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

2

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8

9 **Key words:** Horses, ponies, mycotoxins, roquefortine, laminitis, metabolite profiling, adverse health effects

10

11 **Abstract**

12 The presence of fungi and mycotoxins in silage (fermented maize) for cattle and other ruminants have been
13 studied extensively compared to wrapped haylage (fermented grass) for horses and other monogastric
14 animals. The purpose of this work was to examine the fungal diversity of wrapped haylage and
15 conventional hay and to analyse the forage sample for fungal metabolites. Faeces samples were also
16 analysed to study the fate of fungi and metabolites. Fungal diversity of the samples was determined by
17 direct plating on DG18, V8 and MEA and chemical analyses were done using LC-MS/MS. The results show
18 that *Sordaria fimicola* was common in both hay and haylage, while *Penicillium* spp. was prevalent in haylage
19 and *Aspergillus* spp. in hay. Communiols were found in all types of samples together with gliocladic acid.
20 Roquefortines and fumigaclavines were found in haylage with no visible fungal growth, but not in hay. In
21 haylage hot spot samples a series of *Penicillium* metabolites were detected: Andrastins, fumigaclavines,
22 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.

28

29 Introduction

Equines, being monogastric animals, are considered to be more sensitive to fungi and mycotoxins in their forage than ruminants (Wambacq et al. 2015) 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 hours (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 1980's, 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 (Seguin 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

47 has to dry in the field and is stored indoors under dry conditions (Coblentz and Akins, 2018; Schenk et al.
48 2018).

49 Although mycotoxins in fermented maize (silage) for cattle have been studied extensively (Alonso et al.
50 2013; Storm et al. 2014), less is known about the fungal diversity and fungal metabolites in hay and haylage
51 for equines (Müller et al. 2007; Liesener et al., 2010; Séguin et al. 2010; 2012; Müller, 2018; Schenk et al.
52 2018). Maize ears are prone to *Aspergillus* and *Fusarium* infection in the field (Shotwell et al. 1980; Young
53 and Miller 1985), which can be carried over in the silage, whereas grasses in the field may be colonized by
54 other fungal genera. Silage and haylage, on the other hand, are mostly associated with *P. roqueforti* and
55 roquefortines (Sumarah et al. 2005), but the authors also suggest *P. paneum* and marcfortines are more
56 associated with haylage in colder climates. O'Brien et al. (2006) analysed baled grass silage for *Penicillium*
57 metabolites and found marcfortine A, mycophenolic acid and andrastin A, while Séguin et al. (2012)
58 analysed hay and haylage samples for the classic mycotoxins and found zearalenone. Studies on forage
59 derived from grass have so far focussed on the presence of either specific fungal species or mycotoxins and
60 not the overall fungal growth and metabolite production.

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

66 **Materials and Methods**

67 **Project set-up.** This project commenced in 2011 and comprises 20 Welsh ponies born between 2007 and
68 2009. Throughout the project all 20 ponies were housed in the same barn in two separate pens. One group
69 of 10 ponies was fed haylage and the other 10 ponies were fed hay. When access was allowed to pasture

70 (May 16 to November 22, 2018) the ponies went into separate paddocks in the same field. Before turn out
71 and on return to the barn they were fed the forage appropriate to their group, every day.

72 **Forage samples.** Hay was grown on the study site from permanent pasture containing Creeping Bent
73 (*Agrostis stolonifera*), Rough-stalked meadow grass (*Poa trivialis*) and Yorkshire Fog (*Holcus lanatus*). Less
74 dominant but visible grass species included Meadow Brome (*Bromus comutatus*) and Annual meadow grass
75 (*Poa annua*) and there was very little evidence of any ryegrass (*Lolium* spp.). Grass was cut, spread, rowed
76 up and baled within three to four days. Hay was harvested in 2016 and 2018 and stored as oblong bales
77 (280 kg). Haylage was purchased commercially (Marksway HorseHage, Paignton, Devon) made from short
78 term ryegrass leys (*Lolium* spp). Haylage was provided as small individually wrapped bales (14 kg) produced
79 in 2017. Forage (hay and haylage) samples were collected by randomly grapping handfuls from the bale
80 during distribution to ponies. All sampling to place between March and September 2018 (Table 1).

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

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

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

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

102 **Fungal detection and identification.** The forage sample was aseptically cut onto 2 cm pieces in sterile
 103 plastic bags and the thawed hot spot and faeces samples were broken up in sterile, empty Petri dishes prior
 104 to plating. Each sample was transferred to one plate of V8, DG18 and MEA (approximately 1 g on each
 105 plate) and incubated at 20 °C in the dark. The plates were read after 5 days and representative fungal
 106 colonies were isolated for identification. The plates were read again after 10 days to ensure that slow
 107 growing fungi also were detected. The presence of fungal cultures on each plate was registered
 108 qualitatively at genus level and pooled over the three media. Representative fungal cultures of *Aspergillus*,
 109 *Chaetomium*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Sordaria* and *Wallemia* spp. were isolated, inoculated on
 110 media specific for each genus and identified using micro- and macro-morphological according to Domsch et
 111 al. (2007), Samson et al. (2010) and Seifert et al. (2011). Extraction and metabolite profiling of fungi in pure
 112 culture was done according to Samson et al. (2010) and Frisvad and Thrane (1987; 1993) as amended by
 113 Nielsen et al. (2011), Klitgaard et al. (2014) and Kildgaard et al. (2014). Yeasts were not identified because
 114 they are not known to produce mycotoxins or other secondary metabolites (Frisvad et al. 2008).

115 **Metabolite extraction.** All samples (haylage hot spot, haylage, hay and faeces) for metabolite analyses
 116 were freeze dried and crushed in sterile plastic bags. Each sample (approximately 3 g) was transferred into

117 a 50 mL Falcon tube and added 40 mL of ethyl acetate/isopropanol (3:1 v/v) with 1 % formic acid and
118 extracted ultrasonically for 20 min. Then 10 mL of the extract was evaporated to dryness, re-dissolved in
119 500 μ L methanol and centrifuged. 300 μ L of extract was transferred to a HPLC vial and analysed the same
120 day.

121 **Chemical analyses.** All extracts (pure fungal cultures, hot spot, forage and faeces) were analysed by UHPLC
122 on an Agilent 1290 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 25 cm $\times$ 2 mm ID,
123 2.6 μ M Agilent Poroshell phenylhexyl column which was coupled to an Agilent 6545 quadrupole Time Of
124 Flight (qTOF) high resolution mass spectrometer equipped with an electrospray source. The qTOF was
125 operated in extended dynamic range mode at a resolution of 30,000 FWHM (Full-Width Half-Maximum).
126 Separation of a 1 μ L subsample was performed at 60°C at a flow rate of 0.35 mL/min using a linear gradient
127 consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B and
128 increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and remaining for 3
129 min. The qTOF was operated in both positive and negative modes, scanning from m/z 100 to 1700 four
130 times per second. Automatic MS/MS was also performed using three consecutive collision energies of 10,
131 20 and 40 eV and with an isolation window of $\pm$ 0.7 Da in both positive and negative modes, providing
132 MS/MS spectra (m/z 30-1700) for all major peaks.

133 **Data treatment.** UHPLC-MS/MS data files were inspected for all regulated mycotoxins and all relevant
134 secondary metabolites from the fungal species detected in this study by accurate mass screening of
135 pseudomolecular ions. Furthermore, the MS/MS data was screened against an in-house library of 1600
136 known secondary metabolites and against the metabolite profiles of pure cultures of fungi isolated from
137 the forage samples (Rasmussen et al. 2010; Nielsen et al. 2011; Kildgaard et al. 2014; Klitgaard et al. 2014).
138 Qualitative data matrices were made for both fungal data (binary 1/0 for presence/absence of fungal
139 species) and for metabolite data (binary 1/0 for presence/absence of metabolites) for all 27 samples (4 hay,
140 8 haylage, 8 pooled faeces and 7 hot spot samples). Both matrices were standardized and subjected to

141 Principal Component Analysis (PCA) using The Unscrambler (CAMO, version X 10.0.1) multivariate statistical
142 program package.

143 Results

144 Fungal analyses

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

162 **Faeces samples.** All thawed faeces samples were firm and without any mouldy odour. The 24 faeces
163 samples were analysed individually, but the three samples for ponies in the same group collected at the
164 same time were very similar. Table 2 therefore shows the pooled results for the three ponies in the hay

165 group and in the haylage group at the four different collection dates (A-D). The mycological analyses
166 showed that the fungal composition of the faeces samples from ponies fed hay were different from ponies
167 that had been fed haylage. Faeces from ponies fed on older hay (Fig. 1B) showed a higher load of
168 *Aspergillus* spp. (*A. glaucus*, *A. montevicensis* and *A. versicolor*) compared with the faeces from ponies fed
169 on older haylage (Fig. 1D) where *Penicillium* spp. (*P. expansum*, *P. palitans* and *P. solitum*) were more
170 frequent (Table 2). Both groups of ponies were put on pasture after the C samples had been collected and
171 had access to hay or haylage from newer batches. The faeces samples collected 04 September 2018
172 (sampling D) from both ponies fed hay and haylage (Figs. 2B and D, respectively) were devoid of *Aspergillus*
173 spp. However, in both samples (F-H-D and F-W-D) *P. expansum*, *P. commune*, *P. crustosum* and *S. fimicola*
174 were detected (Table 2). In addition, the two samples contained *Phoma* and *Zygomycetes* spp. and
175 unidentifiable, non-sporulating filamentous fungi.

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

181 A Principal Component Analysis of the qualitative fungal growth (48 species) on all 27 samples (4 hay, 8
182 haylage, 8 faeces and 7 hot spot samples) showed that all faeces samples grouped together next to the
183 three older hay samples and one older haylage sample (W-B3) and the new haylage sample (W-D) (Fig. 3).
184 The new hay sample (H-D) and one haylage sample (W-B2) grouped by themselves and away from other
185 samples since they did not contain any *Aspergillus* or *Penicillium* spp. All seven hot spot samples grouped
186 together with five of the haylage samples, which all contained *P. roqueforti*.

187 **Mycotoxin analyses**

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

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

209 **Haylage hot spots.** The chemical analyses of the haylage hot spot samples showed the same *Chaetomium*,
210 *Podospora* and *Sordaria* metabolites as in the forage samples. Additional *Penicillium* metabolites were,
211 however, detected in the hot spot samples (Table 3). The mycotoxin PR toxin, produced by *P. roqueforti*,

212 was detected in one sample (W-hs-2). Other metabolites from *P. roqueforti* (andrastins, isofumigaclavin A,
213 mycophenolic acid, roquefortines C and D) were found in six of the samples, while *P. solitum* metabolites
214 (cyclopenin, cyclophenol, cyclopeptin, viridicatin and viridicatol) were detected in sample W-hs-2.
215 Metabolites specific for *P. paneum* (asperparaline A, marcfortines A and B) were also detected in three
216 samples. No *Penicillium* metabolites were detected in W-hs-7. None of the *Aspergillus* metabolites that
217 were detected in the forage samples were detected in the hot spot samples. As with forage and faeces
218 samples, the hot spot samples also contained linoleic acid and ergosterol and various derivatives of
219 sphingosine (e.g. dihydro-sphingosine). None of the regulated mycotoxins (aflatoxins, citrinin, fumonisins,
220 ochratoxin or trichothecenes) were detected in any of the 27 samples tested. Screening all 27 samples
221 against the in-house database on fungal metabolites did not reveal any *Alternaria* metabolites (e.g.
222 alternariols or tenuazonic acid). Furthermore, all 27 samples were also screened for the presence of C17-
223 sphinganine, which is a precursor for fumonisin B1, lolitrem B from ryegrass and juglone, but gave negative
224 results.

225 A Principal Component Analysis of the qualitative metabolite production (93 known and unknown fungal
226 compounds) across all 27 samples (4 hay, 8 haylage, 8 faeces and 7 haylage hot spot samples) shows that
227 the old hay samples grouped together due to the presence of cochliodinol, β -cyclopiazonic acid and
228 asperphenamate (Fig. 5). The new hay and haylage samples (H-D and W-D) grouped away from other
229 samples due to the absence of *Penicillium* metabolites and the faeces samples grouped together and away
230 from the forage samples due to the low number of fungal metabolites detected. Again the multivariate
231 analyses showed that all seven hot spot samples grouped together with seven old haylage samples, which
232 all showed similar metabolite profiles including roquefortines, chaetoquadrins and chaetoxanthone.

233 Discussion

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

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

238 The results suggest that the raw materials (grass) for both hay and haylage start out with similar mycobiota
 239 from the field. *Sordariales* (*Chaetomium*, *Podospora* and *Sordaria* spp.) and/or their metabolites were
 240 found in both hay and haylage. This suggests that some *Chaetomium* spp. may live as endophytes in grasses
 241 and survive long enough to produce metabolites, such as 3-epi-aureonitol, longirostrerones and
 242 globosumones (Panthama et al. 2011; Bashyal et al. 2007; Marwah et al. 2007; Shi et al. 2013; Qin et al.
 243 2009). Species of *Sordaria* and the closely related *Podospora*, known as dung fungi (Bills et al. 2013;
 244 Sarrocco 2016), may either contaminate the grass during harvest and drying or may actually also be plant
 245 endophytes. They produce metabolites like appenolides and communiols (Wang et al. 1993; 1997; Che et
 246 al. 2004; 2005), which have antimicrobial effects (Bashyal et al. 2005; Marwah et al. 2007) but nothing is
 247 known about their toxicity to equines.

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

257 β -cyclopiazonic acid and penicillic acid that were found in two hay samples and in one haylage sample may
 258 have some toxic properties. In comparison to α -cyclopiazonic acid (α -CPA), β -cyclopiazonic acid (=

259 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 Ca^{2+} -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 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 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 B₁, diacetoxyscirpenol, fumonisin B₁ and B₂ 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 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

307 al 2011, Gallo et al 2015). Neither fungus nor citrinin were detected in any sample, but the presence of
308 dihydromonacolin suggests that *M. ruber* may be common in haylage.

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

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

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

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

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

344 Little work has been done on the effects on ingestion and inhalation of mycotoxins and other bioactive
345 metabolites in equines. Also the naphthoquinones, similar to juglone, may have negative effects and can be
346 produced by a broad variety of fungi such as *Fusarium* and *Penicillium* (Medentsev and Akimenko1998).
347 More research is needed to disclose if fungal metabolites and mycotoxins – especially metabolites from
348 *Penicillium crustosum*, *P. paneum*, *P. roqueforti* and *P. solitum* together with plant sphingosines and yeasts
349 – are contributory factors to neurologic syndromes like acquired equine polyneuropathy, laminitis and
350 Cushing's disease.

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356 **Compliance with ethical standards**

357 Conflicts of interest

358 The last author is a Trustee of the Laminitis Trust.

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566 **Table 1** The four collection times (A, B, C and D) in 2018 with dates, forage type (hay and wrapped haylage)
 567 and faeces samples, which were analysed for fungal growth and mycotoxin production.
 568

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**	C	08 May and 16 May*	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

569 * Ponies were put on pasture after 16 May 2018

570 ** Batch fed to haylage-ponies the day before faeces sampling

571 **Table 2** Qualitative detection of fungi in the different hay (H), wrapped haylage (W) and faeces (F) samples
572 collected at different dates (A-D) with the most prevalent fungal species marked with ●.
573

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

574 * See Table 1 for collection dates and details.

575 **Table 3** Qualitative detection of fungi (●) and metabolites (+) in the wrapped haylage hot spot (W-hs)
576 collected in 2013 (W-hs-1 to 3), 2014 (W-hs-4 to 6) and 2016 (W-hs-7).
577

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
<i>Penicillium roqueforti</i>	●	●	●	●	●	●	●
(+)-Aristolochene	-	-	+	-	-	-	-
Andrastins B and C	-	-	+	+	+	+	-
Costaclavin	-	-	+	-	-	-	-
Eremofortin A	-	+	-	-	-	-	-
Mycophenolic acid	+	+	-	-	-	-	-
PR toxin	-	+	-	-	-	-	-
Isofumigaclavin A	-	-	+	-	-	-	-
Roquefortines C and D	-	-	+	+	+	+	-
<i>Pen. paneum</i>	-	-	-	●	●	●	●
Asperparaline A	+	+	+	+	-	-	-
Marcfortine A	-	-	-	+	-	+	-
Marcfortine B	-	-	-	+	+	+	-
Marcfortine C	-	-	-	-	-	+	-
<i>Pen. solitum</i>	●	●	-	-	-	-	-
Viridicatols*	-	+	-	-	-	-	-
<i>Paecilomyces variotii</i>	-	●	-	-	-	-	-
<i>Fusarium</i> spp.	-	-	-	-	-	-	-
Dehydrofusaric acid	-	-	+	+	-	+	+
<i>Wallemia sebi</i>	-	-	-	-	-	-	-
walleminol	-	+	+	-	-	-	-
<i>Sordariales</i> spp.	-	-	-	-	-	-	-
(-)-Musanahol	-	-	-	-	+	-	+
3-epi-Aureonitol	-	-	+	+	-	+	-
Anserinone B	+	+	+	+	+	+	-
Chaetoquadrin F and I	+	+	-	+	+	+	+
Chaetosporone	+	-	+	-	-	-	-
Chaetoxanthone B	+	+	+	-	+	+	+
Coarctatin	-	-	-	+	+	+	-
Communiols B, D and F	+	+	+	+	-	+	-
Gliocladic acid	+	+	+	-	-	+	-
Globosumones A and B	-	-	-	+	-	+	-
Heptacyclosordariolone	+	-	-	-	-	+	-
Hydroxysordarin	-	-	-	+	-	-	-
Sordaricin	-	-	-	-	+	-	-
Sordarin	+	+	-	-	-	-	-
<i>Yeast</i> spp.	-	-	●	●	-	-	●
All fungal spp.							
Ergosterol	+	+	+	+	+	+	+
Linoleic acid	+	+	+	+	+	+	+

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

579 **Table 4** Qualitative detection of metabolites and their producers in the different hay (H), wrapped haylage
580 (W) and faeces (F) from ponies fed either hay or haylage collected at different dates (A-D).
581

	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	
<i>Aspergillus</i> spp.																					
Emodins	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	
Nigragillin	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	
<i>Fusarium</i> spp.																					
Enniatins	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	
Sambucinol	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
<i>Monascus</i> spp.																					
Dihydromonacolin	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	
<i>Penicillium</i> spp.																					
β-cyclopiazonic acid	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Asperparaline A	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Asperphenamate	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Eremofortin A	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	
Isofumigaclavin A	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	
Penicillic acid	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	
Roquefortine C	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	
Rugulovaslin A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	
<i>Wallemia sebi</i>																					
walleminol	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Sordariales</i>																					
3-epi-aureonitol	+	+	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-	-	
Anserinones	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	
Appenolides	+	+	-	+	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	+	
Chaetoquadrins	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	+	-	-	
Chaetoxanthones	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	-	-	-	-	
Coarctatin	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
Cochliodinol	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Communiols	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	+	-	+	+	+	
Gliocladic acid	+	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	
Globosumones	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	
Heptacyclosordariolone	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-	+	-	-	-	-	
Hydroxysordarin	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	
Longirostrerones	+	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-	+	+	+	+	
Sordaricin	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	
Sordarin	+	+	-	+	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	+	
All fungal spp.																					
Ergosterol	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	-	-	-	-	
Linoleic acid	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

582 * See Table 1 for collecting dates and details.

583

584

585

586

587

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).

588

589

590

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).

591

592 **Fig. 3** A Principal Component Analysis of the qualitative fungal biota on hay (H), haylage (W) and faeces (F)

593 samples collected early spring (A), intermediate (B and C) and early autumn (D) in 2018. The haylage hot

594 spot (W-hs) samples have been collected in 2013-2016. The most prevalent fungi are given for each sample

595 group. The analysis is based on 27 samples (objects) and 48 fungal species (variables/factors) detected in

596 the samples. Arbitrary axis.

597

598

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

604

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