Detection of Aflatoxin M1 Levels in Cow Raw Milk by using ELISA Techniques in Al-Diwaniyah Governorate

Hussam Hamza Abd and Wisam Raheem Atiyah

College of Veterinary Medicine, University of Al-Qadisiyah, Al Diwaniyah, Iraq

Corresponding author: wisam.atyiah@qu.edu.iq

Received: 11/4/2026 **Accepted:** 11/5/2026 **Publisher:** 15/6/2026

***Abstract*—** The purpose of this study was to assess the prevalence and regional variation of Aflatoxin M1 (AFM1) levels in milk samples taken from five districts. Fifty milk samples were randomly collected from Al-Hamza, AL-Diwaniyha, Al-Sunniyah, Al-Shamiya and Al-Daghara regions from farms and markets of Al-Diwaniyah Governorate for the period between October 2025 and January 2026, in all (n = 10 each district) were examined. Every sample (100%) had a positive AFM1 test. Al-Hamza had mean quantities of 0.411 ± 0.041 ppb, while Al-Diwaniyah had mean values of 0.489 ± 0.048 ppb. Districts differed significantly, according to statistical analysis using one-way analysis of variance (ANOVA) ($F(4,45) = 6.87$, $P = 0.0004$). Al-Diwaniyah and Al-Sunniyah had considerably greater levels than Al-Hamza ($P < 0.05$), according to a post-hoc comparison using Duncan's multiple range test, whereas Al-Shamiya and Al-Daghara showed intermediate concentrations. AFM1 contamination levels may be influenced by management factors, feed storage techniques, and environmental conditions, according to the reported geographical variance. In order to lower exposure hazards and safeguard public health, these findings emphasize the necessity of ongoing monitoring programs and enhanced control mechanisms.

***Keywords* —** Aflatoxine M1, Raw milk, ELISA, Food safty, Iraq.

INTRODUCTION

Mycotoxins are low molecular weight compounds formed by fungi that are among the most considerably harmful naturally occurring substances in food and feed. More than 300 mycotoxins have been found thus far, the majority of which are produced by Fusarium, Aspergillus and Penicillium fungus species (1,2), Inadequate farming methods during harvest, transportation and storage as well as high humidity before and after harvest, which encourages fungal development, are some of the reasons that lead to crop contamination with mycotoxins (3,4). As well as, aflatoxin (AF) has almost 20 different derivatives. Milk and other foods contain aflatoxin B1 (AFB1), which is hydroxylated to produce AFM1. Consuming food tainted with AFM1 may be detrimental to your health. The carcinogenic potential and thermal stability of Aflatoxin M1 make milk contamination a major general health trouble, It has been connected to birth abnormalities and genetic alterations. Moreover it damages DNA and impairs immunity (5,6). Mycotoxins can enter the human body from direct intake of plant-based foods contaminated during prodection and storage or through ingestion of mycotoxin metabolites present in animal-derived products like milk or eggs. Aflatoxin B1 (AFB1)-contaminated feed shifts into aflatoxin M1 (AFM1) by hepatic microsomal cytochrome P450 in the livers of ruminants such as cattle, goats, sheep, etc. (6). AFM1 can be a major health risk to humans and is subsequently expelled in their milk since it has been reported to suppress the immune system, modify DNA structure, and, at high levels, cause serious conditions like liver cirrhosis, hepatocellular carcinoma, and stunted growth in newborn (7). Eliminate AFM1 from dairy products is a challenge because AFM1 keep stable even after thermal treatments like sterilization or pasteurization, which usually occur at much lower temperatures than those used to break down AFM1(237-306 C°) (8). Mycotoxins in milk have an impact on public health, food safety, the economy, agriculture, and international trade. Mycotoxins are among the most controlled natural poisons in the world due to their dangers (9). AFM1 can be found in milk and dairy products using a range of techniques. Chromatographic techniques like mass spectrometry and high-performance liquid chromatography (HPLC) with different several detectors (e.g., Mass Spectrometry) (10,11), Because of its great sensitivity, ease of use, and capacity to analyze several samples quickly, ELISA is still the method of choice for laboratory analysis. AFM1 in food can be determined using a couple currently available ELISA assays(12,13). A number of variables pertaining to cow diet, animal metabolism, and production management circumstances affect the transmission of mycotoxin M1 into milk. The stage of milk production, the species and breed of the animal, its overall health, and its metabolic state are all animal-related elements that affect milk output. Aflatoxin uptake and digestion rate, udder health, toxin

interaction, alveolar cell membrane integrity, and blood-milk barrier alterations are additional aspects to take into account (14,15).

Material and Methods

Fifty random Milk samples (50 samples) were collected from five different regions from farms and markets of Al-Diwaniyah Governorate for the period between October 2025 and January 2026. To prevent aflatoxin degradation, the samples were placed in sterile containers, labeled with unique identification tags, transported under cold chain conditions (4°C), and kept at -20°C until the time of analysis.

Samples extraction:

Extraction procedures were adapted from the Elabscience ELISA kit manual AFM1 (Aflatoxin M1) ELISA Kit (E-TO-E018) and optimized for each sample matrix. All extractions were performed in duplicate.

Reagent and sample preparation

Equilibration: Before using, allow all kit ingredients to come to room temperature (20–25°C) for one to two hours.

A- Sample Pretreatment and homogenizing (Liquid Milk):

1. combine 1 mL with 4 mL of acetonitrile and vortex for five minutes to prepare the sample (liquid milk).
2. Centrifuge at ambient temperature for 10 minutes at 4000 rpm.
3. The residue should be dissolved in 1 mL of Reconstitution Buffer (Solution 1) after 2.5 mL of the supernatant has been dried at 50–60°C.
4. For the assay, use 50 µL of this prepared sample.
5. Reagent Prep: Dilute 20x concentration (1:19 ratio) to create Wash Buffer.

B- Procedure for the ELISA Test:

Run every sample and standard twice .

1. Include Components: Fill each well with 50 µL of Standard/Sample, 50 µL of HRP Conjugate, and 50 µL of Antibody Working Solution.
2. Incubate: Stir thoroughly, cover, and leave in the dark for 30 minutes at 25°C.
3. Wasing: Wash wells five times with 300 µL of Washer Buffer, tapping dry.
4. Added 50 µL of Substrate A and B each, then incubated for 15 minutes in the dark at 25°C to develop the color.
5. Stop & Measure: Within 10 minutes, add 50 µL of Stop Solution and measure the optical density at 450 nm.

C- Analysis of Data

Compute the mean optical density (OD), create a semi-log standard curve, and multiply the determined sample concentration by the dilution factor (3x in this approach).

STATISTICAL ANALYSIS

A one-way analysis of variance (one-way ANOVA) was employed to assess the dataset, while Duncan's multiple range test was used for subsequent post-hoc analysis. The ANOVA detected a statistically significant disparity among the districts (16).

RESULT AND DISCUSSION

Five districts (n = 10 each district) provided a total of 50 samples, all of which (100%) tested positive. From 0.411 ± 0.041 in Al-Hamza to 0.489 ± 0.048 in Al-Diwaniyah, the mean values varied by district. Al-Diwaniyah has the highest mean value (0.489 ± 0.048), closely followed by Al-Sunniyah (0.488 ± 0.031). Al-Shamiya (0.455 ± 0.060) and Al-Daghara (0.453 ± 0.055) had intermediate levels, while Al-Hamza (0.411 ± 0.041) had the lowest.

Table 1. Mean levels among districts and statistical comparison.

District	No. of sam	Positive samples	Range	Mean ± SD	Significance
Al-Diwaniyah	10	10	0.58-0.43	0.048 ± 0.48	a
Al-Sunniyah	10	10	0.52–0.41	0.031 ± 0.48	a
Al-Shamiya	10	10	0.54–0.37	0.060 ± 0.45	b
Al-Daghara	10	10	0.53–0.38	0.055 ± 0.45	b
Al-Hamza	10	10	0.47–0.35	0.041 ± 041	c

A one-way analysis of variance (one-way ANOVA) was employed rigorously assess the data, while Duncan's multiple range test was utili for subsequent post-hoc comparisons.
A statistically significant difference between districts was found by ANOVA:
P = 0.0004, F(4,45) = 6.87
At P < 0.05, means with distinct superscript letters (a–c) differ significantly.

A statistically significant difference across districts was found using one-way analysis of variance (ANOVA) ($F(4,45) = 6.87$, $P = 0.0004$). Al-Diwaniyah and Al-Sunniyah belonged to the same statistical group (a), substantially higher than Al-Hamza (c), according to post-hoc analysis using Duncan's multiple range test, whilst Al-Shamiya and Al-Daghara constituted an intermediate group (b) at $P < 0.05$.

The measured levels may be influenced by environmental or management-related factors, according to the observed geographical variation. The higher levels in Al-Diwaniyah and Al-Sunniyah may be due to variations in handling techniques, climate variance, feed quality, or storage conditions. On the other hand, the lower levels found in Al-Hamza might be the result of better control measures or more favorable environmental conditions. This could be because the cows in these rural agricultural areas are fed green fodder more frequently and don't need to be stored for a long time. Such spatial heterogeneity suggests that in order to better understand pollution patterns and potential risk factors, regional monitoring initiatives are required.

The mean AFM1 concentrations (ppb) ± SD for each of the five districts under study are shown in Figure 1. In line with the previously published statistical analysis, the graphical display amply illustrates district variability. Al-Diwaniyah had the greatest mean concentration, closely followed by Al-Sunniyah, while Al-Hamza had the lowest mean amount.

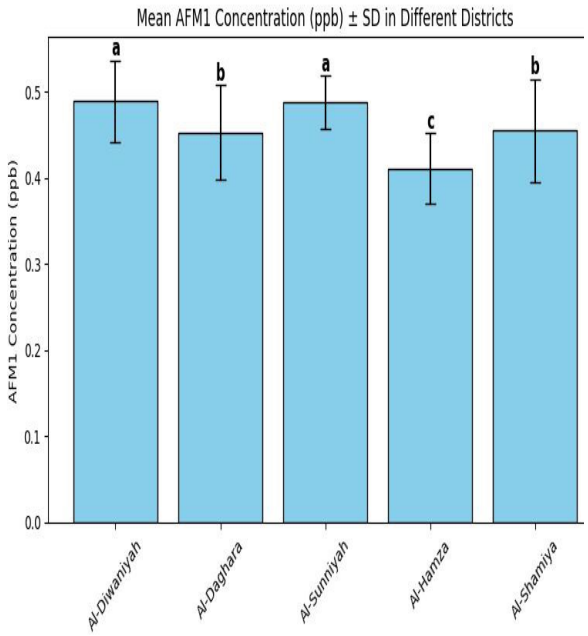

Figure 1. AFM1 concentration (ppb) ± SD in milk samples taken from several districts. According to a one-way ANOVA and Duncan's multiple range test, different letters denote significant differences at $P < 0.05$.

Al-Daghara and Al-Shamiya displayed concentrations that were in the middle. Al-Diwaniyah and Al-Sunniyah's classification into the same statistical group (a) is supported by the error bars (± SD), which show moderate dispersion between groups and comparatively overlapping variability. On the other hand, as previously determined by ANOVA and Duncan's multiple range test ($P < 0.05$), Al-Hamza's statistical separation (group c) is confirmed by the clearly lower bar. As a result, the pictorial trend supports the existence of notable geographic variance between districts.

The most common and extremely toxic mycotoxins in food sources are aflatoxins (17). The system in Iraq generally relies on its own standard specifications (Central Organization for Standardization and Quality Control), which adopt the European Union limit of (0.05 ppb) = 50 nanograms/kg as a health standard (18). The health and productivity of people, animals, and crops can all be affected by mycotoxin poisoning. The economic value associated with human activity and the production of agricultural and livestock items may therefore be impacted by these outcomes (19). Aflatoxin contamination of animal feed crops can occur during cultivation, and seasonal variations have an impact on this contamination. Seasonal fluctuations may also affect the feeding process. Animals can consume feed that contains more fresh food and less stored raw materials when grazing conditions are favorable. It was discovered that stored goods were more frequently contaminated with aflatoxin (20). If conditions remain unchanged, an increase in aflatoxin contamination of animal feed results in increased aflatoxin ingestion and, quantitatively, increased aflatoxin excretion in milk. The particular type of feed utilized determines the degree of contamination. The use of more complex feeds has been associated with aflatoxin contamination in milk. Furthermore, it has been suggested that certain raw materials, such cottonseed, are particularly vulnerable to aflatoxin contamination (18,21).

This study was carried out to assess the contaminated milk samples with aflatoxin M1 (AFM1) in Al-Diwaniyah Governorate. Given the paucity of data and scholarly search regarding the occurrence of AFM1 in dairy products within this administrative region, this study aimed to provide future insights for assessing aflatoxin contamination in milk and to guide research on the importance of this contamination, which is a known carcinogen with cumulative health risks for the population of the governorate and other parts in Iraq. The results were extremely alarming, the 50 milk samples analyzed using the (ELISA) test for AFM1, all samples showed several levels of contamination, show the presence of AFM1 in 100% of the milk, the increased contamination rate may be due to several important reasons, including unsuitable storage conditions for feed (corn, barley, wheat, bread scraps) such as storage with high humidity or poor ventilation in feed warehouses, which promotes fungal growth, or the use of feed contaminated with fungi without treatment, in addition to a lack of control over feed quality.. The samples contained levels of AFM1 exceeding the European Union's permissible limit of 0.05 parts per billion (ppb), a standard accepted for human ingestion. This represents a serious food safety concern for consumer persons in the governorate. The notable variations seen between districts demonstrate how geographic location affects the parameter under study. Previous research have revealed similar regional variances, with storage techniques, humidity, and environmental temperature identified as important contributing factors.

The results of this research are consistent with the results of (18,23,24) indicating that all samples were contaminated with aflatoxin M1, Because the contamination levels were higher than the legally permissible threshold of 50 ng/kg (ppb) as per European legislation, they may be dangerous. Experiments conducted in some European countries have shown elevated levels of aflatoxin M1 contamination, despite preventative measures taken to inhibit aflatoxin growth in poorly stored feed. A study in Hungary indicated that the contamination rate in raw milk reached 68% (25), However, other research conducted in Europe has shown a significant decrease in pollution levels in the Czech Republic, reaching 13%, The Czech Republic has comparatively low aflatoxin levels (more especially, Aflatoxin M1) pollution in milk because of a combination of excellent weather, stringent EU-aligned regulatory monitoring, and strong agricultural feed restrictions. By taking these precautions, milk contamination which happens when cows eat feed tainted with aflatoxin B1 is effectively controlled (26,27).

The comparatively higher means seen in Al-Diwaniyah and Al-Sunniyah could be a sign of increased exposure to risk factors, such as supply chain management or environmental factors. In the meantime, moderate exposure circumstances are suggested by the

intermediate levels seen in Al-Shamiya and Al-Daghara .Al-Hamza's statistically lower level is significant and might be the result of better local management practices or fewer environmental pressures. In order to reduce possible hazards to public health, these findings highlight the significance of putting in place region-specific control measures and ongoing monitoring. To enhance regulatory compliance and food safety monitoring, numerous nations worldwide are developing chromatographic, immunological, and biosensor-based techniques for aflatoxin detection. In order to lessen aflatoxin contamination and the related health and financial costs, this analysis emphasizes the necessity of integrated management techniques and international collaboration (28, 29).

CONCLUSION

All milk tests had AFM1 contamination, which is a major public health risk. There were notable variations between the districts, with Al-Diwaniyah and Al-Sunniyah having higher amounts. Environmental factors and feed storage techniques probably have an impact on these differences. To lower contamination and guarantee milk safety, effective control methods and ongoing monitoring are crucial.

Acknowledgements

N/A

Conflict of Interest

The authors declare no conflict of interest.

REFERENCES

1) Khan, R., Anwar, F., & Ghazali, F. M. (2024). A comprehensive review of mycotoxins: Toxicology, detection, and effective mitigation approaches. Heliyon, 10(8), e28361. https://doi.org/10.1016/j.heliyon.2024.e28361.

2) Szelenberger, R., Cichoń, N., Zajaczkowski, W., & Bijak, M. (2024). Application of Biosensors for the Detection of Mycotoxins for the Improvement ofFood Safety. Toxins, 16(6), 249. https://doi.org/10.3390/toxins16060249.

3) Kafle, S., Paudel, M., Shrestha, C., Kathayat, K. B., Sapkota, R. C., Tiwari, A., & Subedi, D. (2024). Aflatoxin M1 Contamination in Dairy Milk in Kathmandu, Nepal. Toxins, 16(11), 468. https://doi.org/10.3390/toxins16110468.

4) Shekhar, R., Raghavendra, V. B., & Rachitha, P. (2025). A comprehensive review of mycotoxins, their toxicity, and innovative detoxification methods. Toxicology reports, 14, 101952. https://doi.org/10.1016/j.toxrep.2025.101952.

5) Muaz, K., Riaz, M., Oliveira, C. A. F. de, Akhtar, S., Ali, S. W., Nadeem, H., … Balasubramanian, B. (2022). Aflatoxin M1 in milk and dairy products: global occurrence and potential decontamination strategies. Toxin Reviews, 41(2), 588–605. https://doi.org/10.1080/15569543.2021.1873387.

6) Ramani, A., Tanmay H., Anamika D. (2026). Comprehensive Review on the Occurrence of Aflatoxin M1 in Milk, Prevalent Health Issues Associated With It, and Innovative Strategies for Mitigation of Aflatoxin M1 in Processed Milk.FoodSafetyandHealth:4.no.1,67–81. https://doi.org/10.1002/fsh3.70047.

7) Zentai, A., Jóźwiak, Á., Süth, M., & Farkas, Z. (2023). Carry-Over of Aflatoxin B1 from Feed to Cow Milk—A Review. Toxins, 15(3), 195. https://doi.org/10.3390/toxins15030195.

8) Alshannaq, A., & Yu, J. H. (2017). Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. International journal of environmental research and public health, 14(6), 632. https://doi.org/10.3390/ijerph14060632.

9) Kumar, P., Mahato, D. K., Kamle, M., Mohanta, T. K., & Kang, S. G. (2017). Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. Frontiers in microbiology, 7, 2170. https://doi.org/10.3389/fmicb.2016.02170.

10) Esam, R. M., Hafez, R. S., Khafaga, N. I. M., Fahim, K. M., & Ibrahim Ahmed, L. (2022). Assessment of aflatoxin M1 and B1 in some dairy products with referring to the analytical performances of enzyme-linked immunosorbent assay in comparison to high-performance liquid chromatography. Veterinary world, 15(1), 91–101. https://doi.org/10.14202/vetworld.2022.91-101.

11) Chen, M., Liu, X., Yang, S., Chen, Z., Di, B., Liu, W., & Yan, H. (2022). HPLC–MS/MS method for the simultaneous determination of aflatoxins in blood: Toxicokinetics of aflatoxin B1 and aflatoxin M1 in rats. Journal of Analytical Science and Technology, 13(1), 27. https://doi.org/10.1186/s40543-022-00336-3.

12) Herzallah, S. M. (2009). Determination of aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors. Food Chemistry, 114(3), 1141-1146. https://doi.org/10.1016/j.foodchem.2008.10.077.

13) Tarannum, N., Nipa, M. N., Das, S., & Parveen, S. (2020). Aflatoxin M1 detection by ELISA in raw and processed milk in Bangladesh. Toxicology reports, 7, 1339–1343. https://doi.org/10.1016/j.toxrep.2020.09.012.

14) Ferrari, L., Rizzi, N., Grandi, E., Clerici, E., Tirloni, E., Stella, S., Bernardi, C. E. M., & Pinotti, L. (2023). Compliance between Food and Feed Safety: Eight-Year Survey (2013-2021) of Aflatoxin M1 in Raw Milk and Aflatoxin B1 in Feed in Northern Italy. Toxins, 15(3), 168. https://doi.org/10.3390/toxins15030168.

15) Bervis, N., Lorán, S., Juan, T., Carramiñana, J. J., Herrera, A., Ariño, A., & Herrera, M. (2021). Field Monitoring of Aflatoxins in Feed and Milk of High-Yielding Dairy Cows under Two Feeding Systems. Toxins, 13(3), 201. https://doi.org/10.3390/toxins13030201.

16) Armstrong, R.A., Hilton, A.C., Armstrong, R.A. and Hilton, A.C. (2010). One-Way Analysis of Variance (Anova). In Statistical Analysis in Microbiology: Statnotes (eds R.A. Armstrong and A.C. Hilton). https://doi.org/10.1002/9780470905173.ch6.

17) Zentai, A., Jóźwiak, Á., Süth, M., & Farkas, Z. (2023). Carry-Over of Aflatoxin B1 from Feed to Cow Milk-A Review. Toxins, 15(3), 195. https://doi.org/10.3390/toxins15030195.

18) Najim, N. H. (2014). The Occurrence of Aflatoxin M1 in Milk, soft cheese and yoghurt in Baghdad Province

by Using ELISA Test: Najim Hadi Najim and Huda Nsaif Jasim. The Iraqi Journal of Veterinary Medicine, 38(2), 9-16. https://doi.org/10.30539/iraqijvm.v38i2.216.

19) Awuchi, C. G., Ondari, E. N., Ogbonna, C. U., Upadhyay, A. K., Baran, K., Okpala, C. O. R., Korzeniowska, M., & Guiné, R. P. F. (2021). Mycotoxins Affecting Animals, Foods, Humans, and Plants: Types, Occurrence, Toxicities, Action Mechanisms, Prevention, and Detoxification Strategies-A Revisit. Foods (Basel, Switzerland), 10(6), 1279. https://doi.org/10.3390/foods10061279.

20) Umar, A., Qanita, S., Zada, N., & Honey, S.F. (2025). From feed to food – Understanding the impact of aflatoxins consumption by Pakistani livestock. CABI One Health. https://doi.org/10.1079/cabionehealth.2025.0021.

21) Popescu, R. G., Rădulescu, A. L., Georgescu, S. E., & Dinischiotu, A. (2022). Aflatoxins in Feed: Types, Metabolism, Health Consequences in Swine and Mitigation Strategies. Toxins, 14(12), 853. https://doi.org/10.3390/toxins14120853.

22) Al-Rubaye, H., Atiyah, A. J., & Huang, S. . (2023). Comparative Study of Aflatoxin M1 Carry-Over from Feed to Raw Milk in Cow, Buffalo, Sheep, and Goats in Different Areas of Baghdad Province. The Iraqi Journal of Veterinary Medicine, 47(2), 88-93. https://doi.org/10.30539/8svc0t05.

23) Ashraf, W., Rehman, A., Ahmad, M. U., Rabbani, M., Mushtaq, M. H., Aamir, K., Akhtar, F., & Wang, J. S. (2023). Assessment of aflatoxin M1 exposure and associated determinants in children from Lahore, Pakistan. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & riskassessment,40(1),121–133. https://doi.org/10.1080/19440049.2022.2138559.

24) Zhang, C., Qu, Z., Hou, J., & Yao, Y. (2024). Contamination and Control of Mycotoxins in Grain and Oil Crops. Microorganisms, 12(3), 567. https://doi.org/10.3390/microorganisms12030567.

25) Kafle, S., Paudel, M., Shrestha, C., Kathayat, K. B., Sapkota, R. C., Tiwari, A., & Subedi, D. (2024). Aflatoxin M1 Contamination in Dairy Milk in Kathmandu,Nepal.Toxins,16(11),468. https://doi.org/10.3390/toxins16110468.

26) Buzás, H., Szabó-Sárvári, L. C., Szabó, K., Nagy-Kovács, K., Bukovics, S., Süle, J., Szafner, G., Hucker, A., Kocsis, R., & Kovács, A. J. (2023). Aflatoxin M1 detection in raw milk and drinking milk in Hungary by ELISA: A one-year survey. Journal of Food Composition and Analysis, 118, 105178. https://doi.org/10.1016/j.jfca.2023.105178.

27) Hasoňová, L., Samková, E., Baldíková, E., Reindl, K., Nejeschlebová, H., Janoušek Honesová, S., & Hanuš, O. (2025). Occurrence and seasonal variation of aflatoxin M1 in raw cow milk in the Czech Republic, Central Europe.FoodControl,*169*,111028. https://doi.org/10.1016/j.foodcont.2024.111028.

28) Shabeer, S., Asad, S., Jamal, A., & Ali, A. (2022). Aflatoxin Contamination, Its Impact and Management Strategies: An Updated Review. Toxins, 14(5), 307. https://doi.org/10.3390/toxins14050307.

29) Gemede, H. F. (2025). Toxicity, Mitigation, and Chemical Analysis of Aflatoxins and Other Toxic Metabolites Produced by Aspergillus: A ComprehensiveReview.Toxins,17(7),331. https://doi.org/10.3390/toxins17070331.