Acta Veterinaria-Beograd 2019, 69 (1), 106-115
UDK: 636.085.1:582.28
636.52/.57.087.8
DOI: 10.2478/acve-2019-0007

Research article

OCCURRENCE OF AFLATOXIN B1, OCHRATOXIN A AND ZEARALENONE IN MAIZE SILAGE IN THE REGION OF VOJVODINA, SERBIA

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(Received 23 December 2018, Accepted 11 March 2019)

Silage made from the whole-plant maize is one of the most popular forages in Serbia. Consumption of maize silage by cows can be up to 30-35 kg/day. In Serbia in the few last years in the focus of the public and agriculture community were two mycotoxins, aflatoxin B1 and its metabolite aflatoxin M1 due to the outbreak of contaminated maize which affected the Balkans in 2012. Maize is regularly checked on the occurrence of aflatoxin B1, however forages are often neglected as a potential source of mycotoxins in the nutrition of dairy cattle.

In this work, 48 samples of maize silage were analyzed for the occurrence of aflatoxin B1, ochratoxin A and zearalenone. Samples were collected from three regions (Bačka, Banat and Srem) in Vojvodina. In all samples, at least one mycotoxin above the limit of quantification was measured. Aflatoxin B1 was detected in 36 (75%) samples. In two samples from Banat, the concentration of aflatoxin B1 exceeded the maximum level (ML) set by Serbian regulation (30 μg/kg at moisture content of 12%). In seven samples, the concentration of aflatoxin B1 was above 20 μg/kg which is the EU regulated ML. Average concentration of ochratoxin A was 10.4 μg/kg, while the maximum measured value was 34.3 μg/kg. Maximum zearalenone content in all samples was 538 μg/kg while the average zearalenone concentration was 138 μg/kg.

The results from this research point out that mycotoxin contaminated silage in the region of Vojvodina, Serbia can significantly contribute to daily intake of aflatoxin B1 in dairy cattle.

Key words: Vojvodina, maize silage, aflatoxin B1, ochratoxin A, zearalenone

INTRODUCTION

The diet of high yielding dairy cows consists of two classes of feedstuffs – complete feedstuff and forages. Maize, as the main source of energy is often tested on the occurrence of the most investigated mycotoxins. Silage made from the whole-plant

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maize is one of the most popular forages in Serbia. Silage, as often-used forage, can be a significant part of dry matter intake in cows. Also, it can highly contribute as a source to mycotoxins intake.

Mycotoxins are secondary metabolites of fungi. Worldwide surveillance on the occurrence of mycotoxins in food and feed showed that from all the analyzed samples of food and feed from Europe, Asia and Americas (a total of 7049 samples) 81% samples were positive for at least one mycotoxin [1]. Mycotoxins can be carried-over from feed to milk and are considered as a food safety risk, mostly aflatoxin M1 [2]. Ochratoxin A in some cases can be found in milk [3]. Usually ruminal protozoa degrade this mycotoxin. However, drastic changes in feed composition and a high percent of protein-rich concentrate in the diet modify the cleavage capacity of ruminal microorganisms. In Norway, Ochratoxin A was detected in 6 out of 40 conventional cow's milk samples (range 11± 58 ng/l), and in 5 out of 47 organic milk samples (range 15± 28 ng/l) [3]. Zearalenone carry-over from feed to milk is negligible [4]. Its effect on animal health and productivity is more of a concern [4].

In the past few years, the focus of the public and agriculture community in Serbia was on two mycotoxins, aflatoxin B1 and its metabolite aflatoxin M1 due to the outbreak that affected the Balkans in 2012 [5]. High occurrence of aflatoxin B1 in maize due to the favorable climate condition [6] resulted in an increased occurrence of aflatoxin M1 in milk and milk products [7,8-11]. The end result was a rapid decrease in public consumption of milk and milk products.

EU set a maximum level (ML) for aflatoxin M1 at 50 ng/kg [12]. The legislation was established on the ALARA ("As Low As Reasonably Achievable") principle. Countries such as USA and Brazil have set a ML of aflatoxin M1 in milk on the bases of the risk evaluation. These countries allowed ten times higher (500 ng/kg) ML of aflatoxin M1 in milk, compared to EU [2, 11]. Daily intake of aflatoxin B1 should not exceed 40 μg per cow to produce milk with less than 50 ng/kg aflatoxin M1 [13,14-16]. The ML of aflatoxin B1 in complete feedstuffs for cattle, by EU and Serbian legislation is 5 μg/kg while for silage (complementary feedstuffs for cattle) ML by EU legislation is 20 μg/kg [17], while by Serbian regulation is 30 μg/kg [18].

Dairy cattle husbandry in Serbia has some specificity. The majority of farms have a small operation, with up to 10 cows (26%) or from 10 to 50 cows per farm (38%) [19]. Also, numerous farms in Vojvodina and Serbia, formerly state-owned are more or less successfully privatized. The number of animals on such farms is usually large, although agricultural practice varies significantly. Milk processing in dairy plants is privately owned by corporations, and their interest is only to have the best quality of raw milk. After the outbreak of aflatoxin B1 contaminated maize in Serbia and the whole Balkan region during 2012-2013, legislation for aflatoxin M1 in milk has been changed several times in Serbia as an attempt of the government to maintain primary dairy production [20]. ML by the current legislation is 250 ng/kg [18]. Large dairies, after the outbreak of aflatoxin M1 in milk, regularly control raw milk from the primary producers. Some dairies even give a higher price when the level of aflatoxin M1 is below 50 ng/kg. However, primary producers vary greatly in economic resources needed for a good agricultural practice hence producers are struggling to achieve a desirable quality of milk regarding aflatoxin M1.

During 2015 in Banat, Vojvodina an increase in aflatoxin M1 occurrence in milk was observed [10]. Regular control of maize in that area did not show any problems regarding aflatoxin B1. Unofficial data from the Ministry of Agriculture, Forestry and Water Economy indicated that silage can be a problem.

Maize silage consumption in cows can be up to 30-35 kg/day. Some researchers suggested that silage can be cause of occurrence of the aflatoxin M1 in dairy cows [21, 22]. By comparing silage and compound feed as sources of mycotoxins, average dietary intake of two mycotoxins (deoxynivalenol (DON) and zearalenone) was higher in the case of silage 3.5 and 2.9 times, respectively [23].

Data regarding the occurrence of mycotoxins in maize silage in Serbia or the neighboring countries are scarce at best. The aim of this research was to investigate the occurrence of some mycotoxins (aflatoxin B1, zearalenone, ochratoxin A) in silage samples in Vojvodina, Serbia.

MATERIALS AND METHODS

Sampling

A total of 48 samples of silage were taken from 48 randomly chosen dairy farms in Vojvodina (Bačka, Banat and Srem) in December 2017 and January 2018 and analyzed on the presence of aflatoxin B1, ochratoxin A and zearalenone. 20 samples were taken from the farms in Banat, 15 from Bačka and 13 from Srem region. Farms which were included in this research were owned by corporation (previously state-owned) and numerous farms owned by individual farmers with the number of cows from 10 to more than 100 per farm. The majority of individually owned farms had between 20 and 30 cows. One sample of silage was taken from each farm. Sampling was done by taking several sub-samples from the cut edge, from the upper, middle and low section. Sub-samples were homogenized and quartered to get 500 – 1000 g of laboratory sample.

Immediately after sampling, 200 g of each sample were dried at 60 °C overnight and then prepared for analysis by grinding in a laboratory mill in such a way that >93% passed through a sieve with pore diameter of 1.0 mm. Then, the sample was homogenized by mixing and packed in plastic bags. Samples were stored in a freezer at -20ºC until analysis. Prior to each analysis, the samples were allowed to reach room temperature. A portion of sample was used for the determination of moisture content at 105°C.

Extraction

Exactly 20 g of samples were weighed in a 150 ml beaker. Mycotoxins were extracted with 100 ml of 70% methanol on an Ultra Turrax T18 homogenizer for 3 min at 11,000 rpm. Crude extract was then filtered through 6 Advantec filter paper.

Analysis

The immunochemical analysis was performed using the Veratox Quantitative Test Kits (Neogen, Lansing, MI, USA). The analytical procedure was carried out according to the manufacturer's procedure. Optical densities were obtained by using the reader of microtiter plates and 630 nm filter (BioTec Instruments, USA). Limits of quantification (LOQ) for total aflatoxins, ochratoxin A and zearalenone were 5, 2 and 25 μg/kg, respectively.

All results are calculated to 12% moisture content. Moisture content was measured using the NFTA reference method [24].

RESULTS

Results of the occurrence of aflatoxin B1 in maize silage are shown in Table 1.

Table 1. Occurrence of aflatoxin B1 in maize silage (moisture content 12%)

	Aflatoxin B1 μg/kg											
	Total		<LOQ		<30 μg/kg		>20 μg/kg		>30 μg/kg		Range	Average
	N	%	N	%	N	%	N	%	N	%		
Bačka	15	31.2	6.0	12.5	9	18.7	1	2.2	0	0.0	3.5-26.4	11.6
Banat	20	41.7	4.0	8.3	14	29.2	3	6.2	2	4.2	3.5-44.0	14.2
Srem	13	27.1	2.0	4.2	11	22.9	3	6.2	0	0.0	5.3-.25.5	13.5
Vojvodina	48	100	12	25.0	34	70.8	7	14.6	2	4.2	3.5-44.0	13.4

N – number of samples; <LOQ – below limits of quantification

Aflatoxin B1 was detected in 75% of analyzed samples (36 samples). In two samples from Banat, the concentration of aflatoxin B1 exceeded ML set by Serbian regulation (30 μg/kg at moisture content of 12%). In seven samples the concentration of aflatoxin B1 was above 20 μg/kg which is the ML in EU [17]. The average concentration in all positive samples for Banat, Bačka and Srem was 13.4 μg/kg. the maximum measured concentration was 44.0 μg/kg in a sample originating from Banat region.

The occurrence of ochratoxin A and zearalenone in maize silage is shown in Tables 2 and Table 3. All samples were well below the ML. At least one mycotoxin above the limit of quantification was measured in all samples. The average concentration of ochratoxin A in all positive samples was 10.4 μg/kg, while the maximum measured value was 34.3 μg/kg. the maximum measured concentration for zearalenone was 538 μg/kg in a sample from Banat, while the average concentration was 138 μg/kg.

Table 2. Occurrence of ochratoxin A in maize silage (moisture content 12%)

	Ochratoxin A μg/kg									
	Total		<LOQ		<250 μg/kg		>250 μg/kg		Range	Average
	N	%	N	%	N	%	N	%		
Bačka	15	31.2	5.0	10.4	10	20.8	0	0	4.4-25.5	13.0
Banat	20	41.7	1.0	2.1	19	39.6	0	0	2.6-34.3	11.2
Srem	13	27.1	1.0	2.1	12	25.0	0	0	2.6-22.9	6.5
Vojvodina	48	100.0	7.0	14.6	41	85.4	0	0	2.6-34.3	10.4

N – number of samples; <LOQ – below limits of quantification

Table 3. Occurrence of zearalenone in maize silage (moisture content 12%)

	Zearalenone μg/kg									
	Total		<LOQ		<4000 μg/kg		>4000 μg/kg		Range	Average
	N	%	N	%	%	N	N	%		
Bačka	15	31.2	0.0	0.0	15	31.2	0.0	0.0	44-520	154
Banat	20	41.7	0.0	0.0	20	41.7	0.0	0.0	51.9-538	155
Srem	13	27.1	0.0	0.0	13	27.1	0.0	0.0	40.5-161	90.5
Vojvodina	48	100.0	0.0	0.0	48	100.0	0.0	0.0	40.5-538	138

N – number of samples; <LOQ – below limits of quantification

DISCUSSION

As already said the average concentration of aflatoxin B1 in all analyzed samples of maize silage was 13.4 μg/kg, while the maximum value was 44 μg/kg at 12% moisture content. Garon et al. [25] measured aflatoxin B1 in concentrations ranging from 4 to 34 μg/kg (dry matter (DM) basis) in maize silage from Normandy (France). A study from Italy in 2004 pointed out that if silage contained more than 4 μg/kg of aflatoxin B1 it could be considered as dangerous for the nutrition of ruminants. In the analyzed samples, 22% of them contained more than 4 μg/kg [26]. A study from Hungary during 2012 and 2013 revealed that aflatoxin B1 occurred in maize silage in concentrations above the EU ML [27]. However, some researchers have found no samples of maize silage that contained aflatoxin B1 above the limit of detection [23,28,29]. Some of these studies can be explained by climate conditions which are not favorable for *Aspergillus* growth and consequently aflatoxin B1 production. Similar to this, researchers from Israel also did not find aflatoxin in maize silage [28].

Aflatoxin measured in silage could be originating from the pre and post fermentation process and its content increases during storage [30]. Aerobic exposure resulted in the accumulation of aflatoxin in whole crop maize silage [15]. Different ensiling practice (trench silos and silo bags) had effects on mycotoxin contamination [31].

The results from this research point that contaminated silage in the region of Vojvodina, Serbia can significantly contribute to the daily intake of aflatoxin B1 in dairy cattle. The silage process is under the control of the farmer, and the quality of the produced silage can differ significantly among farmers. Any of the numerous factors during the production of silage can be responsible for the increase in the occurrence of aflatoxin B1. In future, it is expected to be more problems with the occurrence of aflatoxin B1 in maize due to the climate changes [32], which means more problems with aflatoxin B1 in silage, as well. Moreover, it seems that the combination of the environmental factors (water activity, temperature and elevated CO_2) have little effect on the growth of *Aspergillus*, however, they do have a significant impact on the aflatoxin biosynthetic gene expression [32]. The results from this study point that there are differences in the occurrence of aflatoxin B1 in maize silage samples in Vojvodina. The climate conditions during the year are more or less very similar; however agricultural practices among farms are very different. Thus, by improving agricultural practices it is possible to influence the occurrence of aflatoxin B1 in maize silage.

It should also be mentioned that some researchers pointed out that in some cases even when aflatoxin B1 levels are within the regulatory limit for feedstuffs, aflatoxin M1 content in milk might exceed ML set by EU legislation [33,34]. This suggests more struggle for farmers to achieve the desired quality of milk.

Average ochratoxin A content in maize silage in positive samples was 10.4 μg/kg. Ingestion of diet containing up to 100 μg/kg ochratoxin A in the period of 28 days did not affected feed intake or milk production in cows, also it was not transferred from feed to milk [35]. Zearalenone concentration was up to 538 μg/kg. This concentration is below the ML set by Serbian regulation [18]. However, guidance value for zearalenone by EU is 500 μg/kg [36]. Moreover, it is known that zearalenone and DON co-occurr [23]. There is no concern about carrying-over from feed to milk regarding ochratoxin A and zearalenone on the base of these results. However, small concentrations of different mycotoxins can have effect osn productivity [37] and consequently profitability.

To some extend good agricultural practice, good storage practice, and the use of the HACCP principle can result in a successful approach to the challenges of climate changes and the occurrence of mycotoxins in food and feed [38].

Results of this study indicate that aflatoxin B1 in silage can be a significant source for milk contamination by aflatoxin M1. Namely, if a dairy cow is fed 1 kg DM of silage where an aflatoxin level of 44 μg/kg (12% moisture content) was determined, milk of that cow would contain 0.05 μg/kg of aflatoxin M1. Also, there is concern that other mycotoxins (even not as contaminants of milk and milk products), can affect the productivity of dairy cows and thus affect profitability.

There is a need for a greater awareness among farmers to which extent silage can be a source of mycotoxins, especially aflatoxin B1. Particularly, if it is known that good agricultural practice can reduce the problem to some extent. Also, there is a need for more research on this topic.

Acknowledgment

This research was financially supported by the Provincial Secretariat for Higher Education and Scientific Research, AP Vojvodina, Serbia (Project numbers: 142-451-2507/2017-02 and 142-451-2636/2017-01/03).

Authors' contributions:

GD project manager, established the design of the research, coordinated and help with draft. PHM carried out sample collection and drafted the manuscript. JI helped to draft the manuscript. KS and GD carried out immunochemical analysis, KS performed the statistical analyses. All authors read and approved the final manuscript.

Declaration of conflicting interests:

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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POJAVA AFLATOKSINA B1, OHRATOKSINA A I ZEARALENONA U KUKURUZNOJ SILAŽI NA TERITORIJI VOJVODINE, SRBIJE

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Silaža napravljena od cele biljke kukuruza je jedno od najpopularnijih kabastih hraniva u Srbiji. Konzumacija kukuruzne silaže po kravi može biti i do 30-35 kg/danu. U Srbiji u zadnjh nekoliko godina u centru poljoprivredne i šire javnosti su bila dva mikotoksina, aflatoksin B1 i njegov metabolit M1 zbog široke pojave kontaminiranog kukuruza koji je pogodio Balkan 2012 godine. Kukuruz se redovno proverava na pojavu aflatoksina B1 u njemu, međutim kabasta hraniva se često zanemaruju kao potencijalno mogući izvor mikotoksina u ishrani mlečnih krava.

U ovom radu, 48 uzoraka silaže cele biljke kukuruza je analizirana na prisustvo aflatoksina B1, ohratoksina A i zearalenona. Uzorci su prikupljeni iz tri regiona (Banat, Bačka i Srem) u Vojvodini. U svim uzorcima je utvrđen barem jedan mikotoksin iznad novoa kvantifikacije. Aflatoksin je detektovan u 36 (75%) uzoraka. U dva uzorka iz Banata utvrđena količina aflatoksina B1 premašivala je dozvoljenu koncentraciju prema važećoj zakonskoj regulative u Republici Srbiji (30 μg/kg, pri sadržaju vlage od 12%). U sedam uzoraka količina aflatoksina je premašivala 20 μg/kg, što je zakonska regulative u EU. Prosečna količina ohratoksina je bila 10,4 μg/kg, dok je maksimalna utvrđena količina bila 34,3 μg/kg. Maksimalna količina zearalenona je bila 538 μg/kg, dok je prosečna koncentracija zearalenona bila 138 μg/kg.

Rezultati ovog istraživanja ukazuju da silaža kontaminirana mikotoksinima u regionu Vojvodine, Srbije može značajno doprinositi dnevnom unosu aflatoksina B1 u ishrani mlečnih krava.