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HYGIENIC SANITARY ESTIMATION OF MAIZE SILAGE IN DAIRY FARMS IN LITHUANIA

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The aim of this study was to estimate maize silage hygienic sanitary parameters, contamination with mycotoxins in dairy farms in Lithuania. In 2011–2012 maize silage samples were collected from 20 dairy farms: prior to ensiling and 3 and 8 months after ensiling. In maize samples, prior to ensiling, *L. monocytogenes* was detected 25.0 % and after 3 months of ensiling – 10 %. Average of yeast and filamentous fungi in raw material samples were respectively – 71.39 ± 32.17 and 47.67 ± 18.31 CFU/g, after 3 months – 50.06 ± 16.09 CFU/g and 28.27 ± 13.18, after 8 months – 213.81 ± 55.98 and 215.08 ± 56.16 CFU/g. In raw maize dominated *Fusarium* spp. and in silage – *Aspergillus, Penicillium* spp.
Average of lactic acid bacteria – 189.01 ± 57.79 (3 months after ensilage) and 436.11 ± 93.97 CFU/g (8 months after ensilage).
Compared prior to ensilage and 3 and 8 months after ensilage maize samples, higher mycotoxins concentrations of DON – 36.96 % ($P < 0.05$) and ZEA – 77.32 % ($P < 0.05$) were detected in 3 month after ensilage, T-2 toxin – 72.69 % ($P < 0.05$), AFL – 94.31 % ($P < 0.05$) were detected in 8 month after ensilage. Concentration of OTA was higher 34.99 % ($P > 0.05$) in samples after 3 months of ensiling.
Dry matter and pH respectively: prior to ensiling – 36.96 % and 5.26, 3 months after ensiling – 33.17 % and 3.84, 8 months after ensiling – 40.52 % and 3.91.
The current results indicate the presence of yeast, viable fungi spores and mycotoxins AFL (total), ZEA, DON, T-2 and OTA contamination in maize silage in Lithuania.

Keywords: fungi, maize silage, mycotoxin, yeast.

INTRODUCTION

Maize silage is a widely used feed product for cattle. In many countries around the world, the production of maize silage is equal to or larger than the production of grass silage (Wilkinson and Toivonen, 2003). In the climatic conditions of Lithuania, fresh fodder needs to be made into silages or another kind of forage to supply cattle with succlent forage during the year (Baležentienė and Mikulionienė, 2006). In Lithuania the production of maize for silage – 31 ton/ha.

The quality of raw milk and dairy products is related primarly to the quality of silage fed to cows. The kind of raw material and environmental conditions determine the composition of epiphytic microflora on the surface of the vegetative parts of green plants, thus affecting the final microbiological quality of silage. One of the quality-related problem is low aerobic stability for silage, which causes an increase in the *Listeria monocytogenes* count (raw material contamination with these microorganisms may cause the occurrence of listeriosis in animals). A high dry matter concentration (>50 %) in the raw material makes silage more susceptible to self-heating and infestations with toxin-producing fungi. The progress observed in silage production technologies primarly concerns maize silage and maize grain silage, whose quality depends upon the growth stage. Delayed harvest aimed at achieving a high dry matter yield results in the mass proliferation of yeasts and mould. Consequently, maize silage has the highest dynamics of changes in microbiological quality (Purwin et al., 2006).

The aim of the current study was to estimate micro-organisms present in maize silage and contamination with mycotoxins during storage period in dairy farms in Lithuania.

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MATERIAL AND METHODS

During 2011–2012 maize silage samples were collected from 20 farms of four geographic regions (five farms in each of the four regions) in Lithuania. Samples were collected at harvest and the again at the same site 3 and 8 months after ensiling. Samples were stored 4 °C prior to processing.

Listeria monocytogenes in raw and ensiled maize silage was detected according LST EN ISO 11290–1:2003/A1:2005.

For identification of yeast, mould count performed according LST EN ISO 7954 „Microbiology. General guidance for enumeration of yeasts and moulds. Colony-count technique at 25 °C". For identification of lactic acid bacteria (LAB) count performed according LST ISO 15214:2009 „Microbiology of food and animal feeding stuffs - Horizontal method for the emumeration of mesophilic lactic acid bacteria – Colony-count technique at 30 °C (ISO 15214:1998, identical)". 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 ± 2 ºC 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 of silage with deoxynivalenol (DON), T-2 toxin, zearalenone (ZEA) and aflatoxins (AFL (total)) Ochratoxin A (OTA) was tested with the RIDASCREEN test kits ('R-Biopharm AG', Germany). Mycotoxin extraction and tests were performed according to manufacturer's instructions. Absorbance was determined using the microwell strip reader (Neogen, USA) at 450 nm. The measured absorbance was converted to the mycotoxin concentration units – µg/kg (ppb).

Dry matter (DM) was analyzed on the day of sampling. To determinate dry matter, samples were chopped in 4 cm –diameter-particles and dried for 18 hours at 55 ºC. After air equilibration, the samples were weighed and then dried again for 20 hours at 103 ºC (AOAC, 2005).The pH was measured in diluted silage with a pH-meter (WTW®inoLab pH 720) fitted with a glass electrode after homogenization of 10 g silage with 40 ml of distilled water.

Statistical analyses were carried out using SPSS software (version 17.0 for Windows, SPSS Inc., Chicago, IL, USA). The data were analyzed by analysis of variance (ANOVA). Specific means differences were identified with LSD 's multiple range tests.

RESULTS

L. monocytogenes were isolated from 25 % prefermented maize samples and from 10 % postfermented samples.

In prior to ensiling and ensiled silage samples taken from a trench-type silo were determined the different number epiphytic microflora (Fig. 1). Filamentous fungi and yeast counts in raw maize were highest respectively 39.61 % ($P > 0.05$) and 29.88 % ($P > 0.05$) then in 3 months silage samples after ensiling and respectively lowest 22.16 % ($P < 0.05$) and 33.39 % ($P < 0.05$) then in samples 8 months after ensiling. LAB count in samples (3 months after ensiling) were lowest 56.66 % ($P < 0.05$) then in samples 8 months after ensiling.

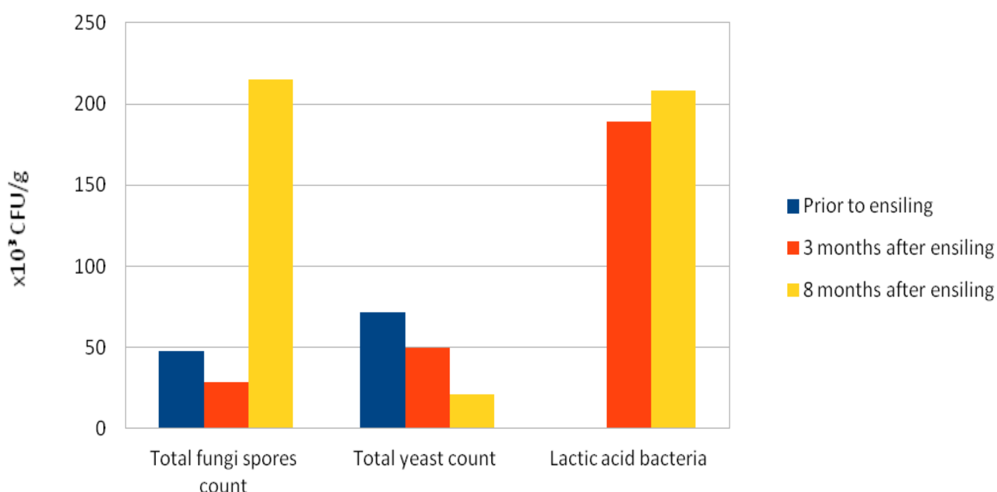

Figure 1. Epiphytic microflora in maize silage samples

Filamentous fungi were isolated from all raw and silage samples. Table 1 shows the isolation frequency (%) of different fungal genera from pre- and postfermented maize samples. Six genera of filamentous fungi were dominated by other fungi. Less frequent species were *Acremonium* spp., *Curvularia* spp., *Rizophus* spp., *Trichoderma* spp. The most commonly isolated filamentous fungi in raw maize were *Alternaria* spp. (73.9 %), *Fusarium* spp. (34.1 %). Predominant *Fusarium* spp. species were *F. sporotrichioides*, *F. poae*, *F. graminearum.* A high frequency of Zygomycetes was also

found, primarily *Mucor*.

The most commonly isolated filamentous fungi (3–months and 8–months after ensilage) were *Aspergillus* spp. and *Penicillium* spp. (Table 1). Predominant *Aspergillus* spp. after the 3–months and 8–months ensilage respectively were *A. fumigatus* (4.88 % and 9.00 %), *A. niger* (15.00 % and 4.29 %). Less frequent species were *Trichoderma* spp. and *Byssochlamys* spp.

Table 1. The frequency of occurrence of fungal genera in pre – and postfermented silage samples

Fungal genera	Frequency of occurrence %		
	Raw (prefermentation)	3 month ensiling	8 month ensiling
Acremoniella	2.6	-	-
Alternaria	73.9	-	-
Aspergillus	-	30.47	61.83
Byssochlamys	-	3.87	3.3
Cladosporium	4.0	-	-
Fusarium	34.1	-	-
Helminthosporium	1.3	-	-
Mucor	21	2.0	45.45
Penicillium	-	22.9	9.59
Trichoderma	-	6.24	-
Other	0.3	2.6	0.6

From twenty tested prefermented silo samples – three samples were positive for AFL (total). Only four samples (20 %) of 3 month postfermented silo and three samples (25 %) of 8 month postfermented silo were negative to AFL (total) (Table 2).

Fusarium mycotoxin (ZEA, DON, T-2) levels in prefermented and postfermented silage samples are shown in Table 2.

Table 2. Concentrations of mycotoxins from pre – and postfermented silage samples

Mycotoxins	Period		
	Raw (prefermentation)	3 month ensiling	8 month ensiling
DON (µg/kg)			
Min–Max	< 18.5–3500.0	480.0–5300.0	100.0–2000.0
Mean ± SD	1640.0 ± 40.0[b]	2600.0 ± 260.0[a]	610.0 ± 17[c]
ZEA (µg/kg)			
Min–Max	0.6–400.0	100.0–1300.0	< 5–700.0
Mean ± SD	206.88 ± 7.31[c]	880.04 ± 60.62[a]	380.42 ± 19.20[b]
T-2 (µg/kg)			
Min–Max	< 20.0–325.0	16.0–394.0	28.0–226.0
Mean ± SD	40.21 ± 18.23[b]	141.48 ± 23.37[a]	147.25 ± 20.8[a]
AFL (total) (µg/kg)			
Min–Max	< 0.0004–6.6	< 0.0004–62.3	< 0.0004–47.5
Mean ± SD	0.94 ± 0.51[b]	16.86 ± 3.96[a]	20.05 ± 5.33[a]
OTA (µg/kg)			
Min–Max		< 2.5–60.0	< 2.5–58.0
Mean ± SD	–	29.15 ± 5.6[a]	18.95 ± 4.86[a]

Values in the same row with different following letters are significantly different (P < 0.05), LSD's multiple range test

ZEA, DON, AFL (total) and T-2 mycotoxin levels between prefermented and postfermented silage samples were significantly different (P < 0.05). T-2, AFL (total) and OTA levels between 3 month and 8 month postfermentation did not showed significant differences (P > 0.05).

In the collected maize samples DM and pH respectively: prior to ensiling 36.96 % and 5.26, after 3 months of ensiling – 33.17 % and 3.84, after 8 months of ensiling – 40.52 % and 3.91.

DISCUSSION

The current study includes 20 dairy farms from four regions of Lithuania. The pH was recorded for all prefermented and postfermented maize samples. The average pH with the *L. monocytogenes* positive prefermented samples was 5.34 ± 0.23 and postfermented – 3.91 ± 0.17. In 2005 year Borucki et al. reported about statistically significant (P < 0.05) the differences between the average pH of negative and positive samples with *L. monocytogenes.* In this study the average pH of the positive silage samples was 4.62 compared to an average pH of 4.4 for the *L. monocytogenes* – negative samples.

In the present study, the biggest total fungi spores and lactic acid bacteria count and the lowest yeast count were detected in samples 8 month after ensilage. In Storm et al. study (2010) the highest numbers of fungi were five to seven and the lowest 11 months after ensiling, while the LAB decreased in numbers during the study.

Making silage in good conditions anaerobiosis and low pH usually prevents the development of fungi (Yiannikouris and Jouany, 2002). *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., the main mycotoxicogenic moulds,

were the prevalent genera. A high frequency of Zygomycetes was also found. These resultas are in agrement with Pereyra et al. (2007) in corn silage samples who found *Aspergillus* spp., *Penicillium* spp. and *Mucor* as prevalent genera. *A. fumigatus* and *A. niger* showed the highest relative density among *Aspergillus* species. The current result agrees with Garon et al. (2006) where *A. fumigatus* were isolated after 4–month sampling of corn silage in France.

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 (Baliukoniene et al., 2012). The analytical results for mycotoxins indicated that raw maize and maize silage samples were contaminated with different AFL (total), OTA, ZEA, DON, T-2 concentrations. 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 the present study, the respectively highest 95.3 % and 15.9 % AFL (total) concentrations were found in silage samples 8 month after ensilage than in raw maize sample or in 3 month after ensilage. The current result did not agree with Pereyra et al. (2006) where prefermented samples did not show AFB_1 contamination and a low percentage of post fermented samples. DON is one of the most common mycotoxins found in forage (Fink-Gremmels, 2008). The highest DON and ZEA concentrations were 3 month after ensilage and this result agreed with Storm et al. (2010) where the detected DON concentration in maize samples collected approximately 3 months after ensilage was 1056 µg kg^{-1}. T-2 was present in some of the prefermented samples and in all samples 3 month after ensilage in study Storm et al. (2010) T-2 was present in two maize silages samples.

CONCLUSIONS

The current results indicate the presence of yeast, viable fungi spores and mycotoxins AFL (total), ZEA, DON, T-2 and OTA contamination in maize silage in Lithuania The main finding of this paper is that the risk of ZEA, DON, OTA mycotoxins contamination was highest in 3 months after ensilage and AFL (total), T-2 in 8 month after ensilage. The risk of fungal spoilage was highest 8 month after ensilage.

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