A Survey on Mycological and Aflatoxin B1 Contamination in Wheat and Maize Harvested during the 2022 Season in Albania

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Article

A Survey on Mycological and Aflatoxin B1 Contamination in Wheat and Maize Harvested during the 2022 Season in Albania

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Abstract: Today, given the paramount importance of food safety to human health, mycotoxins are considered especially important contaminants of foodstuffs. Among the mycotoxins, aflatoxins are potent food contaminants which are present in maize and wheat. Invasions of toxigenic fungi in the field and during storage are directly affected by climate and other environmental factors. A total of 129 samples of maize and grain harvested in 2022 were surveyed for mycological contamination. Aflatoxin B1 contamination was investigated using the enzyme-linked immunosorbent assay (ELISA) method. Our study revealed the presence of fungi from five genera: *Aspergillus, Fusarium, Penicillium, Alternaria*, and *Cladosporium*. We also found that maize was more frequently contaminated than wheat. Fungi of the *Penicillium* genus were the most abundant (77.89%), followed by *Fusarium* (74.73%) and *Aspergillus* (72.63%). Samples originating from the Korça region (10^4 units) exhibited a higher fungal load on the three genera. AFB1 contamination is a more critical issue in maize than wheat. The incidence of AFB1 in maize was 88.23%, with a maximum concentration of 69.12 μg/kg; in contrast, the incidence of AFB1 in wheat was only 4.91%. In wheat, no samples had AFB1 concentrations above the EU Maximum Residue Level (MRL) of 2 μg/kg; in maize, 41.18% of samples exceeded the AFB1 MRL of 5 μg/kg for human food, and 32.25% exceeded the MRL for animal feed. These findings, together with other research results from the last decade, suggest that relevant information should be made available to farmers, and good agriculture practices encouraged, as a matter of priority.

Keywords: maize; wheat; mycotoxigenic fungi; AFB1; ELISA; Albania; southeastern Europe

1. Introduction

Trade globalization has highlighted the importance—for both human and animal health—of safety issues relating to food and feed products [1]. The monitoring of food contaminants and the implementation of safety standards are essential tasks which are now carried out worldwide. However, differences between developed and developing countries with regard to food safety indicate that consumers in developing countries face a greater risk of exposure to food contaminants [2,3]. Among food contaminants, mycotoxins are considered especially important. Natural secondary metabolites produced by certain fungi widely affect food and feed commodities. The most frequently detected mycotoxins are aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), fumonisins (FBs), and deoxynivalenol (DON). The primary mycotoxin-producing fungi belong to the *Aspergillus, Fusarium,* and *Penicillium* genera [4].

Among the hundreds of identified mycotoxins, aflatoxins are a family of compounds structurally related to the substituted difuranocoumarins [5]. In the past, when aflatoxins were found

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only in tropical regions with high levels of temperature and humidity, they represented a threat to food safety at a regional level only; however, because of trade globalization, aflatoxin contamination is today a global health problem which affects food commodities consumed by both humans and animals [6–8]. According to the Rapid Alert System for Food and Feed, between 2011 and 2021, aflatoxins were involved in 95% of notifications and border rejections [9]. Out of a total of 20 aflatoxins so far described, B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) are known to be the most potent toxic compounds, and thus pose the most serious threat to health [10]. The main aflatoxigenic-producing species, *Aspergillus flavus* and *A. parasiticus* are found in primary host cultures of rice, peanuts (groundnuts), oilseeds, wheat, rice, soybeans, cotton, and wheat; as a result, both species are prevalent in tropical and subtropical regions [11]. However, these species are also known to contaminate milk, cheese, and other dairy products [12]. Environmental factors such as temperature, humidity, storage conditions, water activity, concurrent mycobiota, and physical damage all affect the degree to which mycotoxin contamination affects grain commodities [13,14].

Fungal contamination can occur from pre- to post-harvest stages. In stored grain, the incidence and prevalence of mycotoxigenic fungi is influenced by the type and condition of the grain involved, as well as environmental and biological factors. Temperature and water activity (a_w) are the main environmental factors influencing levels of fungi and mycotoxins in stored grain [15]. In maize, strategies for preventing mycotoxin contamination in food- and feed-chains are based on applying the hazard analysis and critical control points (HACCP) approach [16]. Aflatoxin mitigation in the post-harvest stage includes physical methods such as sorting, dehulling, steeping, wet milling, dry milling, heat treatment, and irradiation; chemical methods are based on intervention with chemical agents, e.g., adsorbents, acids, and bases; microbiological methods involve intervention with microbiological agents; finally, genetic engineering methods are based on the regulation mechanism of AF biosynthesis in *A. flavus* strain [7,17].

Aflatoxins are potent liver toxins, immunosuppressants, carcinogens, and mutagens, and they can cause serious public health problems [18]. The World Health Organization (WHO) has classified AFB1 as a Group 1 carcinogenic toxin that can cause human hepatocellular carcinoma (HCC); however, aflatoxin accumulation has also been reported in vital organs such as the kidney, lung, heart, and brain [11,19,20]. The aflatoxins exhibit different strengths of toxicity, carcinogenicity, and mutagenicity, according to the order B1 > G1 > B2 > G2, indicating the importance of chemical-structure specificity to AFB1 and AFG1 [10,21].

The security of staple commodities is expected to be impacted by climate change. Increases in global CO_2 emissions, temperatures, and drought episodes in different regions of Europe have affected crop yields and levels of aflatoxin contamination [22]. In this regard, the Mediterranean basin is expected to be impacted especially seriously, with likely negative effects on food production and an increased risk of AF contamination, especially in maize [23].

Grain cultivation has long been one of the most important agricultural activities in Albania, especially the production of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.). When the communist system collapsed in the 1990s, the administration of arable land underwent a drastic process of change [24]. During this period, state cooperative farms which were capable of implementing good agricultural practices and ensuring food safety on arable land were replaced by smaller family farms. This period was characterized by a decrease in agricultural rentability and an increased level of concern to emerging contaminants in the food-production process. In this study, we sought to investigate the extent of mycotoxin contamination in maize and wheat produced in Albania.

2. Materials and Methods

2.1. Sample collection

Maize (68) and wheat (61) samples were collected during the 2022 harvest period from farms in different regions of Albania: Durrësi, Elbasan, Fieri, Kavaja, and Korça. The first four of these regions are situated in the western part of the country along the Adriatic Sea and are characterized by a

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typical Mediterranean climate. In contrast, a typical continental climate characterizes the Korça region, which is located in the eastern part at a high altitude of 850 m above sea level. Sampling procedures were applied according to EU regulation 2023/915 and submitted to the Laboratory of Toxic Substances and Biomolecules of the Department of Chemistry, in the Faculty of Natural Sciences (FNS). Samples were kept in the dark, under low-humidity, cold-temperature (4°C) conditions until mycological and analytical analysis was finalized.

2.2. Mycological analysis

Isolation and identification of moulds and yeast was carried out by applying the Verband Deutscher Landëirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) procedures [25]. A 20 g quantity of ground sample was added to 180 ml of peptone/water (0.5%). After homogenization, the mixture was diluted to final concentrations of 10^{-2}, 10^{-3}, and 10^{-4}. Aliquots of 1 ml from each dilution were then spread on parallel plates on a solid medium surface composed of deionized water (1000 mL), malt extract (40 g), agar (12 g), yeast extract (2 g), glucose (2 g), Marlophen 810 (1 mL), oxytetracycline (60 mg), and Bengal rose (60 mg). Inoculated Petri dishes were incubated for three days at 25°C, placed in a dark and standard atmosphere, and stored at room temperature for another 2–3 days. Finally, colonies were counted, and the results were expressed as a mean of the colony-forming unit in thousands per gram of sample (10^3 CFU/g) using the following formula:

$$N=(\sum C)/(V\times n\times d)$$

where N = number of colony-forming units per gram of sample (CFU/g); ΣC = sum of all colonies of the count plate; V = volume of the dilution pipetted in the count plate in mL; n = number of count plates that could be evaluated; and d = dilution factor.

Taxonomic fungal genera identification was obtained through a microscope [25].

2.3. Aflatoxin B1 analysis

A grain amount of 1000 g was milled using Laboratory Mill (Perten Lab Mill 120); an additional 100 g of flour was then taken, placed in a plastic jar, and stored in a dark and dry place at a temperature of 4°C. The preparation of wheat and maize samples and the determination of AFB1 using the ELISA method were carried out according to manufacturer's instructions.

According to the described procedure, the ELISA screening method for aflatoxin B1 was conducted using MaxSignal® Aflatoxin B1 ELISA Kit (Catalogue #FOOD-1055-04, PerkinElmer, Waltham, MA, United States). In brief, 5.0 g of milled sample was introduced into a 50 mL conical test tube, adding 25.0 ml of 70% MeOH. The mixture was shaken for 10 min using a multi-tube vortex. The solution was then centrifuged at 2000 x g for 10 min at room temperature. Subsequently, 300 µl of the obtained supernatant was transferred to a 2 ml tube containing 900 µl of solution C, prepared previously, by mixing 100% MeOH with 1x sample extraction buffer, ratio 7:23 v/v, respectively. The sample was vortexed manually for 1 min at maximum speed. Finally, 50µL of diluted supernatant was passed into the well for the test assay.

The parameters were validated using the official European procedures for immuno-enzymatic orientation methods. All values were calculated and expressed in line with the recommendations set out in the European Commission Decision 2002/657 [26]. The method was validated using certified reference material (CRM) from Sigma-Aldrich (A6636).

2.3.1. Enzyme-linked immunosorbent assay procedure

The method uses a competitive, one-step colorimetric enzyme-linked immunosorbent assay. The plate wells have been coated with analyte-specific antibodies. During the analysis, samples with the analyte-conjugated horseradish peroxidase (HRP) are added to the plate wells. Suppose the target analyte is present in the analyzing sample. In that case, it will compete with the antibody by preventing the conjugated HRP from binding with the antibody-coated plate well. After adding the Tetramethylbenzidine (TMB) substrate, the resulting color intensity has an inverse relationship to the target analyte concentration in the sample.

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Microtiter wells were inserted into the microwell holder in sufficient numbers for all standards and specimens. Next, 50 µL amounts of each AFB1 standard solution (0.1, 1, 5, 10, 20, 50, 100 µg/kg) were duplicated to different wells, from lowest to highest concentration levels. Fifty microliters of sample solution were dispersed in duplicate into the remaining plate wells. Next, 100 µL of aflatoxin-HRP Conjugate was added to each well, and the solutions were gently mixed on the plate shaker for 1 min. The cells were then incubated for 30 minutes at controlled room temperature (20–25ºC) in the dark space. Next, the plate was washed thrice with 250 µL of 1X Wash Solution. The plate was then inverted and gently dried. In the next stage, a solution containing 100 µL of TMB substrate was dispersed into each well, followed by incubation at room temperature for 15 min in the dark. A cover was used for the microtiter plate in this step. Finally, 100 µL of Stop Solution was mixed to each well to terminate the substrate reaction. The absorbance was measured at 450 nm using a TECAN reader (Infinite 200 Pro, Nanoquant, Austria).

2.3.2. AFB1 quantification

The results were evaluated using the MagellanTM computer program developed by TECAN, which is compatible with the Infinite 200 Pro microplate reader. The values plotted on the calibration curve were multiplied by a dilution factor of 10. Following the manufacturer's instructions, the LOD for the milk matrix was 0.005 µg kg^{-1}. The analytical quality of ELISA was confirmed using the Certified Reference Material (Sigma Aldrich no. 0476983-7). The validation parameters were calculated and expressed in line with Commission Regulation (EC) No 401/2006 for screening methods [27].

3. Results

3.1. Mycological contamination

Fungal growth and mycotoxin synthesis result from the complex interactions of environmental and biological factors in pre-and post-harvest periods. In the pre-harvest phase, the main determining factors are high temperatures, water stresses, and insect damage; in the post-harvest phase, temperature and water activity are the main determining factors [15,17].

Table 1. The mycological contamination of wheat and maize samples.

Our results indicate that collected wheat and maize samples manifested molds belonging to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, and *Cladosporium* (Table 1). The presence of yeast was also evident. The studied samples indicated a similar distribution of mold infection patterns for the three main genera: *Penicillium* spp. (77.89%), *Fusarium* spp. (74.73%), and *Aspergillus* spp. (72.63%). However, for the genera *Alternaria* and *Cladosporium*, contamination was recorded in only 13.68% of samples in each case, indicating a very different result, compared with the data for the three main genera mentioned previously.

Concerning the two regions used in our study, we found that the incidence of different genera was nonuniform. In the western part of Albania, characterized by low-altitude geography and a Mediterranean climate, all five genera were observed. However, in grain samples from the Korça plain, which is located in the Albanian interior is characterized by high altitude and typical continental climate, the presence of *Cladosporium* was not encountered.

Wheat samples from western regions manifested similar contamination patterns (approx. 10^3 cfu/g), regardless of sampling sites or mold genera.

With respect to maize contamination, our results indicated a highest-overall incidence for *Penicillium* sp. In terms of regions, the highest level of contamination was recorded for *Penicillium* sp. in maize samples from the Korça region ($500x10^4$ cfu/g), followed by *Fusarium* sp. in samples from the Fieri region ($100x10^4$ cfu/g) and the Korça region ($80x10^4$ cfu/g). The third-most prevalent mold belonged to the *Aspergillus* genera, whose highest incidence was in the Elbasan region ($26x10^4$ cfu/g), with contamination levels of 20–$24x10^4$ cfu/g in the regions of Fier, Korça, and Durrës.

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With respect to wheat contamination, we found greatest incidence for *Fusarium*, *Aspergillus*, and *Penicillium* genera, with the highest values recorded for the *Aspergillus* genera (34x10^3 cfu/g), followed by *Penicillium* (10x10^3 cfu/g). In terms of regions, the highest contamination levels were found in wheat samples from the Fieri region. *Alternaria* genera were also present in samples from this region at a level of 1 x10^3 cfu/g. However, wheat samples from the Korça region exhibited a different mold-contamination pattern, with highest counts recorded for the *Fusarium* genera (5x10^2 cfu/g).

3.2. Aflatoxin B1 contamination

AFB1 levels were analyzed in samples of both wheat (61) and maize (68) during the 2022 harvesting season (Table 2). We found that maize samples were more likely to be contaminated than wheat samples; indeed, the incidence of AFB1 in maize (88.23%) was considerably higher than in wheat (4.91%).

Table 2. Aflatoxin B1 in maize and wheat from harvesting season of 2022.

In maize, AFB1 levels varied in a range of 0.390–69.122 μg/kg; only eight samples were not contaminated (11.77%). Because European Regulation 2023/915 states that the maximum residue level (MRL) for AFB1 is 5 μg/kg, we determined that a total of 28, or 41.18%, of the maize samples in the present study, had levels of AFB1 above the MRL, indicating a high degree of risk to consumers were such maize to be consumed as food. In addition, when we considered total aflatoxin exposure with no other aflatoxin homologs, we found that 26 out of 68, or 38.23%, of the analyzed maize samples had levels above the MRL (10 μg/kg).

Table 3. AFB1 intervals in maize and risk assessment in relation to MRL (5 μg/kg) of CE 2023/915 and feed MRL (20 μg/kg) of 2023/915L.

4. Discussion

4.1. Mycological contamination

Mycotoxins are considered one of the most serious contaminants of foodstuffs because of the risk of disease which they pose to both humans and animals. The issue of mycotoxin contamination is addressed in Commission Regulation (EC) No 2023/915, which sets out maximum levels for specific contaminants in food. Specifically, this regulation covers the most significant mycotoxins: AFB1, AFB2, AFG1, and AFG2; deoxynivalenol (DON); the fumonisins B1 (FB1) and FB2; zearalenone (ZEA); the T-2 and HT-2 toxins; and ochratoxin A (OTA) [28]. Two decades prior to this regulation, AFs were not even identified as a concern for primary production in Europe [22,23]. However, in the year 2003, the first alarming contamination of maize was reported in Italy [28].

With regard to specific crops, our findings showed that maize was contaminated at a higher rate compared to wheat. For the three main mold genera, *Aspergillus*, *Penicillium*, and *Fusarium*, a similar pattern distribution was exhibited in both crops. However, we found a different situation for the *Alternaria* and *Cladosporium* genera; in both cases, wheat samples were more likely to be affected than maize samples, with incidence percentages of 19.7%, and 2.90%, respectively.

4.2. AFB1 presence in maize and wheat

Climate change has introduced aflatoxigenic species and increased the incidence of AFB1 in crops grown in Europe, especially southern Europe [30]. Climate-change scenarios involving an increase in temperature of only 2°C suggest an increased probability of aflatoxin contamination—from low to medium—in European countries like France, Italy, and Romania where maize is expected to be cultivated [4,12]. One report on the incidence of AF in maize from the western Balkans found high levels of incidence and contamination during the harvesting season of 2013 [31]. The hot and dry conditions necessary for Aspergillus flavus infestation of maize mainly prevail in Europe at latitudes below 45° N [32]. As a country in southern Europe, Albania has faced climate modification

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in the last decades. Previous publications indicate that mycotoxin contamination in crops presents a critical food safety issue in the country [33,34]. The study of aflatoxin B1 in crops which may also be used as feed is especially important because feed contaminated with the AFB1 metabolite may result in the milk of lactating animals being contaminated with AFM1, another regulated mycotoxin; indeed, the presence of AFM1 in milk produced in Albania has already been reported in the literature [35]. Being toxic, carcinogenic, and immunosuppressive, aflatoxins can produce acute liver toxicoses, liver cancer, and growth impairment in children. Because of this, they are now the subject of ongoing monitoring and evaluation of the risk they pose to consumers worldwide [36].

Maize is used extensively as feed in poultry and cattle farming. In the present study, it was found that 32.35% of Albanian maize samples exceeded the MRL for the use of maize as animal feed (20 μg/kg). In contrast, just 2.1% of samples were found to exceed this level in a previous study of AFB1 in feed in Europe [37]. In addition, compared with data on the incidence of AFB1 from the years 2014 and 2015, we found that samples from 2023 exhibited a much lower level of incidence compared with 2014 (a mean value of 464 μg/kg), but a similar level compared with 2015 (a mean value of 55.7 μg/kg) [34]. This suggests that aflatoxin production may be dramatically influenced by annual climatic fluctuations.

In several regions, mycotoxin concentrations in maize have shown a pronounced year-to-year variation that could be explained by rainfall or temperature conditions during sensitive periods of grain development. Gruber-Dorninger and colleagues (2019) found that, globally, the incidence of AFB1 in maize was 24%. They also found that a large percentage (64%) of maize grains exhibited co-contamination involving two or more mycotoxins. The most frequently observed combinations were mixtures of fusarium toxins, e.g., a combination of deoxynivalenol zearalenone and fumonisins. Co-contaminations with fusarium and aspergillus toxins, e.g., fumonisins and aflatoxin B1, were also reported. In another study carried out in Serbia, an incidence level of 52.5% was reported for AFB1, with total concentrations of AFs in a range of 1–70.3 μg/kg [38],

In the present study, we analyzed wheat samples from three regions: Fieri, Elbasani, and Korça (Table 2). AFB1 was found in only three out of sixty-one samples, giving an incidence level of less than 5%. In these positive samples, concentrations ranged from 0.221-0.401 μg/kg, with a mean of 0.312 μg/kg, indicating that no sample exceeded the MRL (2 μg/kg) [39]. All the contaminated wheat samples originated from the Fieri region. Similar rates of contamination was found in a previous survey of Albanian wheat, with figures of 6.0% and 0.0% reported for the years 2014 and 2015, respectively [34].

Our data indicate a figure for AFB1 incidence in wheat, which lies in the same range as data previously reported in the literature. In a review paper published by Gruber-Dorninger and colleagues (2019), a worldwide AFB1 incidence of 10% was reported. Our data also indicate a level of contamination which is lower than the global referred median (1.0 μg/kg) or maximum (161 μg/kg) values. Compared with data from other countries in southeast Europe, we found a lower level of wheat contamination than that reported for Romania (45.4%) [40], but a similar level to that reported for Serbia [38], Croatia [28], and Italy [41]. Finally, AFB1 has been found to be more prevalent in southern Europe than in other European regions (28.9% compared to 5.9–17.0% positive samples) [37] and in China [42].

Considering different AFB1 contamination levels among maize and wheat samples, the climate conditions are the main factor in the pre-harvesting stage. The wheat commodity cropped in the Summer season, June-July, allowing the crop to reach a moisture level below 12%. In contrast, the maize harvesting stage during the Autumn season, September-October, depended on the climate, exposure to rains, ensuring proper conditions, a_w and increased temperatures, favouring the fungi growth.

This study presents mycological contamination on two main grains grown in the country while also giving their aflatoxin B1 contamination. Battilani and colleagues (2016), and Leggeri and colleagues (2021) have proposed prediction models on aflatoxin maize contamination for southern European countries like Italy. This study on aflatoxin contamination will help to develop prediction models for Albania. *Fusarium* sp., are common fungi in temperate regions, indicating fusarium toxins

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presence in the cereals. Further study needs to focus on giving evidence of co-contamination of corn grains with toxins produced from *Aspergillus*, *Fusarium*, and *Penicillium* genera.

The globalization of trade in food commodities has indirectly increased the possibility that consumers in developing countries will be exposed to mycotoxins, with consequent impact on health and quality of life. Ongoing monitoring of exported food products will ensure that products which do not comply with global food-quality standards will be distributed in local markets. Developing countries still need better monitoring and safety standards [43].

5. Conclusions

Maize and wheat production in Albania has a long tradition. After the communist system collapsed, drastic changes in the administration of arable agricultural land took place, during this period, state-cooperative farms capable of implementing good agricultural practices and ensuring food safety on arable land were replaced by smaller family farms. Although microbiological load does not always indicate mycotoxin contamination, interventions relating to good agricultural practices or selecting resistant cultivars should be considered as options for improving the current situation. In the case of contamination with *Aspergillus* sp., any intervention must be carried out in the field, and subsequent conditions for adequately storing the raw material must be evaluated and continuously controlled.

The data obtained in the present study indicate a low incidence of AFB1 contamination in the wheat commodity; only three out of sixty-one wheat samples were found to have levels of AFB1 contamination above MRL. However, there is a serious risk of AFB1 exposure for both humans and animals in the case of maize, due to its usage in both food and feed products. The concentration levels in the present study may not be considered excessive; however, incidence rates were high, exceeding MRLs for food and feeds.

Future incorporation of DNA techniques for identifying toxigenic versus non-toxigenic Aspergillus species and other molds from genera identified in the study may help farmers implement practices to decrease the incidence of mycotoxin contamination.

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Research involving plants statement: The authors confirm that Experimental research on plants was carried out in accordance with relevant institutional, national, and international guidelines and legislation in the method section. The appropriate permission for the collection of seed specimens was issued by NASRI (National Agency for Scientific Research and Innovation). Authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora..

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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