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MYCOTOXIN CONTAMINATIONS IN COMMERCIALY USED HAYLAGE AND SILAGE

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Abstract

Improvement of sanitary control of grass fodder, taking into account the whole variety of factors that have a negative impact on the animal, is the most important task of agricultural science. In the present study, we investigated mycotoxin contamination of haylage and silage by the method of indirect competitive enzyme-linked immunosorbent assay. In a survey of 30 commercial feed batches from the livestock farms located in the central regions of the European Russia, namely Bryanskaya, Lipetskaya, Moskovskaya, Smolenskaya and Tverskaya provinces, a multiple contamination pattern was shown. In all the samples eight or more mycotoxins were found, and more than half of samples contained 14-15 components. Alternariol was found in all samples of the both types of feeds in amounts from 50 to 1260 µg/kg, while aflatoxin B₁, ochratoxin A, citrinin and ergot alkaloids had extensive distribution with low intensity. In haylage with high incidence of all analyzed fusariotoxins, mass concentration of T-2 toxin was the lowest (4 to 30 µg/kg), and the levels of diacetoxyscirpenol, deoxynivalenol, zearalenone and fumonisins were in the range of 100-1000 µg/kg. Sterigmatocystin, emodin, cyclopiazonic and mycophenolic acids, PR-toxin occurred everywhere, wherein emodin, cyclopiazonic acid and PR-toxin often present in amounts up to 1000 µg/kg. Silage prepared mainly from corn grass, with frequent detection of T-2 toxin and deoxynivalenol in significant content (to 350 and 2820 µg/kg, respectively) revealed similarities with corn grain from the center of European Russia. Sterigmatocystin and emodin were detected in all silage samples, whereas PR-toxin, cyclopiazonic and mycophenolic acids were slightly inferior to them in frequency. Levels of accumulation of these mycotoxins were lower than those found in silage, and for mycophenolic acid they generally remained the same. A botanical composition of herbage and features of toxin-producing fungi complexes, accompanying the growing season and the subsequent process of fermentation, are discussed among possible reasons for the differences in mycotoxin contamination of haylage and silage.

Keywords: haylage, silage, mycotoxins, immunoassay.

Haylage and silage are very close to green fodder in biological value and widely used in animal husbandry. In fact, due to haying during plant growing season the animals are provided with grass feeding at indoor housing. Haylage is mainly prepared from mixed legumes and cereals. At preservation the grasses are chopped, cured, pressed and kept in CO₂ and N₂ evolved by plant cells. By that a high degree of preservation may be achieved on fodder nutrients, particularly proteins and carotene. At ensilaging the corn plants at stages of milk to wax or wax ripeness of grain as well as legumes and cereals or their mix with corn are subjected to lactic fermentation after chopping, pressing, treating with preservative chemical additives and then sealing to keep without air access. Each technological step must be in strict accordance with the approved protocols to provide a high sanitation quality of haylage and silage (1).

Annual or perennial plant type, the terms of sawing and mowing and application of fertilizers are usually considered to estimate the suitability of raw material for preservation (2), and we recognize the absolute importance of these factors. Nevertheless, due attention also should be paid to initial plant contamination with mycotoxins formed in field conditions, as well as their change during feed storage.

It is known that vegetating plants are in contact with phytopathogenic and parasitic fungi, whereupon a mycotoxin contamination can often occur. During fermentation of plant biomass and its further storage at anaerobic conditions a specific mycobiota appears which consists of active toxin-producing species (3-5). Besides, at air access because of improper storage or removing the mold is developed on the surface or in deep layers of herbal substrate that often leads to animal poisoning (6).

Safe use of green fodders was mostly considered in view of definite groups of toxic fungal metabolites, such as fusariotoxins in corn silage (7), or detection of extremely dangerous substances, particularly mycophenolic acid, cyclopiazonic acid, alternariol (8-10). However, the high risks for multiple mycotoxin contamination are increasingly reported in recent years (11).

In the present study we carried out a mycotoxicologic survey of commercial haylage and silage batches from livestock farms based on assay of toxic metabolites produced by fungi which can grow both on plants during vegetation and on herbal substrate preserved.

Technique. A total of 30 commercial batches of haylage and silage were tested, including 9 probes of haylage and 14 probes of silage sampled in 2011-2012 on the livestock farms of Bryanskaya Province (Bryanskii, Zhiryatinskii, Kletnyanskii, Pochevskii, Trubchevskii regions), and the rest received in 2006-2012 from the livestock farms located in Lipetskaya, Moskovskaya, Smolenskaya and Tverskaya provinces. The batches differed in terms of harvesting and storage. The haylage was made from alfalfa, cereals and grass mix (5, 2 and 4 batches, respectively, a total of 11 batches), and the silage was prepared from corn, grasses and the mixes of corn and grasses (8, 2 and 9 batches, respectively, a total of 19 batches). For each batch an average sample was tested.

A total of 15 mycotoxins, namely aflatoxin B₁ (AB₁), T-2 toxin (T-2), ergot alkaloids (EA), sterigmatocystin (STE), ochratoxin A (OA), mycophenolic acid (MPA), citrinin (CIT), alternariol (AOL), zearalenone (ZEN), deoxynivalenol (DON), emodin (EMO), fumonisins (FUM), diacetoxyscirpenol (DAS), cyclopiazonic acid (CPA), PR-toxin (PR), were analyzed according to the procedure described in our previous publication (12). Samples were air-dried, chopped and placed into tubes. The acetonitrile-water mixture (84:16) was added at 1:10 (w/v) ratio of a sample to extragent, and the tubes were shaken. After 12-14 hour extraction they were shaken again, and then the samples were 1:10 diluted with buffer and used in indirect competitive enzyme-linked immunosorbent assay (ELISA).

Results. Our data indicate (Table) that the frequencies of T-2, DAS, DON, ZEN and FUM peculiar to fusariosis agents in legumes and cereals were high in tested haylage and comparable to those in hay from the same areas (12). Among fusariotoxins, the T-2 accumulation was the lowest and much lower compared to hay. In the T-2 positive samples its content was not more than 30 µg/kg and mostly ranged from 4 to 10 µg/kg (see Table), while in hay it varied from 5 to 2240 µg/kg at an average value of 450 µg/kg (12). Besides, in haylage the accumulation of DON and ZEN above a significant level of 1000 µg/kg was not found (see Table), while in hay it, though rare, was detected. The observed peculiarities may result from a botanical composition of herbage and different time before plant harvesting, a partial hydrolysis of the toxins long exposed to acidic conditions at pH 4.4-5.6 in plant biomass, and also from toxin biotransformation by microaerophilic fungi (13).

Other studied mycotoxins were found almost in all tested haylage samples (see Table). The AOL and EMO contamination patterns remained the same as in hay. Probably, the frequency of AOL and EMO producing fungi is the

same in herbage of cereals and legumes. Actually, alternariosis with *Alternaria tenuis* is described for oats and peas (14), and *Cladosporium*, the EMO producing fungi (15), as well as *Alternaria* can develop on different plants (16). Besides, the similarities were revealed in OA and CIT contamination which not exceeded 100 µg/kg in all positive samples, and in AB₁ ranged from 2 to 9 µg/kg, while the clear differences were found for STE, CPA, PR, MPA and EA.

Mycotoxin frequency and content in Haylage and silage samples from the livestock farms located in European Russia

Mycotoxins	n ⁺						Minimal—average—maximal mycotoxin content, µg/kg
	totla/n	mycotoxin content, µg/kg					
		< 10	≥ 10	≥ 100	≥ 1000	≥ 10 000	
H a y l a g e							
T-2	11/11	7	4	—	—	—	4—13—30
DAS	10/11	npe	npe	10	—	—	100—245—420
DON	9/11	npe	—	9	—	—	125—220—330
ZEN	9/11	npe	7	2	—	—	25—135—630
FUM	5/11	npe	—	5	—	—	125—180—245
AOL	10/10	npe	—	9	1	—	110—545—1260
STE	11/11	—	7	3	1	—	20—185—1000
EMO	11/11	npe	—	3	5	3	270—8160—31600
CPA	11/11	npe	npe	3	8	—	130—2480—8900
OA	9/11	—	9	—	—	—	10—23—30
CIT	10/11	npe	8	2	—	—	40—82—160
MPA	11/11	npe	1	10	—	—	35—330—840
PR	9/11	npe	npe	6	3	—	100—660—1500
AB ₁	9/11	9	—	—	—	—	2—4—9
EA	10/11	1	5	4	—	—	6—105—420
S i l a g e							
T-2	19/19	2	9	8	—	—	8—95—350
DAS	11/19	npe	npe	11	—	—	165—265—490
DON	17/19	npe	—	6	11	—	100—1270—2820
ZEN	16/19	npe	10	6	—	—	25—135—740
FUM	15/19	npe	1	14	—	—	80—350—960
AOL	16/16	npe	4	12	—	—	50—315—860
STE	19/19	—	16	3	—	—	10—85—420
EMO	16/16	npe	1	11	4	—	70—1075—5010
CPA	17/19	npe	npe	8	9	—	170—1200—3160
OA	14/19	1	13	—	—	—	9—17—22
CIT	14/19	npe	12	2	—	—	40—75—110
MPA	15/17	npe	5	9	1	—	30—335—2000
PR	12/19	npe	npe	12	—	—	100—310—625
AB ₁	11/19	11	—	—	—	—	3—4—6
EA	13/16	5	6	2	—	—	4—50—270

C o m m e n t s. Aflatoxin B₁ (AB₁), T-2 toxin (T-2), ergot alkaloids (EA), sterigmatocystin (STE), ochratoxin A (OA), mycophenolic acid (MPA), citrinin (CIT), alternariol (AOL), zearalenone (ZEN), deoxynivalenol (DON), emodin (EMO), fumonisins (FUM), diacetoxyscirpenol (DAS), cyclopiazonic acid (CPA), PR-toxin (PR), Dashes mean no positive probe found; *n* — tested probes, *n*⁺ — positive probes; npo — no possible estimation.

A decreased EA level in haylage is probably due to low rate of cereals pre-disposed to development of ergot and specifically colonized by endophytes, however, a mechanical removal of ergot sclerotia during prestorage treatment should not be underestimated. The STE, CPA, MPA and PR were extensively distributed with the above 1000 µg/kg levels of the CPA and PR often detected. A higher STE, CPA, MPA and PR levels may result from activity of microaerophilic fungi able to grow during preservation. Unfortunately, no data about grass haylage microbiota are currently available. In all haylage samples 8 or more mycotoxins were found and more than half of samples contained 14-15 components.

For silage the same frequencies of mycotoxins were characteristic (see Tabke). In all samples 8 or more mycotoxins were found and 9 samples contained 14-15 toxic metabolites.

According to the batch certificates attached, 2 and 8 of 19 silage batches were made from grasses and corn, respectively, and the rest of batches contained corn as a very easy crop to ensile. It is also indicated by frequent occurrence of FUM as well as other fusariotoxins, including T-2, DAS, DON and ZEN.

Fusarium are known to attack lower parts of corn stems since initial stages of plant development, and after flowering the entire colonization of stems occurs resulting in browning lower and then the upper stem nodes. A long term investigations showed the infection to be continued during plant vegetation at 10 % level (17). *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. sporotrichioides*, *F. crookwellense*, *F. oxysporum*, *F. sambucinum* var. *coeruleum* are considered the most frequent causative agents for stem rots, and *F. graminearum* and *F. moniliforme* Sheldon, recently attributed as *F. verticillioides* (Sacc.) Nirenberg, are also found (17).

According to fusariotoxin contamination patterns, the highest frequency of T-2 and somewhat less frequencies of DON, FUM and ZEN indicate a similarity between silage and corn grain from the center of European Russia (18-20). Unfortunately, a comparative study on DAS is still not possible. High ZEN levels in silage were not shown. Perhaps ZEN was not significantly accumulated in the raw material. As reported by P. Lepom et al. (21), the amount of ZEN remained unchanged at ensilaging during 12 weeks of observation.

AOL, a toxin of *Alternaria* fungi, was detected in all silage batches tested. As reported by M. Muller (22), in corn before harvesting a prevalence of *Alternaria* on leaves and cobs is quite significant, accounting for 18 % of the total mycobiota. Most *A. alternata* and *A. tenuissima* isolated strains can produce AOL in vitro, however, the toxin was not analyzed directly in the silage samples the fungi were isolated from (23).

Frequency and accumulation of the rest mycotoxins are obviously determined by fungal colonization of herbage and ensilaging conditions. STE and EMO presented in all tested samples with no exception, and MPA and PR were quite frequent. However, there were no the same high rates as revealed in haylage. CPA contamination in silage was rarer then in haylage, but of the same content rang. MPA contamination remained generally the same, but in one sample reached 2000 µg/kg (see Table). MPA have been found in 32 % silage samples in Germany (24). *Penicillium roqueforti* (24) and *Byssosclamyces nivea* (8) are suggested as candidate MPA-producing fungi.

OA and CIT contamination in the silage were the same as in the haylage (see Table). Quite possibly, they are synthesized by *Aspergillus ochraceus*, as its isolates can produce OA (5), and *Monascus ruber*. In the strains of *A. ochraceus*, isolated from silage in Moscow Province a capability to produce AO has been shown (5), and *M. ruber* producing CIT has been identified in 16 % corn silage and 21 % grass silage samples (24). In 13 samples of 16 tested EA was probably produced by definite *Aspergillus* species and subspecies (25), as well as by *Claviceps purpurea* from occasional herbage components. Particularly, quitch and annual bluegrass predisposed to ergot development are the most common weeds in corn crops in Germany (17).

The final stage of ensilaging was detailed studied for past few decades. Despite of clear differences in mycobiotes of silages due to year-to-year effects, region and technology of preparation, three microaerophilic fungi are shown to predominate, namely *Penicillium roqueforti*, able to grow at pH 3 in 80 % CO₂ and 4-5 % O₂, *Aspergillus fumigatus*, and *Monascus ruber* which can develop at pH 2.5 and 3.5 % lactic acid concentration (26).

Importantly, these fungal species can produce a combination of toxic metabolites. Particularly, *P. roqueforti* produces MPA and PR (8, 27), and *A. fumigatus* produces EMO, CPA and EA (9, 28). Besides, they can compete (26). A mycological study of silages from the livestock farms located in Moscow Province indicated their successful development under anaerobic conditions and also uneven distribution in silage (5). *M. ruber* and *A. fumigatus* contaminate entire vol-

ume, being more characteristic for deeper and surface layers, respectively, while *P. roqueforti* which was found in 22 % samples prevailed in deep layers and close to the walls being rarer on the surface. Therefore, the mycotoxin contamination may differ depending on the part of the silo.

At the same time an influence of plants as main raw materials for haylage and silage preparation on feed contamination is still remains poor studied. Nevertheless, a few available data indicate relevance of such a research. Recently in a plot field experiment with rye grass and two festulolium species the clear dependence of T-2, DON and ZEN levels on harvesting terms was shown. Besides, their sharp increase occurred after the silo sealing at short initial anaerobic phase of ensilaging when the temperature rose (29).

In Russia both natural grasslands and crops such as corn, clover, alfalfa, vetch, oats, timothy grass, hedgehog, or their mixes are traditionally used for preservation. Besides, sunflower, topinambour, soybean, sorghum, galega, peculiar in their biochemical composition and relationship with micromycetes are also involved. Unfortunately, these extremely important fodder crops have not been yet mycotoxicologically assessed. It necessitates their step-by-step multi-level research ranged from plot field experiments to a wide monitoring in the regions of developed fodder production.

Thus, by a mycotoxicological survey of commercial haulage and silage batches first performed in Russia a multiple contamination pattern and accumulation of mycotoxins have been shown at the levels that can present a serious threat to and cause concern over the animal health. Further studies will contribute to prophylaxis of mycotoxicosis in domestic animals whose diet is based on preserved grass feeds.

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