PREVALENCE OF FUNGI AND MYCOTOXINS IN SILAGE AND MILK IN LITHUANIA

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Abstract. The aim of this study was to estimate silage contamination with fungi and mycotoxins and milk contamination with aflatoxin M_1 in Lithuania. During 2008 and 2009, silage samples of different botanical composition and samples of raw milk were collected from different agricultural enterprises in Lithuania. The determined fungi spores count in grass mixture silage from bales was by 23.8% ($p>0.05$) higher than in clover silage and by 86.7% ($p<0.05$) higher than in ryegrass silage from bales. In grass mixture silage taken from trenches, the fungi spores count ranged from 0.0 to 19.0 log 10 CFU/g and in maize silage from trenches it ranged from 0.0 to 175.0 log 10 CFU/g. In the different botanical composition silage samples, the most frequently occurring genera were *Aspergillus* sp. (0.9-15.7%), *Penicillium* sp. (1.2–12.6%), *Rhizomucor* sp. (3.1–15.6%), *Rhizopus* sp. (0.6–14.3%). The highest mean levels of mycotoxins AFL (total), DON, and ZEN were detected in ryegrass silage from bales: AFL (total) – 21.2±3.9 μg/kg, DON – 471.0±65.6 μg/kg; ZEN – 397.5±83.5 μg/kg. By comparison of silage produced using different technologies, contamination with AFL (total) and DON concentrations was detected 14.0% ($p>0.05$) and it was by 24.0% ($p>0.05$) higher in silage from bales. ZEN concentration in silage from bales was by 3.0% ($p>0.05$) lower than in silage from trenches. When the total AFL in ensiled forages of dairy cows ranged from 0.0 to 27.0 μg/kg, the average concentration of AFL M_1 in cows' milk was 0.019±0.01 μg/l. The main finding of this paper is that silage preparation technology had no significant impact on the studied mycotoxicological indicators. Raw materials were more important in this respect.

Keywords: cows, fungi, milk, mycotoxins, silage.

MIKROSKOPINIŲ GRYBŲ IR MIKOTOKSINŲ PAPLITIMAS SILOSE IR PIENE

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Santrauka. Darbo tikslas – nustatyti siloso užterštumą mikroskopiniais grybais ir mikotoksinais bei pieno užterštumą aflatoksinu M_1.

2008–2009 metais skirtingos botaninės sudėties siloso ir pieno mėginiai surinkti Lietuvos pieno ūkiuose. Varpinių žolių silose iš ritinių mikroskopinių grybų sporų nustatyta 23,8 proc. ($p>0,05$) daugiau negu dobilų silose ir 86,7 proc. ($p<0,05$) daugiau negu žolės svidrės silose. Varpinių žolių silose iš tranšėjų mikroskopinių grybų sporų skaičius svyravo nuo 0,0 iki 19,0 log 10 KSV/g, kukurūzų silose iš tranšėjų – nuo 0,0 iki 175,0 log 10 KSV/g. Skirtingos botaninės sudėties siloso mėginiuose daugiausia rasta *Aspergillus* sp. (0,9–15,7 proc.), *Penicillium* sp. (1,2–12,6 proc.), *Rhizomucor* sp. (3,1–15,6 proc.), *Rhizopus* sp. (0,6–14,3 proc.). Didžiausia koncentracija mikotoksinų AFL (bendrai), DON, ZEN nustatyta žolės svidrės silose iš ritinių: AFL (bendrai) – 21,2±3,9 μg/kg; DON – 471,0±65,6 μg/kg; ZEN – 397,5±83,5 μg/kg. Palyginus siloso užterštumą mikotoksinais pagal gamybos technologijas, didžiausias užterštumas AFL (bendrai) – 14,0 proc. ($p>0,05$), DON – 24,0 proc. ($p>0,05$) nustatytas silose iš ritinių. ZEN koncentracija buvo 3,0 proc. ($p>0,05$) mažesnė silose iš ritinių. Kai karvėms skirtame silose AFL (bendrai) nustatyta nuo 0,0 iki 27,0 μg/kg, vidutinė AFL M_1 koncentracija šių karvių piene buvo 0,019±0,01 μg/l. Ištirta, kad siloso paruošimo technologija didelės reikšmės tirtiems mikotoksikologiniams rodikliams neturėjo, daugiau įtakos turėjo silosuojamos žaliavos.

Raktažodžiai: karvės, mikroskopiniai grybai, mikotoksinai, pienas, silosas.

Introduction. Recently, dairy cows health has become an object of concern for frequently elevated milk somatic cell count, increased mastitis, calf deployment, limp cases and developed immunosuppression (Goff, 2006). Each year, an increasing number of dairy cows is kept in barns during the summer where they experience a surplus of heat stress (Kalvolėlis, 2006). However, one of the main factors that govern the health of cows, and good quality products are good quality forages. The diet of high-yielding dairy cows consists of two major classes of feedstuffs, concentrates and forages. Fresh, dried or ensiled forages such as grass, maize and Lucerne constitute the largest fraction (usually 50–75%) of the diet. Forages are generally grown and processed locally, usually at the dairy farm.

Six biological and technological factors affecting forage quality (not yield) are traditionally recognized: crop species, soil fertility and variety maturity stage, harvest and storage techniques, and environment (Frey et al., 2004; Reboux et al., 2006; Fulgueira et al., 2007). Silage production and management practices on farms can influence subsequent fungal development (Skaar, 1996; O'Brien et al., 2007). Moulds, the common term for the large group of fungi *imperfect*, are part of the natural

environment and fulfill many essential functions in the ecological balance as they degrade organic (plant-derived) materials (Fink-Gremmels, 2008). Invasion of living plants by fungi is often detrimental, as many fungal species are common plant pathogens (CAST, 2003). Fungi are known to produce a variety of secondary metabolites that seem to improve their competitiveness in nature (Steyn, 1998). Under field conditions, however, mould and mycotoxin contaminated feed materials, particularly silage, may cause adverse health effects, which do not seem to match the analytical results (O'Brien et al., 2006). The improper ensiling and later improperly stored silage can form the conditions of mycotoxins production (Fink–Gremmels, 2005; Richard et al., 2007).

Ensiled grass may contain a complex mixture of mycotoxins, originating from a pre-harvest contamination by *Fusarium* spp. toxins (Mansfield and Kuldau, 2007), as well as from post-harvest contamination with toxins produced by fungal species that are common in silage (Boysen et al., 2000; Garon et al., 2006). They are: *Aspergillus, Penicillium, Alternaria, Monasacus, Trichoderma, Mucor, Fusarium* and other genera. Following fungi are the potential mycotoxin producers (CAST, 2003; Fink–Gremmels, 2008). Under favorable conditions mycotoxins production continues in produced silage (Fulgueira et al., 2007).

Recently many references have been made to a wide number of fungi species occurring in silage that synthesize and excrete secondary metabolites: *Aspergillus fumigatus, A, flavus, A. parasiticus, A. niger, A. ochraceus, A. carbonarius, Penicillium viridicatum, P. verrucosum, Fusarium culumorum, F. graminearum, F. moliniforme* and other (Cole and Schweikert, 2003; Lugauskas, 2005; Fink–Gremmels, 2008). The latter mycotoxins, such as patulin, aflatoxins, deoxynivalenol, zearalenone and other, exert antimicrobial effects and impair and modify the rumen microflora. The resulting diseases differ from typical mycotoxicoses, and are characterized by a malnutrition syndrome with rumen dysbacteriosis progressing into acidosis, poor feed utilisation, loss of bodyweight and mild diarrhea with undigested fibers in the faces (Escoula, 1992; Puel et al., 2005). During the climate change, the content of mycotoxins including aflatoxins increaseI in the forages in Lithuania. Certain aflatoxins accumulate in forages for dairy cows and are transformed into aflatoxin M_1 and subsequently partially excreted into milk (Kuilman et al., 2000; EFSA, 2004). In particular, this occurs when they are affected by such climatic factors as frequent rainfall and changing environmental temperature. In addition, the processes activate when there is a breach in silage technology, in particularly in ensuring the containment.

The aim of this study was to estimate silage contamination with fungi and mycotoxins and milk contamination with aflatoxin M_1 in Lithuania.

Material and methods. *Samples collection.* During 2008 and 2009, silage samples of different botanical composition were collected from different agricultural enterprises in Lithuania. From bales were taken 54 silage samples (24 silage samples were taken from grass mixtures, 18 from clover silage, and 12 from ryegrass silage), from trenches 26 samples (18 samples from grass mixtures and 18 samples from maize silage). Samples of silage were collected at least 3 weeks after ensiling between September and November, in spring April and May. At the time of sampling, the silage was completely sealed with silo plastic and not yet in use for feeding the dairy cows. Silage was sampled by drawing vertical cores. Three samples of approximately 100 g were taken at different positions and mixed to provide a composite sample. Samples for fungal analysis were collected by using aseptic plastic bags and the samples were stored at 6°C before analysis, which occurred about 12 h after sampling.

In spring 37 samples of raw milk were collected from the farms (in 2008 – 33 samples, in 2009 – 14 samples). The samples were stored in a cool place, and protected from light.

Identification samples contaminatied with fungi. The methods of direct plating and dilution plating were applied for identification of fungi. For determination of fungal colony-forming units per sample (CFU/g), 20 g of each sample was suspended into 180 ml of sterile water and shaken for 20 min. A dilution series (from 10^{-1} to 10^{-3}) was prepared from the obtained suspension. 1 ml of suspension from each dilution series was uniformly dispensed under the surface of acidified malt extract agar (Oxoid) and standard agar Czapek-Dox (Oxoid) supplied with chloramphenicol (50 mg/l) (Sigma) in Petri-dishes and incubated for 5-7 days at 26±2ºC in dark.

In determining the silage sample for direct fungal infection that occur in the parts of the sample surface, the percentage of external exposure to viable mycelium of fungi was estimated.

For purification and identification of fungi isolates, the samples were inoculated on Czapek-Dox agar and malt extract agar and cultivated at 26±2ºC for 5–7 days.

The isolates were ascribed to taxonomic groups following Ainsworth and Bisby's Dictionary of Fungi (Hawksworth et al., 1995). Fungi were identified according to various manuals (Nelson et al., 1983; Pitt, 1997; Klich, 2002; Lugauskas et al., 2002; Samson et al., 2002). The isolation frequency (FO) of genera was calculated.

Mycotoxins analysis. Whole silage samples were air-dried at 70°C for 24 h in an oven, ground to pass a 1 mm screen and homogenized. The silage samples were determined by direct competitive enzyme-linked immunosorbent assays (ELISA) (AOAC, 2000). Contamination with total aflatoxins (AFL), deoxynivalenol (DON) and zearalenone (ZEN) was tested. The Veratox test kits (Neogen Corporation, Scotland), approved by the AOAC Research Institute (Certificate N 950702) were used for the analysis. Mycotoxin extraction and tests were performed according to manufacturer's instructions. Extraction of samples was carried out in distilled water for DON, in methanol: water (70:30 v/v) for AFL, ZEN. The basis of the test is the antigen-antibody reaction. The wells in the microtiter plates were coated with antibodies to each mycotoxin. By adding standards of each mycotoxins or the sample solution the antibody binding sites were occupied in proportion to the concentration of each my-

cotoxin. Any remaining free binding sites were occupied in the next stage by enzyme labeled toxin (enzyme conjugate). Any unbound enzyme conjugate was then removed in a washing step. Enzyme substrate and chromogen were added to the wells and incubated. Bound enzyme conjugate converted the colorless chromogen into a blue product. The addition of the stop reagent resulted in a color change from blue to yellow. Absorbance was determined using the microwell strip reader (Neogen, USA) at 650 nm. The measured absorbance was automatically converted to the mycotoxin concentration units – µg/kg (ppb). The results were estimated taking into account the lowest calibration curve's mycotoxin concentration value (LOD-limit of detection), which is for AFL – 2.0 µg/kg, ZEN – 10.0 µg/kg; DON – 100.0 µg/kg.

The quantitative analyses of aflatoxin M_1 (AFL M_1) in milk were performed with the enzyme linked immunosorbent assays: with test kit RIDASCREEN®Aflatoxin M_1 (R-Biopharm AG, Germany). Absorbance was determined using the microwell strip reader at 450 nm. The mean lower detection limit of the RIDASCREEN®Aflatoxin M_1 is 0.01 µg/l.

Statistical method. Results of microbiological analysis of total fungi spores count in silage, using mathematical method of logarithm, were transferred into log10 CFU/g. Statistical analyses were carried out using SPSS software (version 12.0 for Windows, SPSS Inc., Chicago, IL, USA). The Student´s t-test was used to compare data. Values are presented as means ± standard deviation (SD) and were considered statistically significant when $P<0.05$.

Results. In silage samples taken from bales the different number of viable fungi spores was determined (Fig. 1). In grass mixture silage samples, the fungi spores count ranged from 0.3 to 136.0 log 10 CFU/g. In clover silage, the fungi spores count ranged from 0.0 to 1.0 log 10 CFU/g and in ryegrass silage – from 0.0 to 9.3 log 10 CFU/g. In grass mixture silage from bales, the fungi spores count was determined to be by 23.8% ($p>0.05$) higher than in clover silage and by 86.7% ($p<0.05$) higher than in ryegrass silage from bales.

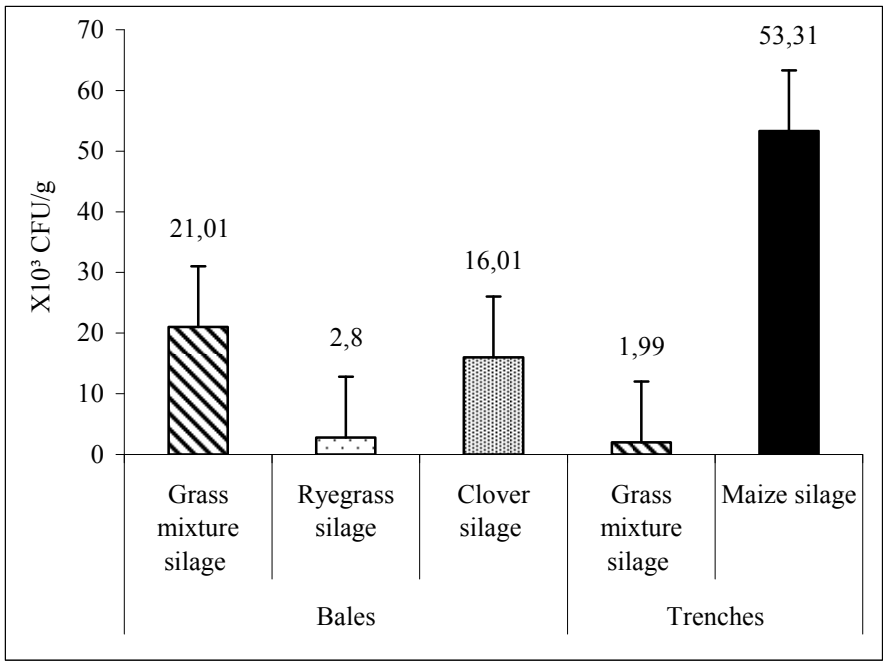

Fig. 1 **Total fungi spores count** (x10^3 CFU/g) **in silage**

In grass mixture silage samples taken from trenches the fungi spores count ranged from 0.0 to 19.0 log 10 CFU/g and in maize silage from trenches the fungi count ranged from 0.0 to 175.0 log 10 CFU/g. In maize silage from trenches the fungi spores count was determined to be by 96.3% ($p<0.05$) higher than in grass mixture silage from trenches.

The counts of viable fungi spores in grass mixture silage produced according different technologies were compared, showing that in grass mixture silage from bales the count of fungi spores was by 90.5% ($p<0.05$) higher than in trenches.

The incidence and total number of fungal genera isolated from the silage samples are given in Table 1.

In the different botanical composition silage samples the most frequently occurring genera were: *Aspergillus* sp. (FO – 0.9-15.7%), *Penicillium* sp. (1.2-12.6%), *Rhizomucor* sp. (3.1-15.6%), *Rhizopus* sp. (0.6-14.3%). The most fungal genera were isolated: from grass mixture silage taken from trenches (seven) and from grass mixture silage taken from bales (twelve).

Aflatoxins (total) and zearalenone were the most frequently detected in silage samples of different botanical composition and produced by different technologies (Table 2).

Table 1. **The frequency of occurrence of fungal genera in silage of different botanical composition and produced by different technologies**

Fungal genera	Frequency of occurrence, %					
	Silage from trenches			Silage from bales		
	Grass mixture	Clover	Maize	Grass mixture	Clover	Ryegrass
Absidia	0.1	-	-	-	0.7	4.5
Acremonium	0.6	-	-	0.6	0.4	-
Actinomycetes	0.1	-	8.7	0.3	1.7	3.1
Aspergillus	1.3	15.7	11.7	3.7	4.2	0.9
Botrytis	-	-	-	-	3.2	-
Cladosporium	-	-	-	0.1	1.3	-
Euriotium	-	-	-	-	0.4	-
Fusarium	-	5.7	6.3	1.8	0.6	0.4
Geotrichum	-	-	-	1.0	0.4	0.9
Gilmaniella	-	-	-	-	1.4	-
Humicola	-	-	-	1.0	1.3	0.3
Mucor	-	0.1	4.0	3,2	1.1	2.4
Penicillium	2.8	1.9	4.3	12.6	1.2	2.1
Rhizomucor	15.6	0.1	13.7	3.2	8.8	4.2
Rhizopus	-	0.6	6.9	2.7	14.3	1.0
Sclerotinia	-	-	-	-	0.6	-
Sporotrix	-	-	-	-	0.6	-
Scopularopsis	0.1	-	-	-	-	-
Sporotrix	-	-	-	0.7	0.6	-
Trichoderma	-	-		-	-	2.1
Ulocladium	-	-		-	-	0.4
Verticillium	-	-	-	-	0.7	-
Other	0.1	-	-	0.6	0.4	0.5

Toxic fungal species were isolated from silage samples: from clover silage – *Aspergillus niger*, *Fusarium poae*, from ryegrass silage – *A. parasiticus*, from grass mixture silage – *A. flavus*, *F. solani,* from maize silage - *A. fumigatus*, *F. sporotrichioides*, *Penicillium expansum*, *Trichoderma harzianum*. These fungal species can synthesize and excrete into environment secondary metabolites of various chemical compositions that worsen silage quality, which becomes hazardous for cattle health.

The highest AFL (total) concentration was detected in ryegrass silage from bales 40.0 μg/kg. The highest ZEN concentration was detected in grass mixture silage from trenches 800.0 μg/kg and the highest DON concentration was 1020.0 μg/kg in clover silage from bales. DON was detected above the LOD (100 μg/kg) in 4% of grass mixture silage and in 6% of clover silage samples taken from bales.

The highest mean levels of mycotoxins were detected in ryegrass silage from bales: AFL (total) – 21.2±3.9 μg/kg, DON – 471.0±65.6 μg/kg; ZEN – 397.5±83.5 μg/kg.

Comparison of silage produced according different technologies showed that contamination with AFL (total) and DON concentrations was by 14.0% ($p>0.05$) and 24.0% ($p>0.05$) higher in silage samples from bales than from trenches. The determined ZEN concentration was by 3.0% ($p>0.05$) lower in silage samples from bales than from trenches.

In problematic cases, when aflatoxins were detected in ensiled forages of dairy cows and mycotoxicosis symptoms for dairy cows were observed, milk samples were taken from these cows, AFL (total) concentration varying from 0.0 to 27.0 μg/kg in the silage samples.

The average concentration of AFL M_1 in milk was 0.019±0.01 μg/l (Fig. 2). The highest AFL M_1 concentration was detected in 2.7% milk samples. 27.0% of milk samples were negative. In 29.7% of milk samples, AFL M_1 concentration varied from 0.02 to 0.03 μg/l.

Discussion. The diet of high-yielding dairy cattle consists of two major classes of feedstuffs, concentrates and forages. Fresh, dried or ensiled forages such as grass, maize and lucerne constitute the largest fraction (usually 50–75%) of the diet. Forages are generally grown and processed locally, usually at the dairy farm itself.

Making silage in good conditions anaerobiosis and low pH usually prevents the development of fungi. The presence of oxygen at the cut edge of the silage or in the silo may favour the growth of fungi (Yiannikouris and Jouany, 2002).

They most often belong to the following genera: *Penicillium, Fusarium, Aspergillus, Mucor, Trichoderma, Byssochlamys* (Amigot et al., 2006; Bíro et al., 2009). Fink-Gremmels (2008) and Richard et al. (2007) distinguish two groups of fungi: field fungi, which develop during vegetation, infect the vegetative mass, and in silage they can be found only if oxygen is present (*Fusarium* sp.) and storage fungi, which are introduced to silage in the form of spores with particles of soil (*Mucor, Penicillium, Aspergillus* and *Monilia* sp.).

Table 2. **Concentrations of total aflatoxins** (AFL)**, deoxynivalenol** (DON)**, zearalenone** (ZEN) (μg/kg) **in silage different botanical composition and produced different technologies**

Mycotoxins	No. of samples	Mean all[1]	Min-Max[2]
Grass mixture silage from bales			
AFL	24	7.7	2.2-14.0
DON	24	328.1	<100.0-1100.0
ZEN	24	22.5	46.0-580.0
Ryegrass silage from bales			
AFL	12	21.2	5.3-40.0
DON	12	471.0	200.0-750.0
ZEN	12	397.5	200.0-700.0
Clover silage from bales			
AFL	18	16.8	12.5-19.9
DON	18	465.4	<100.0-1020.0
ZON	18	334.0	100.0-680.0
Grass mixture silage from trenches			
AFL	18	11.5	6.0-18.0
DON	18	391.3	135.0-500.0
ZEN	18	410.0	30.0-800.0
Maize silage from trenches			
AFL	8	14.0	12.0-16.0
DON	8	435.0	370.0-500.0
ZEN	8	625.0	500.0-750.0

[1]Mean concentration of all samples. The concentration of samples found to contain less than the detection limit was set AFL – 1.0 μg/kg, DON – 100.0 μg/kg, ZEN – 10.0 μg/kg.

[2]Min-Max – Minimum and maximum concentrations (μg/kg) detected.

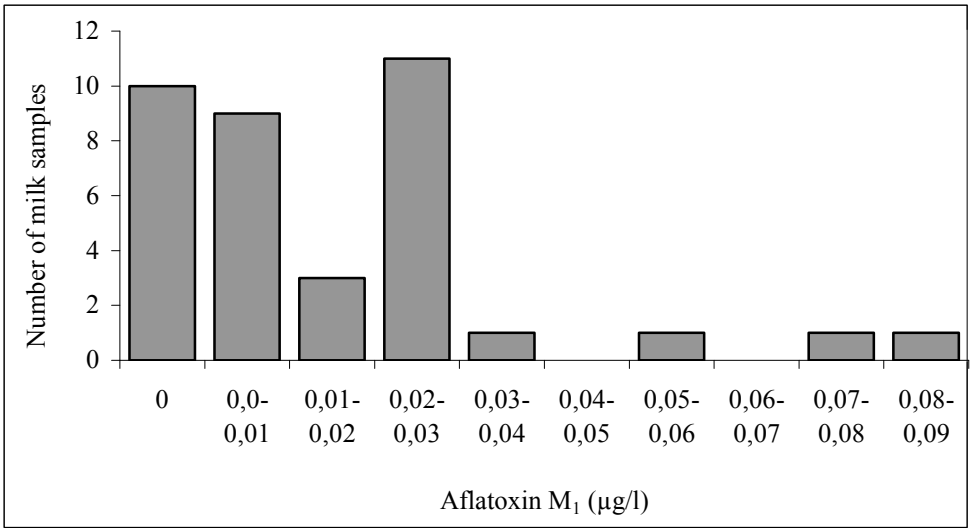

Fig. 2 **Histogram of AFL M_1 concentration in milk**

In this study, the biggest total fungi spores count 53.31 log 10 CFU/g was detected in maize silage from trenches. Similar results concerning the fungal counts in silages were found in farm silages from northern Germany (Auerbach et al., 2000).

During this study, the genera of fungi most frequently observed in silage were *Penicillium*, *Aspergillus*, *Rhizomucor*, *Rhizopus* genera. Visible fungal growth on bales of grass silage on Irish farms is common (O'Brien et al., 2007). The study by Gedek et al. (1981) included 260 farm silages in Germany and showed a high degree of contamination with *P. roqueforti* and *A. fumigatus*. These species were detected in 71.3 and 13.3% of the samples, respectively.

In Lithuania, the weather conditions are favorable for the occurrence fungi. As a result, of fungi growth, the food products and animal feed are often contaminated by mycotoxins.

The analytical results for mycotoxins indicated that silage samples were contaminated with different AFL (to-

tal), DON and ZEN concentrations.

DON is one of the most common mycotoxins found in forages. DON is associated with nausea, vomiting, diarrhea, loss of weight and food refusal. Exposure to grass silage containing high amounts of DON, has caused a toxic syndrome in cattle in North Europe, characterized by an increase in inflammatory reactions in the form of mastitis and laminitis (Fink-Gremmels, 2008). The highest DON concentration 1100.0 µg/kg was in grass mixture silage taken from bales.

Different concentrations of ZEN are found in almost every agricultural product, feed raw materials and produced feed. Similar to DON, ZEN is a frequent contaminant of ensiled feed (Whitlow and Hagler, 2002). ZEN is the cause of hyperestrogenism, vaginal and rectal prolapse, and abortions (CAST, 2003).

The highest ZEN concentration 800.0 µg/kg was in grass mixture silage taken from trenches. ZEN concentration varied from 500.0 to 750.0 µg/kg in maize silage.

The results of this study show the incidence of the *Fusarium* mycotoxins DON and ZEN in silage produced in the Lithuania. Similar contamination levels of maize silage were reported to occur in other countries. In Netherlands, DON concentration in maize silage were 651.0 µg/kg and ZEN 92.0 µg/kg and DON concentration in grass silage 426.0 µg/kg and ZEN 93.0 µg/kg (Driehuis et al., 2008). In Germany, the concentration of DON was higher than 300.0 µg/kg in 79% of maize silage and the concentration of ZEN was above 10.0 µg/kg in 96% of maize silages (Reutter, 1999). In maize silages produced in Austria between 1995 and 1999, DON levels were above 100.0 µg/kg in 91% of samples and ZEN exceeded 10.0 µg/kg in 59% of samples (Hochsteiner and Schuh, 2001).

From aflatoxins (B_1, B_2, G_1 and G_2) AFL B_1 is the most prominent mycotoxin and toxic, human and animal carcinogen. AFL B_1 that escapes rumen degradation is partially converted by hepatic metabolism into AFL M_1 (the 4-hydroxymetabolite of AFL B_1) (Kuilman et al., 2000), which is excreted with dairy milk at a transfer rate that varies between 1% and 6%.

AFL (total) intoxication in dairy cattle is characterized by liver cell injury, a fatty liver syndrome (pale livers), poor feed conversion, and a significant reduction in milk yield (EFSA, 2004).

In this study, the highest found AFL (total) concentration was 27.0 µg/kg in grass mixture silage taken from trenches. AFL M_1 concentration ranged within 0.0-0.019 µg/l in milk. The Commission of the European Communities established a limit of 50.0 ng/l for AFM_1 in milk.

Literary sources indicate that the incidence of AFM_1 contamination is often higher in countries where cows are fed with greater amounts of compound feeds.

Conclusions. Our results indicate that the most viable fungal spores were determined in maize silage from trenches. The most fungal genera were isolated from clover silage from bales. The highest mean concentrations of mycotoxins: AFL (total), DON, ZEN was in ryegrass silage from bales. The main finding of this paper is that silage preparation technology had not a significant impact on the studied mycotoxicological indicators. The type of raw materials is of higher importance.

The average concentration AFL M_1 in milk was 0.019±0.01 µg/l.

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