Effect of Species, Fertilization and Harvest Date on Microbial Composition and Mycotoxin Content in Forage

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Abstract: The aim of the project was to evaluate the potential of microbial threat to feed safety in the year 2018. Analyses of the epiphytic community of several forage species (clovers, cocksfoot, fescue, festulolium, perennial ryegrass, timothy and trefoil) in variants of fertilized and non-fertilized vegetation were performed. The hypothesis is based on the fact that microorganisms are normally present on plant material during its growth all the way from the seed to the senescence; they are influenced by a plant's fitness, and they affect its harvest and utilization. Microflora was analyzed by cultivation on specific substrates, total microbial count and five specific microbial groups were observed and quantified. Forage species did not affect plant microflora. The highest risk factor of microbial contamination of feed was proved to be harvest date. Mycotoxin contamination of fresh feed was determined (deoxynivalenol and zearalenone) using ELISA. Zearalenone (ZEA) levels were negatively correlated to fertilization intensity, although these results were not statistically significant. Deoxynivalenol (DON) levels were the lowest in a moderate fertilization regime. Significant differences in mycotoxin content were found among botanical species.

Keywords: grass; clover; epiphytic microflora; fungi; deoxynivalenol; zearalenone

1. Introduction

Electricity consumption is increasing rapidly in the Czech Republic and decarbonization of its production is under way. One of the more sustainable production methods includes biogas stations [1]. With more than 50% of land being agricultural, possible energy sources for biogas stations are abundant [2]. This, however, presents a challenge of utilization of the biogas secondary product referred to as digestate. Digestate has the potential of becoming a new sustainable form of semi-liquid fertilizer [1].

The surface above ground biomass, such as leaves, stems or reproduction organs, is called phyllosphere [3]. It is colonized by a wide array of microorganisms; approximately 37 bacterial and 12 fungal genera is present on the wheat's leaves [4]. Microbial composition of the surface is affected by weather conditions and geographical location [5].

Microbial contamination of animal feed is closely related to research of nutritional pathology and shows its consequent effects on animal susceptibility to disease and use of antibiotics in livestock breeding [6]. Economic loss due to fungal pathogens is an important factor in feed production, with the main genera causing decreased yield and quality are *Fusarium*, *Aspergillus* and *Penicillium* [7]. Silage is a fermented feed highly used in temperate areas since the 1960s and bacterial species play

a fundamental role in its production [8]. Besides lactic acid bacteria, used as a starter additive for silaging, there are species responsible for spoilage and potential health-deteriorative properties [9].

Many species of filamentous fungi produce secondary metabolites harmful to vertebrates. Also belonging to this category, are mycotoxins, frequently occurring in cereals and other feedstuffs [10]. Mycotoxins are produced by molds under specific conditions and their production is promoted in high humidity, poor agricultural practices (e.g., inadequate fertilization, disuse of crop rotation, contaminated seeds) or damaged and contaminated crops. Although the presence of molds on grains does not necessarily mean there are mycotoxins present, the potential for mycotoxin production does exist. Furthermore, the long-term absence of molds on stored food and feed does not guarantee that the grain is free of mycotoxins [11]. The issue of mycotoxin risk is, therefore, tricky and requires the attention of both agrotechnology specifically and the scientific community generally.

For food and feed safety, the most notable mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, patulin, and zearalenone [12]. However, there are more than 500 mycotoxins known nowadays [13]. Frequently, more than one mycotoxin contaminates feed [14]. Production depends on various factors, including temperature, water activity and genotype of the mycotoxigenic species [15,16]. In silaging, there is clear evidence of fungal and consecutive mycotoxin production inhibition by lactic acid bacteria in the fermentation process [17,18].

There is not sufficient amount of information dedicated to above-ground biomass of grass and clover species, so it is essential to broaden the knowledge on effects of environmental conditions, microbial composition and plant cultivars of the Czech Republic.

2. Materials and Methods

2.1. Experimental Plot Maintenance

This study was conducted on seven forage species and their cultivars: ×*Festulolium* 'Felina', *Dactylis glomerata* L. 'Vega', *Festuca arundinacea* Schreb. 'Prosteva', *Lolium perenne* L. 'Promed' and 'Proly', *Phleum pratense* L. 'Sobol', *Trifolium pratense* L. 'Spurt' and 'Blizard', *Trifolium repens* L. 'Klondike'.

Plots were located in Research Station Vatin, Czech Republic (49°52′ N, 15°96′ E) situated 560 m above sea level, with annual precipitation of 617 mm and mean annual temperature of 6.9 °C. Soil type of the chosen experimental location was Cambisol as a sandy-loam soil on the diluvium of biotic orthognesis. Analyses were conducted in Brno, Czech Republic (49°21′ N, 16°61′ E). A randomized plot design was used and three repetitions of each variant was sown in small plots (1.25×8 m).

Spring fertilization of the experimental field was done by digestate. Input material for digestate provided by biogas station Pikarec was maize silage (13,210 tons were processed in biogas station per year 2018), cow manure (3000 t/year), cereal silage (2000 t/year), grass silage (1000 t/year) and fresh grass forage (500 t/year). Details on chemical composition of the digestate are described in Table 1; pH value was 7.77.

Table 1. Chemical composition of digestate (measured in 100 g of digestate).

Nutrient	Content (%)
Dry matter	5.70
Total nitrogen	0.49
Phosphorus	0.06
Potassium	0.42
Calcium	0.13
Magnesium	0.05

The digestate was applied in October 2017 and after each harvest. Mixed samples were created by blending of samples gathered from fertilization variants A, B and C for purposes of creating reference sample. Fertilization regime on the said three variants of plots were as described below.

- Variant A: non-fertilized control
- Variant B (150 kg N/ha/year): Total amount of nitrogen was applied in three doses, therefore each application dose contained 1/3 of annual dose = 9.43 kg digestate per 10m^2 (equal to 50 kg N/ha/application)
- Variant C (300 kg N/ha/year): Total amount of nitrogen was applied in three doses, therefore each application dose contained 1/3 of annual dose = 18.87 kg digestate per 10m^2 (equal to 100 kg N/ha/application)

Fresh biomass was collected in two harvests: 17 May 2018 and 14 July 2018, subsequently chopped into 3 cm pieces, chilled and immediately transported to specialized laboratory in Brno.

2.2. Microbial Analyses

Sample (10 g) of the original fresh biomass or silage was shaken on a PSU-10i orbital shaker (Biosan, Riga, Latvia) for 10 min with 90 mL of sterile saline. A series of ten-fold dilutions were then prepared from the solution. These groups of microorganisms were determined in the samples after cultivation as follows:

- Total microbial count (TMC) on Plate Count Agar (Biokar Diagnostics, Pantin, France) at 30 °C for 72 h.
- Lactic acid bacteria (LAB) on De Man, Rogosa and Sharpe Agar (Biokar Diagnostics, Pantin, France) at 30 °C for 72 h.
- Enterococcus sp. on COMPASS Enterococcus agar (Biokar Diagnostics, Pantin, France) at 44 °C for 24 h.
- Enterobacteriaceae on Violet Red Bile Glucose Agar (Biokar Diagnostics, Pantin, France) at 37°C for 24 h.
- Micromycetes (yeasts and molds) on Chloramphenicol Glucose Agar (Biokar Diagnostics, Pantin, France) at 25 °C for 120 h.

After the cultivation time, CFUs were counted on ColonyStar colony counter (Funke Gerber, Berlin, Germany) equipped with pressure-sensitive automatic counter and illuminated counting plate. The result was expressed as a number of colony-forming units per gram of sample.

2.3. Mycotoxin Analyses

Samples from species ×Festulolium 'Felina', *Festuca arundinacea* L. 'Prosteva' *Lolium perenne* L. 'Promed' and *Phleum pratense* L. 'Sobol' were prepared by drying fresh biomass at 60 °C. Dried samples were milled to 1 mm particles (Pulverisette laboratory cutting mill; Fritsch, Weimar, Germany) and supernatant was created for further testing by ELISA method. For DON 2 g of milled homogenate were weighted and 20 mL of distillated water was added. In ZEA analysis 2 g of milled sample homogenate were weighted and 8 mL of 90% methyl alcohol was added.

An ELISA method was applied for estimation of the mycotoxin contents. The ELISA assay test was a competitive direct enzyme-linked immunosorbent assay used for the quantitative analysis of mycotoxins. The test kits (MyBioSource, San Diego, CA, USA) were provided in a microwell format. The test was read in a microwell reader. The optical densities of the control formed the standard curve, and the sample optical densities were plotted against the curve to calculate the exact concentration of toxins [19]. Wavelenght in Synergy HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) was adjusted to 450 nm and mycotoxin content was determined.

2.4. Statistical Analyses

Data were evaluated using StatSoft Statistica 12.0 (TIBCO Software Inc., Palo Alto, CA, USA). Data was tested for normality of distribution by Shapiro–Wilk test. Kruskal–Wallis ANOVA analysis

was conducted with microbial data, single-factor ANOVA and Scheffé test were used in mycotoxin data. Significant differences were accepted if $p < 0.05$.

3. Results

3.1. Date of Sampling

The date of sampling does not affect the TMC, but it influences the composition of the microbiome of the plant. No statistical differences were found between the May and July collection dates. TMC samples showed a trend of decreasing microbial counts later in the season. Similarly, there was an increase in micromycetes and also yeasts in later sampling dates. Mean values and statistical differences are summarized in Table 2.

Table 2. Date of sampling effects on microbial counts in plant samples.

Date of Sampling	Microbial Group (CFU/g)	Mean Value	SE	p	H
17.5.2018	Total microbial count	4.39×10^5	1.95×10^8	0.2372 [a]	1.3974
	Lactic acid bacteria	3.83×10^5	1.50×10^5	0.7874 [a]	0.0727
	Enterococcus sp.	5.96×10^5	2.22×10^5	0.5331 [a]	0.3884
	Enterobacteriaceae	4.44×10^5	1.65×10^5	0.0727 [a]	0.7874
	Total micromycetes	6.14×10^5	3.01×10^5	0.0712 [a]	3.2562
	Yeasts	6.00×10^5	3.00×10^5	0.7557 [a]	0.0967
	Filamentous fungi	1.27×10^4	3.29×10^3	0.0001[b]	18.7958
14.7.2018	Total microbial count	2.27×10^8	1.26×10^8	0.2372 [a]	1.3974
	Lactic acid bacteria	3.20×10^3	8.81×10^2	0.7874 [a]	0.0727
	Enterococcus sp.	8.86×10^2	5.28×10^2	0.5331 [a]	0.3884
	Enterobacteriaceae	1.53×10^4	5.66×10^3	0.0727 [a]	0.7874
	Total micromycetes	1.60×10^6	9.00×10^5	0.0712 [a]	3.2562
	Yeasts	1.33×10^6	9.14×10^5	0.7557 [a]	0.0967
	Filamentous fungi	2.61×10^5	7.79×10^4	0.0001[b]	18.7958

SE shows values of standard error of the mean. H shows results of Kruskal–Wallis test. Mean values are statistically significant in $p < 0.05$. These values are marked with a different letter in the upper index.

Statistically significant differences between May and July sample collection dates were found only in filamentous fungi. In May, we measured an amount that was significantly lower (1.27×10^4 CFU/g) compared to July. Moreover, in the second cut grassland, fungal counts increased by more than 100% (2.61×10^5 CFU/g).

3.2. Botanical Species

Statistically significant effects of botanical species on microbial communities of epiphytic plant sections were not observed in our experiment. Highly variable data were gathered in the context of plant species. When assessed from a practical standpoint, it is important to note that higher filamentous fungi count and a, thereby, likely higher in mycotoxin contamination risk was observed in *Lolium perenne* L. 'Proly'.

3.3. Fertilization

The trend of highest microbial counts in fertilization extremes (either unfertilized control or highly fertilized variant C) was observed also in other microbial groups (Table 3). There were high deviations from mean values measured, however, data indicate the optimal fertilization regime is variant B despite the influence of other factors. Especially, amounts of enterococci were minimal in moderate fertilization regime, mean *Enterococcus* sp. was 81 CFU/g. The contrary was visible in case of filamentous fungi, highest values were obtained from a moderately fertilized plot (2.88×10^5 CFU/g). Significant differences were only found among fertilization variants and mixed sample, however mixed sample results were only included as a benchmark for values.

Table 3. Fertilization effects on microbial counts in plant samples.

Fertilization Regime	Microbial Group (CFU/g)	Mean Value	SE	*p*	H
Variant A (0 kg N/ha)	Total microbial count	3.50×10^8	2.41×10^8	0.0001 [a]	20.4679
	Lactic acid bacteria	5.20×10^5	2.65×10^5	0.0040 [a]	13.3123
	Enterococcus sp.	6.86×10^5	3.60×10^5	0.0413 [a]	8.2419
	Enterobacteriaceae	5.05×10^5	2.71×10^5	0.0116 [a]	11.0197
	Total micromycetes	2.24×10^6	1.60×10^6	0.0005 [a]	17.5514
	Yeasts	2.18×10^6	1.59×10^6	0.0035 [a]	13.5785
	Filamentous fungi	6.27×10^4	1.62×10^4	0.0025 [a]	14.3612
Variant B (150 kg N/ha)	Total microbial count	9.53×10^7	3.60×10^7	0.0001 [a]	20.4679
	Lactic acid bacteria	2.62×10^3	9.51×10^2	0.0040 [a]	13.3123
	Enterococcus sp.	81.4	30.5	0.0413 [a]	8.2419
	Enterobacteriaceae	9.72×10^3	3.62×10^3	0.0116 [a]	11.0197
	Total micromycetes	3.54×10^5	1.62×10^5	0.0005 [a]	17.5514
	Yeasts	3.64×10^4	2.62×10^4	0.0035 [a]	13.5785
	Filamentous fungi	2.88×10^5	1.51×10^5	0.0025 [a]	14.3612
Variant C (300 kg N/ha)	Total microbial count	8.34×10^8	3.04×10^8	0.0001 [a]	20.4679
	Lactic acid bacteria	1.99×10^5	9.66×10^4	0.0040 [a]	13.3123
	Enterococcus sp.	4.32×10^5	2.37×10^5	0.0413 [a]	8.2419
	Enterobacteriaceae	3.47×10^5	1.69×10^5	0.0116 [a]	11.0197
	Total micromycetes	1.65×10^6	6.82×10^5	0.0005 [a]	17.5514
	Yeasts	1.39×10^6	7.33×10^5	0.0035 [a]	13.5785
	Filamentous fungi	2.60×10^5	1.22×10^5	0.0025 [a]	14.3612

SE shows values of standard error of the mean. H shows results of Kruskal–Wallis test. Mean values are statistically significant in $p < 0.05$. These values are marked with a different letter in the upper index.

3.4. Mycotoxin Contamination of Fresh Feed

Species and fertilization were tested for affecting the mycotoxin occurrence in plant samples. Zearalenone levels in fresh biomass were generally lower than DON. Practical significance of fertilization related to mycotoxin production was found, although statistically there were no differences proven (Table 4). Both mycotoxins occurred in all fertilization regimes. DON concentrations were lowest in moderately fertilized variant B (5.03 ng/mL). Highest DON contamination was observed the highly fertilized variant C (5.32 ng/mL). ZEA concentration slightly increased with decreased use of digestate, with lowest value of 1.18 ng/mL and highest of 1.39 ng/mL (Table 4).

Table 4. Fertilization effects on mycotoxin concentration in plant samples.

Fertilization Regime	Mycotoxin Concentration (ng/mL)	Mean Value	SE	*p*
Variant A (0 kg N/ha)	Deoxynivalenol	5.0979	1.29	0.4437 [a]
	Zearalenon	1.3984	0.23	0.5574 [a]
Variant B (150 kg N/ha)	Deoxynivalenol	5.0317	1.86	0.4437 [a]
	Zearalenon	1.1825	0.11	0.5574 [a]
Variant C (300 kg N/ha)	Deoxynivalenol	5.3212	1.79	0.4437 [a]
	Zearalenon	1.1820	0.10	0.5574 [a]

SE shows values of standard error of the mean. Mean values are statistically significant in $p < 0.05$. These values are marked with a different letter in the upper index.

There were notable differences in mycotoxin contamination among botanical species (Table 5). Highest levels of DON were found in *Festuca arundinacea* (7.93 ng/mL) and *Phleum pratense* (7.63 ng/mL). Less susceptible to mycotoxin contamination were proved to be *Festulolium* (1.05 ng/mL) and *Lolium perenne* (2.43 ng/mL). Significant differences were found between these two groups, however, there were no statistical differences between ×*Festulolium* and *Lolium* or *Phleum* and *Festuca*.

Table 5. Botanical species effects on mycotoxin concentration in plant samples.

Species	Mycotoxin Concentration (ng/mL)	Mean Value	SE	p
×*Festulolium* 'Felina'	Deoxynivalenol	1.0520	0.52	0.0008 [a]
	Zearalenon	0.9665	0.03	0.4437 [b]
Festuca arundinacea L. 'Prosteva'	Deoxynivalenol	7.9331	0.91	0.0008 [a]
	Zearalenon	1.2893	0.11	0.4437 [b]
Lolium perenne L. 'Promed'	Deoxynivalenol	2.4330	1.43	0.0008 [a]
	Zearalenon	1.3480	0.37	0.4437 [b]
Phleum pratense L. 'Sobol'	Deoxynivalenol	7.6381	0.81	0.0008 [a]
	Zearalenon	1.3209	0.06	0.4437 [b]

SE shows values of standard error of the mean. Mean values are statistically significant in $p < 0.05$. These values are marked with a different letter in the upper index.

4. Discussion

Presence of microbial families and species is geographically specific, which leads us to believe that the same is true for mycotoxins [20]. Study of epiphytic microbial colonies and elements affecting their occurrence is multi-faceted, with high variability due to many apparent factors, such as climatic conditions (e.g., temperature, water activity), microbial tolerance to pH and phytochemicals or mycotoxigenic species genotype [15,16,21,22].

Not many authors have studied epiphytic plant microbiomes, and focus of microbial observations in relation to grassland management techniques is mainly on soil microbiota [23]. Moreover, there are not enough complex studies (including both bacterial and fungal species) done in grassland ecosystems and forage species. Microbial observations are mainly focused on pathogenic *Fusarium* sp. [24]. However, some authors have done experiments on bacterial communities on maize or rice [25,26]. From results gathered in this experimental study, it is possible to conclude that botanical species do not affect microbial composition of a plant's phyllosphere. Species variants with the same climatic and soil factors were observed to have similar CFU counts, which is probably true for the unmonitored weed species as well. However, this conclusion is only applicable in terms of the experimental location of this study and similar weather conditions.

In case of differences between harvest dates, statistical significance between May and July harvests was observed in fungal counts. Lower amounts were found in May, although they were abundant in July samples. It may have been caused by the average monthly air temperature of 18.3 °C in the research location, which has a positive effect on fungal growth and reproduction [27]. This may have also been caused by high pathogenic pressure of the year 2018.

Karlsson et al. [28] had previously recorded a positive correlation between production intensification (increased nitrogen concentrations by fertilization) and higher microbial counts. We found no significant difference between microbial counts in any of the fertilization regimes.

In late autumn, usually, the vegetation of pasture plants gradually decreases and weather conditions stimulate the development of microscopic fungi which, in consequence, may lead to the formation of mycotoxins [29–31]. Besides population density, the formation of mycotoxins additionally depends on several biotic and abiotic factors [32,33]. These metabolites can cause economic losses in animal production and decrease meat quality [34].

DON has an important physicochemical ability of withstanding high temperatures, which increases the risk of its occurrence in food [35]. In another research, it was analyzed that in animals that were exposed to the (DON) a subsequent transfer of this toxin to animal products was found. However, the rate of transmission was low. Overall, the study showed that short-term and sub-chronic exposure to DON decreased body weight, weight gain, and feed consumption in rats and mice. Haematological effects were also observed [36].

Zearalenone is one of mycotoxins produced by the *Fusarium* genus. It can be detected in the forage of grass stands. It exhibits high oestrogenic activity. Apart from the direct impact on ruminants,

the contaminated forage affects rumen microorganisms, too [37]. Forage with a zearalenone content higher than 0.5 mg/kg is not advised for feeding [38].

Many strategies can be employed in the process of decreasing the mycotoxin contamination of animal feed, the most effective is prevention of contamination on the field. One of these strategies may be ensuring optimal nutrient content in soil. There was a visible decreasing trend of ZEA contamination with an increase of digestate amount. From this negative correlation, we can conclude that higher levels of fertilization can help in prevention from fungal degradation of feed in case of ZEA. This, however is not the most efficient way to combat DON accrual in feed. Therefore, the moderate intensity of fertilization is an optimal solution.

The contents of DON and ZEA depended on the course of weather, too. Sutton et al. [39] stated, that rainfall increases the occurrence of zearalenone in corn during summer, although different temperatures did not have an effect on its production. During the growing season, forage grasses may become contaminated with mycotoxins. This phenomenon mainly occurred in May and in July, which means a high risk of mycotoxin input to the food chain.

When cattle are grazing in winter, a higher occurrence of mycotoxins in the feed may be expected. Related damage to animal metabolism may affect the number of diseased animals and/or diagnostics of animal diseases. Consequently, mycotoxins impact not only performance and health of animals, but also overall economy of production [40]. However, some authors estimate that breeding of new species and improvement of currently used forage lines appears to be the most perspective approach [41,42]. From results of our study, we can conclude that in climatic and soil conditions of Vatin (Czech Republic) ×*Festulolium* hybrids appear to be promising due to their lower mycotoxin contamination. Post-harvest technologies have been studied heavily in recent years, including use of essential oils, LAB additives or acidic electrolyzed water [43–45].

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