Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 8, No. 5, 2407-2415
2024
Publisher: Learning Gate
DOI: 10.55214/25768484.v8i5.2007

A survey of natural mycological and AFB1 contamination in wheat and maize during the 2022 harvesting season in Albania

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Abstract: Mycotoxins are considered critical contaminants in food and feed, and aflatoxins are the most potent and ubiquitous in maize and wheat. Invasion of toxigenic fungi is manifested in the field and during storage and is directly related to climate and other ecological factors. Inves-tigation of maize and wheat commodity contamination with Aflatoxin B (AFB1) in Albania was conducted during the harvesting seasons of 2022. AFB1 contamination was investigated using the enzyme-linked immunosorbent assay method. Mycological contamination was stud-ied, and fungi from four genera, Aspergillus, Fusarium, Penicillium, and Alternaria, were re-vealed, with maize 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%). AFB1 contamination is a more critical issue in maize than wheat. The incidence of AFB1 in maize was 88.2%, with a maximum concentration of 69.12 mg/kg; in contrast, AFB1 in wheat was only 4.9%. No samples in wheat had AFB1 concentrations above the EU MRL of 2 µg/kg; in maize, 41.1% of samples exceeded the MRL of 5 µg/kg intended for human con-sumption, and 32.2% exceeded the MRL intended for animal feed. The drastic changes in the administration of arable land from state farms of enormous size to smaller family farms after the '90s have influenced agricultural practices. These findings and climate factors impose that further steps to focus on this issue must be taken by relevant actors to help farmers and in-crease consumer safety.

Keywords: *AFB1, Albania, ELISA, Maize, Mycotoxigenic fungi, Southern Europe, Wheat.*

1. Introduction

Trade globalization has highlighted the importance—for both human and animal health—of safety issues relating to food and feed products [1]. Monitoring food contaminants and implementing safety standards are essential tasks now carried out worldwide. However, differences between developed and developing countries regarding food safety indicate that consumers in developing countries face a greater risk of exposure to food contaminants [2,3]. Among the 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 genera Aspergillus, Fusarium, Penicillium, and Claviceps [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 only in tropical regions with elevated 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

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described, AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are known to be the most potent toxic compounds and thus pose the most severe threat to health [10]. The main aflatoxigenic-producing species, *Aspergillus flavus* and *A. parasiticus*, present in primary host cultures of rice, peanuts, 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 microbiota, 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 are influenced by the type and condition of the grain and environmental and biological factors. Temperature and water activity (aw) are the main ecological 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* [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].

Climate change is predicted to impact staple commodities' security worldwide significantly. 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 adverse effects on food production and an increased risk of AF contamination, especially in maize [23].

Grain cultivation has long been one of Albania's most important agricultural activities, 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 change process [24]. During this period, small family farms replaced the state cooperative farms, which could implement good agricultural practices and ensure food safety on arable land. This period was characterized by a decrease in agrarian rentability and an increased level of concern concerning 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 grain (61) samples were collected during the 2022 harvest period from farms in different regions of Albania, covering western regions: Durrësi, Elbasan, Fieri, and Kavaja and the Eastern region of 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 typical Mediterranean climate. In contrast, a typical continental climate characterizes the Korça region, located in the eastern part of Albania at a high altitude of 850 m above sea level. Sampling procedures were applied according to EU regulation 2023/915. 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

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 8, No. 5: 2407-2415, 2024
DOI: 10.55214/25768484.v8i5.2007

Isolation and identification of molds 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, colony counting 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 sample (CFU/g);
ΣC = sum of all colonies of the count plate;
V = volume of the dilution pipetted in mL;
n = number of count plates; and
d = dilution factor.

Taxonomic fungal genera was identified visually, employing a magnifying glass where applicable. The closest characterizations were obtained using an optical microscope [25].

2.3. Aflatoxin B1 Analysis

A grain amount of 1000 g was milled using a 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 4°C. The preparation of wheat and maize samples and the determination of AFB1 using the ELISA method were carried out according to the manufacturer's instructions.

The ELISA screening method for aflatoxin B1 was conducted using AFB1 test kits (Catalogue Reference #: 1060-09, PerkinElmer, MA, USA) according to the described procedure. In brief, 5 g of milled sample was introduced into a 50 mL test tube, to which 25 ml of 70% EtOH was added. The mixture was shaken for 20 min at room temperature. The solution was then centrifuged at 2000 g/min per 10 min. Finally, 1 mL of the obtained supernatant was diluted with 1 ml of ultrapure water. 50 µL 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 in the European Commission Decision 2002/657 [26]. The method was validated using reference material from the SIGMA company (no. 0476983-7).

2.3.1. Enzyme-Linked Immunosorbent Assay (ELISA) Procedure

Microtiter wells were inserted into the microwell holder in sufficient numbers for all standards and specimens. Next, 50 µL amounts of AFB1 standard solution were added in duplicate to different wells, from lowest to highest levels of concentration. Fifty microliters of samples were dispersed in duplicate into different wells. Next, 100 µL of antibody #1 was added to each well, and the solutions were gently manually mixed on the plate for 1 min. The cells were then incubated for 30 minutes at room temperature (20–25°C) in the dark. Next, the plate was washed thrice with 250 µL of 1 wash solution. The plate was then inverted and gently dried. In the next stage, a solution containing 150 µL of antibody #2 was dispersed into each well, followed by incubation at room temperature for 30 min in the dark. A cover was used for the microtiter plate in this step. After incubation, the plate was washed three times with the washing solution, as described for the first washing procedure. Following this, 100 µL of the tetramethylbenzidine substrate was added and incubated at 20°C for 15 min. Finally, 100 µL of stop buffer (1 N H_2SO_4) was added to each well and mixed to terminate the enzyme reaction. The absorbance was measured at 450 nm using a TECAN reader (Infinite 200 Pro, Nanoquant, Austria).

2.3.2. AFB1 Quantification

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 8, No. 5: 2407-2415, 2024
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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. 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 elevated temperatures, water stresses, and insect damage; in the post-harvest phase, temperature and water activity are the main determining factors [15, 17].

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

3.2. Aflatoxin B1 Contamination

AFB1 levels were analyzed in samples of both wheat (61) and maize (68) during the 2022 harvesting season (Table 1). We found that maize samples were more likely to be contaminated than wheat samples; indeed, the incidence of AFB1 in maize (88.23%) was higher than in wheat (4.91%).

Table 1.
Aflatoxin B1 in maize and wheat from harvesting season of 2022.

	Maize	**Wheat**
Analyzed samples	68	61
Positive samples	60	3
Incidence (%)	88.2	4.9
Mean value (μg kg^{-1})	17.26	0.31
Median value (μg kg^{-1})	0.91	0.31
Minimum value (μg kg^{-1})	0.39	0.221
Maximum value (μg kg^{-1})	69.12	0.40

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Table 2.
The mycological contamination of wheat and maize samples.

Number of samples	Wheat			Maize			Total
	61			68			129
Genus	**Incidence[a] (Percentage, %)**	**Mean/Median[b]**	**Max. level (x 10^3 cfu/g)[c]**	**Incidence (Percentage, %)**	**Mean/ Median**	**Max. level (x 10^3 cfu/g)**	**Incidence, percentage (%)[d]**
Aspergillus	43 (70.5%)	7.43/2.00	34.00	52 (76.5%)	464.2/220.0	1750.0	95 (72.63%)
Penicillium	43 (70.5%)	50.83/6.50	240.00	62 (91.2%)	1078.38/475.0	5000.0	105 (77.89%)
Fusarium	43 (70.5%)	9.79/1.00	60.00	56 (82.4%)	363.32/100.0	1000.0	99 (74.73%)
Alternaria	12 (19.7%)	0.75/0.75	1.00	2 (2.90%)	100/100	100	14 (13.68%)

Note: [a] Number of positive samples.
[b] Arithmetic mean/median of positive samples (x 10^3 cfu/g).
[c] Maximum level detected (x 10^3 cfu/g).
[d] Total number of positive samples referring to a specific genus.

Edelweiss Applied Science and Technology
ISSN: 2576-8484
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In maize, AFB1 levels varied in a range of 0.39–69.12 µg/kg; only eight samples were not contaminated (11.77%). Because European Regulation 2023/915 states that the MRL value for AFB1 is 5 µg/kg, we determined that a total of 28, or 41.2%, 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 38.2%, of the analyzed maize samples had levels above the MRL (10 µg/kg).

Table 3.
AFB1 intervals in maize and risk assessment concerning food MRL (5 µg/kg) and feed MRL (20 µg/kg).

Interval (mg/kg)	Number of samples	Incidence (%)
0	8	11.8
0–5	32	47.16
Over 5 (MRL food)	28	41.2
5–10	2	2.94
Over 10	26	38.2
Over 20 (MRL feed)	22	32.35
Maximum (µg kg^{-1})	69,12	

4. Discussion

4.1. Mycological Contamination

Mycotoxins are considered one of the most serious contaminants of foodstuffs because of their risk of disease to 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 before this regulation, AFs were not even identified as a concern for primary production in Europe [22, 23]. However, in 2003, the first alarming contamination of maize was reported in Italy [28].

Regarding specific crops, our findings showed that maize was contaminated at a higher rate than wheat. A similar pattern distribution was exhibited in both crops for the three main mold genera: Aspergillus, Penicillium, and Fusarium. However, we found a different situation for the Alternaria genera; wheat samples were more likely to be affected in both cases than maize samples, with incidence percentages of 19.7% and 2.9%, respectively.

Concerning the two regions used in our study, we found that the incidence of different genera was nonuniformly observed. All five genera were observed in the western part of Albania, characterized by low-altitude geography and a Mediterranean climate. However, grain samples from the Korça plain, located in the inland Albanian territories, is characterized by high altitude and typical continental climate,

Wheat samples from western regions manifested similar contamination patterns (approx. 103 cfu/g), regardless of sampling sites or mold genera.

To maize contamination, our results indicated the 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 genus, 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.

Concerning wheat contamination, we found the most significant incidence for Fusarium, Aspergillus, and Penicillium genera, with the highest values recorded for the Aspergillus genus ($34x10^3$ cfu/g), followed by Penicillium ($10x10^3$ cfu/g). Regarding regions, the highest contamination levels were found in wheat samples from the Fieri region. The Alternaria genus was 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

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 8, No. 5: 2407-2415, 2024
DOI: 10.55214/25768484.v8i5.2007

different mold-contamination pattern, with the highest counts recorded for the Fusarium genus ($5x10^2$ cfu/g).

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 in 2013 [31]. The hot and dry conditions necessary for Aspergillus flavus infestation of maize prevail in Europe at latitudes below 45° N [32]. As a country in southern Europe, Albania has faced climate modification in the last decades. Previous publications indicate that mycotoxin contamination in crops presents a critical food safety issue in the country [33,34]. Studying aflatoxin B1 in crops that may also be used as feed is essential 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]. Due to their numerous adverse effects, 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 risks they pose to consumers worldwide [36].

Maize is used extensively for poultry and cattle farming. The present study 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 exceeded 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 annual climatic fluctuations may dramatically influence aflatoxin production.

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 sizable 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 were also reported with fusarium and aspergillus toxins, e.g., fumonisins and aflatoxin B1. 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 to 0.40 µg/kg, with a mean of 0.31 µg/kg, indicating that no sample exceeded the MRL (2 µg/kg) [39]. All the contaminated wheat samples originated from the Fieri region. Similar contamination rates were found in a previous survey of Albanian wheat, with figures of 6.0% and 0.0% reported for 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 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 [29, 41]. Finally, AFB1 is 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].

The globalization of trade in food commodities has indirectly increased the possibility that consumers in developing countries will be exposed to mycotoxins, which can consequently impact

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 8, No. 5: 2407-2415, 2024
DOI: 10.55214/25768484.v8i5.2007

health and quality of life. Ongoing monitoring of exported food products will ensure that products that comply with global food quality standards will be distributed in local markets. Developing countries still need better monitoring and safety standards [43].

5. Conclusions

Although microbiological load does not always indicate mycotoxin contamination, interventions relating to good agricultural practices or selecting resistant cultivars should be considered to improve 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 wheat compared with maize commodities of AFB1 contamination. However, their high consumption in the Albanians' diet and application as feed in animal husbandry impose a severe risk of AFB1 exposure for humans and animals, especially maize. The concentration levels in the present study may not be considered excessive; however, incidence rates were high, exceeding MRLs for food and feed products.

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