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MYCOBIOTA AND AFLATOXIN B_1 IN POULTRY FEEDS

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Original scientific paper

Abstract: In this study, a total of 30 poultry (chicken and laying hens) feed samples collected from different poultry farms in Serbia in 2016 were tested for fungal and aflatoxin contamination. Using the plate count and standard mycological methods, total fungal counts and potentially toxigenic fungal genera were determined. Natural occurrence of aflatoxin B_1 (AFB_1) was detected by ELISA (enzyme-linked immune sorbent assay) method.

The total fungal count was in the range from 1 x 10^2 (2 log CFU g^{-1}) to 1.83 x 10^5 CFU g^{-1} (5.26 log CFU g^{-1}). The majority of the chicken feeds (78.57%) had the total fungal count in the ranged from 1 x 10^2 to 4.8 x 10^4 CFU g^{-1}, whereas in 68.75% of the laying hens feeds it was ranged from 5.3 x 10^4 to 1.83 x 10^5 CFU g^{-1}. In 21.43% of the chicken feeds fungal contamination reached the level above the regulation limits. Three potentially toxigenic fungal genera, *Aspergillus*, *Fusarium,* and *Penicillium*, have been identified. In the tested poultry feed samples, more samples contaminated with *Aspergillus* were determined compared to samples contaminated by *Fusarium* and *Penicillium* species. The AFB_1 was detected in concentrations from 1.34 to 18.29 μg kg^{-1}, with an average of 4.47 and 4.56 μg kg^{-1} in the chicken and laying hens feed samples, respectively. In 14.29% of the chicken feeds, the level of AFB_1 was above the regulation limits.

The obtained results confirmed the importance of continuous mycological and mycotoxicological control of poultry feed, as well as need to improve risk assessments of such contaminants along the food chain.

Key words: poultry feed, total fungal count, aflatoxin B_1

Introduction

The majority of cereals (maize, wheat, barley, ray and oats) commonly used as poultry feed may be contaminated with toxigenic fungi, mainly from genera *Aspergillus*, *Fusarium* and *Penicillium* which may produce poisonous secondary metabolites called mycotoxins. Thus mycotoxins can easily enter food chain via meat and meat products produced of animals fed with mycotoxin contaminated feed. Primarily the cereals contamination may appear in the field, where fungal spores are spread by the wind, rain, mechanical injuries or insects to the crops (*Aliyu et al., 2016*). The infection process can be further continued during the grain storage, due to the effect of abiotic and biotic factors (*Krnjaja et al., 2015*).

Aflatoxins (AFs) and ochratoxins have been the most common contaminants of poultry feed. Cereal kernels are a very suitable substrate for the development of *Aspergillus* species (*Fareed et al., 2014*). *Aspergillus flavus* and *A. parasiticus* are the main producers of aflatoxins. Among the different types of aflatoxins (B_1, B_2, G_1, G_2 and M_1), aflatoxin B_1 (AFB_1) has been the most toxic (*Babu et al., 2014*). Consumption of poultry feed contaminated with AFs causes aflatoxicosis in animals and a severe economic losses in the poultry production. Aflatoxins have negative impact on important poultry production parameters such as feed intake, feed conversion, weight gain, etc. An immune response in poultry can also be reduced, which raises the risk to diseases (*Fareed et al., 2014*).

In order to avoid harmful effects of AFs on animal health, the European community set maximum permissible levels for AFB_1 to 20 μg kg^{-1} for complete and complementary poultry feed (except for young animals). The regulation limits for feeds of young animals have been set to 10 μg AFB_1 kg^{-1} (for complete feed) and 5 μg AFB_1 kg^{-1} (for complementary feed) (EC, 2003). In Serbia, according to the Regulation on the quality of feedstuffs (*Službeni Glasnik RS, 4/2010, 113/2012, 27/2014, 25/2015, 39/2016, 54/2017*), the maximum permissible levels in complete and complementary feeding stuffs have been set to 20 μg AFB_1 kg^{-1} for adult poultry, and 5 μg AFB_1 kg^{-1} for young poultry.

Since mycotoxins are inevitable contaminants of cereals as a main constituent of poultry feeds, the aim of this study was to determine the fungal contamination and aflatoxin presence in the samples of poultry feed collected from different farms in Serbia. These investigations are important in order to highlight the importance of quality control along the food and feed chains.

Materials and Methods

In this study, the mycological and mycotoxicological evaluation of 30 poultry feed samples (14 of chicken and 16 of laying hens feed) was performed. The group of the tested chicken feed samples was used for the feeding of the broilers and pullets. The samples were complete or complementary feed mixtures, collected from different poultry farms in Serbia in 2016. The samples of about 1 kg were stored for 2-3 days at 4°C, prior to analysis. The moisture content was determined using a laboratory moisture analyzer (OHAUS MB35, Parsippany, NJ, USA). The presence of fungal species was determined using the ISO 21527-2 method (2008).

Fungal species were identified according to fungal morphology and identification key of *Watanabe* (*2002*). The isolation frequency of potentially toxigenic fungi from genera *Aspergillus, Fusarium,* and *Penicillium* in the tested samples was calculated as the percentage of poultry feed samples contaminated with fungal species in relation to the total number of poultry feed samples.

The presence of aflatoxin B_1 (AFB_1) was detected by ELISA (enzyme-linked immune sorbent assay) method according to the manufacturer's instructions Celer Tecna® ELISA kits. The absorbance was determined at a wavelength of 450 nm on an ELISA plate reader spectrophotometer (Biotek EL x 800TM, Winooski, VT, USA). The lower and upper detection limits of AFB_1 were 1 μg kg^{-1} and 40 μg kg^{-1}, respectively.

The SPSS software (IBM, Statistic 20) was used for data comparison of the tested parameters. The significance levels were determined by t-test and Pearson correlation coefficient.

Results

Total fungal counts in the tested poultry samples are shown in Table 1. In Serbia, according to the Regulation on the quality of feedstuffs (*Službeni Glasnik RS, 4/2010, 113/2012, 27/2014, 25/2015, 39/2016, 54/2017*), the acceptable limits for fungal contamination in plant origin feed mixtures has been set to 200,000 CFU g^{-1} (for adult animals) and 50,000 CFU g^{-1} (for young animals). Following this regulation, in 21.43% (3/14) of the chicken feeds the values were exceeded the permitted limits (Table 1). The mean moisture contents were 10.57% and 10.62% in the chicken and laying hens feed samples, respectively.

Table 1. Level of fungal contamination of tested poultry feed samples

Values above and under regulations limits (CFU g^{-1})	Fungal counts		Fungal frequency (%)/Number of positive samples	
	Colony forming units per g of sample (CFU g^{-1})	log_{10}CFU g^{-1}	Chicken feed	Laying hens feed
> 200,000	0	-	0/0	0/0
> 50,000	$5.3 \times 10^4 – 1.83 \times 10^5$	4.72 – 5.26	21.43/3	68.75/11
< 50,000	$1 \times 10^2 – 4.8 \times 10^4$	2 – 4.68	78.57/11	31.25/5
		Number of total samples	14	16

A significantly higher fungal count was found in the laying hens feeds than in the chiken feeds (Table 2).

Table 2. Statistical analyses of total fungal counts (log_{10}CFU g^{-1}) in tested poultry feed samples

Types of feed	Mean (log_{10}CFU g^{-1} ± S.D.)	Minimum (log_{10}CFU g^{-1})	Maximum (log_{10}CFU g^{-1})
Chicken feed	4.18b ± 0.83	2	4.96
Laying hens feed	4.90a ± 0.26	4.51	5.26
Level of significance	**	-	-

CFU g^{-1}, colony forming units per g of sample; **significant at P<0.01

The occurrence of potentially toxigenic fungal species from the *Aspergillus* genus was more common in the laying hens feeds (93.75% positive samples) than in the chicken feeds (85.71% positive samples). On average, the most number of *Aspergillus* spp. contaminated samples (89.73%) were established, followed by *Fusarium* spp. (79.47%) and *Penicillium* spp. (34.38%) contaminated samples (Table 3).

Table 3. The frequency of contaminated poultry feed samples with potentially toxigenic fungi from genera *Aspergillus*, *Fusarium* and *Penicillium*

Fungal genus	The frequency of fungal contaminated samples (%)		
	Chicken feed	Laying hens feed	Average
Aspergillus	85.71	93.75	89.73
Fusarium	71.43	87.50	79.47
Penicillium	50.00	18.75	34.38

Table 4 shows the frequency, ranges and average concentrations of AFB_1 occurence in the tested poultry feed samples. A higher percentage of positive AFB_1 samples was detected in the laying hens feeds (100%) then in the chicken feeds (85.71%). Average concentrations of AFB_1 investigated in the chicken and laying hens feeds were 4.47 and 4.56 μg kg^{-1} , respectively (Table 4). The level of AFB_1 which was above the regulation limit (5μg kg^{-1}) was recorded in two chicken feed samples (14.29%), whereas in all the laying hens feed samples, the levels of AFB_1 were under the permissible limit (20 μg kg^{-1}).

Table 4. Level of AFB_1 in tested poultry feed samples

Item	Aflatoxin B_1 (AFB_1)	
	Chicken feed	Laying hens feed
Number of positive samples/Number of total samples	12/14	16/16
Frequency %	85.71	100
Range (μg kg^{-1})	1.79 – 16.01	1.34 – 18.29
Average concentration in positive samples (μg kg^{-1})	4.47	4.56

According to data analyses, there was no significant positive correlations between the total fungal counts and the moisture contents (r = 0.39) and between the total fungal counts and the levels of AFB_1 (r = 0.41), while a statistically significant ($P < 0.01$) positive correlation was registered between the levels of AFB_1 and the moisture contents (r = 0.76) in the laying hens feeds. Further, there was no significant negative correlations between the total fungal counts and the moisture contents (r = – 0.15) and the levels of AFB_1 (r = – 0.24), while there was positive but not significant correlation between the levels of AFB_1 and the moisture contents (r = 0.31) in the chicken feeds.

Discussion

Fungi are ubiquitous plant pathogens that are common agents of foods and feedstuffs deterioration. Fungal and mycotoxin contamination of animal feed are the major threats to animal and human health worldwide.

In the tested poultry samples, the lower level of the total fungal counts was 1×10^2 whereas the highest level was 1.83×10^5 CFU g^{-1}. According to the Serbian Regulation the 21.43% of the chicken feeds exceeded the maximum permitted level set to provide food safety and quality assurance. The most of the samples were contaminated with *Aspergillus* spp. with average AFB_1 concentrations from 4.47 μg kg^{-1} in the chicken feeds to 4.56 μg kg^{-1} in the laying hens feeds. These results are similar to those of previous mycological investigations of poultry feed samples in Serbia (*Krnjaja et al., 2010*). However, according to the results of *Cegielska-Radziejewska et al. (2013),* the fungal count was below 1×10^4 CFU g^{-1} in feeds for broilers collected in Poland in 2010, with *Aspergillus* and *Rhizopus* as the most common genera. The same authors observed that fungal contamination in poultry feeds from western Poland in 2010 was much lower than in the period of 2006-2008 which accounted 10^4–10^5 CFU g^{-1}. Additionally, in Argentina, *Monge et al. (2013)* established low values of total fungal counts (1×10^2) in pelleted poultry feed samples with relative high percentages (>40%) of *Aspergillus flavus* and *A. parasiticus* isolates. Similarly, total fungal count ranging from 10–10^6 CFU g^{-1}, and 43.5% of *Aspergillus* spp. isolates have been determined in poultry feed samples by *Greco et al.* (*2014*).

Feed ingredients such as cereals, sunflower, soybean, etc. are suitable for fungal development and mycotoxin contamination. The extreme high aflatoxin levels in maize crops has been recorded in Serbia during the summer of 2012 due to extreme high temperatures and low rainfalls which provoke the high incidence of *Aspergillus* species (*Kos et al., 2012, 2014; Lević et al., 2013; Krnjaja et al., 2013*). In the present study, 85.71% of the chicken feeds and 100% of the laying hens feeds were contaminated with AFB_1. There were 14.29% of the chicken feeds with unacceptable concentrations of AFB_1. Similarly, *Parvathi et al. (2017)*

reported that aflatoxins have been the most common contaminants in different poultry feeds and feed ingredients collected in India. Furthermore, in Pakistan, *Fareed et al. (2014)* reported a higher incidence and contamination levels of aflatoxins then ochratoxin A (OTA) in local poultry feeds and feed ingredients.

The growth of *Aspergillus* species and aflatoxin biosynthesis in cereals and other feed crops are conditioned with suitable environmental factors such as temperature and relative humidity (*Patel et al., 2015*). In addition, water activity (a_w) and temperature of cereal grains are the main factors that influence the fungal growth and mycotoxin synthesis (*Medina et al., 2017*). In warm and humid areas, *A. flavus* and *A. parasiticus* as the main producers of aflatoxins have been dominant species on maize ears. Optimal conditions for their growth are defined with temperature of 35°C and $a_w = 0.95$, whereas the higher value of water activity, $a_w = 0.99$ and temperature of 33°C are necessary for aflatoxin production (*Milani, 2013*). It has also been reported that physical factors, such as moisture, relative humidity, temperature, and mechanical damage are critical for mycotoxin production (*Bryden, 2012)*. Higher values of moisture content (20-25%) provide convenient conditions for fungal infections of crops prior to harvest (*Magan 2006*).

Proper field management practice and use of resistant cereals cultivars are particularly important in mycotoxin control. In addition, during harvest, as a first stage in the cereals production chain, regular and accurate moisture and temperature determination becomes the dominant control measure in the prevention of mycotoxin synthesis. The excessive moisture along the cereal production chain has been the most critical factor affecting the growth and proliferation of fungi, which further increases the risk of feedstuffs mycotoxin contamination (*Kana et al., 2013*). In this study, even the mean moisture content of the tested poultry feeds was relatively low (<11%), toxigenic fungi and AFB_1 were recorded in the most of the samples, confirming that contamination may occur not only during harvest but also during pre-harvest, incorrect storage and transportation conditions or during poultry feed processing (*Binder et al., 2007*). The positive correlations between the moisture content and the total fungal count and the levels of AFB_1 were detected which was in accordance with the observations of *Greco et al. (2014).*

Conclusion

In conclusion, it can be emphasized that the tested poultry feed samples collected in 2016 were mainly contaminated with toxigenic species from the genus *Aspergillus,* followed by *Fusarium* and *Penicillium* genera. In 21.43% of the chicken poultry feeds, the fungal contamination was above the maximum permitted values. The high percentage of positive AFB_1 samples has been registered, whereas in 14.29% of the chicken poultry feeds, the AFB_1 level was also above the regulation limit. These results confirm the necessity of continuous mycological and mycotoxicological control of feeds as the most important measure of control in feed and food safety strategy.

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Mikobiota i aflatoksin B_1 u hrani za živinu

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Rezime

U ovom radu je 30 uzoraka hrane za živinu sakupljenih tokom 2016. godine iz različitih živinarskih farmi u Srbiji, ispitivano na prisustvo gljiva i aflatoksina u uzorku. Primenom metode razređenja i standardnih mikoloških metoda utvrđeni su ukupan broj gljiva i identifikovani su potencijalno toksigeni rodovi gljiva. Prirodna pojava aflatoksina B_1 (AFB_1) utvrđena je primenom biohemijske imunoadsorpcione metode (ELISA).

Ukupan broj gljiva bio je od 1 x 10^2 (2 log CFU g^{-1}) do 1,83 x 10^5 CFU g^{-1} (5.26 log CFU g^{-1}). Najveći broj uzoraka hrane za piliće (78,57%) imao je ukupan broj gljiva u rangu od 1 x 10^2 do 4,8 x 10^4 CFU g^{-1}, dok je 68,75% uzoraka hrane za nosilje imalo ukupan broj gljiva u rangu od 5,3 x 10^4 do 1,83 x 10^5 CFU g^{-1}. U 21,43% hrane za piliće ustanovljen je nedozvoljen ukupan broj gljiva. Identifikovana su tri potencijalno toksigena roda gljiva *Aspergillus*, *Fusarium* i *Penicillium*. Najveći broj ispitivanih uzoraka hrane za živinu bio je kontaminiran *Aspergillus* vrstama, u odnosu na *Fusarium* i *Penicillium* vrste koje su kontaminirale manji broj uzoraka. Rang sadržaja AFB_1 bio je od 1,34 do 18,29 μg kg^{-1}, sa prosečnim sadržajem od 4,47 μg kg^{-1} u uzorcima hrane za piliće, i 4,56 μg kg^{-1} u uzorcima hrane za nosilje. U 14,29% uzoraka hrane za piliće ustanovljen je nedozvoljen sadržaj AFB_1.

Dobijeni rezultati potvrđuju značaj stalne mikološke i mikotoksikološke kontrole hrane za živinu, kao i potrebu za usavršavanjem procene rizika od štetnih (gljivičnih) kontaminenata u lancu ishrane.

Ključne reči: hrana za živinu, ukupan broj gljiva, aflatoksin B_1

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