Monitoring of mycotoxins produced by *Fusarium* and *Aspergillus* spp. in feed materials in Bulgaria (2017−2019)

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Toxins produced by *Fusarium* and *Aspergillus* spp. have been recognized as major contaminants of feed materials. Their detection is very important for feed safety and animal nutrition into the conccept of global health. A complex study in Bulgaria for the presence of these toxins was performed on 221 representative samples. They were collected in the 2017-2019 period, including wheat (85 samples), barley (68 samples), maize (32 samples), and sunflower (36 samples). As diagnostic tool the biochemistry assay − Enzyme Linked Immunisorbent Assay method, was used. The contamination with fumonisins was determined in all of the analyzed samples. The highest fumonisin concentration of 4.82 mg/kg was detected in maize, followed by wheat of 4.67 mg/kg, barley -2.89 mg/kg and the lowest of 2.07 mg/kg was detected in sunflower samples. Our survey revealed that mycotoxins produced by *Fusarium* spp. are frequent contaminants of feed grains in Bulgaria. The highest detected concentration of aflatoxins was 14.49 µg/kg in sunflower samples, 11.55 µg/kg in barley, 10.40 µg/kg in wheat and 9.90 µg/kg in maize. The reported pollution levels of both toxins are lower than those referenced in the European regulations. Despite the low levels, the results show the need for more comprehensive research on these two mycotoxins in the feed chain, which is important for animal and human health. We examined for the first time the presence of the two toxins in a wide range of feed grains, in view of feed safety in Bulgaria for a period of three years.

Keywords: barley, ELISA, maize, mycotoxins, sunflower, wheat

INTRODUCTION

Molds produce secondary metabolites such as mycotoxins, pigments, plant regulators, which do not always have an obvious biological function [1]. Although over 300 different mycotoxins have been identified so far, those of most concern based on their toxicity and occurrence are the aflatoxins, ochratoxin A, zearalenone, deoxynivalenol, fumonisins and T-2 toxin, which cause significant health implications, mainly through food and feed contamination [2]. The presence of aflatoxins and fumonisins in feed materials depends on many factors some of which are weather and storage conditions. Aflatoxins are produced by *Aspergillus flavus* and *Aspergillus parasiticus*. They occur mainly after harvest during the grains storage.

Rabbits, turkeys, chickens, pigs, cows and goats are susceptible to aflatoxins contamination [3], and horses, pigs and rats are the most sensitive animals to fumonisins [4, 5]. Animals fed with food contaminated with aflatoxins can transmit the products of transformation of aflatoxins into eggs, milk, dairy products and meat [6]. Iqbal *et al.* [7] in their study demonstrated that contaminated poultry feed with aflatoxins has a high rate of contamination in chicken and eggs in Pakistan. Most of the cereal grains, oilseeds, tree nuts, and dehydrated fruits are susceptible to fungus contamination and mycotoxin formation.

The most important mycotoxigenic fungus associated with maize and other crops, which produces fumonisins is *Fusarium verticillioides* [8, 9]. In 1988, its chemical structure and biological activity were elucidated in South Africa [10]. Fumonisins are found to be relatively heat-stable [11] and they are also water-soluble.

Toxicological investigations on the fumonisins have focused mainly on fumonisin B1(FB1), the major fumonisin produced in nature. The occurrence of fumonisins in grains and grain products has been a world-wide problem. In wheat plants *Fusarium* infections can lead to diseases such as rot. In view of the difficulty in removing the mycotoxins, monitoring of grains during planting to harvest is important for the control of exposure to these toxins.

Fusarium spp. have an impact on the economy by causing lower grain yields and economic losses [12, 13].

Bulgaria is a country with a temperate-continental climate, which favors the formation of mycotoxins of *Fusarium* spp. and after harvesting under the storage conditions aflatoxins are very likely to be produced. The small number of monitoring programs was the reason to monitor the level of contamination of a wide range representative samples from all regions. It is very important to

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recommend the bussiness operators to carry out constant monitoring in protection of animal and human health.

EXPERIMENTAL

Materials and methods

Feed samples: Freshly harvested 221 representative corn samples collected from different regions of Bulgaria were analyzed: wheat (85 samples), barley (68 samples), maize (32 samples) and sunflower (36 samples).

Samples analyses: 5 g of ground samples were mixed and processed using 70% methanol (Valerus Ltd, Bulgaria) as solvent for extraction. The filtered (Whatman No 1, Merck) samples were screened for fumonisins and aflatoxins by the Enzyme-Linked Immunosorbent Assay (ELISA) method. A commercial kit (R-Biopharm, Germany) was used and the samples were prepared according to the instructions of the manufacturer. The measurement was made at 450 nm. The absorbance was inversely proportional to the fumonisins and aflatoxins concentrations in the sample. The values calculated for the standards were entered into the Ridawin program, Computer Systems (ELx800 Universal Microplate Reader, BIOTEK® Instruments, Inc., USA).

RESULTS AND DISCUSSION

The mycotoxins contamination became a more and more important segment of food chain safety. The research conducted for the period of tree years (2017-2019) allowed us to establish the mycotoxicological status of the evaluated feed materials against aflatoxins and fumonisins. Figure 1 sumarises the results for all samples and their contamination with fumonisins during the study period. The difference in mycotoxins contamination of collected crop samples per year is presented (Table 1).

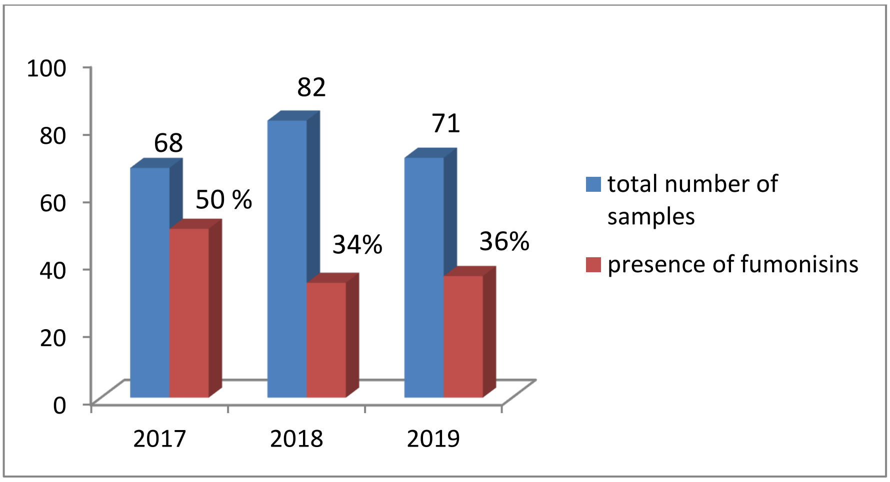

Fig. 1. Number of samples with detected presence of fumonisins in the period 2017-2019

Table 1. Estimated fumonisins contamination of feed materials in the period 2017-2019

Sample	Year	No	% of positive samples	Range (min-max) (mg/kg)	Mean (mg/kg)
Wheat	2017	26	38.5	0.283 - 3.49	1.94±1.19
	2018	34	38.2	0.233 - 4.67	2.1±1.34
	2019	25	28	0.285 - 0.971	0.5±0.21
Barley	2017	22	-	-	-
	2018	24	46	0.300 - 2.89	0.98±0.71
	2019	22	45	0.351-0.738	0.49±0.12
Maize	2017	10	60	1.06 - 4.82	2.60±1.2
	2018	12	33	1.70 - 2.06	1.76±0.25
	2019	10	40	0.380 - 0.520	0.44±0.05
Sunflower	2017	10	50	1.06 - 2.07	1.41±0.36
	2018	12	17	0.782 - 1.86	1.32±0.55
	2019	14	29	0.245 - 0.642	0.38±0.21

Fumonisins were found in all studied materials except of barley samples for 2017 crop and their quantity did not vary widely. This may be caused by the fact that contamination occurred after harvesting, so the *Fusarium* fungi did not have enough time or appropriate conditions to produce detectable amounts of toxins. Higher concentrations of fumonisins in maize were proved to be 4.82 mg/kg for 2017 crop, (Table 1). The maize sample with the found highest concentration of 4.82 mg/kg originates from the Northwestern region of Bulgaria. This is the highest quantity value of this mycotoxin for all investigated feed materials in the studied period. In another study performed by Rubert *et al.* [14] the highest value of fumonisins obtained in Spanish maize samples was 1.20 mg/kg, which is quite lower than this found by us. It could be because the climatic conditions and latitude are important factors for producing fumonisins in cereal grains.

The lowest mean fumonisin level in maize samples found by us was 0.44 mg/kg (Table 1). This value is close to the levels of 0.55 mg/kg reported by Solovey *et al.* [15] and higher than the 0.01 mg/kg found by Scudamore and Patel [16]. On the other side, the main values for maize samples (2017-2018) determined by us are similar to those reported by Vrabcheva *et al.* [17]. They performed the experiment with Bulgarian maize analysed by the ELISA method and found the presence of fumonisins with a mean value of 1.5 mg/kg, 1.8 mg/kg and 2.1 mg/kg while in the collected sunflower samples values close and in the range of 1.06-2.07 mg/kg for 2017 crop were shown (Table 1). Our results show that for the 2018 crop the highest fumonisins concentration is detected in wheat samples (4.67 mg/kg) while in other studied samples the values are lower. It should be mentioned that this trend was also observed in 2019 harvest.

It can be mentioned that for all studied feed materials the content of fumonisins is lower in the 2019 crop. The lowest concentration was found in maize samples (0.380-0.520 mg/kg), followed by sunflower (0.245-0.642 mg/kg), in barley samples (0.351-0.738 mg/kg) and in wheat samples (0.285-0.971 mg/kg) (Table 1).

Similar percent values for positive samples show maize 60% and sunflower 50%, 2017 harvest. The lowest numbers of 17% and 28% positive samples for the study period (2018-2019) were found in sunflower and wheat samples, respectively (Table 1). Mycotoxins produced by *Fusarium* spp. are a serious problem for feed grains and maize.

In another study conducted by Manova and Mladenova [18] on fumonisins contamination in maize in Bulgaria, a wide prevalence (94.7%) was found in the tested samples and only in one of the samples the contamination was found to exceed the maximum limits for untreated corn. In our survey we found that only one maize sample has fumonisins content of 4.82 mg/kg (Table 1). This value is very low compared to the maximum permitted level (60 mg/kg) as referred in EC Recommendation 576/2006 [19]. The presence of fumonisins in almost all of the tested samples could be attributed to the warmer and raining period during the harvest in the studied period. *Fusarium* toxins have been associated with the temperature at which the cultivation, harvest, and storage of cereals occur. As it is known, molds produced by *Fusarium* spp. are so-called "field" mycotoxins and they are formed during growth and harvest. These findings confirm the special importance of *Fusarium* spp. as major contaminants of cereals in areas with temperate continental climates, where our country falls. Our results confirm the opinion that *Fusarium* contamination is high, especially in the northern part of the country (Fig. 3).

Aflatoxins and fumonisins produced by the genera *Fusarium verticillioides* spp. and *Aspergillus flavus* spp. have the ability to infect fodder crops including maize, wheat and barley [20]. This was the reason why feed materials were tested for fumonisins and aflatoxins. Compared to fumonisins the presence of aflatoxins was not registered in all of the studied feed materials. It is evident from the results in Table 2. The highest concentrations are 14.49 µg/kg and 12.45 µg/kg and the highest mean levels are 10 µg/kg and 10.54 µg/kg, respectively. These values were detected in sunflower samples from 2017 and 2018 crops. And the lowest values were found mainly in 2017 crop for maize and wheat samples (1.10-2.73 µg/kg and 2.76-5.65 µg/kg, respectively) with mean levels of 2 µg/kg and 3.9 µg/kg (Table 2).

We found the presence of aflatoxins in all of the studied samples for 2019 crop. It has to be noted that it varies by regions and dominates in the South central part, (Fig. 3). Concerning aflatoxins contamination it should be mentioned that maize, sunflower and feed grains are favorable substrates for the development of aflatoxins. The maximum level of aflatoxins – 14.49 µg/kg found in sunflower samples is below 20 µg/kg as referred to in Ordinance № 10 of 3.04.2009 [21] for the maximum eligible concentrations of unused substances and products feed.

This sample originates from the northwest of the country, while the minimum level of 5.60 µg/kg is for a sample originating from the northeast. It should be mentioned that the mean values of fumonisins for the studied period are considerably lower than these of aflatoxins, which is not in correlation with the established high percent of molds contamination. In Figure 2 are shown the total number of representative samples and their contamination with aflatoxins in percent during the study period. Taking into account the warmer climate in Europe the role of molds of genus *Aspergillus* should not be underestimated which are potential producers of mycotoxins with acute toxic effects.

Figure 3 shows the distribution of both mycotoxins by regions.

Table 2. Estimated aflatoxins contamination of feed materials in the period 2017-2019

Sample	Year	No	% of positive samples	Range (min-max) (μg/kg)	Mean (μg/kg)
Wheat	2017	26	19	2.76-5.65	3.9±1.1
	2018	34	-	-	-
	2019	25	20	8.29-10.40	9.3±0.83
Barley	2017	22	-	-	-
	2018	24	-	-	-
	2019	22	18	2.18-11.55	7.23±3.38
Maize	2017	10	30	1.10-2.73	2±0.68
	2018	12			
	2019	10	20	7.94-9.90	8.92±1.11
Sunflower	2017	10	20	5.60-14.49	10±4.4
	2018	12	16.7	8.63-12.45	10.54±1.65
	2019	14	14.3	7.23-9.23	8.23±1.01

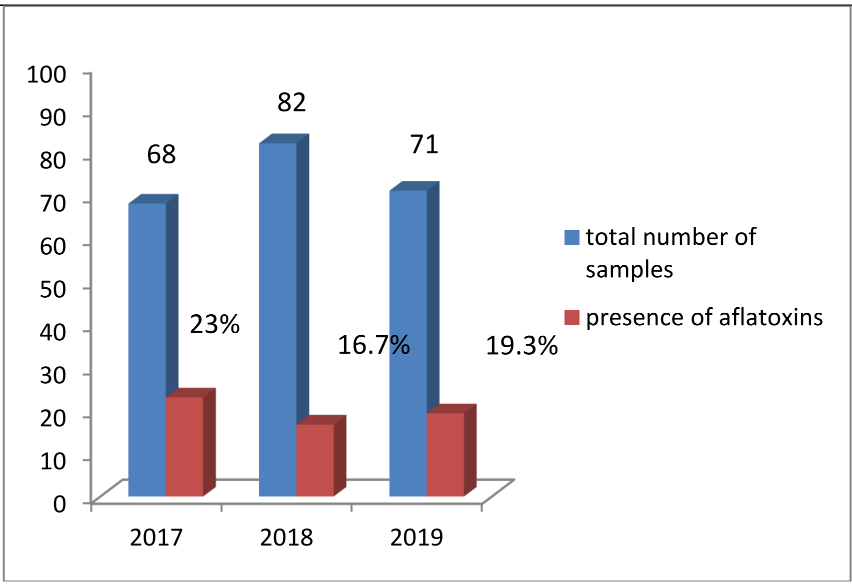

Fig. 2. Number of samples with detected presence of aflatoxins in the period 2017-2019

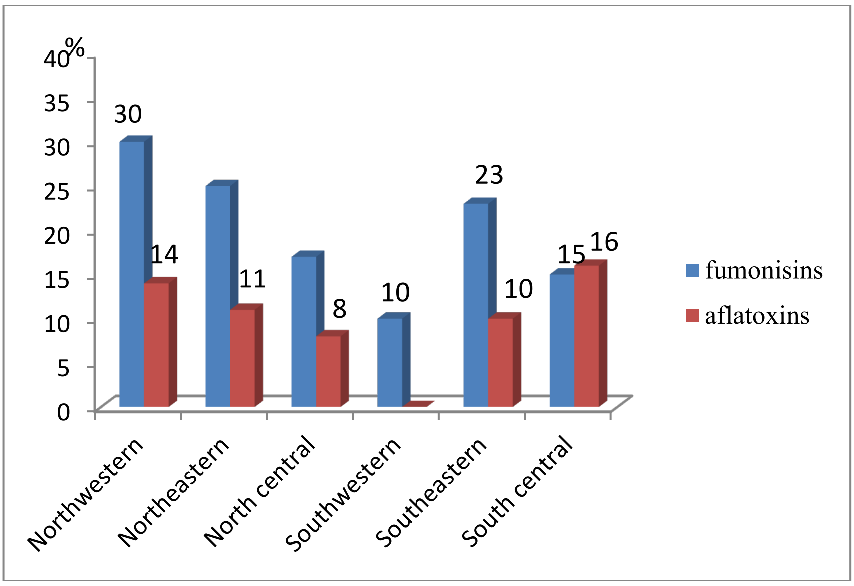

Fig. 3. Fumonisins and aflatoxins occurrence in feed materials and its distribution by regions during 2017-2019

As can be seen, fumonisins predominate. This observation can be explained by latitude and the climatic conditions. If the latitude of the location decreased, the fumonisin content could be increased. Keep in mind that fumonisins are field mycotoxins, climatic conditions could have an important influence in determining the prevalence of the *Fusarium* species.

CONCLUSION

Fumonisins and aflatoxins were found as natural contaminants for wheat, barley, maize and sunflower for the period 2017-2019 while in barley samples their presence was not found in 2017 crop. Maize produced in Northern Bulgaria is more contaminated with fumonisins than that in Southern Bulgaria. Aflatoxin contamination is twice less than that of fumonisins. The presence of fumonisins in feed materials is more widespread than that of aflatoxins in the country. No aflatoxins were detected in the studied representative samples in the south-western region.

Taking into account our results and the values referred in the EC regulation it could be mentioned that the analyzed range of feed grains can be used for animal nutrition because of low levels of contamination.

The contents of mycotoxins measured during the present study did not exceed the permissible levels recommended by the European Commission for animal feed. Analyzing the results according to European regulations, it can be concluded that contamination with aflatoxins and fumonisins does not pose a potential risk to the health and productivity of farm animals. Nonetheless, it is recommended that seeds be monitored continuously to protect against the risk of mycotoxin contamination for safer food and feed.

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