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FUNGAL CONTAMINATION AND NATURAL OCCURRENCE OF OCHRATOXIN A (OTA) IN POULTRY FEED

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Abstract: Total fungal count, the presence of potentially toxigenic fungi and natural occurrence of ochratoxin A (OTA) were studied in 30 poultry feed samples (14 samples of feed for chickens and 16 samples of feed for laying hens), which were collected from different farms in Serbia at the beginning of year 2014. The total number of fungi was determined by the method of dilution and OTA was detected using the imunoadsorption enzymatic assay (ELISA).
In most of the samples of chickens feed (50%) the total number of fungi was 1 - 3 x 10^2 CFU g^{-1}, and in feed for laying hens the highest number of samples (37.50%) had the total fungal count from 1.4 to 4.8 x 10^4 CFU g^{-1}. The species of genera *Aspergillus* and *Penicillium* were identified as producers of OTA in 21.43% and 42.86% of chickens feed samples and in 68.75% and 25% of samples of feed for laying hens. The presence of OTA was detected in 100% of samples of feed for chickens and laying hens, with average concentrations of 34.40 μg kg^{-1} (feed for chickens) and 43.89 μg kg^{-1} (feed for laying hens).
The total fungal count and content of OTA were not above the maximum allowed quantities, even though the presence of *Aspergillus* and *Penicillium* species was found in a large number of samples (up to 68.75%). These results indicate that the tested samples of poultry feed were mycologically and mycotoxicologically correct.

Key words: poultry feed, total fungal count, ochratoxin A (OTA)

Introduction

The fungi are ubiquitous and produce mycotoxins that can occur in all agricultural products in appropriate conditions in the field and in storage. In livestock production, one of the main tasks is to provide healthy food for animals. Feed spoilage by fungi can be a problem for feed security *(Bryden, 2012)*. Poultry

feed is frequently contaminated with mycotoxins. Ochratoxin A (OTA) is a mycotoxin, a secondary metabolite produced by the fungi *Aspergillus* and *Penicillium* species. After aflatoxin, OTA is the second most important mycotoxin in terms of economic losses and is considered the most toxic mycotoxin for birds *(Indresh and Umakanth, 2013).* Ochratoxin often causes lower performances in poultry production, and the level of losses depends on the dose and duration of feeding poultry with contaminated food. The largest amount of this toxin accumulates in the kidneys and liver *(Resanović et al., 2009).* Food contaminated with OTA results in lower egg production, reduced performance and body weight, as well as reduced feed conversion ratio in poultry *(Hassan et al., 2012).*

OTA was detected around the world as a natural contaminant of various grains such as barley, wheat, oats, rye, and maize. OTA is a potent nephrotoxin, immune suppressant, teratogen and carcinogen *(Joo et al., 2013).* Through the food chain, OTA may be involved in the pathogenesis of various forms of human nephropathies, including kidney cancer *(Denli and Perez, 2010).* High concentrations of OTA in food and urine and blood samples in humans are found in rural areas of Bulgaria, Romania, Bosnia and Herzegovina, Croatia, Serbia and Tunisia, where endemic nephropathy (chronic kidney disease) is widespread, where a nephropathy of unknown aetiology occurred. Also, it is considered a possible cause of cancer of the urinary tract in humans and animals *(Klarić, 2013; Kocić-Tanackov and Dimić, 2013)*.

Because of insufficient data on fungal and ochratoxin contamination of poultry feed in Serbia, the aim of the study was to examine the total fungal count, presence of potentially toxigenic fungi, and the level of food contamination with OTA in prepared mixtures of feed for poultry and thereby establish the mycological and mycotoxicological quality of the tested feed samples.

Materials and Methods

At the beginning of 2014 from different poultry farms in Serbia a total of 30 samples of poultry feed (14 chickens and 16 laying hens feed samples) were collected according to *European Commission (2006).* By using a moisture analyzer (Ohaus MB35, USA) the moisture content of tested samples was determined.

According to the pour-plate method, the total fungal count was determined. In the Erlenmeyer bottle the sample (20 g) was homogenized with 180 ml of normal saline (NaCl, 8.5 g/l) in the course of a few minutes on the orbital shaker (GFL 3015, Germany). From homogenate the serial dilutions to 10^{-4} concentration were made and 1 ml of dilutions to 10^{-2}, 10^{-3} and 10^{-4} each, and applied with micro pipette (1000 µl) on Sabouraud maltose agar in a Ø90 mm Petri plates. In incubator (Memmert, Germany) the Petri plates were kept at 25ºC for 5-7 days. Total fungal count was presented as colony-forming units (CFU) per gram of sample. Based on

morphological characteristics the fungal genera were identified according to fungal key of *Watanabe (1994)*.

The presence of ochratoxin A was detected by ELISA assay according to the instructions Tecna S.r.l. (Italy) ELISA kits on an ELISA reader (Biotek EL x 800TM, USA). The limit of detection was 1 μg kg^{-1} for OTA.

Correlation between individual values obtained for total fungal count, concentration of OTA and grain moisture content was determined using Pearson's correlation coefficient.

Results

By analyzing of investigated it was established that the number of fungi ranged from 0 to 14 x 10^4 CFU g^{-1}. Most of chickens feed samples (50%) had a total fungal count from 1 to 3 x 10^2 CFU g^{-1}, whereas 37.50% of laying hens feed samples had a 1.4 to 4.8 x 10^4 CFU g^{-1}. No fungi were detected in 14.29% of chickens feed samples and 6.25% of laying hens feed samples (Table 1). Statistically insignificant negative correlation (r = - 0.31) was determined between the total fungal count and the moisture content in chickens feed samples and positive correlation (r = 0.46) between the total fungal count and the moisture content in samples of feed for laying hens. The moisture content of the chickens feed samples ranged from 8.81 to 12.67% with an average of 11.71%, and of laying hens feed samples ranged from 5.80 to 11.77% with an average of 10.14%.

Table 1. Level of fungal contamination of chickens and laying hens feed samples

Fungal counts (CFU g^{-1*})	Chickens feed	Laying hens feed
	Frequency (%)	
5.4 – 14 x 10^4	0	12.50
1.4 – 4.8 x 10^4	14.29	37.50
1 – 9 x 10^3	21.42	12.50
1 – 3 x 10^2	50	31.25
0	14.29	6.25

*Colony forming units per g of sample

Mycological survey of investigated chickens feed samples identified four fungal genera, *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus*, and in addition to above mentioned genera also *Alternaria* and *Mucor* were identified in feed samples for laying hens. In most chickens feed samples the species from genera *Fusarium* (50% positive samples) and *Penicillium* (42.86%) were isolated, followed by species from genera *Aspergillus* (21.43% positive samples) and *Rhizopus* (7.14% positive samples). In most laying hens feed samples the species from genera *Aspergillus* (68.75% positive samples) and *Fusarium* (43.75% positive samples)

were isolated, followed by species from genera *Alternaria*, *Penicillium* and *Rhizopu*s (each with 25% positive samples) and *Mucor* (18.75% positive samples) (Table 2).

Table 2. Fungal genera in investigated chickens and laying hens feed samples

Fungal genera	Chickens feed	Laying hens feed
	Frequency (%)	
Alternaria	0	25
Aspergillus	21.43	68.75
Fusarium	50	43.75
Mucor	0	18.75
Penicillium	42.86	25
Rhizopus	7.14	25

Mycotoxicological analysis showed the presence of 100% OTA positive samples, with mean concentration of 34.40 μg kg^{-1} (chickens feed samples) and 43.89 μg kg^{-1} (laying hens feed samples) (Table 3). In chickens feed samples between the concentrations of OTA, and the moisture content and the concentration of OTA, and the total fungal count, statistically insignificant positive correlations r = 0.21 and statistically insignificant negative r = - 0.34, respectively, were established. In laying hens feed samples between the concentrations of OTA, and the moisture content and the concentration of OTA, and the total fungal count, statistically insignificant positive correlations r = 0.07 and r = 0.09, respectively, were established.

Table 3. Concentration ochratoxin A (OTA) in investigated chickens and laying hens feed samples

Item	Ochratoxin A (OTA)	
	Chickens feed	Laying hens feed
Sample size[a]	16/16	14/14
Incidence %	100	100
Range (μg kg^{-1})	19.04 – 51.30	28.34 – 65.30
Mean[b] (μg kg^{-1})	34.40	43.89

[a] Number of positive samples/Number of total samples
[b] Mean concentration in positive samples

Discussion

The assesment of total fungal count in animal feed is important criteria in the determination of hygienic quality and a necessary tool for assessing the potential risks and dangers of the increased presence of mycotoxins. According to the Regulation on quality of animal feed *(Službeni glasnik Republike Srbije, 2010)*, mixtures and forage raw materials do not correspond to the hygienic quality if they

contain more than 200,000 spores in 1 g of mixture for older animals or 50,000 spores in feed for young animals. In Serbia the maximum allowed level of OTA is 1000 μg kg^{-1} in chickens feed and 250 μg kg^{-1} in laying hens feed *(Službeni glasnik Republike Srbije, 2010).*

In the present studies, most frequently isolated fungal species were from genera *Aspergillus*, *Fusarium* and *Penicillium*. The values for total fungal count and content of OTA in investigated chickens and laying hens feed samples have not exceeded maximum allowed limit confirmed by the Regulation. In Serbia, there is scant data on the presence of OTA and only in some components of the feed, such as for example wheat. Thus, in the mycotoxicological analysis of wheat originating from different localities in Serbia, *Škrinjar et al. (2005)* have detected the presence of OTA in 5 of 20 samples originating from Niš and Leskovac with concentration from traces to 40 μg kg^{-1} and in 70% of samples originating from Kikinda, with a concentration of 8 to 48 μg kg^{-1}. Also, by studying different types of flour, *Škrinjar et al. (2005)* have found a total fungal count of 10 (graham flour) to 8.5 x 10^3 (rye flour), with the most frequently isolated species from the genera *Aspergillus*, *Eurotium* and *Penicillium*. During the five-year period (2007-2012) *Radulović et al. (2013)* have analyzed 104 samples of poultry (broilers and laying hens) for the presence of OTA and found values for ochratoxin in feed mixtures for broilers which ranged from 2 to 650 μg kg^{-1}, and the values in feed mixtures for laying hens from 4 to 100 μg kg^{-1}. *Milićević et al. (2011)* have found low concentrations of OTA in chicken tissue samples and found that chicken nephropathy observed in Serbia has multitoxic etiology with possible synergistic effect between natural toxins and microorganisms, usually present in low concentrations.

In countries with similar geographical and climatic conditions, there is also little data on contamination of poultry feed with fungi and OTA mycotoxin. Thus, in Poland, *Cegielska-Radziejewska et al. (2013)* have found that the level of fungal contamination in 45 samples of feed for broiler chickens was on average 7 x 10^2 CFU g^{-1}, and the most commonly isolated fungal genera were *Aspergillus* and *Rhizopus*. In most of the studied samples of poultry feed originating from south-western Poland, mycoflora contamination has not exceed the allowed level of 2 x 10^5 CFU g^{-1} and in the majority of cases it was in the range of 10^2-10^4 CFU g^{-1} *(Kubizna et al., 2011).* During the two-year study (2006-2007) of the share of mycotoxins that have a synergistic effect in the etiology of nephropathy from 50 feed samples from various pig/chick farms in Bulgaria, *Stoev et al. (2010)* have determined the presence of OTA in concentrations of 188.8±27.3 μg kg^{-1} (2006) and 376.4±63.9 μg kg^{-1} (2007) and range of fungal contamination (*Aspergillus ochraceus* and *Penicillium verrucosum*) from 4 x 10^3 to 6 x 10^5 CFU g^{-1}. In Croatia, in the analysis of 34 samples of poultry feed, *Pleadin et al. (2012)* have detected low concentrations of OTA with an average of 1.42 μg kg^{-1}.

Conclusion

Given the importance of the OTA as a nephrotoxic and carcinogenic agent that causes many kidney diseases, the best way to control the formation of this mycotoxin is to prevent the growth of fungi on wheat as the main component of poultry feed and on other susceptible commodities. In the fields crops should be protected with appropriate fungicides, and moisture content at harvest should be reduced to a safe level. Regulated aeration of the storage facilities also prevents the increase in fungal contamination. The obtained results revealed the presence of potentially toxigenic fungi and OTA, but the levels of these contaminants did not exceed allowed limits. Since the *Aspergillus* and *Penicillium* species were isolated with relative high frequency (up to 68.75% positive samples) and OTA was present in 100% of investigated samples, it is necessary to emphasize the need for continuous monitoring of the mycologically and mycotoxicologically safe animal feed as an important measure to prevent conditions for increased production of mycotoxins.

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Kontaminacija gljivama i prirodna pojava ohratoksina A (OTA) u hrani za živinu

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Rezime

Ukupan broj gljiva, prisustvo potencijalno toksigenih rodova gljiva i prirodna pojava ohratoksina A (OTA) proučavani su u 30 uzoraka hrane za živinu (14 uzoraka hrane za piliće i 16 uzoraka hrane za nosilje), koji su sakupljeni iz različitih farmi u Srbiji početkom 2014. godine. Ukupan broj gljiva određen je primenom metode razređenja a OTA je detektovan primenom imunoadsorpcione enzimske metode (ELISA).

U najvećem broju uzoraka hrane za piliće (50%) ukupan broj gljiva je bio od 1 - 3 x 10^2 CFU g^{-1}, a u hrani za nosilje najveći broj uzoraka (37,50%) imao je ukupan broj gljiva od 1,4 do 4,8 x 10^4 CFU g^{-1}. Kao producenti OTA

identifikovane su vrste iz rodova *Aspergillus* and *Penicillium* u 21,43% and 42,86% uzoraka hrane za piliće i u 68,75% and 25% uzoraka hrane za nosilje. Prisustvo OTA je detektovano u 100% uzoraka hrane za piliće i nosilje, sa prosečnim koncentracijama od 34,40 μg kg^{-1} (hrana za piliće) i 43,89 μg kg^{-1} (hrana za nosilje).

Vrednosti za ukupan broj gljiva i sadržaj OTA nisu bile iznad maksimalno dozvoljenih količina, iako je ustanovljeno prisustvo *Aspergillus* i *Penicillium* vrsta u velikom broju uzoraka (do 68,75%). Ovi rezultati ukazuju da su ispitivani uzorci hrane za živinu mikološki i mikotoksikološki ispravni.

References

BRYDEN L.W. (2012): Mycotoxin contamination of the feed supply chain: implications for animal productivity and feed security. Animal Feed Science and Technology, 173, 134-158.

CEGIELSKA-RADZIEJEWSKA R., STUPER K., SZABLEWSKI T. (2013): Microflora and mycotoxin contamination in poultry feed mixtures from western Poland. Annals of Agricultural and Environmental Medicine, 20, 1, 30-35.

DENLI M., PEREZ J.F. (2010): Ochratoxins in feed, a risk for animal and human health: control strategies. Toxins, 2, 1065-1077.

EUROPEAN COMMISSION (2006): Commision regulation (EC) No. 401/2006. Official Journal of the European Union, L70, 12.

HASSAN Z.U., KHAN M.Z., KHAN A., JAVED I., SADIQUE U., KHATOON A. (2012): Ochratoxicosis in white leghorn breeder hens: production and breeding performance. Pak Vet J, 32, 4, 557-561.

INDRESH H.C., UMAKANTHA B. (2013): Effects of ochratoxin and T-2 toxin combination on performance, biochemical and immune status of commercial broilers. Veterinary World, 6, 11, 945-949.

JOO Y.D., KANG C.W., AN B.K., AHN J.S., BORUTOVA R. (2013): Effects of ochratoxin A and preventive action of a mycotoxin-deactivation product in broiler chickens. Veterinarija ir zootechnika, 61, 83, 22-29.

KLARIĆ, M.Š., RAŠIĆ D., PERAICA M. (2013): Deleterious effects of mycotoxin combinations involving ochratoxin A. Toxins, 5, 1965-1987.

KOCIĆ-TANACKOV S., DIMIĆ G.R. (2013): Gljive i mikotoksini – kontaminenti hrane. Hemijska industrija, 67, 4, 639-653.

KUBIZNA J., JAMROZ D., KUBIZNA J.K. (2011): Contamination of feed mixtures with mycoflora in South-Western Poland. Electronic Journal of Polish Agricultural Universities, 14, 2, #08.

MILIĆEVIĆ D., JOVANOVIĆ M., MATEKALO-SVERAK V., RADICEVIĆ T., PETROVIĆ M.M., LILIĆ S. (2011): A Survey of spontaneous occurrence of ochratoxin A residues in chicken tissues and concurrence with histopathological

changes in liver and kidneys. J. Environ. Sci. Health C. Environ. Carcinog. Ecotoxicol. Rev., 29, 2, 159-175.
PLEADIN J., PERŠI N., VULIĆ A., ZADRAVEC M. (2012): Survey of mycotoxin feed contamination in Croatia. Biotechnology in Animal Husbandry, 28, 2, 167-177.
RADULOVIĆ S.S., MARKOVIĆ R.V., MILIĆ D.D., JAKIĆ-DIMIĆ D.P., ŠEFER D.S. (2013): Degree of mycotoxicological contamination of feed and complete feed mixtures for pigs and poultry during the period 2007-2012. on the territory of the Republic of Serbia. Jour. Nat. Sci., Matica srpska Novi Sad, 124, 153-169.
RESANOVIĆ R.M., NEŠIĆ K.D., NEŠIĆ, V.D., PALIĆ T.D., JAĆEVIĆ V.M. (2009): Mycotoxins in poultry production. Jour. Nat. Sci., Matica srpska Novi Sad, 116, 7-14.
SLUŽBENI GLASNIK REPUBLIKE SRBIJE (2010): Pravilnik o kvalitetu hrane za životinje, br. 4.
STOEV S.D., DUTTON M.F., NJOBEH P.B., MOSONIK J.S., STEENKAMP P.A. (2010): Mycotoxic nephropathy in Bulgarian pigs and chickens: complex aetiology and similarity to Balkan Endemic Nephropathy. Food additives and Contaminants, 27, 1, 72-88.
ŠKRINJAR M., MAŠIĆ Z., KOCIĆ-TANACKOV S. (2005): Fungi and ochratoxin A – frequency in food and raw materials for their production in Serbia. Jour. Nat. Sci., Matica srpska Novi Sad, 108, 9-15.
WATANABE T. (1994): Pictorial atlas of soil and seed fungi: Morphologies of cultured fungi and key to species. Lewis Publishers, Boca Raton, Boston, London, Washington D.C. pp. 410.

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