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PRELIMINARY REPORT ON AFLATOXIN M1 IN PIG URINE AS A MARKER OF AFLATOXIN B1 EXPOSURE IN SERBIA

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Abstract

In contemporary agricultural practices, it is increasingly difficult imagine production processes that are unaffected by the challenges posed by climate change. The aflatoxin B1 (AFB1) is a mycotoxin which was not very prominent in food and feed in Europe. However, in recent decades, climate change has emerged as a significant issue, raising concerns about food safety and animal production. One of the main problems regarding AFB1 is an uneven distribution in feed samples, and quite often feed sample analysis does not provide a proper insight into the exposure to this mycotoxin. This study aimed to investigate the occurrence of AFB1 in feed and its biomarker aflatoxin M1 (AFM1) in urine of fattening pigs on one small family farm. Urinary biomarker levels were subsequently used to estimate the probable daily intake and concentration of AFB1 in feed, employing established mathematical models. Four samples of a complete feeding mixture for fattening pigs originating from one farm were analyzed and the average AFB1 concentration of 0.046.3±0.0048 mg/kg was determined. Seven urine samples collected from the slaughter fatteners that were fed the above mentioned feed were analyzed for AFM1 levels. All samples contained AFM1, with an average concentration of 6.7±2.7 µg/l. The estimation of the AFB1 daily intake enabled us to assess the level in feed. The mathematical model revealed a level of 0.269 mg/kg that overestimated the AFB1 content obtained by analyzing the feed.

Key words: feed, daily intake, mathematical model, mycotoxins

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PRELIMARNI IZVEŠTAJ O ANALIZI AFLATOKSINA M1 U URINU SVINJA U SRBIJI KAO MARKERA IZLOŽENISTI AFLATOKSINU B1

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Kratak sadržaj

U savremenoj poljoprivrednoj praksi, skoro je nemoguće zamisliti proizvodnju bez nekog problema prouzrokovanog klimatskim promenama. Aflatoksin B1 (AFB1) je mikotoksin koji nije bio previše izražen u hrani i hranivima u Evropi. Međutim poslednjih nekoliko decenija usled klimatskih promena, postao je veliki problem i izazvao zabrinutost u pogledu bezbednosti hrane i animalne proizvodnje. Jedan od glavnih problema vezanih za AFB1 je neravnomerna distribucija u bilo kom uzorku hraniva, i često se dešava da analiza uzoraka hraniva ne da odgovarajući uvid izloženosti ovom mikotoksinu. Ova studija je imala za cilj da istraži pojavu AFB1 u hranivu i njegovog biomarkera aflatoksina M1 (AFM1) u urinu kod tovljenika na maloj porodičnoj farmi. Nivoi biomarkera u urinu su korišćeni za izračunavanje verovatnog dnevnog unosa i koncentracije AFB1 u hrani za tovljenike korišćenjem prethodno utvrđenih matematičkih modela. Četiri uzorka kompletne smeše za ishranu tovljenika sa jedne farme je analizirano i utvrđena prosečna koncentracija AFB1 je iznosila 0.046.3±0.0048 mg/kg. Sedam uzoraka urina uzetih sa linije klanja od tovljenika hranjenih pomenutom hranom je analizirano na AFM1. Svi uzorci su sadržali AFM1, prosečne koncentracije 6.7±2.7 µg/l. Procenjen dnevni unos AFB1 korišćen je za procenu koncentracija u hrani za tovljenike. Matematički model je ukazao na nivoe od 0.269 mg/kg što premašuje nivoe AFB1 dobijene analizom hrane za životinje.

Ključne reči: hrana za životinje, dnevni unos, matematički model, mikotoksini

INTRODUCTION

Aflatoxin B1 (AFB1) is a mycotoxin that has garnered significant attention in this region of Europe over the past two decades. (Cavallarin et al., 2004; Medina et al., 2014; de Rijk et al., 2015). At the beginning of the research of AFB1 in Europe, its occurrence in feed or food was linked to the imported food and feed in Europe from other continents (Blount 1961; EFSA 2005). However, the occurrence of AFB1 in corn produce in some European countries like Italy (EFSA 2005) or some countries in the Balkans (including Serbia) became frequent with occasional incidences of major outbreaks in recent decades (de Rijk et al. 2015; Horvatovic et al. 2018). The outbreak consequently influenced dairy production by reducing demand for milk products. The ultimate result was a significant reduction in the number of dairy herds in Serbia. There are a lot of published studies on the occurrence of aflatoxin M1 (AFM1) in milk or milk products in Serbia (Kos et al. 2013; Kos et al. 2014; Jajić et al. 2019).

At the same time, corn was used in the rearing and fattening of monogastric animals such as pigs. There is no concern regarding food safety issues when it comes to the occurrence of AFB1 in animal feed because there are no significant residues in the meat of these animals (Pleadin et al. 2021). Moreover, due to the controls of feed, cases of acute poisoning were rare even in the years the aflatoxins outbreak in corn. However, it is widely recognized that mycotoxins negatively impact production, influencing the immune status of the affected animals and key performance parameters, such as weight gain (Kanora and Maes 2009; Pu et al. 2021).

In intensive animal production, such as pig farming, animals are often at the brink of their biological limits. This mode of production encounters various challenges, with mycotoxin-related issues frequently being suspected. However, due to the uneven distribution of mycotoxins in feed, the results of feed analyses can vary significantly. For instance, during the outbreak of AFB1 in the Balkans in 2013, mycotoxin levels among replicated sample test results ranged from 21 to 204 μg/kg (de Rijk et al. 2015), all exceeding the European Union (EU) legal limit for AFB1 in corn for feed, set at 20 μg/kg (EC 2002). Despite this, the contaminated corn successfully passed import controls and was distributed to farms, raising concerns about how such substantial shipments with elevated AFB1 levels evaded detection by the EU.

AFM1 is produced from AFB1. It is excreted in urine, feces, and milk. In order to determine the mycotoxin AFB1 intake through feed, urine concentration of AFM1 can be used (Thieu and Pettersson 2009; Gambacorta et al., 2019; Tkaczyk and Piotr 2021). A good correlation was observed between the

ingested amount of mycotoxin and excreted biomarkers in the urine of pigs (Gambacorta et al. 2013). The mean percentage of dietary AFM1 excreted as a biomarker in 24 h post-dose urine was 2.5% for AFB1 (Gambacorta et al. 2013; Gambacorta et al. 2019).

The aim of this paper was to examine the concentration of AFM1 in urine and employ the existing mathematical model (Gambacorta et al., 2019) to evaluate exposure to AFB1 on a pig farm in Serbia. The samples were taken from a small-scale producer, that fed pigs with corn produced locally on their land on a farm in Vojvodina province, Serbia. At the same time, the complete feeding mixture produced by a farmer for fattening pigs was analyzed for AFB1.

MATERIAL AND METHODS

Animals

The animals (N=7) originated from a small farm in Sot, Srem, Vojvodina, Serbia. The animals were reared in controlled conditions on the farm. The study included piglets-fatteners obtained from F1 generation sows and terminal sires. All fattening pigs were reared on the same premises and fed the same feed. Fattening of the hogs was divided into three stages: the starting, growing, and finishing stage. After the end of the fattening period, when they were approximately six months old (weighing 105-110 kg), they were taken to the local slaughterhouse where the samples were taken.

Sampling

Urine samples were collected from seven randomly chosen pigs from the slaughter line. The whole bladders full of urine were taken and frozen at -20 °C After thawing the required volume of urine, the samples were taken for analysis. The samples of feed were taken randomly from the feeding place.

Determination of AFB1 in feed

Precisely 20 g of samples were measured and placed in a 150 mL beaker. AFB1 was extracted with 100 mL of 70% methanol in the water on an Ultra Turrax T18 homogenizer (IKA, Germany) for 3 min at 11,000 rpm. The crude extract was then filtered through a quantitative slow filtration filter paper (Filtros Anoia, Spain). The immunochemical analysis was performed using the Veratox, Aflatoxin (Total), and Quantitative Test Kit (Neogen, Lansing, MI,

USA) with four calibration standard solutions (0, 5, 15 and 50 μg/kg). The analytical procedure was carried out according to the manufacturer's procedure. AFB1 quantification was carried out on an ELISA reader equipped with a 630 nm filter (BioTec Instruments, USA).

Determination of AFM1 in urine

After defrosting, 12 mL of urine was incubated with the β-glucuronidase (obtained from Sigma Aldrich, USA) for 14 hours in order to clear conjugates. Following the incubation with β-glucuronidase, the samples were cleaned up by immunoaffinity columns AflaStar R M1 (Romer Labs, USA). Immunoaffinity columns were dried using a nitrogen stream, and the AFM1 bound to the columns was eluted with 3 mL of methanol. The methanol extract was evaporated in a stream of nitrogen, and all remains were dissolved in 200 μL of n-hexane (Sigma Aldrich, USA). Also, 200 μL of trifluoroacetic acid (Sigma Aldrich, USA) was added for derivatization. This mixture was heated for 10 minutes at 40 °C. After 10 minutes, the mixture was evaporated in a stream of nitrogen, dissolved in 300 μL of acetonitrile/water (75:25), and transferred to a vial for analysis. The analysis was carried out on HPLC with fluorescent detection (1260 Infinity, Agilent Technologies, USA), using the following conditions: column flow was set to 1.25 mL/min. The mobile phase was acetonitrile/water 75:25. Acetonitrile (HPLC grade) was purchased from Sigma Aldrich, USA. FLD excitation wavelength was set at 365 nm, and the emission wavelength was set at 440 nm. The chromatography column used for the analysis was C18 Zorbax Hypersil ODS 150×4,6 mm, 5 μm in particle size (Agilent Technologies, USA). The column was kept at 30 °C. All the analyses were done in triplicate.

Method quality control was confirmed by carrying out recovery (standard addition method), precision, and linearity tests. Recovery was tested at 1, 10, and 50 μg/L resulting in an average recovery rate of 91.3%. Precision was calculated after 6 consecutive injections of spiked pig urine samples for repeatability and another 6 injections on the next day for interlaboratory reproducibility calculation. Relative standard deviations for repeatability and interlaboratory reproducibility were 4.9% (N=6) and 7.7% (N=12), respectively. Linearity was calculated after injecting a series of 7 standard solutions (ranging from 2.5 to 25 μg/L), resulting in a regression coefficient of R^2=0.9993. Limits of detection and quantification were 0.3 and 1.0 μg/L, respectively. They were calculated based on the signal-to-noise ratio.

Mathematical model

The mathematical model applied in this study was adapted from a previous paper that used in vivo experiment to determine the relationship between the intake of AFB1 through feed and the excretion of its metabolite AFM1 in urine over a 24-hour period (Gambacorta et al. 2019). That study established the average excretion rate of AFM1 in urine based on different AFB1 concentrations in the feed. It was conducted on pig fatteners weighing 110 kg on average, which is also a typical weight at the end of the fattening phase in the Republic of Serbia. Therefore, in our study, we also used pig fatteners with an average body weight of 110 kg. The formulas used to calculate the probable daily intake of AFB1 were sourced from this earlier research, which also analyzed other mycotoxins in urine (although we only used data for AFB1). The average 24-hour urine excretion values incorporated into the formula are from the studies conducted by Gambacorta et al. (2013; 2019).

Estimation of mycotoxin intake (Gambacorta et al. 2019) was as follows:

$$PDI = (C \times V \times 100) / (W \times E)$$

Where:
PDI - probable daily intake of AFB1 mycotoxin (μg/kg body weight - BW)
C - pig urinary concentration (μg/L)
V - mean 24 h pig urine volume (2.5 l)
W - mean pig body weight
E - mean urinary excretion rate of 2.5% for AFB1 excreted as AFM1

Estimation of feed contamination:

$$ML = PDI \times W / V$$

Where:
ML - mycotoxin level in the feed (μg/kg)
PDI - probably daily intake of each mycotoxin (μg/kg body weight)
W - mean pig body weight
V- mean 24h pig urine volume (2.5 L)

RESULTS AND DISCUSSION

The results of the analyzed feed and pig's urine are presented in Tables 1 and 2. Prior to the analysis of feed and pig urine samples, the farmer reported issues, including a decline in production parameters. The occurrence of AFB1 in feed was from 0.041 - 0.051 mg/kg. The analyzed feed samples revealed

concentrations two or more times higher than the maximum permissible level of 0.02 mg/kg established by Serbian legislation. (Serbian Regulation, 2014).

Table 1. AFB1 (mg/kg) content in complete feeding mixtures for fattening pigs (N=4).

Samples	AFB1 (mg/kg)
1	0.041 ± 0.009
2	0.051 ± 0.013
3	0.042 ± 0.011
4	0.051 ± 0.010
Average	0.046 ± 0.005

Table 2 shows the results of analyzed samples of pig urine. Based on the average weight on the slaughter line and probably daily intake, the occurrence of AFB1 in feed was calculated. The presumed concentrations of AFB1 in feed were higher than the levels actually determined in the complete feed mixture.

Moreover, the average concentration was five times higher than the one analyzed in feed and almost 13 times higher than allowed for this category of animals.

Table 2. AFM1 content in pig's urine and AFB1 levels in feed calculated using the mathematical model (N=7)

Samples	AFM1 in urine (μg/l)	PDI (μg/kg BW)	ML (mg/kg)
1	6.33 ± 1.7	5.75	0.253
2	12.21 ± 2.9	11.10	0.488
3	8.41 ± 2.2	7.65	0.336
4	7.18 ± 2.3	6.53	0.287
5	5.17 ± 0.8	4.70	0.207
6	4.44 ± 0.8	4.04	0.177
7	3.32 ± 0.6	3.02	0.133
Average	6.72 ± 2.7	6.11	0.269

PDI: calculated probable daily intake of AFB1 (μg/kg body weight)
ML: calculated AFB1 level in the feed (mg/kg)

The detected concentrations of urinary AFM1 were surprisingly high. The concentrations were much higher than in the study of Gambacorta et al. (2019). However, this study was done in Sweden, a country with very good agricultural practices and traditionally this region of Europe is not linked with a high level of aflatoxin in feed. On the other hand, the study done in Vietnam indicated that pigs are commonly exposed to aflatoxin and the highest found concentration in 15 samples of AFM1 in pigs' urine was 7.8 ng/ml (Thieu and Pettersson, 2009) or 13.66 μg/kg (Lee et al., 2017).

The use of mycotoxin biomarkers in pig urine is commonly employed in certain countries as an indicator of mycotoxin exposure. (Vidal et al., 2018; Lee at al., 2017). However, to our knowledge, this approach has not been utilized in Serbia. It is widely recognized that in recent years, farmers in Serbia have struggled to meet the permissible concentration of AFM1 in milk due to the prevalence of AFB1, primarily in corn. (Kos, et al. 2013; Kos et al. 2014; Kos et al. 2018; Jajić et al. 2019). Small family farms produce relatively small amounts of feed which they use as feed locally for their animals. Feed or feed ingredients are not commonly tested. However, there is no actual data regarding the occurrence of aflatoxin in feed for fattening animals such as pigs on such farms. On small farms, when producers are aware of corn contamination, they typically avoid including it in the feed mixture for dairy cattle; however, there are no instances of completely excluding corn from the diet. It is used for animals for which it is thought that are less susceptible to mycotoxins. The farmers do not have enough economic power to completely avoid the contaminated corn.

It is nearly inconceivable that this farm experienced no issues related to the presence of AFB1 in the feed. This may be attributed to either a lack of viable options for the farmer to mitigate the problem or a complete unawareness of the associated risks. AFB1 is recognized for its impact on production in intensively fattened pigs (Smith et al., 2005). By utilizing biomarkers in urine to assess exposure to AFB1, it is possible to monitor the effects of various mycotoxin deactivators in pig feed. (Lauwers et al., 2019).

CONCLUSION

The results of this study indicate high concentrations of AFM1 in pig urine. However, it is important to note that the samples were taken from animals already suspected of consuming contaminated corn. Feed samples indicated AFB1 levels that were double the legal limit, while urine samples suggested that AFB1 concentrations in the feed that exceeded the permitted limit by more than twelve times. This discrepancy raises the question of whether urine

biomarkers overestimate AFB1 exposure or if AFB1 levels in feed are being underestimated due to inadequate sampling methods.

Given that AFM1 in urine has been established as a reliable indicator of AFB1 exposure (Gambacorta et al., 2019), we believe that the AFB1 levels in complete feed mixtures should align more closely with those suggested by the urine samples. Serbia, as one of the world's leading corn exporters, faces significant economic consequences due to AFB1 contamination. This issue disproportionately affects smaller producers who may lack the financial resources to address the problem effectively. While AFB1 contamination in fattening animals does not pose a direct safety risk to consumers, it still negatively impacts animal production. Further extensive research, particularly at slaughter lines, is necessary to better understand the incidence of AFB1 contamination in fattening pigs and to determine the full scope of the problem.

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Author's Contribution:

MPH wrote the manuscript with input from all authors; SK verified the analytical methods; DG and MD performed the analysis; MM sampling and statistics; IJ contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript All authors discussed the results and approved the final manuscript.

Competing interest

The authors declare that there is no conflict of interest.

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