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The horse's hidden threat: occurrence and exposure risks to the tremorgenic alkaloid paxilline in central European grasslands

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ABSTRACT

Background: Paxilline is a tremorgenic indole-diterpenoid produced by toxin-producing fungal endophytes in meadows and pastures. It is currently understudied in European grasslands.
Aims/objectives: This study evaluated the occurrence of paxilline in selected European grasslands and estimated the exposure risks for horses.
Methods: The risk of the paxilline exposure was estimated based on the analyses made from a total of 392 grass samples from grasslands. The grass species of *Lolium perenne, Lolium multiflorum, Festuca pratensis, Festuca arundinacea, Festuca rubra, Festuca nigrescens,* and *Festulolium* × *loliaceum* were sampled from grasslands in Austria (n=42 samples, 1 location), France (n=134, 1 location), Hungary (n=14, 1 location), and Germany (n=192, 4 locations). Concentrations were determined using ultra-performance liquid chromatography–mass spectrometry.
Results: Per location and year, the positive samples for paxilline ranged from 0 to 45% of tested samples. Associations with location and plant species were observed ($p < 0.001$). Model estimates using logistic procedures indicated high likelihoods (up to 75%) for detecting paxilline in certain grasslands, such as in France and Austria, especially for *F. rubra,* followed by *L. multiflorum* and *L. perenne*. Temperatures measured two months before sampling were more positively associated with the concentration of paxilline than that at sampling week.
Conclusions: Horses are exposed to paxilline in central European grasslands, but the exposure risk differs with their location and plant species. The study suggests that besides preserving the biodiversity of grasslands, routine monitoring for alkaloid occurrence may be needed, especially when *F. rubra, L. multiflorum* and *L. perenne* are present.

1. Introduction

Pastures and meadows are the cornerstone of equine feeding, used for grazing and producing roughage. However, inopportune grassland management hides several threats for animal health, especially if present vegetation is contaminated with toxin-producing fungal endophytes. These are fungi that form a mutualistic relationship with their host plant, and may infect a range of grasses, including meadow fescue grass (*Festuca pratensis*) and perennial ryegrass (*Lolium perenne*). The two aforementioned cool-season grasses combine several beneficial traits in respect to productivity, including high nutritional value and weather resistance, and making up a large percentage of grasses on European grasslands [1,2]. Endophytes of the genus *Epichloë,* such as *E. coenophiala* and *E. festucea var. lolii,* can infect theses grasses and produce various endophyte alkaloids, being an increasing threat to animal health [3,4].

Among alkaloids produced by endophytes, ergot alkaloids and indole diterpenes are known to be (neuro)toxic to mammals [3–5]. Nevertheless, most of the research undertaken in grazing ruminants and horses so far has mainly focused on ergot alkaloids, especially the ergopeptine ergovaline, the main cause of equine fescue toxicosis [3,4]. Illnesses caused due to consumption of endophyte-contaminated grass are referred to as "endophyte toxicity", which is a widely known phenomenon in the USA, Australia, and New Zealand, and spontaneous cases are

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also described in Europe [6,7].

In Europe, research on indole-diterpenoid lolitrem B has recently attracted increasing attention [1,8,9]. Lolitrem B has tremorgenic properties and is seen as the primary cause of ryegrass staggers in livestock and horses [10]. Lolitrem B and paxilline share a common moiety, with paxilline also shown to be tremorgenic, thus potentially amplifying the effects of lolitrem B [11,12]. Besides endophytes, paxilline is also produced by the filamentous ascomycete *Penicillium paxilli* [13]. Although incidences of endophyte toxicosis seems to be rare in Europe, studies have documented occurrence of "cocktails" of ergot alkaloids and lolitrem B in European grasslands [1,8,9,14,15]. However, to our knowledge, the occurrence of paxilline has not yet been studied, and this information is of importance for predicting the tremorgenic exposure and health risk to livestock. This study aimed to evaluate the occurrence and risk of encountering paxilline on selected central European horse grasslands. Our hypothesis was that there are exposure risks of paxilline in central European grasslands, but different based on the location, year, and vegetation (i.e., plant type).

2. Materials and methods

2.1. Locations of the pastures and meadows

Over the course of three years (2021 - 2023), 7 horse studs (i.e., locations) were selected in Austria (1 in the province of Salzburg), Germany (3 from the federal state of Baden-Württemberg, 1 in Bavaria), France (1 in the Auvergne-Rhône-Alpes district), and Hungary (1 in the Veszprém region), and included in this study. Most of the grass samples analyzed in this study were collected in an earlier study reporting ergovaline and lolitrem B concentrations [1]. Further details of the study area and locations, including specific information of each grassland evaluated have been reported [1]. In brief, in each location, 5–10 grasslands used as equine pastures and meadows for hay production were sampled, with 5 plots per grassland, yielding a total of 392 grass samples. Supplementary Table 1 gives an overview of sampling location and sampling times per location, including the number of plots per location and the number of samples taken. The specific location (longitude, latitude, altitude, slope), date, and time for each sampled grassland were noted. The mean altitude of the grasslands in the study varied from 235 m (Hungary) to 650 m (Austria), and the average slope ranged from 0 to 13%. All vascular plant species of each sampled plot were identified and documented by an expert. The phenological growth stadium of the plants was monitored according to the scaling recommendations of the Biologische Bundesanstalt Bundessortenamt and Chemische Industrie (BBCH scale; [16]). Samples of *Festuca* and *Lolium* species, genera which include known hosts of *Epichloë* endophytes, were collected and sent to the lab for analysis.

2.2. Sampling and analyses

Details for sampling were reported earlier [1]. In brief, from each vegetation plot of 10 m^2, only samples of *Lolium* and *Festuca* species were collected, with a focus on inflorescences, seeds, and lower leaf sheaths. Samples were stored in paper bags and left to dry at room temperature until sample preparation and analysis. Due to time and budget constraints, only a subset of collected samples could be tested. The number of grass species per location was different, but in general 143 *Lolium* and 112 *Festuca* samples were analyzed, whereas the rest of the species were less represented.

A total of 392 samples were analyzed for paxilline. Alkaloids were extracted from dried plant material as follows: first, 20 mg of tissue was ground to a fine powder using a mixer mill (Retsch MM400) and two UFO stainless steel beads (5.6 and 3.2 mm diameter). One mL of methanol:water:formic acid (70:30:0.1, v/v/v) was added and the mixture was shaken in the mixer mill for 4 min at 30 Hz. Following centrifugation, the supernatant was placed into an HPLC vial and 1 μL injected in a UPLC I-Class (Waters) system coupled to a QTRAP 6500+ (Sciex). The column for separation was an Acquity UPLC BEH C18 50mm x 2.1 mm internal diameter, 1.7 μm particle size (Waters). The mobile phases were (A) H_2O + 0.05% formic acid and (B) acetonitrile + 0.05% formic acid. A gradient from 5% B to 93.2% B in 6.5 min at 0.4 mL/min was applied, followed by a hold at 100% B for 2 min and reequilibration at 5% B for 1.5 min. The column temperature was 25°C. Alkaloids were monitored in positive electrospray ionization using the following transitions: Q and q transitions were 436.2-421.2 and 436.2-130.1, respectively. Source parameters were IS 5500 V, TEM 400°C, GS1 50 psi, GS2 40 psi, and CUR 30 psi. The system was controlled by Analyst 1.7.1. Quantification was done by external calibration using reference standards of paxilline.

Since one of the objectives was to discover possible correlations between paxilline concentrations and weather conditions preceding the sampling period, weather data were included in our analyses. Using a meteorological website (https://open-meteo.com/), hourly weather data including lowest temperature (in°C), highest temperature (in°C), duration of sunshine (in hours), and amount of precipitation (in mm) were compiled.

To ensure the possibility of studying long-term effects of the influence of weather conditions on toxin concentration, data were compiled starting from 1, 2, and 3 months prior to sampling. A weekly average was calculated using daily weather data. In addition, monthly averages were calculated for three months prior to the month in which the sample was taken.

2.3. Statistical analysis

The data were statistically analyzed using the SAS program (version 9.4, SAS Institute Inc., Cary, NC, USA). The likelihood of detecting paxilline as affected by various factors (i.e., BBCH scale, month of sampling, location, slope, plant species) was tested with the logistic procedure (PROC LOGISTIC). The analysis of maximum likelihood estimates (Wald Chi-Square test) with a model of backward selection method was used to evaluate potential influencing factors (i.e., month of sampling, plant species, location, slope, BBCH scale), by selecting only those showing a significant relationship ($p < 0.05$).

The correlation analysis was performed using CORR procedure to evaluate potential associations between weather factors and the alkaloid concentrations. For this, the Pearson correlation coefficient (*r)* and *p*-value were computed. Only *r* with $p < 0.10$ were considered in the interpretation of the relationships as follows: $r = 0.45–0.50$, the relationship was considered good; $r = 0.50–0.70$, the relationship was considered strong; $r > 0.70$, the relationship was considered very strong. All results with $p \geq 0.10$ were considered not statistically significant.

3. Results

3.1. Paxilline occurs in grasslands at all locations

Data of the descriptive analysis with number of samples, positive samples, and the concentration measured are shown in Table 1. All concentrations of paxilline are reported on a dry matter (DM) basis. In 2021, paxilline was detected in 11 samples (26.8%). Of these, two detections were in Austria (maximum concentration: 14 μg/kg) and 9 detections in France (mean concentration: 20.6 μg/kg and maximum concentration: 165.6 μg/kg). Only 16 samples (15.4%) were positive in 2022. Austria had 9 positive samples (25.7%) with a maximum concentration of 215.4 μg/kg, and a mean concentration of 16.2 μg/kg. DE-4 showed 5 positive samples (15.6%), with a maximum concentration of 9.3 μg/kg and a mean concentration of 0.7 μg/kg. DE-3 had 2 positive samples (5.4%), with a maximum concentration of 1.3 μg/kg and a mean concentration of 0.1 μg/kg. In 2023, 50 out of 247 screened samples were positive, whereby the majority (39) were found in France. Overall, the highest concentration was found in DE-1 location in Germany with

Table 1
Descriptive statistics of paxilline data at various locations and years.

Year/ Location	n	Number positive	% positive	Concentration (μg/kg DM)		
				mean	SD	Max
2021	41	11	26.8	10.4	31.1	165.6
AT	7	2	28.6	2.2	5.2	14.0
DE-4	10	0	0.00	0.0	0.0	0.0
HU	4	0	0.00	0.0	0.0	0.0
FR	20	9	45.0	20.6	42.6	165.6
2022	104	16	15.4	5.7	29.0	215.4
AT	35	9	25.7	16.2	48.7	215.4
DE-4	32	5	15.6	0.7	1.9	9.3
DE-3	37	2	5.4	0.1	0.3	1.3
2023	247	50	20.2	8.1	26.2	206.9
DE-1	61	5	8.2	10.7	41.4	206.9
DE-2	52	5	9.2	2.8	11.8	71.1
HU	10	1	10.0	0.7	2.2	7.1
FR	124	39	31.4	7.9	19.1	129.9

n = number of samples analysed; DM = dry matter; SD = standard deviation; Max = maximum concentration; AT = Austria; DE = Germany (4 different locations), FR = France, HU = Hungary (details on locations are provided in Supplementary Table 1).

206.9 μg/kg.

3.2. The exposure of horses to paxilline depends on location and grass species found in grasslands

The exposure risk of horses to paxilline was estimated based on the predicted probability for detecting it. The effect of factors on the exposure risk to paxilline, including values of the Wald Chi-Square and *p,* are shown in Table 2. This analysis revealed that the factors "location" and "plant species" were significantly related to the exposure of horses to paxilline. However, phenological growth stadium of plants, month of sampling, type of grassland (pasture or meadow), and slope did not play a significant role in the probability of encountering the toxin. As illustrated in Fig. 1a, the risk for detecting paxilline was the highest in France, where also the highest concentration of paxilline was measured (Fig. 1b). Further, the analysis showed the highest probability (72%) to detect paxilline when *F. rubra* was present in the pastures and meadows (Fig. 1a). *F. rubra* also showed the highest paxilline concentration (Fig. 1c). A high risk (60%) for paxilline was also found for the same plant species in the grasslands of Austria. Another species with relatively high risk for paxilline was *L. multiflorum*, and then *L. perenne*, especially in France and Austria. In contrast, other plant species and sites (independent of plant species) showed low probability to detect paxilline.

Table 2
Effects of different factors on the concentrations of paxilline measured from samples collected in pastures and meadows in Austria, Germany, France, and Hungary.

Factors	Descriptive statistics			Data of the model	
	min	max	DF	Wald Chi-Square	*p* value
BBCH scale[1]	7	99	24	24.58	0.428
Month of sampling	May	July	3	2.498	0.475
Pasture vs. Meadow			2	0.087	0.957
Slope (%)	0	13	13	13.41	0.416
Species[2]			6	55.79	<0.001
Location[3]			6	26.87	0.001

[1] The phenological growth stadium of the plants was monitored according to the scaling recommendations of the Biologische Bundesanstalt Bundessortenamt and Chemische Industrie (BBCH scale; Hess et al., 1997).

[2] *Festuca arundinacea, Festulolium × loliaceum, Festuca pratensis, Festuca rubra, Lolium multiflorum, Lolium perenne,* others.

[3] Details on locations are provided in Supplementary Table 1.

3.3. Air temperature at 2 months before sampling correlates with paxilline concentrations

This analysis showed that the maximum air temperature measured at 2 months before sampling date correlated positively ($r = 0.65$) with the paxilline concentration of grass samples (Table 3). Temperature at the time of sampling did not correlate with concentrations of paxilline. Other climatic variables, such as sun duration or rainfall, did not correlate with the paxilline concentration at any of the sites.

4. Discussion

Grazing or consuming hay coming from grasslands contaminated with indole-diterpenoids, such as lolitrem B and paxilline, can cause tremorgenic disorders in animals. The presence of lolitrem B in grasslands has been recently reported in Europe [1,8,9]; however, there is limited information about the occurrence of paxilline in European grasslands. To help bridge this gap, we evaluated the occurrence and risk of encountering paxilline in selected grasses of central European grasslands used for hay production or grazing by horses. Our study revealed the occurrence of paxilline from 0 to 45% of the samples tested differing among locations and years of sampling, with major differences between tested locations. Interestingly, our findings confirmed our hypothesis about the exposure risks of paxilline in central European grasslands, highlighting that the risk of encountering the alkaloid differs by location and plant species. In our analysis, the grass samples from France had the highest risk for detecting paxilline, followed by Austria, and one location in Germany. Indeed, our analysis revealed that the effect of grassland location on paxilline occurrence was potentiated by the plant species of the grasslands, whereby the presence of species like *F. rubra* increased the risk of paxilline occurrence, especially in the pastures and meadows in France and Austria. Interestingly, as with the risk of detecting paxilline, *F. rubra* aggr. was also associated with increased risk for ergovaline detection, and even lolitrem B was found in *F. rubra* aggr., as reported in the study by Seiler et al. [1]. *F. rubra* aggr. is a ubiquitous species aggregate in grasslands throughout Europe. Previous studies have confirmed that *F. rubra* can be infected by the endophytic fungus *E. festucae* [17] and an association between *E. festucae* and *F. rubra* is described as common and at a high frequency in Mediterranean permanent grasslands [18]. Thus, our study suggests a similar risk of *F. rubra*-related endophytes in the grasslands of central European countries as is the case in Mediterranean permanent grasslands. Among the other species with increased risk for paxilline occurrence were *L. multiflorum* and *L. perenne.* The latter is best known for infection by the endophyte *E. festucea var. lolii*, producing the alkaloid lolitrem B that is consequently responsible for ryegrass staggers in mammals. Indeed, ryegrass staggers have been described in horses grazing on perennial ryegrass (*L. perenne*) infected with *E. festucae var. lolii* in Australia, New Zealand, and the United States [7,10], and sporadic cases of intoxication have also been reported in Europe.

The strong effect of location on paxilline responses, especially the fact that the grass samples of some sites showed low probability to detect paxilline, independent of plant species, indicates that other local factors beyond those evaluated in the study might have played a role in this variation. We did not find an effect of the sampling month and phenological growth stadium (based on the BBCH scale) or the use of the grassland (pasture vs. meadow); however, it is known that in vegetative tissues (BBCH stage 2) endophytes are mostly located at the base of the tiller, but as the floral tiller develops (BBCH stage 3 and 4) the endophyte grows with the developing floral tiller, infecting the developing seed for subsequent transfer to following generations. The alkaloid levels are highest in the mature seed [8]. Endophytes change their relative concentrations within different tissues of the grass plant during their growth. At the beginning, endophytes are mostly located at the base of the plant, and while growing, the fungus moves towards the growing stem until it reaches the seedheads, so that in the fully ripe

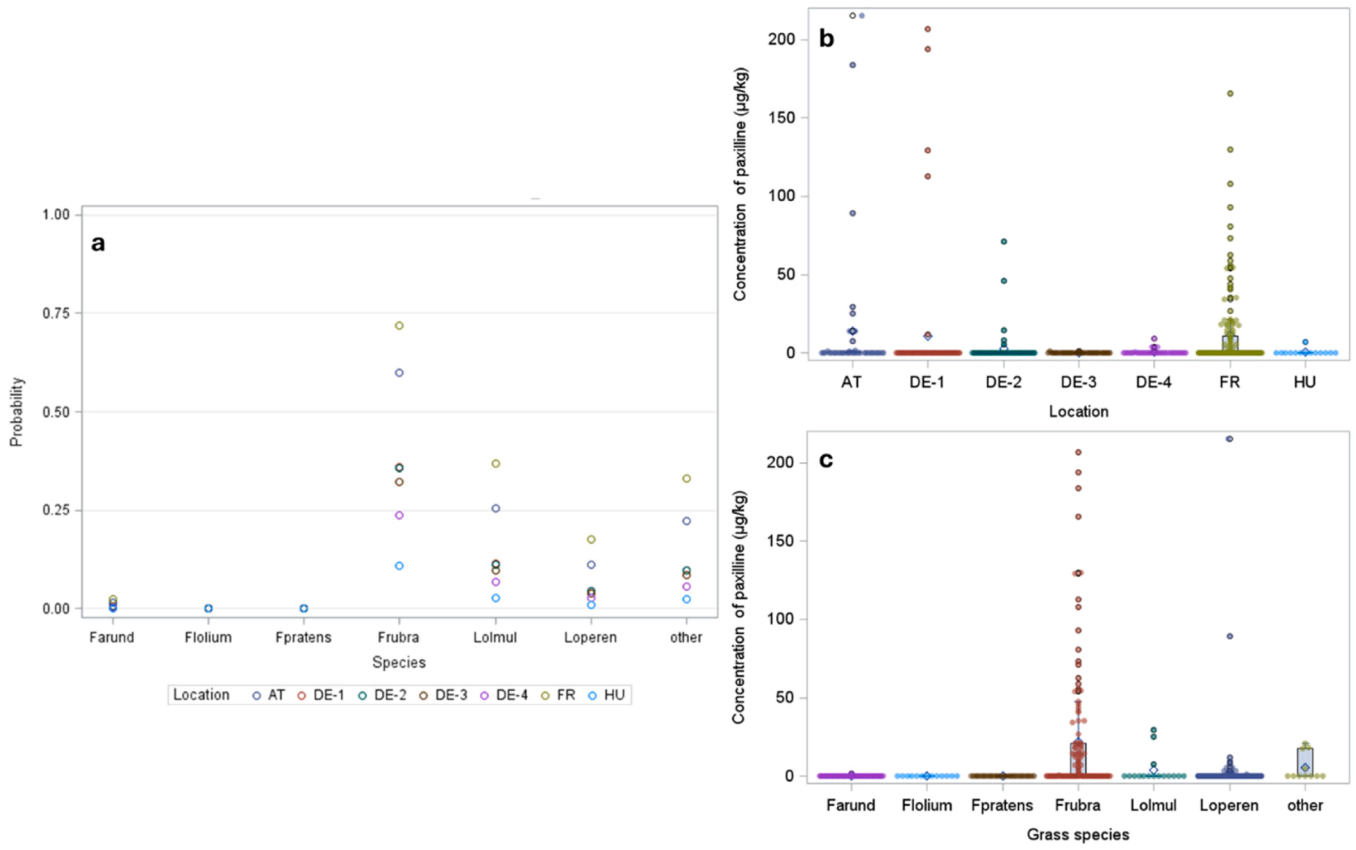

Fig. 1. Predicted probability to detect paxilline (a), the concentrations of paxilline at different locations (b) and within different grass species (c) [(locations: AT = Austria; DE = Germany (4 locations), FR = France, HU = Hungary) (plant species: Farund = *Festuca arundinacea,* Flolium = *Festulolium loliaceum,* Fpratens = *Festuca pratensis,* Frubra = *Festuca rubra,* Lolmul = *Lolium multiflorum,* Loperen = *Lolium perenne.* Other = other than previous)].

Table 3
Descriptive statistics and Pearson correlation coefficients (r) with p value between various weather data and the concentrations of paxilline of the horse pastures and meadows.

	Descriptive statistics		Statistics parameters	
Weather variable[1]	min	max	*r*	*p* value
Minimal temperature (°C)				
3 mo	-0.6	4.2	0.37	0.24
2 mo	0.4	10.1	0.49	0.08
1 mo	5.0	14.1	0.38	0.18
At sampling	8.5	17.2	0.01	0.96
Maximal temperature (°C)				
3 mo	7.1	15.6	0.54	0.07
2 mo	10.8	19.4	0.65	0.01
1 mo	14.5	26.0	0.44	0.11
At sampling	17.9	28.5	0.11	0.72
Sun duration (h/d)				
3 mo	4.9	11.2	-0.12	0.71
2 mo	6.3	11.7	-0.11	0.71
1 mo	7.7	13.1	0.09	0.76
At sampling	9.2	14.3	-0.24	0.41
Rainfall (mm)				
3 mo	0	4	0.32	0.31
2 mo	0.9	4.7	0.05	0.85
1 mo	1.1	5.5	0.27	0.42
At sampling	0	4.6	0.36	0.20

3 mo = 3 months before sampling time; 2 mo = 2 months before sampling time; 1 mo = 1 month before sampling time; At sampling = measurement at sampling time (details on sampling times are provided in Supplementary Table 1).

stage, the toxin level is highest [8]. For example, Repussard et al. [8] found that grasses reach maximum lolitrem B concentrations at the fully ripe stage, and that the concentration is higher in the inflorescence than other parts of the plant. In our study, we collected basal plant parts, inflorescences and seedheads, as long they were available, and attempted to have all these plant parts equally represented for the analysis.

In terms of toxicity risks, the measured concentration of the alkaloid plays an important role together with individual characteristics of animals and their overall feed intake. There is no threshold of toxicity for paxilline, neither for livestock, nor horses. In terms of concentration, the levels of paxilline measured in our study can be considered as rather low (<300 µg/kg DM), when relating to the existing data of lolitrem B. The level of toxicity for lolitrem B in bovines is 2000 µg/kg [19], whereas for horses there is no recommendation on lolitrem B threshold. In horses, Johnstone et al. [10] tested the effects of lolitrem B at doses of 2070 to 2170 µg/kg for two weeks (which is at the threshold established to display neurological signs in bovines), and reported tremors, ataxia, and other important biochemical disturbances. These symptoms were reversible following the removal of the toxin exposure. In the study conducted by Seiler et al. [1], the reported level of lolitrem B was also low (<1100 µg/kg) in all 4 countries, being similar to the levels measured for lolitrem B in a study conducted earlier in southern France, measuring lolitrem B in cultured perennial ryegrass [8]. The low concentrations of lolitrem B and paxilline which were measured seem to fit with the low incidence rate of ryegrass staggers on the European continent. However, a study conducted on 3 sites in Germany observed that 14% of samples exceeded the toxicity thresholds of lolitrem B set for ruminants [9]. This was similar to the levels measured in inflorescences of *L. perenne* in France [8].

The risk for encountering elevated concentrations of tremorgenic alkaloids such as paxilline and lolitrem B that could cause ryegrass staggers in livestock and horses seems to be rather low in the grasslands tested in this and other studies [1,8,9]. Although our analysis revealed

no effect of sampling month on paxilline concentration, the weather data analysis revealed that temperature measured at 2–3 months before sampling correlated positively to paxilline, suggesting longer-term effects of temperature on endophyte growth. These and research data reported previously [8,9,14,15] indicate that higher temperatures in European grasslands are associated with greater endophyte alkaloid concentrations. For example, Penagos-Tabares et al. [14] found that the concentration of the ergot alkaloids in pastures increased exponentially with increasing temperatures. König et al. [9] measured mean concentrations of lolitrem B in *L. perenne* samples and reported significantly higher concentrations of lolitrem B in summer vs. spring (mean of 3400 vs. 500 mg/kg). In the light of climate warming, it thus may be advisable to monitor meadows and pastures for the presence of fungal endophytes and their toxic alkaloids in order to avoid potential intoxication of animals. Overall, there is a knowledge gap regarding the relative toxicity and tremorgenic potential of paxilline as compared to lolitrem B to horses. Although the results from mouse and sheep studies indicate that paxilline is 2-14 times less tremorgenic that lolitrem B [11,12,20], this has not yet been confirmed for horses.

5. Conclusion

This study revealed that horses are exposed to paxilline in selected grasses from central European grasslands, but the exposure risk differs from location to location. Although there are no defined toxicity threshold levels for horses thus far, the measured concentrations are probably too low to cause acute toxicity. This study confirmed that occurrence risk is higher in specific grass species, especially *F. rubra*, particularly at higher temperatures. Thus, our data suggest the need for regular monitoring of grasslands for indole diterpenes, including paxilline, especially when the risk factors reported in this study are present.

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Statement for animal/human studies

This research did not involve animal or human studies.

CRediT authorship contribution statement

Q. Zebeli: Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Resources, Conceptualization. **R. Khiaosa-ard:** Writing – review & editing, Visualization, Validation, Project administration, Methodology, Formal analysis. **V. Milojevic:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **R. Vanselow:** Writing – review & editing, Project administration, Investigation. **B.U. Metzler-Zebeli:** Writing – review & editing, Visualization, Resources, Conceptualization.

Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jevs.2026.106206.

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