APPRECIATIONS REGARDING THE EFFECT OF SOME FUNGAL MYCOTOXINS ON DAIRY COWS FORAGE

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ABSTRACT

The contamination of the forage crops with mycotoxigenic fungi before and after harvesting is a world problem. Most of the species producing mycotoxins are filamentous ascomycetes, basidiomycetes or deuteromycetes. Aspergillus, Fusarium and Penicillium are the most important types.

By analysing the proportion of different types of fungi in the 7 analysed forage samples, we found that 100% of the samples were contaminated with Aspergillus spp., 57,14% with Penicillium spp, 42,85% with Cladosporium spp. And Mucor spp., 44,28% with Rhizopus spp. The Aspergillus and Penicillium species were found on maize grains and sunflower seeds. There were 7x103 of them per forage gram.

In general, we isolated more than 50 fungi species from the cereal seeds. It is worth to note that barley, sunflower meal and peas had fungal contamination of 2x103, 2x103 and 1x103 per forage gram and they respect the maximum limits of 5x103 colonies per forage gram.The great effect of 100% of the Aspergillus species and of 57,14% of the Penicillium species, both of them having mycotoxigenic potential, suggests that these forage samples should be further researched due to the natural presence of mycotoxins

INTRODUCTION

According to FAO data (2001), about 20% of the food is mycotoxin contaminated. Within an assessment made by Pittet (1998), the presence of mycotoxins was found as 40% in 25.000 food and concentrate forage samples in 30 countries. Cereal grains and nuts were the most affected ones, as they are the ingredients having the highest risk for the human food chain and for monogastric animal chain.The situation is much more complicated for ruminants whereas their rations contain both volume forage and concentrate forage and thus multi-contamination may take place (Cola M., Cola F.,2013).

The contamination of forage crops and of the forage ensiled with toxic fungal metabolites and their consequences were ignored in the past. The pastures may be infested with: *Clavicespurpurea*, which is responsible for ergotism; *Pithomyceschartarum* produces sporidesmin which causes facial eczema; *Neotyphodium,* which causes dry gangrene and *Rhizoctonia* (slaframine producer) induces sialorrhea in ruminants (Abarca M.L., 2001).

Hay may be contaminated by the fungi of three ecological types: field fungi during the pre-harvesting phase, intermediary fungi during the harvesting phase and storage fungi (warehouse)(Cola M., Cola F.,2013).

The intermediary fungi and the storage fungi are completely missing in the hay harvested and stored in dry conditions, but they become numerous and diverse in incompletely dry hay. Both the hydrophilic and heat-tolerant species such as *A. fumigatus* which produces gliotoxin and *Stahybotrysatra* which produces satra, G and H toxins by inducing stahibotriotoxicose, are predominant in hay that is harvested and stored in humid conditions.In theory, preserving forage as silo or in hay close systems is safe regarding the mycotoxin risk.

The lack of oxygen during the first phase of forage ensiling completely inhibits the growing of the *Fusaria* species (Damaglou et al., 1984; Lepom et al., 1988).

There are fungi, *A. Fumigatus*, or some *Penicillium* species which tolerate the partial lack of oxygen, and others, such as *P. varioti*, *P. roqueforti* which are indifferent to the presence of oxygen. These fungi resist in silos, and then they develop after the opening or when the anaerobiosis is not complete.
Organic acids such as formic acid, propionic acid or butyric acid have antifungal properties.

Within an assessment made in France and Italy in three successive years, Pelhate (1975) analysed 1230 silo samples for the presence of 70 species of fungi. The incidence of *P. roqueforti*, *Byssochlamys*, *Monascus*, *Aspergillus* and *Paecilomyces* was 76%, 41%, 31%, 21% and respectively 27%.

We are not certain about what happens in silos to the mycotoxins derived from field fungi. Some studies say that DON stays constant, while ZEN and AFB_1 go down during storage (Kalac and Woolford, 1982).

Many species of fungi produce the mould formation in cereal grains and they are unspecificThe fungi associate to the cereal grains both on the field and during transportation and storage, when the environmental conditions (humidity and temperature) are favourable (Bonciu,E.2020).
Insect-caused destructions predispose cereal grains to fungal colonisation. Insects may be spore vectors or may create lesions, allowing fungi to access the endosperm (Bonciu,E.2020)

The genetic improvement of cereal grains regarding the insect resistance makes the cereal grain consumption safer (Munkvold et al., 1999). *Fusarium, Gibberella, Penicillium, Aspergillus, Cladosporium* and *Clariceps* are among the most prevalent fungi before cereal harvesting. After harvesting, these fungi resist in the rests left on the field and the resulted spore contaminate the soil. Thus, the crop rotation is a method to reduce the risk of mycotoxin contamination of cereal grains(Bonea,D.,2013).

The infections caused by contaminated soil may be diminished by cutting cereal plants 7-19 cm from the soil when harvesting. Also, cereal humidity should be reduced under 15% in 48 hours from harvesting. The good air circulation in cereal silos is beneficial because it achieves grain drying and prevents temperature from raising. The mould formation in grains happens in dispersed areas in the silo(Bonea,D.,2020).

Cereal grains are considered to be the main mycotoxin vector in food or forage (Pfohl-Leszkowicz, 2000).

These researches aim to establish, in the first place, the most important fungi producing mycotoxins in dairy cows forage.

Toxicogenesis or mycotoxin elaboration is a complex phenomenon. It is well known that the presence of mycotoxigenic fungi in food products or forage does not also indicate the production of these mycotoxins. Mycotoxin biosynthesis is tightly regulated and depends on the environmental conditions: the growing substratum, the pH, the water activity, the temperature. This suggests that, for a complete assessment of the mycotoxicological status of a type of food or forage, detecting the mycotoxigenical fungus is not enough, but we also need to know its ability to activate its genes of mycotoxin biosynthesis in the environmental conditions suitable to the food or forage chain.

MATERIAL AND METHOD

The researches carried out between 2020-2021 at the Fenov dairy cow farm from Robanesti, Doljcounty, based on forage samples.
For the extraction, we chose two ways, either by means of a high-speed blender with solvents for a few minutes, or by shaking with solvents starting from 30 minutes and up to 2 hours. Different solvents are used for the extraction (table 1).

Choosing the solvent is usually a compromise between the solvent strength needed for the efficient extraction of toxins and its compatibility with the analytical testing system. Once the solid sample to be analysed was mixed with the extraction solvent, the liquid was separated from the solid fragments by means of filtration or centrifugation. The resulted extract was either further cleansed in order to isolate the mycotoxins, or directly applied according to the procedures.

Solvent systems used to extract mycotoxins

Table 1

Mycotoxin	Sample to be analysed	Solvent system
Aflatoxin B_1	Forage	Chloroform, methanol-water
Aflatoxin M_1	Cheese	Acetone-water(86:14)
Deoxynivalenol	Cereals	Acetonitrile-water(85:15)
Trichotecenes	Cereals	Organic solvents
Zearalenones	Fermented maize	Acetone
Patulin	Food	Ethyl acetate

Once the mycotoxin was extracted from the solid matrix, the liquid extract is cleansed in order to remove the impurities before determination or quantification. Many “screening” methods such asELISA do not need a cleansing different than diluting the extract and/or filtering before the analysis.

The methods used for the qualitative and quantitative determination of mycotoxins were thin-layer chromatography (TLC) and the ELISA technique.

The thin-layer chromatography (TLC) consists of a chromatographic separation on a stationary phase looking like a layer of about 0,25 mm, placed on a plaque made of an inert material whose sizes are 5 x 20 cm, 10 x 20 cm or 20 x 20 cm.

The ELISA technique is based on the interaction between specific antibodies and the mycotoxin of interest. The mycotoxin detection is achieved by means of some colour reactions whose intensity is measured by means of a spectrophotometer.

The fungi-infected material was examined in the forage control laboratory within DSVSA Dolj, and in order to ease the identification of the microorganism, the materials submitted to analysis were kept in optimal temperature and humidity conditions in order to create development and fruition conditions for the fungus.

The material that we used was firstly washed and disinfected on the surface in order to remove the external flora, which would have developed abundantly, stopping thus the externalisation of the real pathogen agent that could have been found inside tissues.

The pathogen material was placed in Petri dishes, crystallizers and desiccators, on whose bottom we placed filter paper soaked in sterile water.

The temperature needed for the development was provided by introducing the dishes in the thermostat (24-26 ^{o}C), or by leaving them in the laboratory at room temperature (20-22 ^{o}C). After developing the mycelium, the fungi identification was made either directly under the microscope, or by means of

isolation on a suitable environment. The fungi isolated on a nutritional environment are named “isolated elements”.

RESULTS AND DISCUSSIONS

The following forage types were submitted to be controlled: alfalfa, concentrate mix of maize grains and sunflower seeds, sunflower meal, barley grains, combined forage, peas and wheat grains (table 2)

By analysing the proportion of different types of fungi in the 7 forage sampleswe found that 100% of the samples were contaminated with Aspergillus spp., 57,14% with Penicilliumspp,42,85% with Cladosporium spp. and Mucor spp., 44,28% with Rhizopusspp (figure 1).

Fungal contamination starts on the field before mowing. The surface of the leaves and of the stems is mainly covered with bacteria which protect the plants from mycotic infections and yeasts that seem to have a protection part against the effects of visible light.

After mowing, the water content decreases fast and the competition between bacteria and yeasts diminishes. In these conditions, some new species of bacteria, yeasts and many moulds begin to multiply on the plant exudates during the drying process. The faster they dry, the least is lost from the dry substance of the hay because of these fungi. In this phase, there are about 10 fungus types that prevail. While hay is placed into bales, a new group of microorganisms (especially fungi) begins to multiply, especially if the hay has 20-30% humidity. These fungi, named “storage fungi” mainly belong to Aspergillus, Fusarium and Penicillium species

Table 2

The fungal contamination level and the types of fungi isolated from forage

Nr. crt.	Types fourage	Fungal contamination						Nr. drojdiișimuce gaiuri/g
		Aspergillus spp.	Penicillium spp.	Cladosporium spp.	Rhizopus spp.	Mucor spp.	Rhodotorula spp.	
1	Alfalfa *	+	+		+	+		7x103
2	Concentrate mix of maize grains and sunflower seeds **	+	+					7x103
3	Sunflower meal *	+	+	+			+	2x103
4	Barley grains *	+				+		2x103
5	Combined forage ***	+	+	+			+	17x103
6	Peas *	+				+		1x103
7	Grains ***	+		+			+	12x103

(+ presence of the fungus type)

*** samples having a fungi charge/g over 10x103

** samples having a fungi charge/g between 6 x 103 and 9x103

* samples having a fungi charge/g up to the maximum admitted limit of 5x103

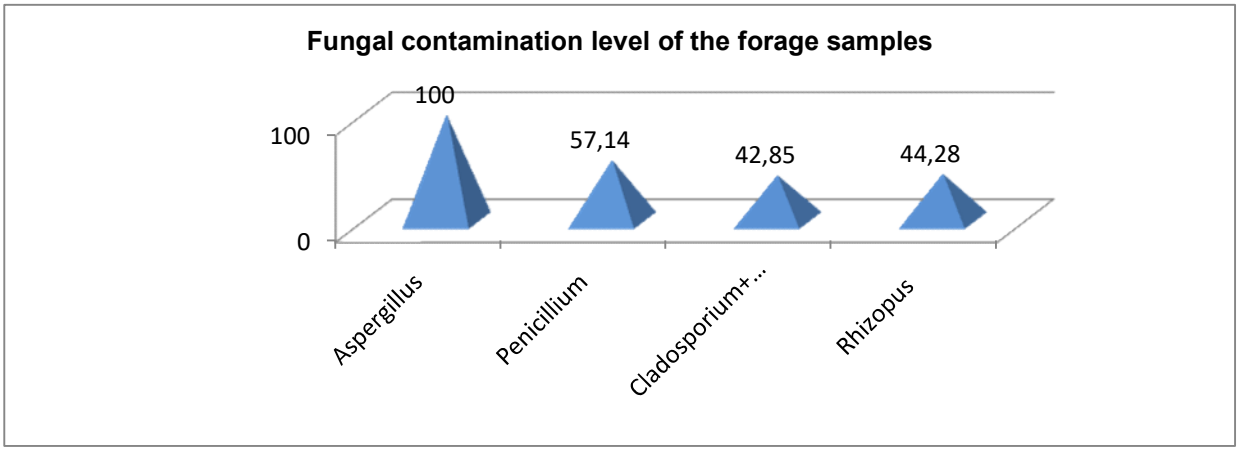

Figure. 1. Fungal contamination level of the forage samples

In the alfalfa made at S.C.FENOV DOLJ in 2020 and analysed in March 2021, we found the following fungus species: Aspergillus, Penicillium, Rhizopus and Mucor. Their number was $7x10_3$ per gram, and the maximum admitted limit is $5x10_3$ per gram. Some researchers also determine the total amount of fungal biomass. In order to do that, they measure the components of the fungal cell wall (glucosamins or chitines).

According to these tests, hay contains, in the best conditions, 1-2% of total fungal biomass.

A glucosamine unit represents almost 10 units of fungal biomass. Strongly moulded hay has over 10% fungal biomass.

Such a contamination shows that the hay was placed into bales at a much higher humidity. In such situations, the temperature inside the hay bale increases very much and, thus, the proteins degrade and there is a massive increase of mycotoxigenic fungi able to produce mycotoxins.

The Aspergillus and Penicillium species were found on maize grains and sunflower seeds. Their number was $7x10_3$ per forage gram.

Generally, over 50 fungus species were isolated from the cereal seeds.

The seed fungi classify into “field” fungi and “storage” fungi. The “field” fungi invade the seed during its maturation and development before harvesting. The specific species are Alternaria, Helminthosporium and Fusarium.

The “storage” fungi are those fungi developing on stored seeds. The main species are Aspergillus and Penicillium.

The infection incidence and the severity of storage fungi depend on numerous factors, including here the storage temperature, the grain humidity, the relative air humidity and the cereal mass, the mechanical destructions during harvesting or handling, the present species and the level of fungal infections during the pre-harvesting phase.

Generally, maize that is harvested late in the autumn and kept in humidity conditions for a long time determines the mould formation in grains.The species isolated from sunflower seeds were: Aspergillus and Penicillium.

It is noteworthy that barley, sunflower meal and peas had fungal contamination of $2x10_3$, $2x10_3$ and $1x10_3$ per forage gram and are within the maximum admitted limits of $5x10_3$ colonies per forage gram.

The highest mycotic charge belonged to the combined forage of $17x10_3$ colonies per gram.

The analysed combined forage is a locally prepared forage containing a mixture of cereal grains, peas and sunflower meal.

Wheat, the main component of combined forage, had very high fungal contamination of $12x10_3$ colonies per gram. The analysed wheat resulted from conditioning wheat for seed and it is generally a thin, fragile, parasitized type of wheat.

The species of isolated fungi were: Aspergillus, Cladosporium and Rhodotorula.

Cladosporium produces cereal darkening. The attacked plants have a darker colour. There are black spots on the plants representing the fungus sporulation. The attacked grains become more fragile.

Rhodotorula is a yeast species.

This yeast is oxidative and it may synthesize carotenoid pigments, changing the colony into a brick red colour.

The Aspergillus species were dominant in the 7 analysed forage samples, with 100% incidence.

Harley grains had a fungal charge of $2x10_3$ colonies per gram. The isolated species were Aspergillus and Mucor.

The Mucor spp. colonies are typically white or beige and their height increases with a few centimetres.

Table 3

Incidence of fungal contamination of the investigated forage samples

Number of fungus colonies/ gram	Number of samples	Incidence %
1x103	7 / 1	14,28
2x103	7 / 2	28,58
7x103	7 / 2	28,58
12x103	7 / 1	14,28
17x103	7 / 1	14,28

Table 4

The mycotoxin content in the forage samples

No.	Forage type	Content of total aflatoxin	Content of ochratoxin A (OTA)
1	Alfalfa	Under the Kit detection limit	0,0088 mg/kg ± 0,0018 mg/kg
2	Mixture of maize and sunflower	Under the Kit detection limit	0,0014 mg/kg ± 0,0003 mg/kg
3	Sunflower meal	Under the Kit detection limit	0,0003 mg/kg ± 0,0007 mg/kg
4	Barley	Under the Kit detection limit	0,0016 mg/kg ± 0,0003 mg/kg
5	Combined forage	Under the Kit detection limit	0,0006 mg/kg ± 0,0013 mg/kg
6	Peas	Under the Kit detection limit	0,0013 mg/kg ± 0,0003 mg/kg
7	Wheat	0,0018 mg/kg ± 0,0003 mg/kg	Under the Kit detection limit

In the forage coming from the cattle farm of S.C.FENOV DOLJ, the content of total aflatoxin was under the maximum admitted limit in dairy cows forage of 5 ppb (0,005 ppm). It is extremely important to consider the fact that this forage is also consumed by dairy cows and it may cause diseases or may make milk unsuitable for consumption.

Regarding the content of ochratoxin A, all the analysed sampleshave a content of ochratoxin A under the maximum admitted limit (3 ppb).

Combined forage and especially their ingredients have been excellent environments for fungus growth.

The high incidence of Aspergillus species did not determine a high incidence of total aflatoxin (aflatoxins B_1, B_2, G_1 and G_2).

Ochratoxin A is a nephrotoxic, hepatotoxic and teratogenicmycotoxin produced by the Aspergillus and Penicillium species. The content of ochratoxin A in the investigated forage was between 0,0 µg/kg and 8,8 µg/kg.

Knowing closely the ecology of the fungi contaminating the crops with mycotoxins may be the key for an improved management of solving the problems caused by these mycotoxins. The production area of the analysed forage contains a diverse agricultural system with complex rotation of the crops. The Aspergillus species are largely dispersed into agriculturally used and unused soils and they are associated to the decomposition of the vegetal debris from where they migrate to the crops.

Being able to reproduce in any ecological niche, the Aspergillus species use a large variety of natural substrata. The extension of these atoxigenic stems on soils and on crops shows that they are well adapted to the environmental conditions. This suggests that atoxigenic stems may compete with toxin-producing stems and the result could be the

removal of the toxin producers and the decrease of the aflatoxin contamination.

The aflatoxin-preventing technologies based on using atoxigenic stems of A. flavus or A. parasiticus have already been elaborated for some crops. Atoxigenic stems are considered biopesticides

CONCLUSIONS

The yeast and mould contamination has crossed the maximum admitted limit of $5x10^3$,in over 57,14% of the analysed samples.

In the forage coming from the cattle farm, the aflatoxin content was under the maximum admitted limit in dairy cows forage of 5ppb(0,005ppm).

Regarding the ochratoxinA content, all the analysed samples have a content of ochratoxin A under the maximum admitted limit (3ppb).

It is noteworthy the fact that some samples may show a low charge of fungi and a high content of mycotoxins, but there is also the possibility for a product to present a high number of yeasts and moulds/g and a low content or even the complete absence of mycotoxins.

Monitoring the sanitation of food and forage from the mycological and mycotoxicological perspective is an important way to prevent disease both in humans and in animals.

There is not enough awareness regarding the mycotoxin impact on human food compared to the effects of additives, pesticides, heavy metals or microbial agents.On the other hand, the diseases produced by mycotoxins rarely manifest in acute forms, thus their real action on human organism or on animal organism is less obvious compared to the action of other contaminating agents. Mycotoxins may penetrate the human organism by food – cereal, seeds, spices, fruit, beverage, coffee and indirectly by products obtained from contaminated animals – milk, meat, eggs and their by-products.

BIBLIOGRAPHY

1. **Abarca M.L**., 2001. Current importance of ochratoxinA producing *Aspergillus* spp., Journal of food protection, 64, 903-907

2. **Bonciu, E.,**2020, Study regarding the cellular activity in garlic (A. sativum) bulbs affecting by Sclerotium cepivorum. Scientific Papers. Series A. Agronomy, Vol LXIII, No. 1: 186-191.

3. **Bonciu, E.,** 2020, Aspects of the involvement of biotechnology in functional food and nutraceuticals, Scientific Papers. Series A. Agronomy, Vol LXIII, No. 2: 261-266.

4**.Bonea D.,** 2020. Phenology, yield and protein content of maize (*Zea mays* L.) hybrids as affected by different sowing dates. Scientific Papers. Series "Management, Economic Engineering in Agriculture and rural development", Vol. 20(3), 145-150.

5**.Bonea D.,** 2013. Managementul calităţii produselor alimentare de origine vegetală. Editura ProUniversitaria Bucureşti şi Editura Universitaria Craiova

6**.Cola M., Cola F.,** 2013- The effect of including peas in the cows' ration on the milk production- Annals of the university of craiova, series agriculture, montanology, cadastre, XLIII, vol.1, 118-121,

7.**Cola M., Cola F.** 2013. Results regarding the effect of peas in the cows' ration on the forage efficiency. Annals of the university of craiova, series agriculture, montanology, cadastre, XLIII, vol.1,122-127

8**.Damaglou, A. P. – Shannon, W. – Downey, G. A**.,1984. The interactions between Fusaria and their mycotoxins in grass silage. J. Sci. Food Agric., 35, 1984: 279–284

9.**Kalac, P. and Woolford, M.K.,** 1982. A review of some aspects of possible associations between the feeding of silage and animal health. Br. Vet. J., 138: 305-320.

10**.Lepom, P. – Baath, H. – Knabe, O**.,1988. Occurrence of Fusar-of silaging on the zearalenone content of CCM maize. Arch. Anim. Nutr., 38, 1988: 817–823

11 **Munkvold, G. P., McGee, D. C., and Carlton, W. M.,** 1997.Importance of different pathways for maize kernel infection by Fusariummoniliforme. Phytopathology 87:209-217
12.**Pelhate, J.,** 1977. Maize silages: Incidence of moulds during conservation. Folia Vet. Lat., 7:
13.**Pfohl-Leszkowicz A, Bartsch H, Azemar B, Mohr U, Esteve J, Castegnaro M. MESNA**,2002. Protects rats against nephrotoxicity but not carcinogenicity induced by ochratoxin A, implicating two separate pathways. FactaUniversitatis, Ser Med Biol ;9:57-6
14.**Pfohl-Leszkowicz A**.,2000.Mycotoxicological risks for health in animals and humans
15.**Pittet, A.** ,1998. Natural occurrence of mycotoxins in foods and feeds – an updatedreview. Revue de MédecineVétérinaire **149**, 479– 492
16.**FAO**-2001 http://www.fao.org/faostat/en/#data/