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## Aflatoxin M1 levels in sow milk

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**ABSTRACT.** Aflatoxins (AFs) are one of the most known and investigated group of mycotoxins, which can be found as contaminants in different types of food and feed. Animals are exposed to AFs mainly through the consumption of contaminated feed, particularly products of plant origin. Among AFs, aflatoxin M1 (AFM1) is the monohydroxylated derivative of AFB1 formed in the liver and excreted into the milk of lactating animals. This study encompassed the Vojvodina region of Serbia and was aimed at determining the levels of AFM1 excretion in sows' milk in the first 3–5 days of lactation, after consumption of naturally contaminated with AFB1 maize. A total of 110 sows' milk samples from 11 swine farms in the specific region were analyzed by Enzyme Linked Immunosorbent Assay (ELISA). Different levels of AFM1 were detected in the majority (97%) of the examined milk samples. The obtained results showed AFM1 levels ranging from 5 to 165.4 ng/L. The results of this study pose special health concern associated with aflatoxin contamination of swine feed raw materials in this particular part of Serbia. Moreover, such high incidence of AFM1 detection in sows' milk may suggest the occurrence of long-term low level aflatoxicosis clinical cases.

**Key words:** aflatoxin M1, sow milk, Vojvodina region

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## INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by certain fungi belonging predominantly to the *Aspergillus*, *Penicillium* and *Fusarium* genera, and may cause a variety of adverse effects on both humans and animals (Kabak, 2012). Depending on classification, 300–400 mycotoxins are known today (Streit et al., 2012). Among them, aflatoxins (AFs) are the most abundant and toxic metabolites produced primarily by two closely related *Aspergillus* moulds, *A. flavus* and *A. parasiticus* (Marin et al., 2002) and in rare cases, by *A. nomius* (Ertas et al., 2011). There are at least 20 different types of naturally-occurring AFs and the most recognized are B1, B2, G1 and G2 (Diekmann and Green, 1992; Kabak, 2012). Animals are exposed to AFs mainly through the consumption of contaminated feed, particularly products of plant origin such as cereal grains (Hussein and Brasel, 2001; Weaver et al., 2013a; Manouras and Malissiova, 2015).

Among different metabolites that arise from the biotransformation of Aflatoxin B1 (AFB1) and Aflatoxin B2 (AFB2) in mammals, AF-8, 9-epoxide aflatoxicol and AFQ1, two hydroxylated metabolites of the parent compounds indicated as Aflatoxin M1 (AFM1) and Aflatoxin M2 (AFM2) are of major concern. The parent AFs are absorbed through the lining of the intestinal tract and transported by blood stream into the liver where bioactivation occurs (the formation of a reactive epoxide at the 8, 9-position of the terminal ring). They can bind covalently to nucleic acids, alter structure and function of proteins, block RNA polymerase and ribosomal translocase. Liver metabolism of AFs can result in the production of M1 and M2 metabolites, which can be incorporated into milk and milk products (Bognanno et al., 2006; Prandini et al., 2008; Chaytor et al., 2011; Bianco et al., 2012). The amount of converted AFB1 from feed in AFM1 in milk is influenced by several factors including breed, health, type of diet, milk production, rate of ingestion and digestion, etc. (Duarte et al., 2013). Depending on the level of feed contamination, approximately 0.3–6.3% of AFB1 ingested by livestock is transformed to AFM1 in milk (Prandini et al., 2008; Kabak, 2012). The AM1 has been detected in the milk of cows, sheep, goats, buffalos, camels and women (Galvano et al., 1996; Duarte et al., 2013; Bilandzic et al., 2014).

Swine is one of the most susceptible animal species to AFs with toxic effects in the intestine and the liver (Hussein and Brasel, 2001; Weaver et al., 2013a). Acute toxicity following consumption of high doses of AF in pigs is characterized by clinical manifestations such as feed refusal and reduced weight gain, as well as histological findings associated with liver dysfunction (Miller et al., 1981). However, the effects of chronic low-level exposure to AFs are usually clinically “silent” and more difficult to document (Weaver et al., 2013a). The results of recent studies also indicate negative effects of AFs on platelet count, serum albumin and calcium homeostasis in nursery pigs (Sun et al., 2015). In the study of Marin et al. (2002) it was concluded that even subclinical exposure to AFs may result in economic losses due to decreased performance and impairment of immune functions (immunosuppression). Experimental intoxications (sows receiving diets with 800 ppb AFB1, or 800 ppb AFG1 or 400 ppb AFB1 and 400 ppb AFG1) have shown damaged lymphocytes and macrophages in suckling piglets, indicating a loss of immune-competence due to exposure of sows to AFs (Silvotti et al., 1997; Kanora and Maes, 2009).

Swine breeding is an important part of Serbian economy, especially in the northern part of the country (Vojvodina Province) (Prodanov-Radulović et al., 2015a). However, according to the report of the Republic Hydrometeorological Service of Serbia (RHSS), climatic changes resulted in specific extreme conditions in 2011/12 crop production year in Serbia. Extremely high air temperatures during June, July and August 2012 as well as precipitation deficit resulted in extreme draughts in Vojvodina region with an average precipitation rate of only 25% (RHSS, 2012). The above-mentioned climatic conditions favored AFB1 production in swine feedstuffs such as maize, as observed by Jaksic et al., 2015 (61.2% highly AF-contaminated maize detected in 2012–2013). Other authors (Kos et al., 2013; Levic et al., 2013) reported presence of AFs in 68.5% of examined maize at concentration levels from 1.01 to 86.1 mg/kg in the same region and detection of AFM1 in cows' milk from that particular region during 2013–2014 (Bilandzic et al., 2014; Kos et al., 2014).

The aim of the present study was to investigate levels of AFM1 in sows' milk originating from animals bred in the Vojvodina Province and fed with

**Table 1.** Aflatoxin M1 levels and distribution in sow milk samples from farms in the Vojvodina Province.

| Farm | N  | Min – Max<br>(ng/L) | Mean<br>value<br>(ng/L) | Variance | C.V. | ±SD  | Mode     | Frequenci<br>of Mode | p-Value |
|------|----|---------------------|-------------------------|----------|------|------|----------|----------------------|---------|
| A    | 10 | 11.1 – 29.6         | 17.0                    | 34.0     | 34.3 | 5.8  | Multiple | 1                    | 0.1     |
| B    | 10 | 9.0 – 47.3          | 35.1                    | 145.3    | 34.4 | 12.1 | Multiple | 1                    | 0.1     |
| C    | 10 | 5.0 – 95.7          | 71.0                    | 1254.8   | 49.9 | 35.4 | 5,0      | 2                    | 0.4     |
| D    | 10 | 14.0 – 124.7        | 64.1                    | 1433.9   | 59.1 | 37.9 | Multiple | 1                    | 0.9     |
| E    | 10 | 5.0 – 25.2          | 13.5                    | 41.3     | 47.7 | 6.4  | 12,3     | 2                    | 0.5     |
| F    | 10 | 24.7 – 52.9         | 41.0                    | 74.3     | 21.0 | 8.6  | Multiple | 1                    | 0.4     |
| G    | 10 | 7.1 – 33.7          | 18.2                    | 86.4     | 51.1 | 9.3  | Multiple | 1                    | 0.7     |
| H    | 10 | 64.8 – 162.6        | 90.8                    | 773.2    | 30.6 | 27.8 | Multiple | 1                    | 0.0     |
| I    | 10 | 61.7 – 165.4        | 105.4                   | 976.2    | 29.6 | 31.2 | Multiple | 1                    | 0.3     |
| J    | 10 | 34.4 – 125.2        | 78.7                    | 632.5    | 31.9 | 25.1 | Multiple | 1                    | 0.4     |
| K    | 10 | 50.4 – 121.8        | 81.9                    | 474.8    | 26.6 | 21.8 | Multiple | 1                    | 0.4     |

A, B, C, D, E, F –swine farms located in Juznobacki district

G, H, I, J, K - swine farms located in Sremski district

N - total number of examined milk samples from each farm

Min – Max –minimum and maximum concentrations detected in the samples from each farm

C.V. - coefficient of variation

±SD - standard deviation

Mode – most frequent value in data set (the frequency of determined AF concentration in milk samples)

p-Value – probability of correctness of the calculated average value of AF concentration in examined milk samples

the AF-contaminated maize harvested at the end of August 2012 and was provided as feedstuff on eleven swine farms in the specified area.

## MATERIALS AND METHODS

### Sample collection and records

In the present study, we examined total 110 samples of sows' milk derived from 11 farrow-to-finish swine farms (10 samples from each farm A, B, C, D, E, F, G, H, I, J and K) in two districts (Juznobacki and Sremski district) of Vojvodina Province (Serbia) during spring and summer 2013. The number of sows under production on the farms included in the study ranged from 330 (farm A) to 1200 (farm E).

All samples were collected from sows during 3rd to 5th day of the lactation period. Farm selection criteria included the presence of AF-contaminated maize (2012 crops from specific regions) in sows' diets and occurrence of specific health problems in suckling piglets, i.e., reduced growth rates, persistent diarrhea, and absence or weak response to applied antimicrobial therapy (Prodanov-Radulovic et al., 2013; Prodanov-Radulovic et al., 2015b). All tested farms implemented regular vaccination programs against major viral and bacterial pathogens in sows during gestation.

Samples were collected by milking each sow separately, during lactation time, so as to receive 15-20 ml of milk that was maintained at -20°C

thereafter. In particular cases, artificial stimulation by intramuscular administration of Oxytocine (Belapharm) was performed according to manufacturer's instruction. Prior to analysis, milk samples were centrifuged for 10 min at 3500 g at 10 °C. The upper cream layer was removed by aspiration through a Pasteur pipette. Skimmed milk was used directly in the test (100 µl per well).

### Sample analysis

The presence of AFM<sub>1</sub> in sows' milk was analyzed by enzyme-linked immunosorbent assay method (ELISA), using Ridascreen®Aflatoxin M<sub>1</sub> (Art. No. R1121) test kit (R-Biopharm, Germany). All reagents used for analysis were included in the kit. For sample preparation and test procedure, calibrate pipettes Eppendorf Research® volumes 10–100 µl and 100–1000 µl were used. In order to separate the fat content, samples were centrifuged in Hermle Z 206 A centrifuge (HERMLE Labortechnik, GmbH). The color intensity was measured photometrically at 450 nm (Multiskan FC, Thermo Scientific, China) and was inversely proportional to the mycotoxin concentration in the sample.

According to the manufacturer's description, the detection limit (DL) for AFM<sub>1</sub> determination was 5 ng/L (ppt). Laboratory limit of detection (LOD) and limit of quantification (LOQ) were determined by six repeated measurements of blind sample. LOD was calculated as a sum of the mean value for blind sample and three standard deviations, and LOQ as a sum of the mean value and tenfold standard deviation. In this way, the values obtained for LOD and LOQ were 2.6 ng/L and 5.2 ng/L, respectively. The determination range specified in manufacturer's instruction was 5–80 ng/L. In the case of higher concentrations of AFM<sub>1</sub> that were out of the range of determination, the sample was additionally diluted two and three times with the sample dilution buffer included in the kit, so that the determination levels ranged from 10 to 160 ng/L and 15–240 ng/L, respectively.

The analytical quality of the ELISA method was assured by participation in proficiency testing scheme (milk powder sample FAPAS 04224). Recovery of the method was 105% for AFM<sub>1</sub>. Special software the Rida®Soft Win (Art. No. Z9999, R-Biopharm, Germany) was used for the evaluation of enzyme immunoassays. Additionally, recovery determined

using spiked milk samples (with Supelco standard AFM<sub>1</sub> U-46319) was 107%. Milk powder sample (FAPAS 04224) was used for determination of precision as relative standard deviations under reproducibility conditions, RSD<sub>R</sub> (14%) and repeatability conditions, RSD<sub>r</sub> (33%). The values were compared with those derived from Horwitz equation (39% and 58%). The obtained precision and recoveries comply with the requirements of the European Commission concerning development of analytical methods (EC, 2006).

### Statistical analysis

Data were analyzed using Basic statistic package (Statistics10, Copyright of StatSoft GmbH, Hamburg). The results were presented as the minimum and maximum concentrations of AFM<sub>1</sub>, the mean value, variance, coefficient of variation, the mean ± standard deviation (SD), range, mode and frequency of mode, and the p-value. The level of significance was set at 0.05.

## RESULTS

The results of the study established the occurrence of AFM<sub>1</sub> in milk samples from all farms tested (Table 1). In 97% of all tested samples, the levels of AFM<sub>1</sub> were above LOD. Non detectable levels of AFM<sub>1</sub> were observed in only 3 samples (two samples from farm C and one from farm E, respectively). The AFM<sub>1</sub> levels in positive samples ranged from 5 to 165.4 ng/L. More than a half of tested farms (54.5%) revealed mean AFM<sub>1</sub> values above 60 ng/L. At least one milk sample from each of five farms (D, H, I, J, and K) had AFM<sub>1</sub> levels above 120 ng/L.

## DISCUSSION

The occurrence of AFM<sub>1</sub> in milk of different animal species has been reported in many countries (WHO, 2010). However, literature data on AFM<sub>1</sub> concentrations in sow milk are scarce, especially in comparison to cow milk (Silvotti et al., 1997; Bertuzzi et al., 2003; Weaver 2013b). In the present study, AFM<sub>1</sub> was detected in sows' milk samples at all swine farms' included in the study.

It is known that climatic conditions, i.e., high temperature and humidity in tropical and subtropical regions favor the growth of toxigenic *Aspergillus* fungi (Kabak, 2012). Also, long periods of high temperatures



and long-lasting drought in summer in other regions can also promote the development of particular mycotoxins (Bilandzic et al., 2014; Manouras and Malissiova, 2015). In the northern region of Serbia, extremely warm and dry 2011/12 crop production year was characterized also by high aflatoxin levels in maize and consequent detection of high levels of AFM1 in cow milk (Kos et al., 2013; Kos et al., 2014; Jaksic et al., 2015). All maize used as feedstuff for all tested farms originated from Juznobacki and Sremski district. Although not in the direct scope of this study, 7 maize samples from farms A, B, D, F, H, J were investigated by another research group that reported AF contamination greater than 65 µg/kg in samples from farms D, H, J as well as lower contamination levels of 4.3 µg/kg; 8.4 µg/kg; 11.8 µg/kg; 12.4 µg/kg in farms A, B, F, G, respectively (Jaksic et al., 2015). Our results show that consumption of such AF-contaminated feedstuffs during the study period resulted in the detection of the M1 metabolite in sow milk at all examined swine farms.

Sow milk is not used for human consumption, thus, regulatory levels for AFM1 do not exist. However, according to our results, determination of an AFM1 threshold level in sow milk as a quick guide for the detection of chronic aflatoxicosis in sows should be further investigated and suggested. According to Dowling and Brown (2012), any level of AFM1 represents the risk for the newborns since milk is their main food at this age. Levels of AFM1 in milk reach their highest concentration 3 days after consumption of contaminated feed, while AFM1 is undetectable 4-5 days after AFB1-contaminated feed withdrawal (Galvano et al., 1996). It is considered that the rate of absorption of AFB1 and excretion of AFM1 in milk varies from day to day and from one milking to the next (Duarte et al., 2013; Kos et al., 2014). Feed contaminated with AF may cause a dose-related decrease in feed intake and reduced weight gain, liver damage, alterations of the immune response (immunosuppression) and eventually significant economic losses in swine production (Bryden, 2012; Marin et al., 2002; Weaver et al., 2013a). However, under field conditions, mycotoxins usually occur in concentrations leading to reduced animal performance and variable immunosuppression without causing any obvious clinical symptoms (Kabak, 2012; Prodanov-Radulovic et al., 2014). It is especially important to point out that impairment of immune functions might

culminate in decrease of host resistance to infections and possibly compromise vaccine-induced immunity even in properly vaccinated animals (Marin et al., 2002). The results of this study are indicative of the usefulness of AFM1 detection in sow milk as an easy tool for detection of aflatoxin contamination in sows and consequently the interpretation of possible piglet's growth impairment. Such a tool would be even more helpful if implemented as an easy-to-use on-farm quick test for sow milk, in order to further improve accurate clinical diagnosis and reduce antibiotic usage in pigs.

Furthermore, according to Serbian feed regulation, maximum permitted level of total AFs in corn was 50 mg/kg, while in swine feed it was 20 mg/kg (Serbian Regulation, 2010). The present as well as future in-depth investigations of AF contamination of swine feed and sow milk in Serbia may contribute to the full harmonization of the specific Serbian regulation with the relative EU Regulation (European Commission, 2002).

## CONCLUDING REMARKS

The vast majority of tested milk samples (97%) were positive for the presence of AFM1 above detection level. The detected levels of AFM1 in examined sow milk samples clearly indicate the consumption of contaminated feedstuff at all swine farms during the study period. Further steps are needed in order to avoid usage of such contaminated maize as swine feedstuff. The possibility of AFM1 contamination of sow milk should be taken into consideration when interpreting growth retardation cases in piglets.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

## Statement of ethical compliance

All experimental procedures were performed in compliance with Serbian Law on Animal Welfare (Official Gazette of the Republic of Serbia No 41/09).

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