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Occurrence of moulds and mycotoxins in grass-legume silages influenced by nitrogen fertilization and phenological phase at harvest

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Abstract

The aim of this study was to determine the presence of fungi and concentrations of mycotoxins, zearalenone, fumonisin and deoxynivalenol, in lucerne and grass-legume silages, under the influence of different doses of N fertilization and harvesting at different stages of phenological development of the plants. Studies included pure lucerne crop and mixtures of lucerne with cocksfoot, tall fescue and sainfoin, sown in different ratios, fertilized with 0, 70, 140 or 210 kgN ha^{-1} and harvested in the butonization phase or at 50% flowering of lucerne plants. Results showed that the total fungi count in the silage depended on the three investigated factors. The highest total fungi count was determined in the mixture silages of lucerne, cocksfoot and tall fescue, which was well treated with different nitrogen quantities, harvested and prepared in later stages of plant development. The most commonly-occurring fungi were Fusarium species (85.5% of fungi were this genus). Of the studied mycotoxins, only DON depended on fertilization, as it reduced the concentration of DON from 0.15 to 0.07 mg kg^{-1}.

Preventing appearance of fungi and their mycotoxins in forage and silage should begin in the field as well as during the process of preparation of silage.

Keywords: microbiology, mycotoxicology, silage quality, fertilization, phenological development

1. Introduction

Ensiling is a specific form of conservation of roughage based on a series of complex chemical and microbiological responses. However, successful ensiling can be suppressed as a result of many problems, such as high moisture, high buffer capacity of forage, insufficient quantity of fermentable carbohydrates, the level of pollution in the soil and the presence of harmful microorganisms, resulting in diminished quality and spoilage of the silage. The quality of silage can be evaluated based on the presence of harmful microorganisms, of which the most important are toxigenic fungi. Mycotoxigenic fungi belong to different genera; however, the most important and most common are representatives of the genera *Fusarium*, *Alternaria*, *Aspergillus* and *Penicillium*. Significant research into silage mycoflora prepared from various forage crops has been conducted in Europe. The composition of mycoflora is very diverse and depends on type of crops, climatic conditions, silage preparation and storage. In Austria, 455 samples of grass and maize silage were studied of which 53.6% were infected with *Penicillium roqueforti, Byssochlamys nivea, Aspergillus glaucus* and *Monascus ruber*. Fungi of other genera, such as *Absidia, Mucor* and *Rhizopus* were present in a small percentage (6.4%) of the silages [1]. In a mycological study of 98 samples of grass silage in Germany, it was concluded that three fungi genera were the most common: *P. roqueforti* (30%), *M. ruber* (19%) and *A. fumigatus* (9%) [7]. Finally, in a comprehensive study of grass

silages in the EU [22], researchers concluded that fungi of the genera *Penicillium, Aspergillus* and *Fusarium* were the most common.

In the Republic of Serbia, it was established that animal feed was most often contaminated with fungi of the genera *Fusarium* spp. (51.4-84.3%), *Aspergillus* spp. (54.4-79.2%), *Penicillium* spp. (30.9-68.1%) as well as *Mucor* spp. and *Rhizopus* spp. [27]. Late spring or early summer periods are the most suitable times for preparation of silage from grass-legume mixtures, as both weather conditions and the physiological properties of plants (increased sugar content) are then favourable. At the same time, these periods are when fungi belonging to the genera *Fusarium, Mucor* and *Penicillium* occur the most frequently [17]. On legume plants, such as lucerne and plants of *Trifolium* sp., the most frequent toxigenic genera are *F. oxysporum* and *F. solani* [26]. If the silage has been well prepared, the presence of species of *Fusarium* genus should not be expected, since these are entirely aerobic microorganisms. However, if the silage has not been sufficiently compressed, and there are some remaining air pockets, or the closing of the silo facility is prolonged, their presence in silage is possible [8]. Presence of fungi in the silage has the consequence of heat production and dry matter (DM) losses which lower the nutritional value of the silage. Also, fungal development can cause changes of color, texture and flavour of the animal feed that are often associated with a decrease in the palatability and feed intake by animals [10]. Mycotoxins are secondary fungal metabolites, of varying chemical composition and molecular weight. Mycotoxins are chemically very stable substances, so if they occur in the field on the plant, they can be transferred into silage. The most frequent producers of fusarium toxins in Serbia, such as zearalenone (ZON), trichothecenes and fumonisins, are the species *F. graminearum, F. verticillioides, F. subglutinans, F. oxysporum, F. proliferatum, F. avenaceum, F. tricinctum* and *F. solani* [25]. In livestock, mycotoxins can cause alterations in hormonal functions, poor feed utilization, lower rates of body weight gain and in some cases death. Also certain mycotoxins may pass into milk and can cause further problems in the food chain [4; 12]. In the Republic of Serbia, the presence of fusarium toxins has been studied in maize grain, wheat and barley [21]. There are relatively few studies, worldwide and in the Republic of Serbia which focus on contamination of grass-legume silages with toxigenic fungi and their mycotoxins, and especially on the effect of agro-technical measures on their incidence. Therefore, aim of this study was to determine the presence of fungi and mycotoxins in lucerne silage and silage made of grass-legume mixtures, under the influence of different quantities of nitrogen mineral fertilizer applied during vegetative growth, and harvest of crops at different stages of phenological development.

2. Materials and Methods

Field trial

The study was carried out in the Institute for Animal Husbandry, in 2005 and 2006, in two phases: field and laboratory conditions. The field trial lasted two years and it was designed in 4 replicates, with basic parcels of 10 m^2. The study included pure lucerne crop, a mixture of lucerne and cocksfoot (S1), a mixture of lucerne, cocksfoot and tall fescue (S2) and a mixture of lucerne, cocksfoot, tall fescue and sainfoin (S3). Fertilization was carried out using four different nitrogen doses: 0, 70, 140 and 210 kg ha^{-1} in two terms, at the beginning of vegetation and after the first cut. Crops were harvest at two phenological phases: in the phase of butonization and when 50% of plants were flowering.

Preparation of silage

Silage was prepared from the second cut so that the total effect of nitrogen fertilization could be observed and the effect of increased presence of weeds from the first cut on the quality of

ensiled material avoided. Ensiling was carried out in experimental silos of 10 dm^3 volume, in four replicates. In order to realize better quality of fermentation before filling the silo, the plant material was treated with microbiological preparation Sil-All 4x4 (Danstar Ferment A.G., Zug, Switzerland), which is mixture of four homofermentative strains of lactic acid bacteria (*Lactobacillus plantarum*, *Pediococcus acidilactici*, *Streptococcus faecium*, *Lactobacillus salivarius*) and four different enzymes (cellulase, hemicellulase, amylase, xylanase), at the rate of 10 g + 2 l H2O t^{-1} of green mass. Sampling of silage was carried out 90 days after closing the silos.

Microbiological analyses

A total of 96 silage samples, per study year, were subjected to a total fungi count using standard microbiological methods for fungi in the Microbiology Laboratory of the Institute for Animal Husbandry, Belgrade-Zemun. Ten grams of the raw silage sample was taken, chopped and homogenized with 90 ml of sterile saline solution. Serial dilutions of the homogenized silage/saline solutions were prepared. Volumes (1ml) of 10^{-3} and 10^{-4} dilutions were transferred into Petri dishes with Sabouraud maltose agar, which were subsequently incubated at 25±2°C for 5-7 days. Identification of fungal genera was performed based on morphological traits of fungi described by [3], [15] and [24].

Mycotoxicological analyses

Analysis of fusarium toxins, zearalenone (ZON), fumonisin (FB1) and deoxynivalenone (DON), was performed with an Enzyme-Linked Immunosorbent Assay (ELISA) using Celer®Techna test kits. For date analysis, not detected levels were based on the quantification limits for all measured toxins: ZON< 0.01 mg kg^{-1}, FB1< 0.75 mg kg^{-1} and DON < 005 mg kg^{-1}. Samples for mycotoxin analyses were prepared in the following steps: 50 g of raw silage was mixed with 10 g NaCl and 250 ml of the appropriate solvent. For quantification of ZON and FB1, 70% methanol solution was used as the solvent, and distilled water for DON quantification. Samples prepared in this way were homogenized in a Osterized blender at 1300 rpm for 3 minutes and filtered through Whatman No. 1 filter paper. For analysis of FB1, filtrate was diluted in an adequate ratio according to the manufacturer's instructions. Concentrations of mycotoxins in the extracted samples were quantitatively determined with an ELISA reader at 450 nm wave lengths (BioTek EL x 800TM).

Statistical analyses

Raw data from the analysis of the total fungi count in silage were transformed to log10 CFU(colony-forming units) g^{-1} to normalize the data and facilitate their processing. Transformed values, as well as results of toxicological analysis were processed using non-parameter statistics with the Kruskal-Wallis (ANOVA) test of median significance (StatSoft. Inc., STATISTICA 8, 2007).

3. Results

Mycoflora of silage made from grass-legume mixtures and pure lucerne

Total fungi counts in silages made from grass-legume mixtures and pure lucerne, produced under differing nitrogen doses and different utilization stages are presented in Table 1. There was a significant difference between the silages made from grass-legume mixtures and pure lucerne in 2005 in regard to total fungal count. In lucerne silage from 2005, there were fewer fungi (2.57 log10 cfu g^{-1}) compared to other silage prepared from crop mixtures (3.13-3.38 log10 cfu g^{-1}). The highest fungal count among all mixed silages was determined in silage made from lucerne, cocksfoot and tall fescue (S2) (3.38 log10 cfu g^{-1}). N fertilization caused a very significant increase of total fungi in the silages in 2005. Increasing the N dose from 0 to 210 kg ha^{-1} caused the total fungal count to increase from 2.54 to 3.74 log10 cfu g^{-1}.

In 2006, very significant differences were determined between the silages. The highest total fungal count, as in 2005, was found in silage made from mixture of lucerne, cocksfoot and tall fescue (S2) (3.90 log10 cfu g^{-1}), whereas samples of silage made from other mixtures, as well as pure lucerne silage, had significantly lower total fungal counts (1.39-2.02 log10 cfu g^{-1}). Delaying harvesting also increased the total fungi count in the silages, as silage made from crops cut in the butonization phase (F1) had an average total fungal count of 1.76 log10 cfu g-1, while in the later F2 stage, the count was 2.86 log10 cfu g^{-1} (Table 1).

Table 1. Total fungal count (log_{10}cfu g^{-1}) in lucerne and grass-legumes silage affected by N fertilization and phenological phase at harvest in the two years study

Treatments	Total fungal count			
	2005		2006	
	Average	SE	Average	SE
Mixture (S)				
S1	3.13	±0.42	2.02	±0.43
S2	3.38	±0.37	3.90	±0.33
S3	3.27	±0.40	1.39	±0.42
Lucerne silage	2.57	±0.36	1.94	±0.42
N fertilization (N)				
0	2.54	±0.38	1.97	±0.45
70	2.95	±0.41	2.06	±0.44
140	3.12	±0.42	2.01	±0.44
210	3.74	±0.31	3.22	±0.41
Utilization phase (F)				
F1	3.17	±0.27	1.76	±0.30
F2	3.00	±0.28	2.86	±0.30
Average	3.09		2.31	
Level of significance				
S	*		*	
N	*		ns	
F	ns		*	

S1 – mixture of lucerne and cocksfoot; S2 - mixture of lucerne, cocksfoot and tall fescue; S3 - mixture of lucerne, cocksfoot, tall fescue and sainfoin; F1 – butonization phase; F2 – phase of 50% flowering of plants; ns- non significant; *-p≤0.05

Figure 1 shows the percentage, in the two-year period, of all isolated and identified fungi species found in the silages, regardless of the type of crop mixture, N fertilization and phase of harvesting.

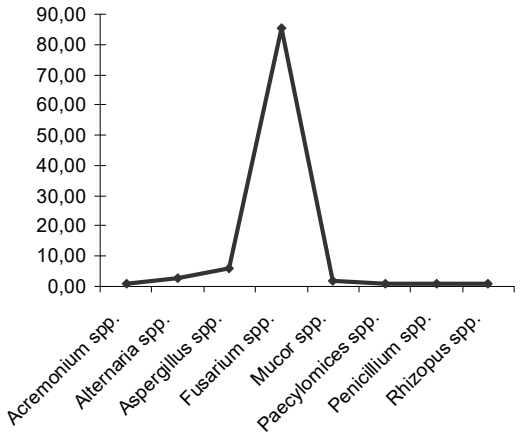

Figure 1. Percentage of fungi species in silage samples in two years study

In the examined silages, the genus *Fusarium* (85.5%) were the most common fungi found. Other species were much less common, and included species of genera *Aspergillus* (5.9%), *Alternaria* (2.8%), *Mucor* (1.8%), *Rhizopus, Paecilomyces, Penicillium* and *Acremonium* (1%).

Mycotoxins in silage made from grass-legume mixtures and pure lucerne crop

The concentrations of zearalenone (ZON), fumonisin (FB1) and deoxynivalenol (DON) in silage made from grass-legume mixtures and lucerne, depending on the level of N fertilization and the phenological stage of crops at harvest, in 2005 and 2006, are presented in Table 2. The concentrations of ZON and DON in the silages were not significantly affected by either of the studied factors. On average, the concentration of ZON was higher in samples from 2005 (0.188 mg kg^{-1}) compared to 2006 (0.092 mg kg^{-1}). Based on presented mycotoxicological analysis of silage samples, it is observed that none of the investigated factors, as in case of ZON, demonstrated a significant effect on the concentration of FB1 in silage made from grass-legume mixtures and pure lucerne. In 2005, the FB1 concentration in silages, on average, was 7.34 mg kg^{-1}, which was significantly higher compared to the FB1 concentration observed in the following year (2.05 mg kg^{-1}). The concentration of DON was only significantly affected by fertilization in 2006. Non fertilized crops had the highest DON content of 0.15 mg kg^{-1} while crops treated with 70 kgN ha^{-1} produced silages with the lowest average concentration of DON (0.07 mg kg^{-1}). In the other two fertilization treatments, the concentration of DON ranged from 0.08-0.11 mg kg^{-1}. In regard to years, the second year was characterized by a higher concentration of DON in silage compared to the first year (0.10 and 0.07 mg kg^{-1}, respectively).

Table 2. Level (mg kg^{-1}) of ZON, FB1 and DON in lucerne and grass-legumes silages affected by N fertilization and phenological phase at harvest

Treatments	ZON		FB1		DON	
	2005	2006	2005	2006	2005	2006
Mixture (S)						
S1	0.025	0.024	8.93	1.33	0.08	0.12
S2	0.066	0.184	9.40	1.31	0.08	0.07
S3	0.054	0.131	2.03	1.96	0.06	0.07
Lucerne silage	0.327	0.031	9.00	3.58	0.06	0.11
N fertilization (N)						
0	0.255	0.154	5.41	1.44	0.08	0.15
70	0.075	0.042	5.13	2.10	0.07	0.07
140	0.047	0.119	7.78	2.50	0.07	0.08
210	0.096	0.055	11.04	2.16	0.07	0.11
Utilization phase (F)						
F1	0.128	0.115	9.00	2.66	0.07	0.07
F2	0.109	0.070	5.68	1.43	0.07	0.14
Average	0.118	0.092	7.34	2.05	0.07	0.10
Level of significance						
S	ns	ns	ns	ns	ns	ns
N	ns	ns	ns	ns	ns	*
F	ns	ns	ns	ns	ns	ns

S1 – mixture of lucerne and cocksfoot; S2 - mixture of lucerne, cocksfoot and tall fescue; S3 - mixture of lucerne, cocksfoot, tall fescue and sainfoin; F1 – butonization phase; F2 – phase of 50% flowering of plants; ns- non significant; *-$p \leq 0.05$

4. Discussion

The fungal count is a technique used to estimate the hygienic quality of the substrate. Fungal concentrations over 1 x104 cfu g^{-1} or 4 log10 cfu g^{-1} in forage, may cause a lot of health problems in animals, so it can be considered as a limit for indicating good manufacturing practices in animal feed [2]. In the present study, total counts of fungi in silage were below this limit, and therefore, they can be classified as good quality silage. Lucerne silage had lower fungal counts compared to silage made from its mixtures, which is likely due to the lower content of fermentable sugars in lucerne [18], [19]. Published data confirm that, depending on the method of silage preparation, the total number of fungi in silage can be either less [16] or significantly more [23] than was found in the current study. Nitrogen fertilization in both years increased the total fungal counts in silage, as did delaying the time of harvesting in 2006. There are few studies of the effect of fertilization on the occurrence and number of fungi on other animal feeds, such as wheat and maize. According to data in [22] and [29], fertilization significantly increased the incidence of Fusarium spp. on wheat, whereas in maize, it did not have any significant effect [28]. Most literature on contamination of feedstuffs by fungi refer to stored grains or maize silage whereas very few refer to the mycoflora present in forages and haylage. In a study of the presence of microbes in silages throughout Europe, fungi of the genera *Penicillium*, *Aspergillus* and *Fusarium* were the most common [20]. In our research, the most common fungi were genus *Fusarium* (85.5%). The remaining part of the mycological population consisted of following species: *Aspergillus* spp., *Alternaria* spp., *Mucor* spp., *Rhizopus* spp., *Paecilomyces* spp., *Penicillium* spp., *Acremonium* spp. The studied factors had no effect on the concentration of mycotoxins in silages made from grass-legume mixtures or pure lucerne, except in the case of DON and the effect of N fertilization in 2006. Some authors have examined the impact of N fertilization on the concentration of DON in the plants, but the results vary [11], [14], although it is largely agreed that the effect of N fertilization on the concentration of DON is not clear and depends on the type of applied N fertilizer, the resistance of the variety, duration of phenological phases and agro-climatic conditions [13]. Mycotoxicological analysis of samples of silage made from lucerne and grass-legume mixtures in the Czech Republic, [9] confirmed that lucerne silage and silage made from grass-legume mixtures contained higher concentrations of ZON and DON compared to our study, with 0.577 and 0.179 mg kg^{-1} (ZON) and 0.5 and 0.63 mg kg^{-1} (DON), respectively and lower concentration of FB1 ,0.05 and 0.47 mg kg^{-1}, respectively. The presence of mycotoxins was investigated in 140 samples of maize silage, 120 samples of grass silage and 30 samples of wheat silage [6]. Contrary to our investigated samples, DON was not detected in grass silage [6]. ZON was identified in 49% of maize and 6% of grass silage, with average concentrations of 174 and 93 μg kg^{-1}, respectively, and maximum concentrations of 943 and 308 μg kg^{-1}, respectively. FB1 was detected in 1.4% cases, in two samples of grass silage; in one sample, FB1 and fumonisin B2 concentrations were 26 200 and 7 800 μg kg^{-1}, respectively, and in the other sample only FB1 was found, at a concentration of 1 600 μg kg^{-1}[6]. The European Commission [5] has determined the allowed mycotoxin values in animal feed as follows: ZON 0.1-3.0 mg kg^{-1}; fumonisins (FB) from 5-60 mg kg^{-1}, and; DON from 0.9-12 mg kg^{-1}. The US Food and Drug Administration (FDA) regulated the limit of FB to 50 mg kg^{-1}, while DON is permitted up to 5 mg kg^{-1} in animal feed [30]. In the Republic of Serbia, there is a regulation limiting the presence of mycotoxins in concentrated animal feed, but not in fodders or silage. The concentrations of mycotoxins found in silages in the current study do not exceed the limits laid down by European Commission, and therefore, these silages can be used in animal nutrition as conserved animal feed of high quality.

4. Conclusion

Based on our two-year study of the effect of nitrogen fertilization and phenological phase on microbiological and mycotoxicological quality parameters of silage made from grass-legume mixtures and pure lucerne, the following conclusions can be drawn. The highest total fungal count in both years occurred in silage made from a mixture of lucerne, cocksfoot and tall fescue. Silages made from other mixtures, as well as pure lucerne silage, had significantly lower total fungal counts. Fertilization with nitrogen was associated with an increase of total fungal count in silage, as was delaying the harvesting of the crops. The most common fungus was Fusarium species, which comprised 85.5% of the total fungi counted in the silages. Concentrations of ZON and FB1 in the silages were not dependent on N fertilization nor on the phenophase of crops, and their values were below the EU allowed limits. Fertilization decreased the concentration of DON in the silages, and the values obtained were also below the EU limit allowed in animal feeds. Preventing appearance of fungi and their mycotoxins in forage and silage should begin in the field, using appropriate agro-technical measures and varieties that are well adapted to given climatic conditions and are resistant to fungal diseases. Also great attention should be directed towards ensuring good conditions during the process of the silage preparation.

5.Acknowledgements

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