Detection of Aflatoxin B₁ in Fodders Correlated With the Level of Aflatoxin M₁ in Milk from Transylvanian Dairy Cow Farms

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Abstract

A number of 22 feedstuffs samples were collected from dairy cow farms in four Transylvanian counties and analyzed for mycological and mycotoxicological content. The mycological examination was performed by cultivation on Sabouraud medium. The quantitative identification of Aflatoxin B₁ from samples was performed by means of ELISA, a directly competitive immunoenzymatic test. Our purpose was to determine the mycotic load, to identify the Fungi genera in analyzed samples and to assess Aflatoxin B₁ level in fodders. We correlated the contamination degree of fodders with the level of Aflatoxin M₁ in milk, with and without mycotoxins inhibitors. A moderate and intense mycotic charge was found in a number of 15 samples. The concentrations of Aflatoxin B₁ in samples ranged between 1.13 and 92.92 µg/kg. The maximum level of Aflatoxin M₁ in milk samples was 160 ng/kg. The high level of Aflatoxin B₁ from analyzed samples was correlated with increased values of Aflatoxin M₁ in the milk of dairy cows in investigated areas. The removal of the contaminated fodders from the combined fodder and administration of mycotoxins inhibitors led to a decreased level of Aflatoxin M₁ in milk.

Key words
mycetes, mycotoxins, dairy-cow, Aflatoxin B₁, Aflatoxin M₁

INTRODUCTION

Aflatoxins are a group of mycotoxins produced by several species of Aspergillus, mainly A. flavus, A. parasiticus and A. nomius, which contaminate the feeds and fodders. They often occur in crops prior to harvest. Postharvest contamination can occur if crop drying is delayed, or during storage if water is allowed to exceed critical values for mould growth. Aflatoxin mycotoxins are toxic to humans and even more toxic to animals. They could produce cancer in humans and animals (Pitt, 2014). The aflatoxin family comprise about 20 similar compounds belonging to a group called difuranocoumarins, but only four are naturally found in foods. The most important ones are Aflatoxins B₁, B₂, G₁, G₂. Aflatoxin B₁ is considered the most toxic one. Lactating animals or humans exposed to food containing Aflatoxins B₁, B₂, G₁, or G₂ can secrete Aflatoxins M₁ (AFM₁) or M₂ (AFM₂) in milk. AFM₁ and to a lesser extent AFM₂ are the main metabolites, which represent the hydroxylated forms of the parent toxins. In the liver, AFB₁ is transformed into Aflatoxin M₁ (AFM₁), which is then excreted through milk (1-3%), as a part of the detoxification process (Masoero, 2009). The extent of transfer from feed to milk could vary widely between animals and between milking. Other factors include: nutritional factors, the date of the milking, milk production or other individual factors. A number of mycotoxin inhibitors, based on yeast cell wall, are used in farms in order to inactivate the mycotoxins in the gut (Di Natale, 2009).
MATERIALS AND METHODS

Feedstuffs (22 samples) were collected from dairy cow farms in four Transylvanian counties between January and August 2013 and analyzed for mycological and mycotoxicological content. Nine corn samples including HMC (high moisture corn), corn grits, kernels, 2 samples of corn silage, 3 samples of sunflower cakes, 4 samples of concentrated fodder for dairy cows, 1 sample of wheat bran, 1 sample of wheat kernel and 1 sample of milled dry Alfalfa were collected. The mycological examinations of analyzed samples were carried out by insemiinating them on sterile Petri dishes, 10 cm in diameter, two per each dilution, by introducing 1 ml suspension of each two dilutions, $10^{-4}$ and respectively, $10^{-5}$. The cultivation was performed on Sabouraud medium.

The samples were ground to 1.0 mm particle size. For mycological examination the samples were inseminated on solid Sabouraud medium and incubated at 24°C (by the STAS norms). The results were read at 3- and 5 days of incubation and were expressed as CFU/g product. Identification of prevailing fungi was carried out by stereomicroscopic examination of the cultural characters and by microscopic examination of the preparations on slides stained with Bleu Cotton. The quantitative identification of Aflatoxin $B_1$ from feed was performed by means of ELISA, a directly competitive immunoenzymatic test. Samples (5 g) were taken for each assay, processed by grinding and then submitted to extraction with 25 ml of methanol 70%. The extracts obtained were filtered through filter paper. Standards, assay extract and the mycotoxin conjugate with the enzyme were mixed and added in the buckets coated with antibodies.

After rinsing, the enzymatic substrate was added, thus the intensity of the blue colour that was obtained was inversely proportional with the mycotoxin concentration. After the addition of the stopping solution, the colour changed from blue to yellow, its intensity was measured by means of spectrophotometry with the help of a microplate reader, using a 450 nm filter. The optical densities (OD) of the samples were compared with those of the standards, and used for determination of mycotoxin concentrations in the samples. The interpretation of the results was performed according to the EC Regulations No. 1881/2006 and No. 1126/2007 regarding the limits of mycotoxins in fodder and food. The statistical analysis was performed using t student by Anova system in GraphPad InStat.

RESULTS AND DISCUSSIONS

Following the mycological examination, there have been found:
- Moderate mycotic charge in a number of 6 samples; the CFU/g product was comprised between 60 thousand and 200 thousand
- Intense mycotic charge in 9 samples; the CFU/g varied between 200 thousand and 1.100 thousand
- 7 samples contained <60 thousand CFU/g product

![Fig. 1 Apergillus flavus, Penicillium spp. (Sample 1: dilution $10^{-5}$)](image1)

![Fig. 2 Aspergillus niger, Aspergillus verzicolor (Sample 9: dilution $10^{-5}$)](image2)

According to the EU Mycotoxins Legislation (2006), the maximum level approved for Aflatoxin $B_1$ in all feed materials and complete feeding stuffs for dairy cows is 20 µg/kg. The maximum level accepted for Aflatoxin $M_1$ in milk is 50 ng/kg.
Sample number 1, HMC (high moisture corn) which goes into the structure of a TMR (total mixed ration) coming from a dairy farm in Bihor county, showed the highest level of Aflatoxin $B_1$, respectively 92.92 μg/kg (Tab.1, Figure 3). The value of Aflatoxin $M_1$ in the milk obtained from the cows fed with this TMR comprised values between 100-160 ng/kg, exceeding the maximum values admitted by the European legislation for Aflatoxin $M_1$ in milk, respectively 50 ng/kg. The fodder entering the structure of the TMR in this farm were represented by HMC, corn grits, corn kernels, sun-flower grits and corn silage.

Administration of mycotoxin inhibitors based on glucomanans like Mycosorb did not decrease the level of Aflatoxin $M_1$ in milk. Excluding HMC from the TMR and continuing to use the mycotoxin inhibitor determined a decrease in the level of Aflatoxin $M_1$ under the maximum limit. In another farm in Bihor County, milk obtained from dairy cows contained Aflatoxin $M_1$ level at 80 ng/kg. After analyzing the different fodder types in the TMR, it was determined that Aflatoxin $B_1$ concentration was 51.18 μg/kg in corn kernels. Administration of another mycotoxin inhibitor, respectively Agricell, containing both yeast derivatives and sodium hydrated aluminosilicate associated with the elimination of infested corn in the TMR led to a decrease in Aflatoxin $M_1$ level in milk (42 ng/kg), reaching EU standards. The analysis of 3 samples of concentrated fodder used in a dairy cow farm from Alba county, revealed Aflatoxin $B_1$ levels of 20.07, 25.44 and 27.06 μg/kg. These values were in the limit or slightly exceeding the admitted limit for Aflatoxin $B_1$ and led to an increase value of Aflatoxin $M_1$ in milk (Figure 4).

Tab. 1 Aflatoxin $B_1$ content in analyzed samples (μg/kg)

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of positive samples</td>
<td>100</td>
</tr>
<tr>
<td>Range</td>
<td>1.02-92.92</td>
</tr>
<tr>
<td>Mean</td>
<td>16.33</td>
</tr>
<tr>
<td>Median</td>
<td>10.16</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>± 20.56</td>
</tr>
</tbody>
</table>

Fig. 3 Aflatoxin $B_1$ level in fodder samples (μg/kg)
Statistical analysis of correlation between Aflatoxins B<sub>1</sub> and M<sub>1</sub> showed a linear correlation coefficient r = 0.9803, coefficient of determination r squared = 0.9610, and P = 0.0033, considered very significant. A study conducted in Brazil showed a contamination by AFM<sub>1</sub> in 10 (24%) samples of raw milk from the farms producing type B milk in the North of Paraná state, and 3 (7%) of the analyzed samples were above the maximum limit allowed of 0.5 μg/l (Sassahara, 2005). In another study performed in Spain, AFM<sub>1</sub> was detected in 94.4% (68/72) of whole UHT milk samples, in 2.8% (2/72) of yoghurt samples and not detected in cheese. The maximum level was detected in one yoghurt sample with 51.58 ng/kg, only this sample being over the legal EU limit of 50 ng/kg (Can-Sancho, 2010). In Serbia, AFM1 was detected in 98.7% of analyzed cow's milk samples in concentrations ranged from 0.01 to 1.2 μg/kg. A number of 129 (86.0%) cow’s milk samples contained AFM1 in concentration greater than maximum residue levels (MRL) of 0.05 μg/kg defined by European Union (EU) Regulation (Kos, 2014).

CONCLUSION
A large number of samples (50%) were found to have a higher than normal humidity level, which reveals a problem with the production, storage and handling of samples. The main mycetes found belonged to the Aspergillus and Penicillium genera. Results of the mycological examinaton performed on the analyzed samples have shown high and very high mycotic loads in 68 % of all samples. Aflatoxin B<sub>1</sub> was detected in all analyzed samples, with values between 1.02 and 92.92 μg/kg, average content being 16.33 μg/kg. Administration of glucomannan-based mycotoxin inhibitors in fodders with high Aflatoxin B<sub>1</sub> levels did not decrease Aflatoxin M<sub>1</sub> levels in milk. Addition of glucomannans and aluminosilicate-based mycotoxin inhibitors, associated with the elimination of Aflatoxin B<sub>1</sub> infested fodders eventually dropped Aflatoxin M<sub>1</sub> levels under the maximum permitted limit. In situations where Aflatoxin M<sub>1</sub> levels were well above the maximum limit of 50 ng/kg, reaching up to 160 ng/kg, the administration of mycotoxin inhibitors had no effect, making the Aflatoxin B<sub>1</sub>-infested fodder elimination the only solution for dropping Aflatoxin M<sub>1</sub> in milk.

REFERENCES
