Mycotoxin Research (2020) 36:159–172
https://doi.org/10.1007/s12550-019-00377-5

ORIGINAL ARTICLE

Fungal and chemical diversity in hay and wrapped haylage for equine feed

Birgitte Andersen[1] · Christopher Phippen[1] · Jens C. Frisvad[1] · Sue Emery[2] · Robert A. Eustace[2]

Received: 1 August 2019 / Revised: 17 October 2019 / Accepted: 21 October 2019 / Published online: 27 November 2019

Abstract

The presence of fungi and mycotoxins in silage (fermented maize) for cattle and other ruminants have been studied extensively compared to wrapped haylage (fermented grass) for horses and other monogastric animals. The purpose of this work was to examine the fungal diversity of wrapped haylage and conventional hay and to analyse the forage sample for fungal metabolites. Faeces samples were also analysed to study the fate of fungi and metabolites. Fungal diversity of the samples was determined by direct plating on DG18, V8 and MEA and chemical analyses were done using LC-MS/MS. The results show that *Sordaria fimicola* was common in both hay and haylage, while *Penicillium* spp. was prevalent in haylage and *Aspergillus* spp. in hay. Communiols were found in all types of samples together with gliocladic acid. Roquefortines and fumigaclavines were found in haylage with no visible fungal growth, but not in hay. In haylage hot spot samples, a series of *Penicillium* metabolites were detected: Andrastins, fumigaclavines, isofumigaclavines, marcfortines, mycophenolic acid, PR toxins, and roquefortines. *Penicillium solitum* was found repeatedly in haylage and haylage hot spot samples and viridicatols were detected in a hot spot sample, which has not been reported before. Even haylage with no visible fungal growth contained more metabolites than hay. Individually, the metabolites detected in haylage may, in high doses, be mutagenic, neurotoxic or immunosuppressive; but the synergistic effect of small doses may also have other or greater negative health effects on equines than on ruminants.

Keywords Horses · Ponies · Mycotoxins · Roquefortine · Laminitis · Metabolite profiling · Adverse health effects

Introduction

Equines, being monogastric animals, are considered to be more sensitive to fungi and mycotoxins in their forage than ruminants (Wambacq et al. 2016) and several studies describe cases of leukoencephalomalacia and stachybotryotoxicosis in equines caused by fumonisins (*Fusarium* spp.) and trichothecenes (*Fusarium* and *Stachybotrys* spp.) resulting in both acute and chronic toxicoses and death (Le Bars and Le Bars 1996; Liesener et al. 2010; Vendruscolo et al. 2016; Pitt and Miller 2017). In vitro studies on lameness (laminitis) in horses found that fumonisin B1 induced lamellar separation in the hoofs (Reisinger et al. 2016) and in vivo studies found that juglone, the active compound in Black walnut (*Juglans nigra*) husks, could cause laminitis within 12 h (Galey et al. 1991). Furthermore, Hanche-Olsen et al. (2008) suggested that forage of poor microbial quality may be involved in hind limb lameness (acquired equine polyneuropathy).

Traditionally, equine forage has been hay, grain and grass, but since the 1980s, fermented grass, wrapped in bales (haylage) has partially or totally replaced hay for the feeding of equines in Northern Europe (Müller 2005, 2018; Müller et al. 2011; Schenk et al. 2018). One reason for replacing hay with haylage is that if the animal has respiratory problems (e.g. equine asthma or chronic obstructive pulmonary disease), feeding haylage avoids the dust from old or mouldy hay (Thomson and McPherson 1983). Hay dust has been shown to contain high levels of *Aspergillus* and *Penicillium* spp. spores, but no mycotoxins (Séguin et al. 2010). Another reason why haylage production and consumption has increased is convenience; grass for wrapping can be processed when it is moist and bales kept in plastic can be stored outside, whereas conventional hay has to dry in the field and is stored

✉ Robert A. Eustace
rae@equilife.co.uk

[1] Department of Biotechnology and Biomedicine, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

[2] The Laminitis Clinic, Mead House, Dauntsey, Chippenham, Wiltshire SN15 4JA, UK

indoors under dry conditions (Coblenz and Akins 2018; Schenk et al. 2018).

Although mycotoxins in fermented maize (silage) for cattle have been studied extensively (Alonso et al. 2013; Storm et al. 2014), less is known about the fungal diversity and fungal metabolites in hay and haylage for equines (Müller et al. 2007; Liesener et al. 2010; Séguin et al. 2010, 2012; Müller 2018; Schenk et al. 2018). Maize ears are prone to *Aspergillus* and *Fusarium* infection in the field (Shotwell et al. 1980; Young and Miller 1985), which can be carried over in the silage, whereas grasses in the field may be colonized by other fungal genera. Silage and haylage, on the other hand, are mostly associated with *P. roqueforti* and roquefortines (Sumarah et al. 2005), but the authors also suggest that *P. paneum* and marcfortines are more associated with haylage in colder climates. O'Brien et al. (2006) analysed baled grass silage for *Penicillium* metabolites and found marcfortine A, mycophenolic acid and andrastin A, while Séguin et al. (2012) analysed hay and haylage samples for the classic mycotoxins and found zearalenone. Studies on forage derived from grass have so far focussed on the presence of either specific fungal species or mycotoxins and not the overall fungal growth and metabolite production.

The purposes of this study were therefore (1) to characterize the fungal diversity of hay and haylage and isolate the most frequent occurring filamentous fungi, (2) to qualitatively analyse hay and haylage samples for mycotoxins and other bioactive fungal metabolites produced by the most frequent fungi and (3) to characterize and analyse the faeces from ponies fed either hay or haylage for residual fungal spores and metabolites.

Materials and methods

Project set-up This project commenced in 2011 and comprises 20 Welsh ponies born between 2007 and 2009. Throughout the project, all 20 ponies were housed in the same barn in two separate pens. One group of ten ponies was fed haylage and the other ten ponies were fed hay. When access was allowed to pasture (May 16 to November 22, 2018) the ponies went into separate paddocks in the same field. Before turn out and on return to the barn, they were fed the forage appropriate to their group, every day.

Forage samples Hay was grown on the study site from permanent pasture containing Creeping Bent (*Agrostis stolonifera*), Rough-stalked meadow grass (*Poa trivialis*) and Yorkshire Fog (*Holcus lanatus*). Less dominant but visible grass species included Meadow Brome (*Bromus comutatus*) and Annual meadow grass (*Poa annua*) and there was very little evidence of any ryegrass (*Lolium* spp.). Grass was cut, spread, rowed up and baled within 3 to 4 days. Hay was harvested in 2016 and 2018 and stored as oblong bales (280 kg). Haylage was purchased commercially (Marksway HorseHage, Paignton, Devon) made from short-term ryegrass leys (*Lolium* spp). Haylage was provided as small individually wrapped bales (14 kg) produced in 2017. Forage (hay and haylage) samples were collected by randomly grapping handfuls from the bale during distribution to ponies. All sampling to place between March and September 2018 (Table 1).

Faeces samples Faeces from three ponies in each group were included in the study in order to follow the fate of fungi and metabolites found in the forage. Faeces were collected within 2 min after normal defaecation, four times during 2018 (Table 1) from ponies fed with hay (# 1, 6 and 15) and ponies fed haylage (# 12, 13 and 14).

Haylage hot spot samples Hot spot samples (haylage with visible fungal/mould growth) from older haylage bales sampled in 2013, 2014 and 2016 were also included in the study as worst case samples in order to know which fungi and metabolites to analyse for in the forage samples. Hot spot samples had been frozen at − 80 °C in the interim.

Sample delivery and treatment Samples of hay and haylage were shipped from England to Denmark via courier in four instalments (Table 1) in chilled boxes and kept at 0 °C until the next day when samples were treated. Haylage hot spots and faeces samples were shipped frozen and left to thaw in the fridge overnight prior to treatment.

Growth media All samples were plated out onto three different media for fungal detection: V8 agar (Campbell's V8 juice: 175 mL V8® vegetable juice original, 3 g calcium carbonate, 20 g agar, 0.05 g chloramphenicol, 0.01 g zinc sulphate, 0.005 g copper sulphate, 825 ml water, 0.05 g chlortetracycline (added after autoclaving), Samson et al. 2010), DG18 agar (Dichloran 18 % Glycerol: 31.5 g dichloran-glycerol-agar-base (Oxoid), 220 g glycerol (anhydrous), 0.05 g chloramphenicol, 0.01 g zinc sulphate, 0.005 g copper sulphate, 1000 mL water, 0.05 g chlortetracycline (added after autoclaving), Samson et al. 2010) and MEA agar with antibiotics (Malt extract agar: 25 g malt extract (Oxoid), 10 g agar, 0.05 g chloramphenicol, 0.01 g zinc sulphate, 0.005 g copper sulphate, 1000 mL water, 0.05 g chlortetracycline (added after autoclaving)).

Fungal detection and identification The forage sample was aseptically cut into 2 cm pieces in sterile plastic bags and the thawed hot spot and faeces samples were broken up in sterile, empty Petri dishes prior to plating. Each sample was transferred to one plate of V8, DG18 and MEA (approximately 1 g on each plate) and incubated at 20 °C in

Table 1 The four collection times (A, B, C and D) in 2018 with dates, forage type (hay and wrapped haylage) and faeces samples, which were analysed for fungal growth and mycotoxin production

Crop/production date		Collection dates in 2018		Forage		Faeces (F)	
Hay (harvest year)	Haylage (batch number)	Code	Dates	Hay (H)	Haylage (W)	Hay-ponies (F-H, n = 3)	Haylage-ponies (F-W, n = 3)
2016	543-062465	A	06 March and 07 March	H-A	W-A	F-H-A	F-W-A
2016	543-062312 543-062314 543-062280	B	31 March and 09 April	H-B	W-B1 W-B2 W-B3	F-H-B	F-W-B
2016	543 072634 543 078625 543 062391[b]	C	08 May and 16 May[a]	H-C	W-C1 W-C2 W-C3	F-H-C	F-W-C
2018	FE AA48 15232	D	04 September	H-D	W-D	F-H-D	F-W-D

[a] Ponies were put on pasture after 16 May 2018

[b] Batch fed to haylage-ponies the day before faeces sampling

the dark. The plates were read after 5 days and representative fungal colonies were isolated for identification. The plates were read again after 10 days to ensure that slow-growing fungi also were detected. The presence of fungal cultures on each plate was registered qualitatively at genus level and pooled over the three media. Representative fungal cultures of *Aspergillus*, *Chaetomium*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Sordaria* and *Wallemia* spp. were isolated, inoculated on media specific for each genus and identified using micro- and macro-morphological according to Domsch et al. (2007), Samson et al. (2010) and Seifert et al. (2011). Extraction and metabolite profiling of fungi in pure culture was done according to Samson et al. (2010) and Frisvad and Thrane (1987, 1993) as amended by Nielsen et al. (2011), Klitgaard et al. (2014) and Kildgaard et al. (2014). Yeasts were not identified because they are not known to produce mycotoxins or other secondary metabolites (Frisvad et al. 2008).

Metabolite extraction All samples (haylage hot spot, haylage, hay and faeces) for metabolite analyses were freeze dried and crushed in sterile plastic bags. Each sample (approximately 3 g) was transferred into a 50-mL Falcon tube and added 40 mL of ethyl acetate/isopropanol (3:1 v/v) with 1 % formic acid and extracted ultrasonically for 20 min. Then 10 mL of the extract was evaporated to dryness, re-dissolved in 500 μL methanol and centrifuged. Three hundred microliters of extract was transferred to a HPLC vial and analysed the same day.

Chemical analyses All extracts (pure fungal cultures, hot spot, forage and faeces) were analysed by UHPLC on an Agilent 1290 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 25 cm × 2 mm ID, 2.6 μM Agilent Poroshell phenylhexyl column which was coupled to an Agilent 6545 quadrupole Time Of Flight (qTOF) high-resolution mass spectrometer equipped with an electrospray source. The qTOF was operated in extended dynamic range mode at a resolution of 30,000 FWHM (full-width half-maximum). Separation of a 1-μL subsample was performed at 60 °C at a flow rate of 0.35 mL/min using a linear gradient consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B and increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and remaining for 3 min. The qTOF was operated in both positive and negative modes, scanning from m/z 100 to 1700 four times per second. Automatic MS/MS was also performed using three consecutive collision energies of 10, 20 and 40 eV and with an isolation window of ± 0.7 Da in both positive and negative modes, providing MS/MS spectra (m/z 30–1700) for all major peaks.

Data treatment UHPLC-MS/MS data files were inspected for all regulated mycotoxins and all relevant secondary metabolites from the fungal species detected in this study by accurate mass screening of pseudomolecular ions. Furthermore, the MS/MS data was screened against an in-house library of 1600 known secondary metabolites and against the metabolite profiles of pure cultures of fungi isolated from the forage samples (Rasmussen et al. 2010; Nielsen et al. 2011; Kildgaard et al. 2014; Klitgaard et al. 2014). Qualitative data matrices were made for both fungal data (binary 1/0 for presence/absence of fungal species) and for metabolite data (binary 1/0 for presence/absence of metabolites) for all 27 samples (4 hay, 8 haylage, 8 pooled faeces and 7 hot spot samples). Both matrices were standardized and subjected to Principal Component Analysis (PCA) using The Unscrambler (CAMO, version X 10.0.1) multivariate statistical programme package.

Results

Fungal analyses

Forage samples The four hay samples (labelled H) were dry and without any smell when they arrived, whereas the eight haylage samples (labelled W) were moist and most samples had a weak sour smell. None of the forage samples had any visible mould growth. The mycological analyses showed that hay samples in general had a larger biodiversity in fungal species than the haylage samples. The older hay samples (H-A to H-C) *Aspergillus* spp., especially *A. glaucus*, *A. montevidensis* and *A. pseudoglaucus* (formerly known as *Eurotium herbariorum*, *E. amstelodami* and *E. repens*, respectively) (Fig. 1A) were prevalent followed by *Chaetomium globosum, Penicillium solitum* and *Wallemia sebi* (Table 2). The older haylage samples (W-A to W-C) *P. roqueforti* (Fig. 1C) was prevalent followed by yeast (not identified), *A. pseudoglaucus, Chrysosporium* spp and *Paecilomyces variotii.* In one haylage sample (W-B2) only *A. cristatus* was detected whereas *Fusarium poae* was the most prevalent fungus. *Aspergillus flavus* (aflatoxin producer (Samson et al. 2010)) and *A. niger* (fumonisin and ochratoxin producer (Samson et al. 2010)) were only detected in low numbers in hay samples H-A and H-C, respectively. *Aspergillus fumigatus* was only seen in low levels in older hay samples (H-A to H-C) and in one haylage sample (W-B3). The newer hay and haylage samples (H-D and W-D) were very different from their older counterparts. The hay sample again showed the greatest biodiversity with *Alternaria* and *Epicoccum* spp. as the most frequently occurring fungi compared to the haylage sample, which was low in fungal biodiversity (Figs. 2A and C). Common for the two types of forage were the presence of *Sordaria fimicola* in both older and newer samples (Table 2).

Faeces samples All thawed faeces samples were firm and without any mouldy odour. The 24 faeces samples were analysed individually, but the three samples for ponies in the same group collected at the same time were very similar. Table 2 therefore shows the pooled results for the three ponies in the hay group and in the haylage group at the four different collection dates (A–D). The mycological analyses showed that the fungal composition of the faeces samples from ponies fed hay were different from ponies that had been fed haylage. Faeces from ponies fed on older hay (Fig. 1B) showed a higher load of *Aspergillus* spp. (*A. glaucus*, *A. montevidensis* and *A. versicolor*) compared with the faeces from ponies fed on older haylage (Fig. 1D) where *Penicillium* spp. (*P. expansum, P. palitans* and *P. solitum*) were more frequent (Table 2). Both groups of ponies were put on pasture after the C samples had been collected and had access to hay or haylage from newer batches. The faeces samples collected 04 September 2018 (sampling D) from both ponies fed hay and haylage (Figs. 2B and D, respectively) were devoid of *Aspergillus* spp. However, in both samples (F-H-D and F-W-D) *P. expansum*, *P. commune, P. crustosum* and *S. fimicola* were detected (Table 2). In addition, the two samples contained *Phoma* and *Zygomycetes* spp.and unidentifiable, non-sporulating filamentous fungi.

Haylage hot spots samples All seven thawed samples of the haylage hot spots had visible fungal growth and a strong mouldy and sour smell. The results from the mycological analyses showed that *Penicillium roqueforti* was found in all samples. In some samples *P. paneum*, *P. solitum* and *Paecilomyces variotii* were also detected (Table 3). No *Aspergillus* spp. or field fungi (*Alternaria*, *Cladosporium* and *Fusarium* spp.) were detected in any of the hot spot samples.

A Principal Component Analysis of the qualitative fungal growth (48 species) on all 27 samples (4 hay, 8 haylage, 8 faeces and 7 hot spot samples) showed that all faeces samples grouped together next to the three older hay samples and one older haylage sample (W-B3) and the new haylage sample (W-D) (Fig. 3). The new hay sample (H-D) and one haylage sample (W-B2) grouped by themselves and away from other samples since they did not contain any *Aspergillus* or

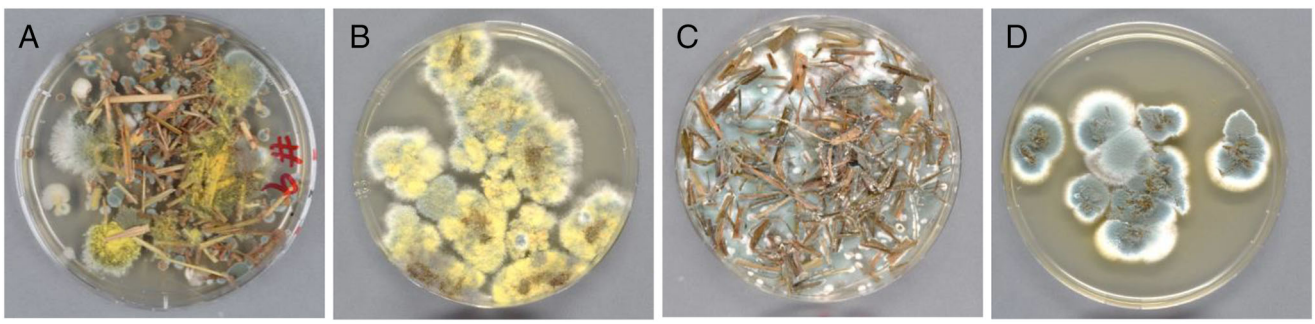

Fig. 1 Forage and faeces samples from spring. Direct plating on DG18. **A**: Hay (H-A), **B**: Faeces from pony #1 fed hay (F-H-A), **C**: Haylage (W-C2) and **D**: Faeces from pony #14 fed haylage (F-W-C)

Table 2 Qualitative detection of fungi in the different hay (H), wrapped haylage (W) and faeces (F) samples collected at different dates (A–D) with the most prevalent fungal species marked with ●

	Hay (H)				Faeces-Hay (F-H)				Haylage (W)								Faeces-haylage (F-W)			
Fungi	A	B	C	D	A	B	C	D	A	B1	B2	B3	C1	C2	C3	D	A	B	C	D
Aspergillus																				
Asp. cristatus	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Asp. flavus	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Asp. fumigatus	+	+	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Asp. glaucus	+	●	+	−	●	●	●	−	−	−	−	−	−	−	−	−	+	+	−	−
Asp. montevidensis	+	+	●	−	●	−	●	−	−	−	−	−	−	−	−	−	−	−	−	−
Asp. niger	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Asp. pseudoglaucus	●	●	+	−	−	●	−	−	−	−	−	+	+	−	−	+	+	−	+	−
Asp. versicolor	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Penicillium																				
Pen. astrolabium	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	●	−	−	−
Pen. brevicompactum	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pen. chrysogenum	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pen. commune	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+
Pen. crustosum	●	−	−	−	+	−	−	+	−	−	−	●	−	−	−	−	+	−	+	+
Pen. echinulatum	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	+	−
Pen. expansum	−	−	−	−	+	●	●	+	−	−	−	−	−	−	−	−	+	●	●	+
Pen. majusculum	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−
Pen. palitans	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−
Pen. polonicum	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pen. roqueforti	−	−	−	−	−	−	−	−	●	+	−	−	●	●	+	−	−	−	+	−
Pen. solitum	−	+	+	−	−	+	+	−	−	−	−	−	−	−	−	−	●	●	●	−
Pen. verrucosum	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Field and other fungi																				
Alternaria spp.	−	−	−	●	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−
Arthrinium spp.	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Cephalotrichum sp.	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Chaetomium globosum	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Chrysosporium sp.	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−
Cladosporium spp.	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−
Engyodontium album	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Epicoccum nigrum	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fusarium poae	−	−	−	+	−	−	−	−	−	−	●	−	−	−	−	−	−	−	−	−
Harzia acremonioides	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Nigrospora sp.	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Phoma spp.	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+
Sordaria fimicola	+	+	+	+	−	−	−	+	+	+	+	−	+	−	−	−	−	−	−	+
Paecilomyces variotii	−	−	−	−	−	−	−	−	−	−	−	−	−	●	+	−	−	−	−	−
Wallemia sebi	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Zygomycetes	−	−	−	−	+	+	−	●	+	−	−	+	−	−	−	−	−	+	−	●
Yeasts	−	−	−	−	●	+	+	−	+	●	●	−	+	●	+	−	+	−	−	−
Sterile mycelium	−	−	−	−	+	+	+	●	−	−	−	−	−	−	−	−	−	−	+	●

See Table 1 for collection dates and details

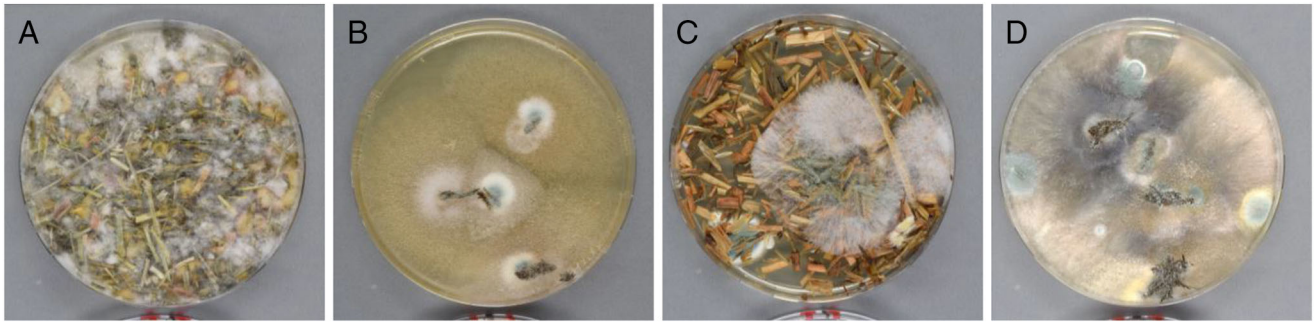

Fig. 2 Forage and faeces samples from autumn. Direct plating on DG18. **A**: Hay (H-D), **B**: Faeces from pony #1 fed hay (F-H-D), **C**: Haylage (W-D) and **D**: Faeces from pony #14 fed haylage (F-W-D)

Penicillium spp. All seven hot spot samples grouped together with five of the haylage samples, which all contained *P. roqueforti*.

Mycotoxin analyses

Forage samples Metabolites associated with different species of *Chaetomium* and *Sordaria/Podospora* (*Sordariales*) were prevalent in both old and new forage types (Table 4), but lower in numbers in the new hay (H-D) and haylage (W-D) samples. Sordaricin and/or sordarins were found in all samples but one (H-C). Cochliodinol was found in the three older hay samples, while chaetoxanthone B was detected in all haylage samples, except W-B3. Metabolites from different *Penicillium* spp., on the other hand, varied with forage type and age. Asperphenamate, β-cyclopiazonic acid and penicillic acid were detected in the older hay samples (H-A to H-C) and roquefortine C and isofumigaclavine A in two haylage samples (W-C2 and W-C3). No *Penicillium* metabolites were detected in the two new hay and haylage samples. Furthermore, *Aspergillus* metabolites such as emodin, methyl-emodin and nigragillin, were detected in both hay and haylage samples, whereas dihydromonacolin, associated to *Monascus* spp., was found in older haylage samples (W-B1, W-B3 and W-C1). Figure 4 compares the chromatograms of hay sample H-C and haylage sample W-C2, but generally all haylage samples had the same profile with the same 6–8 peaks of sphingosine derivatives. All samples also contained communiols, linoleic acid and ergosterol and many peaks that could not be identified as fungal metabolites.

Faeces samples Only a few fungal metabolites were detected in the faeces samples of ponies fed hay, while in faeces from ponies fed haylage more metabolites were detected (Table 4). Most common were metabolites produced by fungi in *Sordariales*: the *Chaetomium* metabolites communiols, gliocladic acid and longirostrerone A and the *Podospora*/*Sordaria* metabolite appenolide A together with various derivatives of sphingosine (e.g. dihydro-sphingosine) and linoleic acid. However, no ergosterol was detected. Enniatins were detected in faeces samples from animals feeding on the new forage samples (F-H-D and F-W-D) in both groups.

Haylage hot spots The chemical analyses of the haylage hot spot samples showed the same *Chaetomium*, *Podospora* and *Sordaria* metabolites as in the forage samples. Additional *Penicillium* metabolites were, however, detected in the hot spot samples (Table 3). The mycotoxin PR toxin, produced by *P. roqueforti*, was detected in one sample (W-hs-2). Other metabolites from *P. roqueforti* (andrastins, isofumigaclavin A, mycophenolic acid, roquefortines C and D) were found in six of the samples, while *P. solitum* metabolites (cyclopenin, cyclopenol, cyclopeptin, viridicatin and viridicatol) were detected in sample W-hs-2. Metabolites specific for *P. paneum* (asperparaline A, marcfortines A and B) were also detected in three samples. No *Penicillium* metabolites were detected in W-hs-7. None of the *Aspergillus* metabolites that were detected in the forage samples were detected in the hot spot samples. As with forage and faeces samples, the hot spot samples also contained linoleic acid and ergosterol and various derivatives of sphingosine (e.g. dihydro-sphingosine). None of the regulated mycotoxins (aflatoxins, citrinin, fumonisins, ochratoxin or trichothecenes) were detected in any of the 27 samples tested. Screening all 27 samples against the in-house database on fungal metabolites did not reveal any *Alternaria* metabolites (e.g. alternariols or tenuazonic acid). Furthermore, all 27 samples were also screened for the presence of C17-sphinganine, which is a precursor for fumonisin B1, lolitrem B from ryegrass and juglone, but gave negative results.

A Principal Component Analysis of the qualitative metabolite production (93 known and unknown fungal compounds) across all 27 samples (4 hay, 8 haylage, 8 faeces and 7 haylage hot spot samples) shows that the old hay samples grouped together due to the presence of cochliodinol, β-cyclopiazonic acid and asperphenamate (Fig. 5). The new hay and haylage samples (H-D and W-D) grouped away from other samples due to the absence of *Penicillium* metabolites

Table 3 Qualitative detection of fungi (●) and metabolites (+) in the wrapped haylage hot spot (W-hs) collected in 2013 (W-hs-1 to 3), 2014 (W-hs-4 to 6), and 2016 (W-hs-7)

Fungi/metabolites	Haylage hot spot						
	W-hs-1	W-hs-2	W-hs-3	W-hs-4	W-hs-5	W-hs-6	W-hs-7
Penicillium roqueforti	●	●	●	●	●	●	●
(+)-Aristolochene	−	−	+	−	−	−	−
Andrastins B and C	−	−	+	+	+	+	−
Costaclavin	−	−	+	−	−	−	−
Eremofortin A	−	+	−	−	−	−	−
Mycophenolic acid	+	+	−	−	−	−	−
PR toxin	−	+	−	−	−	−	−
Isofumigaclavin A	−	−	+	−	−	−	−
Roquefortines C and D	−	−	+	+	+	+	−
Pen. paneum	−	−	−	●	●	●	●
Asperparaline A	+	+	+	+	−	−	−
Marcfortine A	−	−	−	+	−	+	−
Marcfortine B	−	−	−	+	+	+	−
Marcfortine C	−	−	−	−	−	+	−
Pen. solitum	●	●	−	−	−	−	−
Viridicatols*	−	+	−	−	−	−	−
Paecilomyces variotii	−	●	−	−	−	−	−
Fusarium spp.	−	−	−	−	−	−	−
Dehydrofusaric acid	−	−	+	+	−	+	+
Wallemia sebi	−	−	−	−	−	−	−
Walleminol	−	+	+	−	−	−	−
Sordariales spp.	−	−	−	−	−	−	−
(-)-Musanahol	−	−	−	−	+	−	+
3-epi-Aureonitol	−	−	+	+	−	+	−
Anserinone B	+	+	+	+	+	+	−
Chaetoquadrin F and I	+	+	−	+	+	+	+
Chaetospirone	+	−	+	−	−	−	−
Chaetoxanthone B	+	+	+	−	+	+	+
Coarctatin	−	−	−	+	+	+	−
Communiols B, D and F	+	+	+	+	−	+	−
Gliocladic acid	+	+	+	−	−	+	−
Globosumones A and B	−	−	−	+	−	+	−
Heptacyclosordariolone	+	−	−	−	−	+	−
Hydroxysordarin	−	−	−	+	−	−	−
Sordaricin	−	−	−	−	+	−	−
Sordarin	+	+	−	−	−	−	−
Yeast spp.	−	−	●	●	−	−	●
All fungal spp.							
Ergosterol	+	+	+	+	+	+	+
Linoleic acid	+	+	+	+	+	+	+

All precursors (cyclopenins and viridicatin) to viridicatol were detected in sample W-hs-2

and the faeces samples grouped together and away from the forage samples due to the low number of fungal metabolites detected. Again the multivariate analyses showed that all seven hot spot samples grouped together with seven old haylage samples, which all showed similar metabolite profiles including roquefortines, chaetoquadrins and chaetoxanthone.

Discussion

The results of the chemical analyses did not show any of the regulated mycotoxins (aflatoxin, fumonisins, ochratoxin, patulin or trichothecenes) in any samples in this study, but fungi capable of producing these mycotoxins (*Aspergillus*

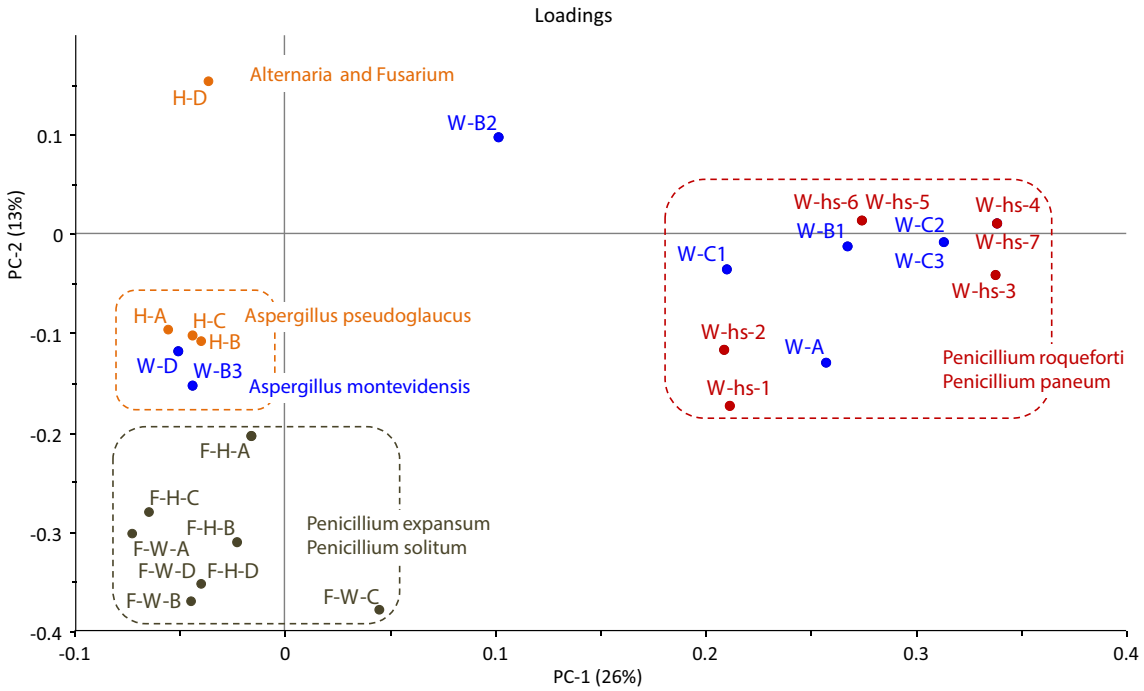

Fig. 3 A Principal Component Analysis of the qualitative fungal biota on hay (H), haylage (W) and faeces (F) samples collected early spring (A), intermediate (B and C) and early autumn (D) in 2018. The haylage hot spot (W-hs) samples have been collected in 2013–2016. The most prevalent fungi are given for each sample group. The analysis is based on 27 samples (objects) and 48 fungal species (variables/factors) detected in the samples. Arbitrary axis

flavus, *A. niger, Fusarium poae* and *Penicillium paneum*) were detected together with a broad range of field and storage fungi and their metabolites.

The results suggest that the raw materials (grass) for both hay and haylage start out with similar mycobiota from the field. *Sordariales* (*Chaetomium, Podospora* and *Sordaria* spp.) and/or their metabolites were found in both hay and haylage. This suggests that some *Chaetomium* spp. may live as endophytes in grasses and survive long enough to produce metabolites, such as 3-epi-aureonitol, longirostrerones and globosumones (Panthama et al. 2011; Bashyal et al. 2007; Marwah et al. 2007; Shi et al. 2013; Qin et al. 2009). Species of *Sordaria* and the closely related *Podospora*, known as dung fungi (Bills et al. 2013; Sarrocco 2016), may either contaminate the grass during harvest and drying or may actually also be plant endophytes. They produce metabolites like appenolides and communiols (Wang et al. 1993, 1997; Che et al. 2004, 2005), which have antimicrobial effects (Bashyal et al. 2005; Marwah et al. 2007) but nothing is known about their toxicity to equines.

Hay Different treatments and storage of grass favour different fungal contaminants. Hay, which is aerobic, dry and neutral in pH, is more likely to be contaminated with fungal species that tolerate lower water activities, such as *Aspergillus* spp., formerly known as *Eurotium* spp., and *Wallemia sebi*. These species are not known to produce mycotoxins (Moss 1998; Chen et al. 2017), but they are able to produce exorbitant amounts of fungal spores that become airborne when the dry hay bales are moved and broken up. Walleminol, detected in one hay sample, is toxic to rat liver cells and baby hamster kidney cells, but no information is available on the effects on living animals (Moss 1998). Asperphenamate, emodin and nigragillin, found in both hay and haylage samples, are not considered toxic and have even been used in Oriental medicine (De Vries et al. 2005; Lin et al. 2014; Dong et al. 2016).

β-cyclopiazonic acid and penicillic acid that were found in two hay samples and in one haylage sample may have some toxic properties. In comparison to α-cyclopiazonic acid (α-CPA), β-cyclopiazonic acid (=bissecodehydrocyclopiazonic acid) has been characterized as relatively non-toxic (Cole and Cox 1981; Ostry et al. 2018). α-CPA is a specific inhibitor of sarco-plasmic reticulum Ca2+ -ATPase and has been studied in chickens, guinea pigs, mice, rats, pigs, dogs, monkeys, but not equines, and affects the alimentary tract, heart, kidney, liver, skeletal muscles and the nervous system (Ostry et al. 2018). Penicillic acid is cytotoxic (Cole and Cox 1981; Gräbsch et al. 2006), but has a low oral toxicity (Malekinejad et al. 2015), and has mostly been recognized as a mycotoxin because of its co-occurrence with ochratoxin A (Stoev et al. 2004; Stoev 2015) and because of its toxicity to broiler chickens (Pazhanivel et al. 2015). It may indirectly affect equines because of its antimicrobial effect and its quorum sensing inhibition of bacteria (Rasmussen and Givskov

Table 4 Qualitative detection of metabolites and their producers in the different hay (H), wrapped haylage (W) and faeces (F) from ponies fed either hay or haylage collected at different dates (A-D)

	Hay (H)				Faeces-hay (F-H)				Haylage (W)								Faeces-haylage (F-W)			
Fungi/metabolites	A	B	C	D	A	B	C	D	A	B1	B2	B3	C1	C2	C3	D	A	B	C	D
Aspergillus spp.																				
Emodins	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−
Nigragillin	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−
Fusarium spp.																				
Enniatins	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+
Sambucinol	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Monascus spp.																				
Dihydromonacolin	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−
Penicillium spp.																				
β-cyclopiazonic acid	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Asperparaline A	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Asperphenamate	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Eremofortin A	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+	−
Isofumigaclavin A	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Penicillic acid	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Roquefortine C	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Rugulovasin A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Wallemia sebi																				
Walleminol	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sordariales																				
3-epi-aureonitol	+	+	−	−	−	−	−	−	−	+	−	+	+	+	−	+	−	−	−	−
Anserinones	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−
Appenolides	+	+	−	+	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−	+
Chaetoquadrins	−	−	−	−	−	−	−	−	+	+	−	+	−	−	+	−	−	+	−	−
Chaetoxanthones	−	−	−	−	−	−	−	−	+	+	+	−	+	+	+	+	−	−	−	−
Coarctatin	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Cochliodinol	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Communiols	+	+	+	+	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+
Gliocladic acid	+	−	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	+	+
Globosumones	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
Heptacyclosordariolone	−	−	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	−	−	−
Hydroxysordarin	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	+	−	−	−	−
Longirostrerones	+	−	−	−	−	−	+	−	−	+	+	+	−	−	−	−	+	+	+	+
Sordaricin	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−
Sordarin	+	+	−	+	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−	+
All fungal spp.																				
Ergosterol	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−
Linoleic acid	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

See Table 1 for collecting dates and details

2006), possibly changing the gut microbiota. In general, dry hay seems more resistant to mycotoxin production compared to the humid haylage, which is corroborated by Gallo et al. (2015), which also showed that hay samples usually have lower toxin contents than their haylage counterparts.

Haylage Haylage, as opposed to hay, is semi-anaerobic, moist and low in pH, and therefore prone to fungal contaminants, such as *Penicillium roqueforti* that is known for its ability to grow at pH 2.7–3.0 (Kalai et al. 2017). *Penicillium roqueforti* and the closely related species *P. paneum* are particularly

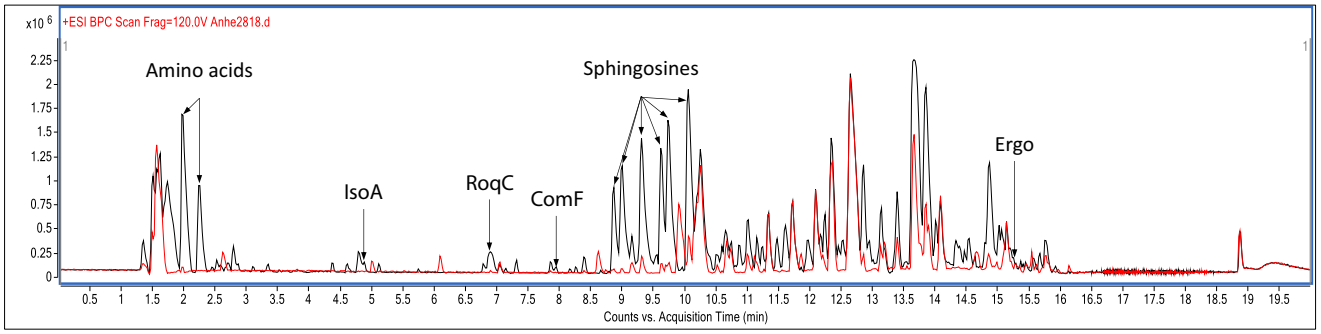

Fig. 4 Liquid Chromatography chromatograms of haylage sample W-C2 (black line) and hay sample H-C (red line). The majority of compounds (peaks) are plant compounds, like the sphingosines. Three fungal metabolites are marked: Isofumigaclavine A (IsoA), roquefortine C (RoqC) in the haylage and communiol F (ComF) and ergosterol (Ergo) in both haylage and hay. The peaks of communiol F and ergosterol are higher in the haylage than in the hay

common in silage due to their tolerance to acetic acid, lactic acid, and quite high levels of carbon dioxide (Nielsen et al. 2006; O'Brien et al. 2006; Ogunade et al. 2018). In earlier studies on whole-plant maize silage aflatoxin B_1, diacetoxyscirpenol, fumonisin B_1 and B_2 and zearalenone, have been detected (Alonso et al. 2013; Ogunade et al. 2018), but *Aspergillus flavus* and *A. niger* were not detected in any haylage samples and *Fusarium poae* only in one sample in this study. It is also known that *A. fumigatus* can grow in maize silage, when the pH is higher than 3.5 (Alonso et al. 2017); it was found in one haylage sample, but the acidic conditions are usually not conducive for mycotoxin production (Northolt and Bullerman 1982).

However, in the haylage samples in this study, cocktails of bioactive *Penicillium* metabolites were detected. This was especially evident in the haylage hot spot samples where *P. roqueforti, P. paneum* and *Paecilomyces variotii* were found. These species are known to produce mycotoxins, such as PR-toxin, patulin and viriditoxin (Cole and Cox 1981; Samson et al. 2010). PR-toxin was detected in one hot spot sample in this study, but other *Penicillium* metabolites like andrastins, asperparaline A, isofumigaclavine, marcfortines, mycophenolic acid, rugulovasines and roquefortines, were detected in haylage and/or haylage hot spots. *Penicillium solitum*, which has not previously been associated with silage or haylage, was found in two hot spot samples and its

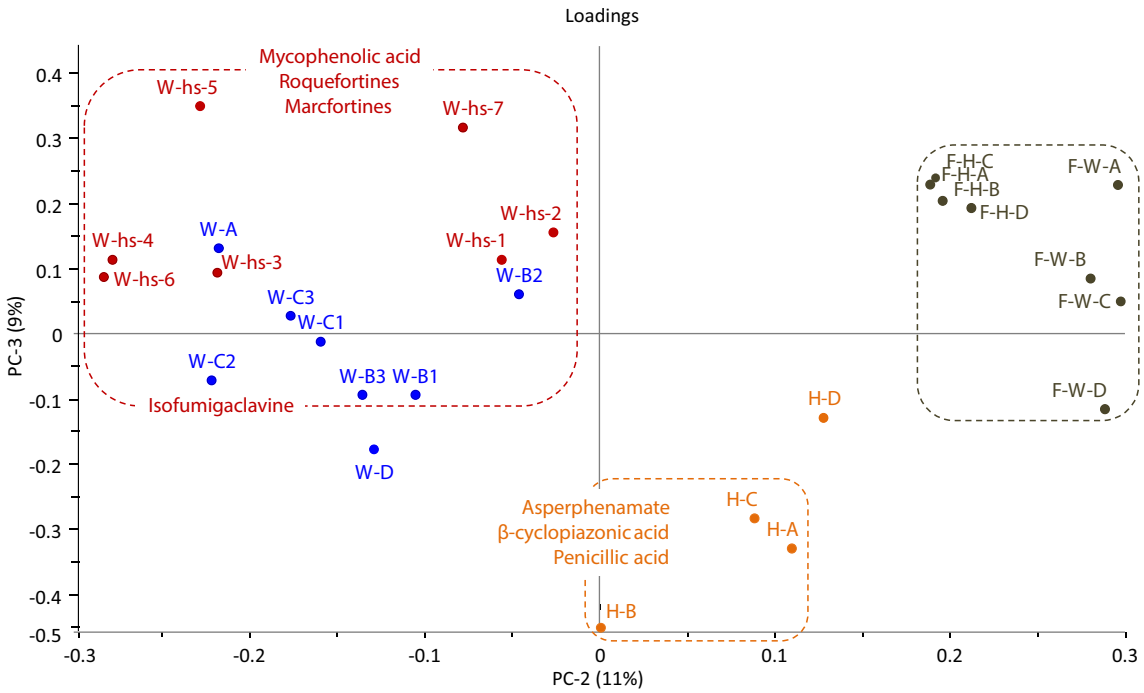

Fig. 5 A Principal Component Analysis of the qualitative metabolite production on hay (H), wrapped haylage (W) and faeces (F) samples collected early spring (A), intermediate (B and C) and early autumn (D) in 2018. The haylage hot spot (W-hs) samples have been collected in 2013–2016. Key fungal metabolites are given for each sample group. The analysis is based on 27 samples (objects) and 93 mycotoxins and other compounds (variables/factors) detected in the samples. Arbitrary axis

metabolites, viridicatols (=cyclopenins (Samson et al. 2010)), in one hot spot sample. These metabolites can also be produced by *P. crustosum* (Samson et al. 2010), which was detected in a haylage sample. These findings suggest that viridicatols should be taken in to consideration when assessing the cocktail effect.

PR-toxin, possibly the most toxic metabolite from *P. roqueforti*, is rarely detected in silage (Gallo et al. 2015), whereas roquefortine C and mycophenolic acid are encountered often (Auerbach et al. 1998; O'Brien et al. 2006; Storm et al. 2008, 2010, 2014; Dubey et al. 2018). PR-toxin is hepato- and nephrotoxic to mice and has been claimed to be potentially carcinogenic (Dubey et al. 2018). The data on ruminants indicate toxic effects, but without affecting the gut microbiota (Dubey et al. 2018). Roquefortine C was initially considered to be neurotoxic, but the data were based on intraperitoneally injected samples (Polonsky et al. 1977). Recent data suggest that roquefortine C is not cytotoxic (Larsen et al. 2002) and that ingestion of roquefortine C has little toxic effect on cows and sheep (Ogunade et al. 2018). Mycophenolic acid appears to be orally non-toxic for vertebrates, but it has a strong immune-lowering effect (Bentley 2000). Rugulovasines A and B produced by *P. commune* (Frisvad et al. 2004) are ergot alkaloids that have been shown to be acutely toxic to poultry (Dorner et al. 1980; Fabian et al. 2018), and have been classified as mycotoxins (Skóra et al. 2017). There is no reliable toxicity data on the andrastins (both *P. paneum* and *P. roqueforti*), isofumigaclavine (*P. roqueforti*) or on the marcfortines (*P. paneum*). In general, little is known about the toxicity of bioactive fungal metabolites and mycotoxins to equines.

Dihydromonacolin, which is produced by *Monascus ruber* (Nakamura et al. 1990), was detected in three haylage samples. *Monascus ruber* is known to contaminate maize silage and produce citrinin (Rasmussen et al. 2011; Gallo et al. 2015). Neither fungus nor citrinin were detected in any sample, but the presence of dihydromonacolin suggests that *M. ruber* may be common in haylage.

Penicillium solitum and *P. crustosum,* found in haylage or hot spot samples, have not previously been regarded as resistant to acetic acid. The occasional appearance of these Penicillia in haylage suggests that the pH and oxygen levels were not as low as they should be to prevent fungal growth and metabolite production. In general, moist haylage is more susceptible to mycotoxin production than dry hay; it is therefore very important that the fermentation has been successful and that the plastic wrapping has not been compromised.

During work with the haylage samples in the laboratory it became evident that especially *P. roqueforti* spores became airborne when the samples were handled, similar to *Aspergillus* and *Wallemia* spp. in the hay samples. This adds another complicating factor to equine health, since inhalation increases the toxicity of roquefortine C and mycophenolic acid. These metabolites can induce significant inflammatory responses in mouse lungs if inhaled (Rand et al. 2005). Thus inhaling spores coated with roquefortine C, mycophenolic acid and other bioactive metabolites from *P. roqueforti* and *P. paneum* growing and sporulating in haylage may pose an additional health risk to equines.

Faeces The diversity and succession of fungal species, especially *Aspergillus*, *Penicillium* and *Sordariales* spp. in the faeces seem to reflect the different forage types fed to the ponies as well as the age/quality of the forage. These results suggest that fungal spores can survive the travel through the gut, as seeds can in birds, and that the spore composition in the faeces may be predictive for forage type and quality. The results also show that the number of different fungal metabolites, including linoleic acid, in the faeces samples is low, suggesting that most metabolites and ergosterol present in the forage have either been broken down by the anaerobic microbes in the gut or been taken up by the ponies together with nutrients in the forage.

Metabolite cocktails and adverse health effect in equines The results in this study indicate that the distribution of metabolites can be very inhomogeneous within a haylage bale and between bales. Furthermore, metabolite production can have occurred in bales even when there is no visible fungal growth, which O'Brien et al. (2006) also found. The principal component analyses (Figs. 3 and 5) show that haylage samples have the same profile of fungal metabolites as the hot spots, which suggest that haylage bales may contain many of the same fungal species and metabolites as the hot spot samples.

Equines that are fed haylage are therefore exposed to a different cocktail of fungal metabolites via the forage than equines fed hay, which may—over the years—have a negative impact of their health. Whether the presence of immunosuppressive mycophenolic acid in some haylage samples, is paving the way for equine infections, is not known either. Suppressed immune function by fungal metabolites may eventually decrease resistance to, or reactivate chronic exposures to, mycotoxins (Oswald et al. 2005). Furthermore, the lactic acid bacterial fermentation seems to release large amounts of sphingosines from the plant material and promote growth of yeasts, but it is not known if these compounds and organisms have a positive or a negative health effect on equines.

Little work has been done on the effects on ingestion and inhalation of mycotoxins and other bioactive metabolites in equines. Also the naphthoquinones, similar to juglone, may have negative effects and can be produced by a broad variety of fungi such as *Fusarium* and *Penicillium* (Medentsev and Akimenko 1998). More research is needed to disclose if fungal metabolites and mycotoxins—especially metabolites from *Penicillium crustosum*, *P. paneum*, *P. roqueforti* and *P. solitum*

together with plant sphingosines and yeasts—are contributory factors to neurologic syndromes like acquired equine polyneuropathy, laminitis and Cushing's disease.

Acknowledgements The authors wish to thank The Laminitis Trust for their financial support and Lisette Knoth-Nielsen for technical assistance.

Funding information This study was supported by The Laminitis Trust.

Compliance with ethical standards

Conflicts of interest The last author is a Trustee of the Laminitis Trust.

References

Alonso VA, Pereyra CM, Keller LAM, Dalcero AM, Rosa CAR, Chiacchiera SM, Cavaglieri LR (2013) Fungi and mycotoxins in silage: an overview. J Appl Microbiol 115:637–643. https://doi.org/10.1111/jam.12178

Alonso V, Cavaglieri L, Ramos AJ, Torres A, Marin S (2017) Modelling the effect of pH and water activity in the growth of *Aspergillus fumigatus* isolated from corn silage. J Appl Microbiol 122:1048–1056. https://doi.org/10.1111/jam.13395

Auerbach H, Oldenburg E, Weissbach F (1998) Incidence of *Penicillium roqueforti* and roquefortine C in silages. J Sci Food Agric 76:565–572. https://doi.org/10.1002/(SICI)1097-0010(199804)76:4<565::AID-JCFA990>3.0.COI2-6

Bashyal BP, Wijeratne EK, Faeth SH, Gunatilaka AL (2005) Globosumones A− C, cytotoxic orsellinic acid esters from the Sonoran desert endophytic fungus *Chaetomium globosum*. J Nat Prod 68:724–728. https://doi.org/10.1021/np058014b

Bashyal BP, Burns AM, Liu MX, Paranagama PA, Seliga CJ, Turbyville TJ, Wijeratne EMK, Zhan J, Gunatilaka MK, Arnold AE, Faeth SH, Whitesell L, Gunitilaka AAL (2007) Discovery of small molecule bioactive agents from endophytic fungi of the Sonoran Desert. 6th International Symposium on fungal endophytes, Grassland Research Practices Series No. 13, New Zealand Grassland Association, Dunedin, pp 211-214.

Bentley R (2000) Mycophenolic acid: a one hundred year odyssey from antibiotic to immunosuppressant. Chem Rev 100:3801–3825. https://doi.org/10.1021/cr9900976

Bills GF, Gloer JB, An Z (2013) Coprophilous fungi: antibiotic discovery and functions in an underexplored arena of microbial defensive mutualism. Cur Op Microbiol 16:549–565. https://doi.org/10.1016/j.mib.2013.08.001

Che Y, Gloer JB, Scott JA, Malloch D (2004) Communiols A–D: new mono-and bis-tetrahydrofuran derivatives from the coprophilous fungus *Podospora communis*. Tetrahedron Lett 45:6891–6894. https://doi.org/10.1016/tetlet.2004.07.093

Che Y, Araujo AR, Gloer JB, Scott JA, Malloch D (2005) Communiols E − H: new polyketide metabolites from the coprophilous fungus *Podospora communis*. J Nat Prod 68:435–438. https://doi.org/10.1021/np049592f

Chen AJ, Hubka V, Frisvad JC, Visagie CM, Houbraken J, Meijer M, Varga J, Rasine D, Jurjević Ž, Kubátová A, Sklenář F, Samson RA (2017) Polyphasic taxonomy of *Aspergillus* section *Aspergillus* (formerly *Eurotium*) and its occurrence in indoor environment and food. Stud Mycol 88:37–135. https://doi.org/10.1016/i.simyco.2007.07.001

Coblenz WK, Akins MS (2018) Silage review: Recent advances and future technologies for baled silages. Journal of Dairy Science 101:4075–4092. https://doi.org/10.3168/jds.2017-13708

Cole RJ, Cox RH (1981) Handbook of toxic fungal metabolites. Academic Press, New York

De Vries RP, Frisvad JC, van de Vondervoort PJ, Burgers K, Kuijpers AF, Samson RA, Visser J (2005) *Aspergillus vadensis*, a new species of the group of black Aspergilli. Antonie Van Leeuwenhoek 87:195–203. https://doi.org/10.1007/s10482-004-3194-y

Domsch KH, Gams W, Anderson T-H (2007) Compendium of soil fungi, 2nd edn. IHW-Verlag, Eching

Dong X-X, Fu J, Yin X, Cao S, Li X-B, Lin L, Cao S-L, Li X-C, Lin L-F, Huyiligeqi, Ni J (2016) Emodin: a review of its pharmacology, toxicity and pharmacokinetics. Phytother Res 30:1207–1218. https://doi.org/10.1002//ptr.5631

Dorner JW, Cole RJ, Hill R, Wicklow D, Cox RH (1980) *Penicillium rubrum* and *Penicillium biforme*, new sources of rugulovasines A and B. Appl Environ Microbiol 40:685–687

Dubey MK, Aamir M, Kaushik MS, Khare S, Meena M, Singh S, Upadhyay RS (2018) PR Toxin–Biosynthesis, Genetic Regulation, Toxicological Potential, Prevention and Control Measures: Overview and Challenges. Front Pharmacol 9:288. https://doi.org/10.3389/fphar.2018.00288

Fabian SJ, Maust MD, Panaccione DG (2018) Ergot Alkaloid Synthesis Capacity of *Penicillium camemberti*. Appl Environ Microbiol 84: e01583–e01518. https://doi.org/10.1128/AEM.61583-18

Frisvad JC, Thrane U (1987) Standardized High Performance Liquid Chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone indices and UV VIS spectra (diode array detection). J Chromatogr 404(195):214. https://doi.org/10.1016/S0021-9673(01)86850-3

Frisvad JC, Thrane U (1993) Liquid column chromatography of mycotoxins. In: Betina V (ed) Chromatography of mycotoxins: techniques and applications. Journal of Chromatography Library 54. Elsevier, Amsterdam, pp 253–372

Frisvad JC, Smedsgaard J, Larsen TO, Samson RA (2004) Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Stud Mycol 49:201–241

Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res 112:231–240. https://doi.org/10.1016/j.mycres.2007.08.018

Galey FD, Whiteley HE, Goetz TE, Kuenstler AR, Davis CA, Beasley VR (1991) Black-walnut (*Juglans nigra*) toxicosis –a model for equine laminitis. J Comp Pathol 104:313–326. https://doi.org/10.1016/S0021-9975(08)80043-6

Gallo A, Giuberti G, Frisvad JC, Bertuzzi T, Nielsen KF (2015) Review on mycotoxin issues in ruminants: occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effect. Toxins 7: 3057–3111. https://doi.org/10.3390/toxins7083057

Gräbsch C, Wichmann G, Loffhagen N, Herbarth O, Müller A (2006) Cytotoxicity assessment of gliotoxin and penicillic acid in *Tetrahymena pyriformis*. EnvironToxicol 21:111–117. https://doi.org/10.1002/tox.20162

Hanche-Olsen S, Teige J, Skaar I, Ihler CF (2008) Polyneuropathy associated with forage sources in Norwegian horses. J Vet Intern Med 22:178–184. https://doi.org/10.1111/j.1532-950X.2008.00396.x

Kalai S, Anzala L, Bensoussan M, Dantigny P (2017) Modelling the effect of temperature, pH, water activity, and organic acids on the germination time of *Penicillium camemberti* and *Penicillium roqueforti* conidia. Int J Food Microbiol 240:124–130. https://doi.org/10.1016/j.ijfoodmicro.2016.03.024

Kildgaard S, Mansson M, Dosen I, Klitgaard A, Frisvad JC, Larsen TO, Nielsen KF (2014) Accurate dereplication of bioactive secondary metabolites from marine-derived fungi by UHPLC-DAD-QTOFMS and MS/HRMS library. Mar Drugs 12:3681–3705. https://doi.org/10.3390/md12063681

Klitgaard A, Iversen A, Andersen MR, Larsen TO, Frisvad JC, Nielsen KF (2014) Aggressive dereplication using UHPLC-DAD-QTOF –

screening extracts for up to 3000 fungal secondary metabolites. Anal Bioanal Chem 406:1933–1943. https://doi.org/10.1007/S00216-013-7582-x

Larsen TO, Gareis M, Frisvad JC (2002) Cell cytotoxicity and mycotoxin and secondary metabolite production by common Penicillia on cheese. J Agric Food Chem 50:6148–6152. https://doi.org/10.1021/jf020453i

Le Bars J, Le Bars P (1996) Recent acute and subacute mycotoxicoses recognized in France. Vet Res 27:383–394

Liesener K, Curtui V, Dietrich R, Märtlbauer E, Usleber E (2010) Mycotoxins in horse feed. Mycotoxin Res 26:23–30. https://doi.org/10.1007/S12550-009-0037-8

Lin S, He J, Jiang Y, Wu F, Wang H, Wu D, Sun J, Zhang D-D, Qu H-X, Yang B (2014) Production of nigragillin and dihydrophaseic acid by biotransformation of litchi pericarp with *Aspergillus awamori* and their antioxidant activities. J Funct Foods 7:278–286. https://doi.org/10.1016/j.jff.2014.02.001

Malekinejad H, Aghazadeh-Attari J, Rezabakhsh A, Sattari M, Ghasemsoltani-Momtaz B (2015) Neurotoxicity of mycotoxins produced in vitro by *Penicillium roqueforti* isolated from maize and grass silage. Human Exper Toxicol 34:997–1005. https://doi.org/10.1177/0960327114565493

Marwah RG, Fatope MO, Deadman ML, Al-Maqbali YM, Husband J (2007) Musanahol: a new aureonitol-related metabolite from a *Chaetomium* sp. Tetrahedron 63:8174–8180. https://doi.org/10.1016/j.tert.2007.05.119

Medentsev AG, Akimenko VK (1998) Naphthoquinone metabolites of the fungi. Phytochem 47:935–959. https://doi.org/10.1016/S0031-9422(98)80053-8

Moss MO (1998) Recent studies of mycotoxins. J Appl Microbiol 84: 62S–76S. https://doi.org/10.1046/j.1365-2672.1998.0840s162S.x

Müller CE (2005) Fermentation patterns of small-bale silage and haylage produced as feed for horses. Grass Forage Sci 60:109–118. https://doi.org/10.1111/j.1365-2494.2005.00457.x

Müller CE (2018) Silage and haylage for horses. Grass Forage Sci 73: 815–827. https://doi.org/10.1111/gfs.12387

Müller CE, Pauly TM, Udén P (2007) Storage of small bale silage and haylage–influence of storage period on fermentation variables and microbial composition. Grass Forage Sci 62:274–283. https://doi.org/10.1111/j-1365-2494.2007.00580x

Müller CE, Hultén C, Gröndahl G (2011) Assessment of hygienic quality of haylage fed to healthy horses. Grass Forage Sci 66:453–463. https://doi.org/10.1111/j.1365-2494.2011.00803x

Nakamura T, Komagata D, Murakawa S, Sakai K, Endo A (1990) Isolation and biosynthesis of 3α-hydroxy-3, 5-dihydromonacolin L. J Antibiot 43:1597–1600

Nielsen KF, Frisvad JC, Sumarah M, Miller JD (2006) Production of metabolites from the *Penicillium roqueforti* complex. J Agric Food Chem 54:3756–3763. https://doi.org/10.1021/jf060114f

Nielsen KF, Månsson M, Rank C, Frisvad JC, Larsen TO (2011) Dereplication of microbial natural products by LC-DAD-TOFMS. J Nat Prod 74:2338–2348. https://doi.org/10.1021/np200254t

Northolt MD, Bullerman LB (1982) Prevention of mold growth and toxin production through control of environmental conditions. J Food Prot 45:519–526. https://doi.org/10.4315/0362-028X-45.6.519

O'Brien M, Nielsen KF, O'Kiely P, Forristal PD, Fuller HT, Frisvad JC (2006) Mycotoxins and other secondary metabolites produced in vitro by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland. J Agric Food Chem 54:9268–9276. https://doi.org/10.1021/jf0621018

Ogunade IM, Martinez-Tuppia C, Qeiroz OCM, Jiang Y, Drouin P, Wu F, Vyas D, Adesogan AT (2018) Silage review: Mycotoxins in silage: Occurrence, effects, prevention and mitigation. J Dairy Sci 101: 4034–4059. https://doi.org/10.3168/jds.2017-13788

Ostry V, Toman J, Grosse Y, Malir F (2018) Cyclopiazonic acid: 50th anniversary of its discovery. World Mycotoxin J 11:135–148. https://doi.org/10.3290/WMJ2017.2243

Oswald IP, Marin DE, Bouhet S, Pinton P, Taranu I, Accensi F (2005) Immunotoxicological risk of mycotoxins for domestic animals. Food Addit Contam 22:354–360. https://doi.org/10.1080/02652030500058320

Panthama N, Kanokmedhakul S, Kanokmedhakul K, Soytong K (2011) Cytotoxic and antimalarial azaphilones from *Chaetomium longirostre*. J Nat Prod 74:2395–2399. https://doi.org/10.1021/np2004903

Pazhanivel N, Balachandran C, Muralimanohar B, Dhinakarraj G, Balakrishnan V, Kirubaharan JJ, Raja A (2015) Alleviative effect of gingerol on cell mediated and humoral immunity and immune organs against penicillic acid mycotoxicosis in broiler chickens. Int J Life Sci Pharma Res 5:L28–L34

Pitt JI, Miller JD (2017) A concise history of mycotoxin research. J Agric Food Chem 65:7021–7033. https://doi.org/10.1021/acs.jafc.6b64494

Polonsky J, Merrien MA, Scott PM (1977) Roquefortine and isofumigaclavine A, alkaloids from *Penicillium roqueforti*. Ann Nutr l'Aliment 31:963–968

Qin JC, Gao JM, Zhang YM, Yang SX, Bai MS, Ma YT, Laatsch H (2009) Polyhydroxylated steroids from an endophytic fungus, *Chaetomium globosum* ZY-22 isolated from *Ginkgo biloba*. Steroids 74:786–790. https://doi.org/10.1016/j.steroids.2009.04.011

Rand TG, Giles S, Flemming J, Miller JD, Puniani E (2005) Inflammatory and cytotoxic responses in mouse lungs exposed to purified toxins from building isolated *Penicillium brevicompactum* Dierckx and *P. chrysogenum* Thom. Toxicological Sciences 87: 213–222. https://doi.org/10.1093/toxsci/kfi223

Rasmussen TB, Givskov M (2006) Quorum-sensing inhibitors as anti-pathogenic drugs. Int J Med Microbiol 296:149–161. https://doi.org/10.1016/j.ijmm.2006.02.005

Rasmussen RR, Storm IMLD, Rasmussen PH, Smedsgaard J, Nielsen KF (2010) Multi-mycotoxin analysis of maize silage by LC-MS/MS. Anal Bioanal Chem 397:765–776. https://doi.org/10.1007/S00216-010-3545-7

Rasmussen RR, Rasmussen PH, Larsen TO, Bladt TT, Binderup ML (2011) In vitro cytotoxicity of fungi spoiling maize silage. Food Chem Toxicol 49:31–44. https://doi.org/10.1016/j.fct.2010.09.007

Reisinger N, Dohnal I, Nagl V, Schaumberger S, Schatzmayr G, Mayer E (2016) Fumonisin B1 (FB1) induces lamellar separation and alters sphingolipid metabolism of in vitro cultured hoof explants. Toxins 8:89. https://doi.org/10.1016/j.toxlet.2019.01.013

Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010) Food and indoor fungi. 1st edn CBS Laboratory Manual Series 2. CBS-Fungal Biodiversity Centre, Utrecht.

Sarrocco S (2016) Dung-inhabiting fungi: a potential reservoir of novel secondary metabolites for the control of plant pathogens. Pest Manag Sci 72:643–652. https://doi.org/10.1002/PS.4206

Schenk J, Djurle A, Jensen DF, Müller C, O'Brien M, Spörndly R (2018) Filamentous fungi in wrapped forages determined with different sampling and culturing methods. Grass Forage Sci 74:29–41. https://doi.org/10.1111/gfs.12399

Séguin V, Lemauviel-Lavenant S, Garon D, Bouchart V, Gallard Y, Blanchet B, Diquelou S, Personeni E, Gauduchon P, Ourry A (2010) An evaluation of the hygienic quality in single-species hays and commercial forages used in equine nutrition. Grass Forage Sci 65:304–317. https://doi.org/10.1111/j.1365-2494.2010.00751

Séguin V, Garon D, Lemauviel-Lavenant S, Lanier C, Bouchart V, Gallard Y, Blanchet B, Diquelou S, Personeni E, Ourry A (2012) How to improve the hygienic quality of forages for horse feeding. J Sci Food Agric 92:975–986. https://doi.org/10.1002/jsfa.4680

Seifert KA, Morgan-Jones G, Gams W, Kendrick B (2011) The genera of Hyphomycetes. CBS-Fungal Biodiversity Centre, Utrecht

Shi Y, Zhang X, Lou K (2013) Isolation, characterization, and insecticidal activity of an endophyte of drunken horse grass, *Achnatherum inebrians*. J Insect Sci 13:151

Shotwell OL, Bennet GA, Goulden ML, Plattner RD, Hesseltine CW (1980) Survey for zearalenone, aflatoxin, and ochratoxin in U.S. grain sorghum from 1975 and 1976 crops. J Assoc Off Anal Chem 63:922–926

Skóra J, Sulyok M, Nowak A, Otlewska A, Gutarowska B (2017) Toxinogenicity and cytotoxicity of *Alternaria*, *Aspergillus* and *Penicillium* moulds isolated from working environments. Int J Environ Sci Technol 14:595–608. https://doi.org/10.1007/S13762-016-1172-3

Stoev SD (2015) Foodborne mycotoxicoses, risk assessment and underestimated hazard of masked mycotoxins and joint mycotoxin effects or interaction. Environ Toxicol Pharmacol 39:794–809. https://doi.org/10.1016/j.etap.2015.01.022

Stoev SD, Stefanov M, Radic B, Domijan AM, Peraica M (2004) Experimental mycotoxicosis in chickens induced by ochratoxin A and penicillic acid and intervention with natural plant extracts. Vet Res Commun 28:727–746. https://doi.org/10.1023/B:VERC.0000045960.46678.d3

Storm IM, Sørensen JL, Rasmussen RR, Nielsen KF (2008) Mycotoxins in silage. Stewart Postharvest Rev 6:1–12

Storm IMLD, Kristensen NB, Raun BML, Smedsgaard J (2010) Dynamics in the microbiology of maize silage during whole-season storage. J Appl Microbiol 109:1017–1026. https://doi.org/10.1111/j.B65-2672.2010.04729x

Storm IMLD, Rasmussen RR, Rasmussen PH (2014) Occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in Danish maize silage. Toxins 6:2256–2269. https://doi.org/10.3390/toxin6082256

Sumarah MW, Miller JD, Blackwell BA (2005) Isolation and metabolite production by *Penicillium roqueforti, P. paneum* and *P. crustosum* isolated in Canada. Mycopathologia 159:571–577. https://doi.org/10.1007/s11046-005-5257-7

Thomson JR, McPherson EA (1983) Chronic obstructive pulmonary disease in the horse 2: therapy. Equine Vet J 15:207–210. https://doi.org/10.1111/j.2042-3306.1983.tb01766.x

Vendruscolo CP, Frias NC, de Carvalho CB, de Sá LRM, Belli CB, Baccarin RYA (2016) Leukoencephalomalacia outbreak in horses due to consumption of contaminated hay. J Vet Int Med 30:1879–1881. https://doi.org/10.1111/jvim.14588

Wambacq E, Vanhoutte I, Audenaert K, De Gelder L, Haesaert G (2016) Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: a review. J Sci Food Agric 96:2284–2302. https://doi.org/10.1002/jsfa.7565

Wang Y, Gloer JB, Scott JA, Malloch D (1993) Appenolides A, appendiculede B, appendiculide C: three new antifungal furanones from the coprophilous fungus *Podospora appendiculata*. J Nat Prod 56:341–344. https://doi.org/10.1021/np50093a005

Wang HJ, Gloer KB, Gloer JB, Scott JA, Malloch D (1997) Anserinones A and B: new antifungal and antibacterial benzoquinones from the coprophilous fungus *Podospora anserina*. J Nat Prod 60:629–631. https://doi.org/10.1021/np970071k

Young JC, Miller JD (1985) Appearance of fungus, ergosterol and *Fusarium* mycotoxins in the husk, axial stem and stalk after ear inoculation of field corn. Can J Plant Sci 65:47-53. https://doi.org/10.4141/cjps85-007