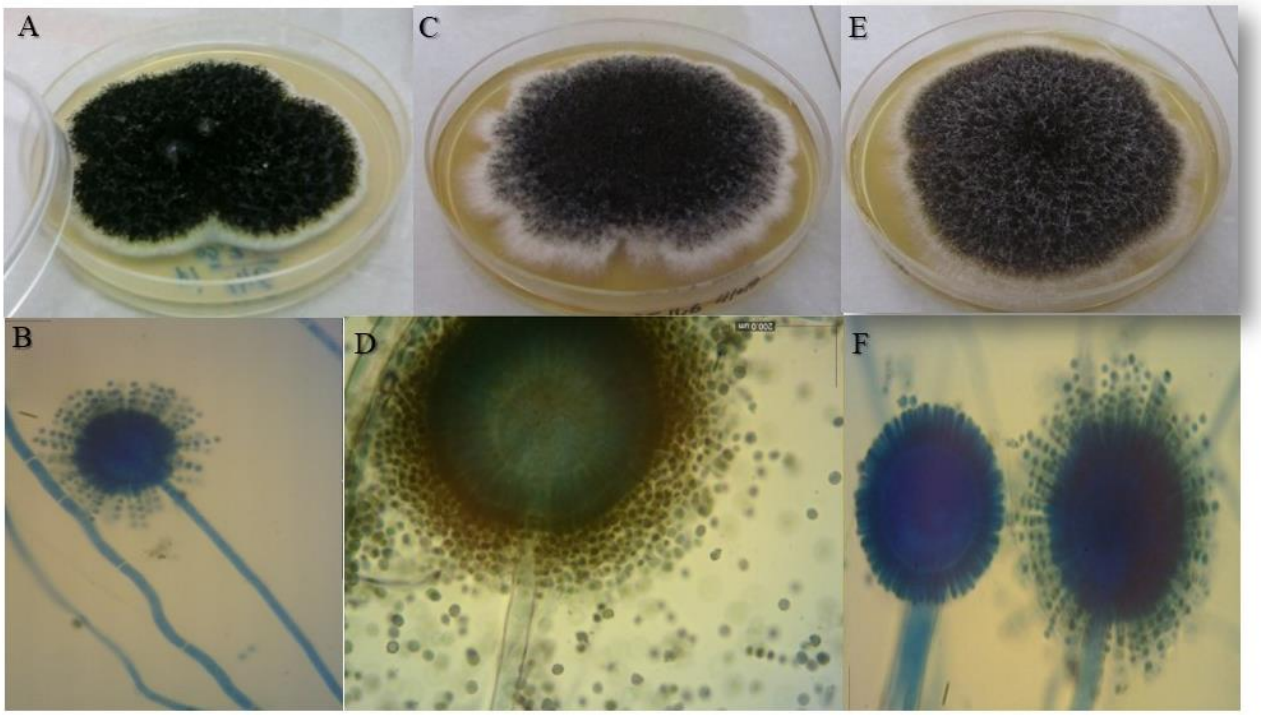

Figure 3. Microscopic image of *Aspergillus tubingensis* (A: *conidiophore* (x40) B: Isolated image on the plate), *Aspergillus niger* (C: *conidiophore* (x40) D: isolate image on the plate) and *Aspergillus sp.* (E: *conidiophore* (x40) F: isolate image on the plate)

Results of Mycotoxins Assay

The presence of mycotoxins in imported cereals was evaluated qualitatively and quantitatively. From winter to autumn 2016, the type and amount of mycotoxin were measured in 2750 samples of cereals referred to customs in three laboratories in Ahvaz. These included corn, wheat, and barley. The mycotoxins tested on these cereals were aflatoxins, zearalenone, deoxynivalenol, and ochratoxin, according to applicants.

The results of the mean cereal contamination of different mycotoxins and their comparison with the national standard of Iran (5925) are presented in Table 1, 2 and 3 respectively (one sample t-test) (Graph pad prism 8). The results of statistical analysis showed that the mean of mycotoxins in different grains had a significant difference with the maximum national standard of each mycotoxin (P<0.05)

Out of 2111 corn samples, 8.4% (689) were over contaminated (5 ppb) in AFB1 and 0.4 (9), 3.3 (2), and 0.1 (3) of samples were over contaminated in AFB2, AFG1, and AFG2, respectively.

The levels of ZEN, DON and OTA mycotoxins in 1139 corn samples were lower than the national standard and lower than 200 ppb, 1000 ppb, and 50 ppb, respectively. Of 166 wheat samples, 4.2% (1.2%) and 1.2% (2%) of samples were contaminated at concentrations below 5 ppb with AFB2 and AFG1, respectively. Also, 13.8% (16) of the samples were contaminated with OTA at less than 5 ppb.

None of the wheat samples showed contamination with DON, ZEN, AFB1, and AFG2. Of the 149 barley samples imported during the sampling interval of this study, only aflatoxins were detected in which no contamination was observed in any of the samples. ANOVA and LSD post hoc tests were used to compare the mean levels of mycotoxins in different months of the year (January to December 2015). According to the results of this test, there was a significant difference between the mean of other mycotoxins in different months of the year (P<0.05).

Of the 50 rice samples tested for aflatoxins, 16 were contaminated with aflatoxin B1, of which only 1 showed more than 5 ppb. Samples 2 and 4 were contaminated with AFB2 and AFG1, respectively, (<5 ppb). The results of the study of mean rice contamination with aflatoxins and comparison with the national standard of Iran are presented in Table 4 (one sample t-test).

Table 2. Comparison of mean mycotoxin contaminants of imported corn with the national standard of Iran in winter and spring 2016

Grains	mycotoxins	National standard maximum allowable (ng /g)	Winter2016			Spring 2016			Maximum contamination(ng/g)
			Average (ng/g)	Standard deviation	P-value	Average (ng/g)	Standard deviation	P-value	
Corn	DON	1000	105.8	62.31	<0.0001	329.9	278.2	0.0007	**868.57**
	ZEN	200	13.47	13.35	<0.0001	33.58	4.582	0.0003	**123.731**
	OTA	50	0.035	0.1385	<0.0001	0.71	0.6621	<0.0001	-
	AFB1	5	1.151	2.669	<0.0001	0.05	0.0734	<0.0001	**169.08**
	Total AFs	20	0.8	0.973	<0.0001	1.25	2.253	<0.0001	**266.77**

Table 3. Comparison of mean mycotoxins of imported corn contaminants with the national standard of summer and autumn 2016

Grains	Mycotoxins	National standard maximum allowable (ng /g)	Summer2016			Autumn2016		
			Average (ng/g)	Standard deviation	P-value	Average (ng/g)	Standard deviation	**P-value**
Corn	DON	1000	496.6	175.6	<0.0001	0	0	**NC**
	ZEN	200	54.43	30.89	<0.0001	0	0	**NC**
	OTA	50	0.97	0.73	<0.0001	0.98	0.71	**<0.0001**
	AFB1	5	7.03	19.23	0.28	7.553	18.52	**0.0025**
	Total AFs	5	11.91	32.86	<0.0001	11.02	29.32	**<0.0001**

Table 4. Comparison of mean mycotoxins contaminated by imported wheat with Iranian national standard in spring 2016

Grains	mycotoxins	National standard maximum allowable (ng /g)	Spring 2016			Maximum contamination (ng/g)
			Average (ng/g)	Standard deviation	P-value	
Wheat	DON	1000	0	0	NC	-
	ZEN	200	0	0	NC	-
	OTA	5	2.67	1.053	<0.0001	**4**
	AFB1	5	0	0	NC	-
	Total AFs	20	0.21	0.07	0.0016	-

Table 5. Comparison of the average Aflatoxins of Khuzestan rice production contaminants with the national standard of Iran

mycotoxins	National standard maximum allowable (ng /g)	Average(ng/g)	Standard deviation	P-value	Maximum contamination(ng/g)
AFB1	5	2.45	4.42	0.036	18.612
Total AFs	20	2.60	4.52	<0.00001	-

Discussion

In this study, Aspergillus susceptible to toxin production was identified and isolated from rice seeds of Khuzestan province (Champa and Anbarboo). Results showed high percentages of *Aspergillus flavus* (42.1%) followed *by Aspergillus niger, Aspergillus tubingensis, Aspergillus terreus,* and *Aspergillus nidulans* with 21.5%, 15.7%, 10.52%, and 5.26%, respectively. The findings of Nyongesa *et al.* (2015), Riba *et al.* (2010) and Gao *et al.* (2007) showed that *Aspergillus flavus* was the main pollutant in oilseeds (pistachio, almond, hazelnut), corn, wheat, cereals, and beans, respectively. The results of the studies of Makun *et al.* (2007), Amadi *et al.* (2009) were also consistent with this study (13, 14, 15, 16, 17). Because of the saprophyte and widespread toxinogenic fungi such as Aspergillus, rice and cereal products have some relative contamination from the beginning.

The presence of spores and their propagation during transport and storage of grains or contamination of the storage environment with these fungi can lead to the spread of contamination. As Magnussen & Parsi (2013) found out, drought stress is one of the factors increasing the susceptibility of plants to Aspergillus and as a result of aflatoxin infection (18), the results of this study also suggest that the rate of these fungi can be increased with high heat conditions and increased storage time.

By stage of infection, mycotoxins are "field mycotoxins" produced mainly by Fusarium species and "Mycotoxins in storage", which are produced by Aspergillus and Penicillium species in the pre-harvest period or immediately after harvest during storage **(19,20)**.

The growth of fungi and the accumulation of mycotoxins in food and feed are influenced by various factors which in general relative humidity and temperature are critical factors in the drying and storage period **(21)**. Findings from the study of the amount and type of cereal mycotoxins imported from Imam Khomeini port, mainly from South American countries, showed that the contaminations were not nearly acceptable by Iranian national standard, and corn samples showed the highest levels of mycotoxins, especially aflatoxins. The findings of a study by Mazaheri *et al.* (2018) also confirmed that corn is most susceptible to mycotoxins, especially aflatoxins **(22)**. The findings of this study indicate that the maximum concentration of mycotoxins is in September and November. These results illustrate the impact of the duration of storage of grain in shipments or in warehouses. This is confirmed by Najafian *et al.* (2014) **(25)**.

In a study, Ranjbar *et al.* (2010) showed that Aspergillus and Aflatoxin levels decrease in spring and summer and due to the use of stocked livestock feed and the lack of proper storage conditions, the rate of milk contamination with aflatoxin M1 increases in the autumn and winter. They stated that inappropriate storage, in addition to contaminating livestock feed with Aspergillus and subsequent aflatoxin production, also transmitted it to *packaged* milk **(26)**.

Cano-Sancho *et al.* (2013) also stated that the presence of mycotoxins in food depends on many conditions such as season, weather (temperature, humidity), target area, harvesting method, storage and processing **(27)**. Lahouar *et al.* (2015) studied the effect of temperature and incubation time on the growth rate of *Aspergillus flavus* and AFB1 production on sorghum seeds, and reported that the flavus isolate from Tanzanian sorghum has the ability to grow over a wide range of temperatures (15-37°C), however, aflatoxin production occurs at a lower temperature range (25-37°C) **(28)**. Ghali *et al.* (2010) showed that the highest aflatoxin production occurred at 24°C but the highest flavus strain growth occurred at -35°C **(19)**.

In this study, the presence of Aspergillus did not indicate the presence of aflatoxins and, conversely, the presence of aflatoxins was not a reason for the presence of Aspergillus.

Conclusion

Given the widespread presence of mycotoxins in foods and their harms, especially AFB1, which is a contaminant in food products, control of AFB1 contamination in foodstuffs requires monitoring different factors such as raw materials and food supply, food processing, finished products, and storage.

Acknowledgment

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Conflict of Interest

Authors declared no conflict of interests.