Hay vs. haylage: forage type influences the equine urinary metabonome and faecal microbiota

Article

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SUMMARY

Background: Microbial communities are increasingly being linked to diseases in animals and humans. Obesity and its associated diseases are a concern for horse owners and veterinarians, and there is a growing interest in the link between diet, the intestinal microbiota and metabolic disease.

Objectives: Assess the influence of long-term hay or haylage feeding on the microbiota and metabolomes of 20 Welsh mountain ponies.

Study design: Longitudinal study.

Methods: Urine, faeces and blood was collected from 20 ponies on a monthly basis over a 13 month period. Urine and faeces were analysed using proton magnetic resonance (^{1}H NMR) spectroscopy and faecal bacterial DNA underwent 16S rRNA gene sequencing.

Results: Faecal bacterial community profiles were observed to be different for the two groups, with discriminant analysis identifying 102 bacterial groups (or operational taxonomic units, OTUs) that differed in relative abundance in accordance with forage type. Urinary metabolic profiles of the hay and haylage fed ponies were significantly different during 12 of the 13 months of the study. Notably, the urinary excretion of hippurate was greater in the hay fed ponies for the duration of the study, while ethyl-glucoside excretion was higher in the haylage fed ponies.

Main limitations: The study was undertaken over a 13 month period and both groups of ponies had access to pasture during the summer months.

Conclusions: The data generated from this study, suggests that the choice of forage may have implications for the intestinal microbiota and metabolism of ponies and therefore, potentially their health status. Understanding the potential implication of feeding a particular type of forage will enable horse owners to make more informed choices with regard to feed, especially if their horse or pony is prone to weight gain.

Keywords: equine, forage, hay, haylage, microbiota, metabonomics

INTRODUCTION

Obesity is of rising concern for the health and well-being of the horse, with a reported prevalence of 31% in the United Kingdom [1]. This has led to an increase in the occurrence of laminitis, pituitary *pars intermedia* dysfunction (PPID) and equine metabolic syndrome (EMS) [2], which all have economic and welfare implications. EMS has been described as an endocrinopathy grouping insulin dysregulation, obesity or regional adiposity and a predilection to laminitis in the equine species [3]. However, more recently the definition has been adapted, since insulin dysregulation can occur with or without obesity or regional adiposity [4]. At present the definition of EMS refers to a group of endocrine abnormalities including abnormal glucose homeostasis, insulin dysregulation, dyslipidaemia (with or without obesity or regional adiposity), dynamic adipokine concentrations and a predilection to laminitis [5,6].

Ponies and horses that are overweight are at increased risk of developing EMS [7]. However, currently there is a paucity of data regarding whether forage choice and the relationship with the intestinal microbiota has implications on equine obesity or the potential for development of metabolic disease. Interestingly, numerous studies have reported a correlation between diet and metabolic disease syndromes in humans [8,9]. Despite dietary and digestive differences, the microbial community of the equine intestine has some similarities to that of humans and is dominated by bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* [10][11].

The intestinal bacterial community within the equine hindgut has previously been shown to be stable However, diet has the potential to influence the composition of the equine intestinal bacterial community, as previously it has been recognised as a major factor influencing the bacterial community of the intestine of humans [12,13]. The majority of the bacteria that reside in the intestine are obligate anaerobes and therefore, cannot always be analysed using culture techniques [14]. However, sequencing of the 16S rRNA gene present in bacteria allows for an overview of the bacterial community. Using this approach, differences in bacterial community profiles have previously been observed, between healthy horses and those with intestinal disease [15–19].

Metabolites that are produced from co-metabolism between bacteria and the equine host are present in the biofluids of horses and can be measured using metabolic profiling techniques such as proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Previously, metabonomic approaches have been used to identify changes in bacterial metabolites within the urine of horses with equine grass sickness [18], in faecal water in relation to impact of age and obesity on the microbiome [20] and in the lipid composition of horse blood following induction of laminitis using oligofructose [21]. In combination, these analytical techniques empower our understanding of the relationship between the equine intestinal microbiota, diet and disease.

The primary objective of this study was to evaluate the impact of long-term hay or haylage feeding on the equine faecal microbiota and associated metabolome. A group of 20 Welsh Mountain ponies maintained in separate hay and haylage groups for the preceding five years were studied monthly over a 13 month period (July 2016 to July 2017). This native UK breed was selected as they are known to be predisposed to obesity and to obesity-related diseases, such as laminitis, PPID and EMS [4,22,23]. High-resolution metabolic and bacterial profiling techniques were applied in parallel to identify variation in the intestinal microbiota and the metabolic system of the ponies receiving two different forage types (hay and haylage).

METHODS

Animals and husbandry

Twenty Welsh Mountain ponies (aged 7-9 years at the start of the study) were included in the study. Animals were divided into two equally-sized and gender-balanced (geldings and mares) groups 5 years prior (2011) to the onset of the study. From the point at which these groups were established, the animals were group housed and turned out to pasture as separate groups. No direct interactions between animals in the separate groups were permitted.

When not at pasture, both pony groups were loose-housed within the same spacious, well-ventilated barn. Group pens allowing adequate space for free movement, modest exercise and social interactions. From the time of group establishment (5 years prior to the study), One group was fed

exclusively haylage, commercially produced from short-term rye grass leys made by the same company. For the purposes of this study, all haylage offered was from the same batch. The second group only received hay grown on the study site (Wiltshire, UK) during the previous year.

During the winter (October – March), when the pasture was too wet to allow access, each group was fed its relevant forage, hay or haylage. Between April and September ponies were turned out to graze for ~ 8 hours daily, *ad libitum* in adjacent paddock systems that maintained group separation at all times. Group-specific forages were available during the nocturnal housed periods.

Any illness, changes in demeanour or laminitis (diagnosed by a veterinary surgeon using established criteria including measuring the intensity of the digital pulse technique [24]) were noted. This information, alongside any medication administered, age and group assignment of each study pony is listed in Table S1. This study was conducted under the jurisdiction of the ASPA (1986), Home Office licence number 30/3370.

Equine biofluid sample acquisition

Once a month, over the duration of the study (July 2016 – July 2017), urine, faeces and blood samples were collected from all ponies. Mid-stream urine was collected between 8am and 2pm and stored at -80ºC in 2 ml aliquots. Fresh faeces were collected between 8am and 12pm, no more than five minutes after evacuation from multiple sites in the faecal ball. Blood samples were collected between 8am and 9 am (not used for this part of the study) from the jugular vein directly into the respective vacutainers. Following collection, all samples were immediately frozen at -80°C, until required for analyses.

Equine biofluid analysis by ^{1}H NMR spectroscopy

Urine samples were prepared for ^{1}H NMR analysis by adding 200 µl of phosphate buffer (pH 7.4; 100% D_2O) containing 1 mM of the internal standard 2-trimethylsilyl-1-[2,2,3,3,-$^{2}H_4$] propionate (TSP) to 400 µl of each sample. Faecal samples (100 mg) were combined with 1.7 mm Zirconia beads and

700 µl phosphate buffer and subjected to lysis by bead-beating for 10 minutes. The homogenate was centrifuged for 30 minutes at 10,000 *g* at 4°C and the supernatant (600 µl) was transferred to 5 mm NMR tubes prior to ^{1}H NMR analysis. Spectroscopic analysis of all samples was performed using a 600 MHz Bruker NMR spectrometer operating at 300 K for urine and faeces. Standard 1D ^{1}H NMR spectra were acquired for all urine and faecal samples. For all samples, 8 dummy scans were followed by 32 scans and these were collected in 64 K data points.

Multivariate statistical analysis of ^{1}H NMR spectra

Multivariate statistical models were built in the Matlab environment (R2014a, Mathsworks) using in-house scripts to identify metabolic variation in the biofluids between the two groups of ponies. Principle component analysis (PCA) was initially used to identify metabolic variation between the two groups. Pair-wise orthogonal projection to latent structures-discriminant analysis (OPLS-DA) models were then constructed to compare the metabolic profiles of each dietary group at each month. Metabolites were assigned to peaks identified by models using the database of equine metabolites found in the study published by Escalona *et al*. 2015 [25] and Chenomx (NMR suite 8.2). Metabolic time series plots were generated in R using the SANTA-R package.

Faecal sample DNA extraction and submission for 16S bacterial gene sequencing

DNA was extracted from all faecal samples collected using the PSP® Spin Stool DNA Kit (Stratech). Extractions were performed with the manufactures instructions and DNA concentrations were quantified. All extracts were sent to the Animal and Plant Health Agency (APHA, Weybridge, UK) for sequencing on the Miseq Illumina platform. The V4 and V5 regions of the 16S rRNA gene were amplified using the following primers: U515F (GTGYCAGCMGCCGCGGTA) and U927R (CCCGYCAATTCMTTTRAGT), which produced a fragment 300 base pairs in length [26]. Amplification was performed using the following conditions: 95°C for 3 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 35 seconds and 72°C for one minute, followed by 72°C for 8 minutes. Amplicons were purified using Ampure XP magnetic beads (Beckman Coulter). Each sample was

subsequently tagged with a unique pair of indices and sequencing primer using Nextera XT v2 Index kits and 2x KALPA HiFi HotStart ReadyMix. The following PCR conditions were used for this: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, followed by 72°C for 5 minutes. The resulting amplicons were purified using Ampure XP magnetic beads. The concentration of each sample was quantified using the Quantiflour assay (Promega) and concentrations were normalised before pooling all samples. Sequencing was performed on an Illumina MiSeq with 2 x 300 base reads according to the manufacturer's instructions (Illumina, Cambridge, UK).

Analysis of 16S sequencing files

Sequence files were uploaded onto a remote linux server and quantitative insights into microbial ecology 2 (QIIME2) was used for all processing and analyses carried out (qiime2-2018.4) [27]. Files were imported and converted into a QIIME2 file (qiime tools import). Quality control programme DADA2 [28] was used to trim reads at positions 6 and 260 to remove low quality reads. Alignment was performed on the sequences (qiime alignment mafft) and this alignment was masked to remove positions that were highly variable (qiime alignment mask). FasTtree was used to generate a phylogenetic tree from this masked alignment (qiime phylogeny fastree) and midpoint rooting was applied (qiime phylogeny midpoint-root). Core metrics were generated at a sampling depth of 30,000 reads. Alpha rarefaction boxplots using the observed_otus measure were generated and significant differences in alpha rarefaction between groups assessed (qiime diversity alpha-group-significance). The reference database greengenes [29] was utilised and trained on the sequences generated from the study (qiime feature-classifier classify-sklearn). Taxonomic composition of all samples and samples by groups were generated (qiime taxa barplot). Any differences observed within taxa summary plots were confirmed using Mann-Whitney U test for significance. To identify bacterial groups that differed between groups of samples the BIOM table was downloaded as text and analysed using linear discriminate analysis effect size (LEfSe) [30]. The data from this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical reasons.

RESULTS

The faecal bacterial communities of ponies fed on hay or haylage did not differ significantly in diversity

A total of 260 faecal samples were subjected to bacterial DNA sequencing, which returned a total of 18,287,205 sequences, with a mean of 65,533 sequences per sample. Sequence files from four of the samples were not taken forward for further analyses as they returned less that 30,000 sequences per sample (P8 - month 12, P11 – month 11 and P13 - month 11). Boxplots were drawn to identify any differences in alpha diversity (measured as observed OTUs) between the different groupings of samples. When samples were grouped by hay or haylage group and month there was no significant differences between the bacterial diversity of the hay and haylage groups in any of the 13 months of the study ($p > 0.05$, Figure 1). Additional boxplots were constructed to explore whether other variables were linked to differences in the diversity of faecal bacterial communities. No differences in bacterial diversity were observed when samples were grouped by forage and by the presence of laminitis ($p > 0.05$, Figure S1A and B). When samples were grouped by pony, significant differences were observed between several ponies ($p < 0.05$, see asterisks in Figure S1C). Bacterial diversity of faecal samples taken from all ponies was significantly higher in month 6 (December 2016, $p < 0.05$) when all samples from this month were grouped together (Figure S1D).

Faecal bacterial community profiles oscillate throughout the year, irrespective of forage fed

Bacterial community profiles were drawn as a mean for the two groups of ponies at class (Figure 2A and 2B), order and family level (Figure S2) of taxonomic classification. Overall, there was little difference between the percentage abundance of the two dominant classes, *Clostridia* and *Bacteroidia*, in the hay or haylage fed ponies. However, when the number of reads for *Clostridia* for all samples were compared between the two groups of ponies there was a significant difference between the hay and haylage fed ponies ($p < 0.05$), whereas there was no significant difference for *Bacteroidia* reads ($p > 0.05$). When the number of reads for *Clostridia* and for *Bacteroidia* were compared between hay fed and haylage fed ponies for each month of the study no significant differences were

observed ($p > 0.05$). Overall, there were significantly more reads identified belonging to the bacterial classes *Alphaproteobacteria*, *Planctomycetia* and *Mollicutes* in the ponies fed haylage, compared to those fed hay. In addition, significantly more reads were identified as belonging to *Verruco5* in the ponies fed hay ($p < 0.05$) compared to those fed haylage. Bacterial community profiles at order and family levels demonstrated a similar trend to those at phyla and class level. The bacterial order *Bacteroidales* and bacterial family *Lachnospiraceae* were at a higher percentage abundance in the hay fed ponies, whereas the order *Clostridiales* and family *Rumminococcaceae* were at a higher percentage abundance in the haylage fed ponies.

Over the 13 month duration of the study the percentage of reads identified as *Bacteroidia* and *Clostridia* fluctuated in both groups of ponies (Figure 2A and 2B). The percentage of reads identified as belonging to *Bacteroidia* was, on average, the highest in the hay fed and haylage fed ponies in month 4 (October 2016, 40 % and 38 %, respectively), whereas this bacterial class was at the lowest percentage in the hay fed ponies in month 7 (January 2017, 31 %) and lowest for the haylage fed ponies in month 1 (July 2016, 29 %). The percentage of reads identified as belonging to *Clostridia* was, on average, the highest in the hay fed ponies in month 7 (January 2017, 56 %) and in the haylage fed ponies in month 13 (July 2017, 58 %). However, this bacterial class was at the lowest percentage in the hay fed and haylage fed ponies in month 6 (December 2016, 44 % and 46 %, respectively). When the raw number of reads for the two groups of ponies were analysed, the highest number assigned to *Bacteroidia* and *Clostridia* were identified in the samples from month 6 (December 2016). The mean bacterial community profiles at phyla level for month 1 (July 2016) revealed the presence of the bacterial class *Bacilli* in both hay (2 %) and haylage fed ponies (4 %). However, this bacterial class was observed at < 1% of the overall bacterial profile for both groups in the remainder of the 12 study months.

Similar oscillations were observed in the dominant bacterial orders (*Clostridiales and Bacteroidales*), and a higher average relative abundance of the bacterial family *Bacillales* in the haylage fed group in July 2016 (month 1, 4 %) compared to the hay fed group in the same month (< 1 %), but this was not significant ($p > 0.05$, Figure S3A). At the family level the bacterial communities became more complex with the two dominant phyla splitting into a number of different bacterial families (Figure S3B). A

noticeable difference was observed at family level and this was associated with the higher abundance of *Planococcaceae* in month 7 (January 2016) in haylage fed ponies (3 %) compared to hay fed ponies (< 1 %), but this difference was not significant ($p > 0.05$).

Faecal bacterial groups differed between the hay fed and haylage fed ponies, but these differences were not universal

LEfSe analysis (Figure 3) identified 61 OTUs that were significantly higher in relative abundance in samples from the hay ponies and 41 OTUs that were significantly higher in the haylage fed ponies. The bacterial phyla that had the highest percentage of these discriminatory bacterial groups for the hay fed group were *Firmicutes* (36 %), *Bacteroidetes* (14 %) and *Tenericutes* (11 %). For the haylage fed group the highest percentage of discriminatory bacterial groups belonged to *Firmicutes* (36 %), *Proteobacteria* (34 %) and *Bacteroidetes* phyla (12 %). There were a number of bacterial groups belonging to the classes *Fibrobacteria and Spirochaetes* associated with the hay fed ponies and the bacterial classes *Epsilonproteobacteria* and *Gammaproteobacteria* associated with the ponies fed haylage (Figure 3A). The relative abundance of the two bacterial groups with the strongest association with hay or haylage fed ponies is visualised in Figures 3B and C. These figures illustrate that differential bacterial groups were not highly abundant in all samples, but there were a small number of samples which exhibited very high relative abundance of these bacterial groups.

Forage supplementation with hay or haylage resulted in a shift in the urinary metabolome

Metabolic signatures were captured from urine and faecal samples collected from all ponies over the 13 month duration of the study. Multivariate modelling revealed urinary metabolic differences between ponies fed hay or haylage (Figure S3A). Ponies fed haylage excreted higher quantities of creatinine while those fed hay excreted higher amounts of hippurate in their urine (Figure S3B). A supervised OPLS-DA model was constructed to further investigate the biochemical differences in urinary metabolic profiles between hay and haylage fed groups. This model highlighted that feeding hay

resulted in a greater urinary excretion of hippurate and trimethylamine-*N*-oxide (TMAO), whereas haylage intake resulted in a greater urinary excretion of ethyl glucoside ($Q^2Y = 0.60$; Figure S3C).

PCA models were constructed using the urinary metabolic spectra from samples taken each month to investigate urinary metabolic variation between the dietary groups by month. Separation was observed between the two groups in the scores plots for every month except for month 12 (June 2017, Figure S4). OPLS-DA models were then built on the urinary profiles comparing the treatment groups at each month. An example for month 9 (March 2017) is provided in Figure 4A. During this month haylage ponies excreted higher ethyl-glucoside and *p*-cresol sulfate, whereas the hay fed ponies excreted greater amounts of hippurate, *p*-cresol glucuronide, TMAO and dimethyl sulfone. The urinary metabolites identified by the OPLS-DA models to differ between the two groups are provided in Table S2 along with the predictive ability (Q^2Y value) of the model. Hippurate was found to be excreted in higher amounts in the urine of ponies fed on hay compared to haylage for every month of the study (13 months total) except for month 12 (June 2017) where no metabolic differences were observed. Other metabolites that were observed in higher abundance in the urine of hay fed ponies at specific points over the 13 months were TMAO, phenylacetylglycine (PAG), dimethyl sulfone, and *p*-cresol glucuronide. Metabolites that were found to be increased in the urine of haylage fed ponies were PAG, glucose, creatinine, *p*-hydroxyphenylacetate, *p*-cresol sulphate and quinate. The model constructed with the strongest predictive ability was with the samples collected in month 6 (December 2016, $Q^2Y = 0.94$) and the weakest predictive ability was with the samples collected in month 11 (June 2017, $Q^2Y = 0.22$).

To further analyse the temporal changes in the metabolites, the peaks that represent metabolites identified as differing between two groups were integrated. Integrals for these metabolites were plotted as an average of the two groups of ponies over the 13 months of the study. The relative abundance of these metabolites differed from month to month throughout the study (Figure 4B). Metabolites identified in higher abundance in the urine of hay fed ponies (hippurate, PAG, dimethyl sulfone and *p*-cresol glucuronide) peaked at month 10 (April 2017). Ethyl-glucoside was higher in the urine of the haylage fed ponies at all months compared to the hay fed ponies and was at its highest in month 7 (January 2017). Urinary glucose was highest in the haylage fed ponies at months: 1, 7 and

12 (July 2016, January and June 2017). Although *p*-hydroxy-phenylacetate and *p*-cresol sulphate were identified as significantly higher in the urine of haylage fed ponies in months 5, 6 and 9 (November 2016, December 2016 and March 2017) of the study, the highest mean integrals of these metabolites could be seen in hay fed ponies in month 10 (April 2017).

Differences in faecal metabolome between ponies fed on hay or haylage were only observed in three sample months

A PCA model was constructed using all faecal NMR spectra and showed no separation between samples from the hay fed and haylage fed ponies (Figure S5). PCA models were also built on the monthly sample sets and separation was only observed in the PCA scores plot between the dietary groups at month 9 (March 2017). From the OPLS-DA models comparing the metabolic profiles at each month, a significant model was obtained for six of the study months (months 6, 8, 9, 10, 11 and 13; Table S3). From these models, the faeces of the haylage fed ponies were noted to contained higher quantities of acetate in month 9 (March 2017), whereas the faeces of the hay fed ponies contained higher quantities of acetate in month 13 (July 2013), malonate in months 9 and 10 (March and April 2017) and propionate in months 10 and 13 (April and July 2017), respectively.

Correlations present between bacterial groups and biofluid metabolites

A correlogram was constructed using the number of counts for the ten OTUs with the highest LDA score for the two groups of ponies and the integrals of the metabolites identified by the monthly OPLS-DA models (Figure 5). Strong positive correlations could be seen between bacterial groups of the same taxonomic lineage and between aromatic urinary metabolites (PAG, *p*-cresol sulphate, hippurate and *p*-hydroxy phenylacetate). Faecal propionate was found to be negatively correlated with faecal acetate and malonate. There were a number of weaker negative correlations including: urinary metabolites (including hippurate and PAG) to a number of bacterial groups (including *Oscillospira* and *Eubacterium*) and faecal metabolites (acetate and malonate) to *Bacteroidia* bacterial groups.

294

295 *Laminitis was diagnosed in three of the study ponies*

296 Three ponies were diagnosed with laminitis following examination by a veterinary surgeon (RAE),
297 during the 13 month duration of the study: P12 (months 8 and 9), P13 (months 2, 3 and 10) and P17
298 (months 3 and 4). Interestingly, these ponies all belonged to the group of ponies fed haylage as
299 forage (Table S1).

300

301 **DISCUSSION**

302 This study identified no statistically significant differences in bacterial community profile (at class
303 level) or bacterial diversity of equine faeces from ponies fed on hay vs those fed on haylage.
304 However, taxonomic resolution to the level of bacterial order revealed an increased abundance of
305 *Bacteroidales* (*Lachnospiraceae*) in the faeces of hay fed ponies and an increased abundance of
306 *Clostridiales* (*Rumminococcaceae)* in the faeces of haylage fed ponies. Distinct urinary metabolic
307 phenotypes were associated with each of the two forage types; hay fed ponies had consistently
308 higher abundance of urinary Hippurate and haylage fed ponies had consistently higher abundance of
309 urinary ethyl glucoside. These data indicate significant differences in host-microbial co-metabolism
310 associated with feeding the two different types of forage (hay vs haylage).

311 Haylage is an ensiled hay product created to allow bacterial fermentation of the grasses' natural
312 sugars and the subsequent production of lactate. Haylage is cost effective and of a higher nutritional
313 value (higher in readily available sugars) compared to hay. Moreover, the high moisture content and
314 low dust content of haylage makes it the forage of choice for horses with dust allergies and those that
315 prefer moist feed. Although an abrupt change to a haylage diet has previously been shown to
316 increase the numbers of lactobacilli in the intestinal microbiota of horses [31], few studies have
317 explored the influence of feeding ponies hay or haylage on the faecal microbiota.

318 Faecal bacterial diversity and community profiles of the two groups of ponies oscillated over the 13
319 month duration of the study. There was a significant increase in the faecal bacterial diversity of the

ponies in month six, however this could not be explained by changes to the ponies' management or diet. Equine faecal bacterial communities have previously been reported to change over the course of a year [32]. These changes are likely to be associated with seasonal variations in the nutritional content of the grass from the pasture, including grass used to make hay and haylage which is fed during winter months.

A large number of differences in the relative abundance of bacterial groups between the hay fed and the haylage fed ponies were identified by discriminant analysis (LEfSe). The *Bacteroidia* class and *Bacteroidales* order of bacteria were most strongly associated with hay fed ponies, with a number of bacterial groups belonging to the *Fibrobacteria and Spirochaetes*. *Fibrobacteria* bacteria within the horse intestine are essential for horses to breakdown their highly fibrous diets and have previously been reported to increase in relative abundance when forage was introduced to a population of horses [32]. The association of this group of bacteria with ponies fed hay illustrates that there are greater numbers of bacteria breaking down cellulose within the large intestine of these ponies, which may influence the relative abundance of dietary by-products. The *Oscillospira* genus, belonging to the *Clostridia* class of bacteria, was more abundant in haylage fed ponies than hay fed. This genus of bacteria has previously been reported to be increased in the faecal microbiota of obese humans consuming a low fat, high carbohydrate diet [33]. Ponies fed on haylage may have a higher abundance of *Oscillospira* due to the increased availability of sugars in this forage type.

Epsilonproteobacteria and *Gammaproteobacteria* are classes of *Proteobacteria* that we found to be associated with feeding haylage. Both have previously been reported to be present in the faeces of healthy horses [33,34], but in increased relative abundance after anthelmintic treatment [35], preceding a colic episode [36] and in elderly horses [20]. However, the reasons underlying these associations are currently unknown.

Multivariate models identified metabolic profiles that differed between hay and haylage fed ponies in each month of the study. Over the 13 months ponies fed on hay excreted higher quantities of urinary hippurate, TMAO, PAG, p-cresol glucuronide and dimethyl sulfone whereas the haylage fed group excreted more ethyl glucoside, PAG, glucose *p*-hydroxy-phenylacetate, creatinine, *p*-cresol sulphate and quinate. Differences in urinary hippurate and ethyl glucoside were consistently detected

throughout the study period, when comparing urine samples from hay and haylage fed ponies sampled in the same month. Both hippurate and TMAO are the products of bacterial-host co-metabolism. Hippurate has previously been reported as a marker of “healthy microbiota” [37] and has been found in reduced abundance in the urine of horses with equine grass sickness compared to healthy matched controls [18]. The current study identified reduced hippurate excretion in the urine of haylage fed ponies compared to those fed hay. Ethyl glucoside is a metabolite that is derived from the diet [38] and so the difference in forage supplementation may have resulted in the higher excretion of this metabolite in the urine of the haylage fed ponies. Interestingly, glucose was found to be at a higher concentration in the urine of the haylage fed ponies in one of the study months. This may suggest that these ponies have higher levels of circulating glucose which could lead to the development of PPID and EMS [39].

Oscillations in the abundance of urinary metabolites throughout the thirteen month study period were most likely associated with changes in feeding and changes in pasture nutritional content. Examination of urinary metabolic profiles by month revealed clear separation of hay fed ponies vs haylage fed ponies for all months except month 12. Exploration of metadata failed to reveal any confounding factors that might explain this finding.

Faecal metabolite profiles demonstrated no clear differences between hay fed and haylage fed ponies. This is consistent with other studies which suggest that faeces is an insensitive matrix for metabolic profiling [25]. However, the negative correlation between acetate/malonate and propionate does suggest functional variation in SCFA production by hindgut bacteria.

The results presented here, though interesting and potentially meaningful for understanding the role of forage in EMS, are preliminary and there are several limitations to the current study. The study we report here benefited from age and breed matched ponies maintained in tightly controlled conditions. However, ponies had access to pasture during the summer months, whereas in the winter they were housed environment with controlled feeding and this may have influenced the results. Moreover, if resource had permitted, it would have been useful to have sampled faeces from the ponies more frequently and over a longer period of time. This would have allowed for the detection of any further oscillations in bacterial communities and metabolite concentrations between current sample points.

Moreover, it would have helped elucidate if differences identified between the two groups persisted longer than the 13 month study reported here. The 16S data generated from the faecal samples provides a clear overview of the bacterial communities present in the ponies, but in order to detect more subtle differences a shotgun metagenomics approach would have been useful.

CONCLUSION

This study has demonstrated the potential impact of forage choice on the metabolic phenotype of ponies maintained under controlled conditions. Although significant differences in the diversity and high-level taxonomic composition of the faecal microbiota were not detected, discriminant analysis was able to identify a large number of bacterial groups in the faeces that varied between the two forage type groups. Furthermore, metabonomic analysis demonstrated that forage type had a consistent and measurable effect on host-microbial metabolism. Interestingly, glucose was found to be at a higher concentration in the urine of the haylage fed ponies in one of the study months, suggesting that forage type may impact on the potential development of obesity-related diseases, such as laminitis and equine metabolic syndrome. However, as this was only detected in one of the study months, further studies are required to verify this finding.

FIGURE LEGENDS

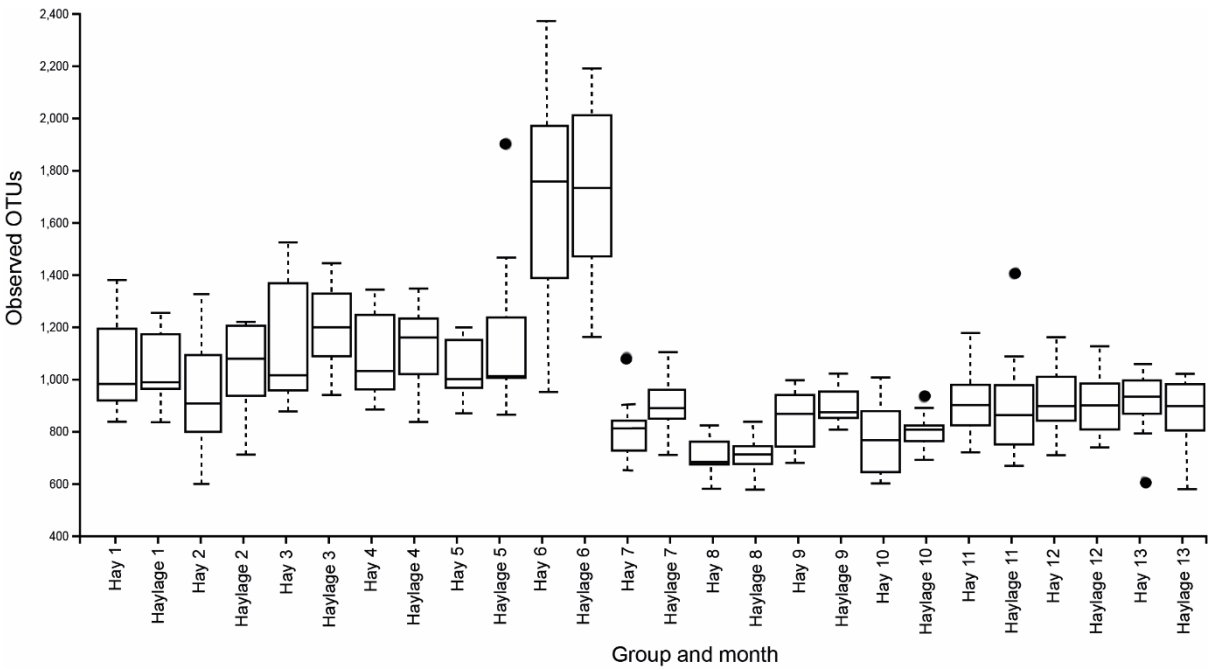


Figure 1: Alpha diversity as boxplots showing samples by group (hay/haylage) and the month the sample was collected. The number of observed OTUs per sample was taken at 30,000 reads per sample. Differences in bacterial diversity between hay and haylage groups when comparing observed OTUs per month were not significant ($p > 0.05$).

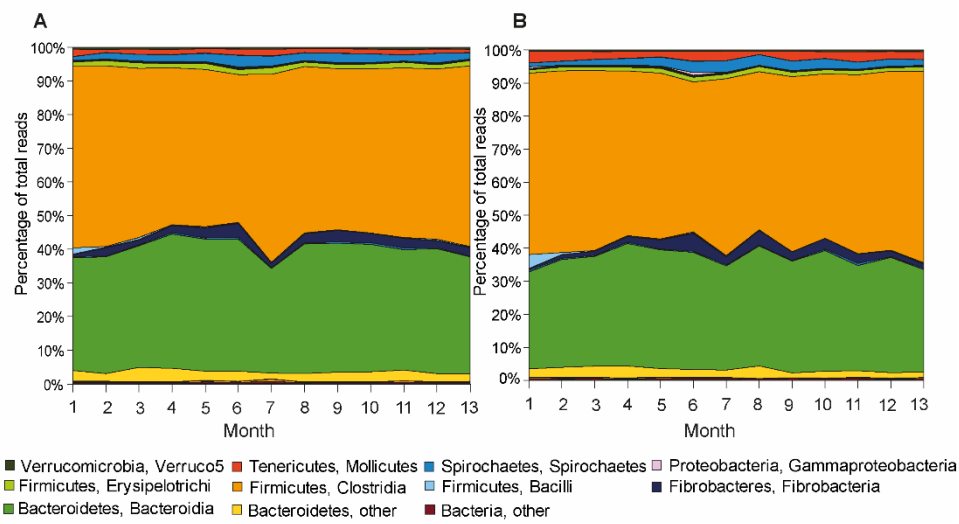
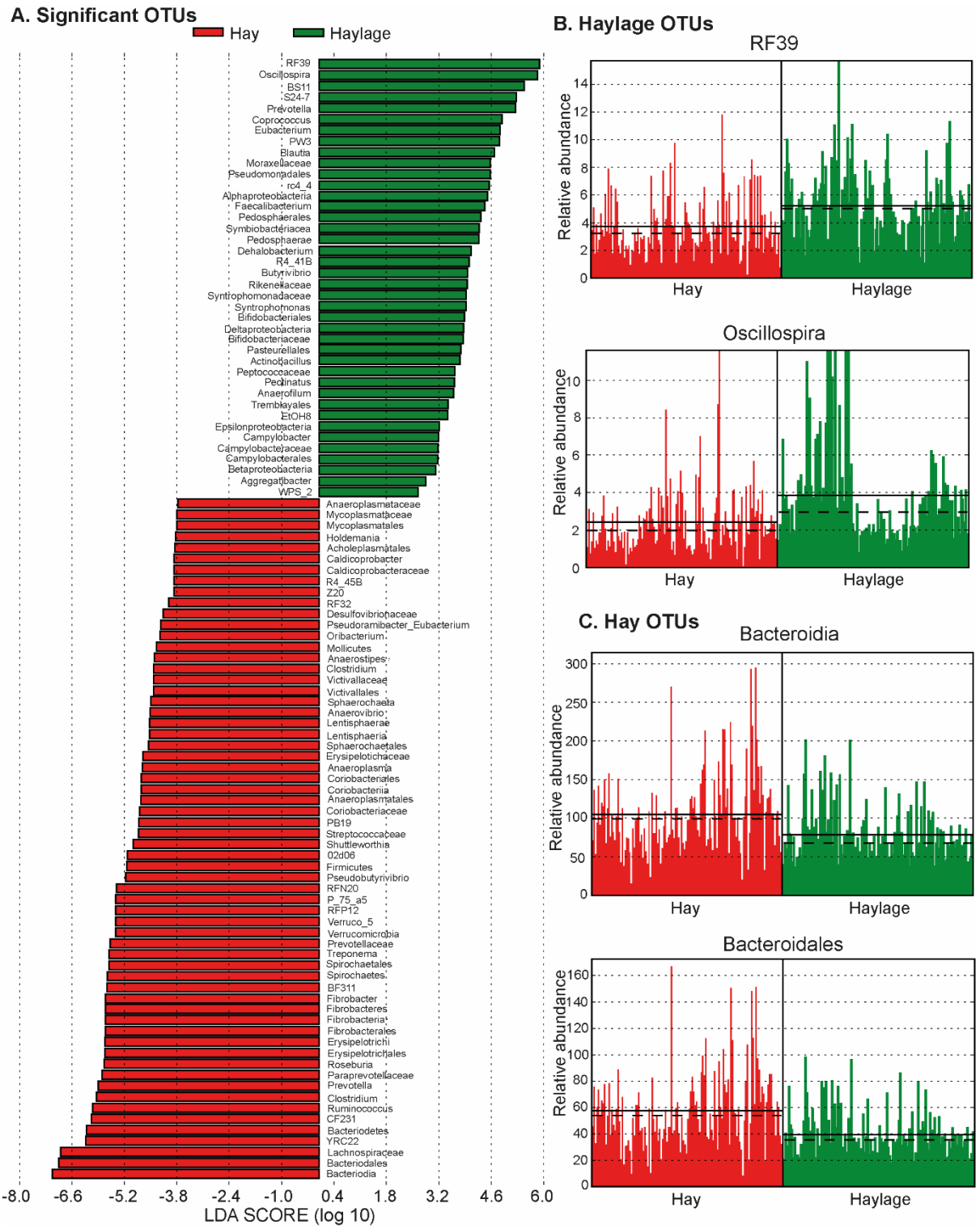


Figure 2: Mean bacterial community profiles at class level over the 13 months of the study. A) As means for the hay fed ponies and B) as means for the haylage fed ponies.



407 **Figure 3:** OTUs that were identified by LEfSe analysis as significantly different when comparing the
408 faecal microbiota of the ponies fed on hay or haylage. A) LDA scores plot indicating the strength of
409 the association of an OTU with the two groups, B) relative abundance in all samples for the two OTUs

410 with the strongest association to the haylage group and C) the two OTUs with the strongest
411 association to the hay fed ponies.

412

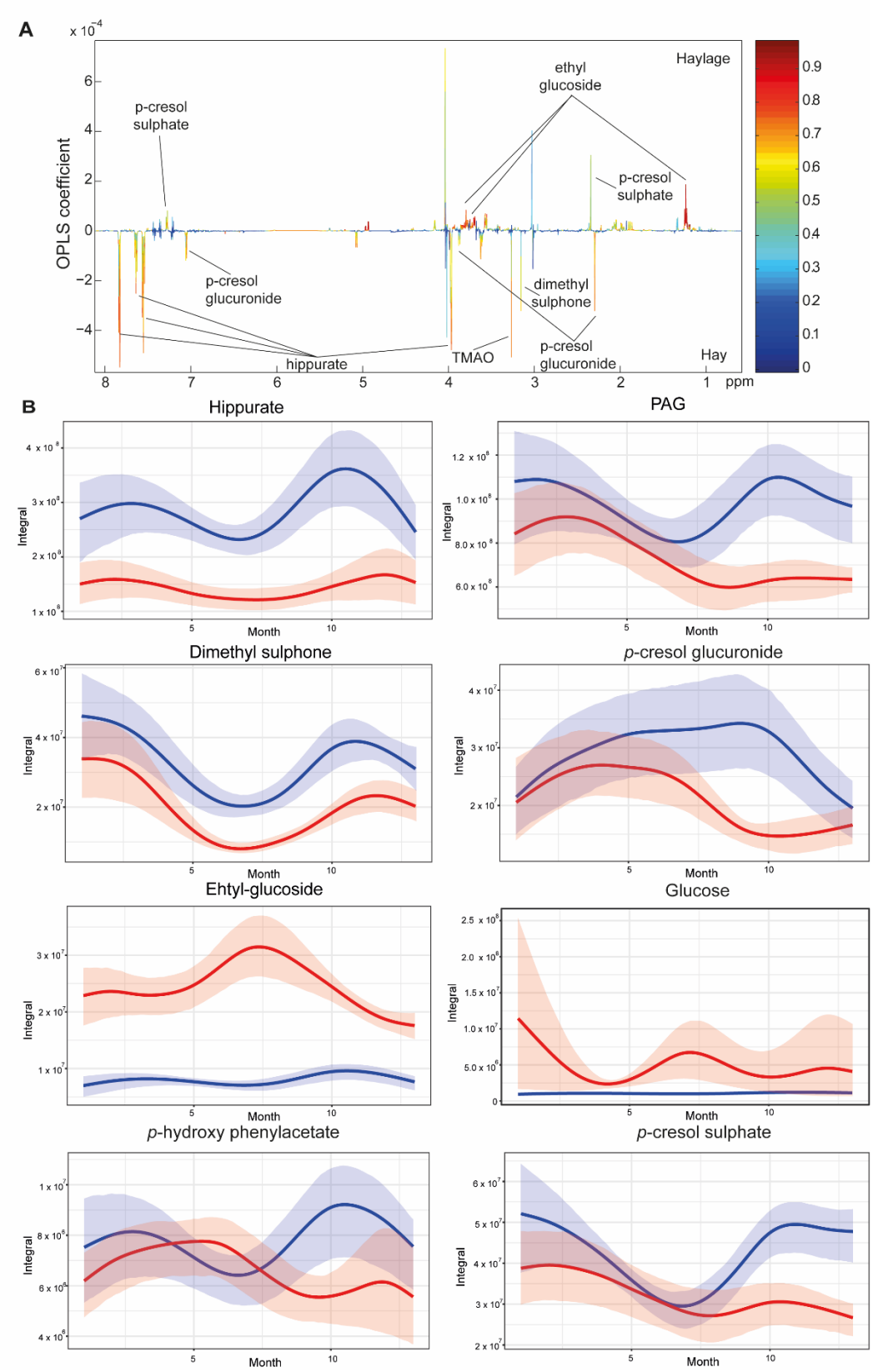


Figure 4: A) Example of an OPLS-DA model built with urinary spectra from each month; this model was built with the samples taken from hay fed and haylage fed ponies in month 9 (March 2017). B) The integrals for each metabolite found to be different between the ponies in the hay and haylage fed groups by the monthly OPLS-DA models. Lines illustrate the mean for the hay (blue) and the haylage (red) groups and shaded areas around the mean lines represent bands of confidence. Integrals were significantly different between the two groups for all metabolites ($p < 0.05$), except those for glucose, *p*-hydroxy phenylacetate and *p*-cresol sulphate ($p > 0.05$).

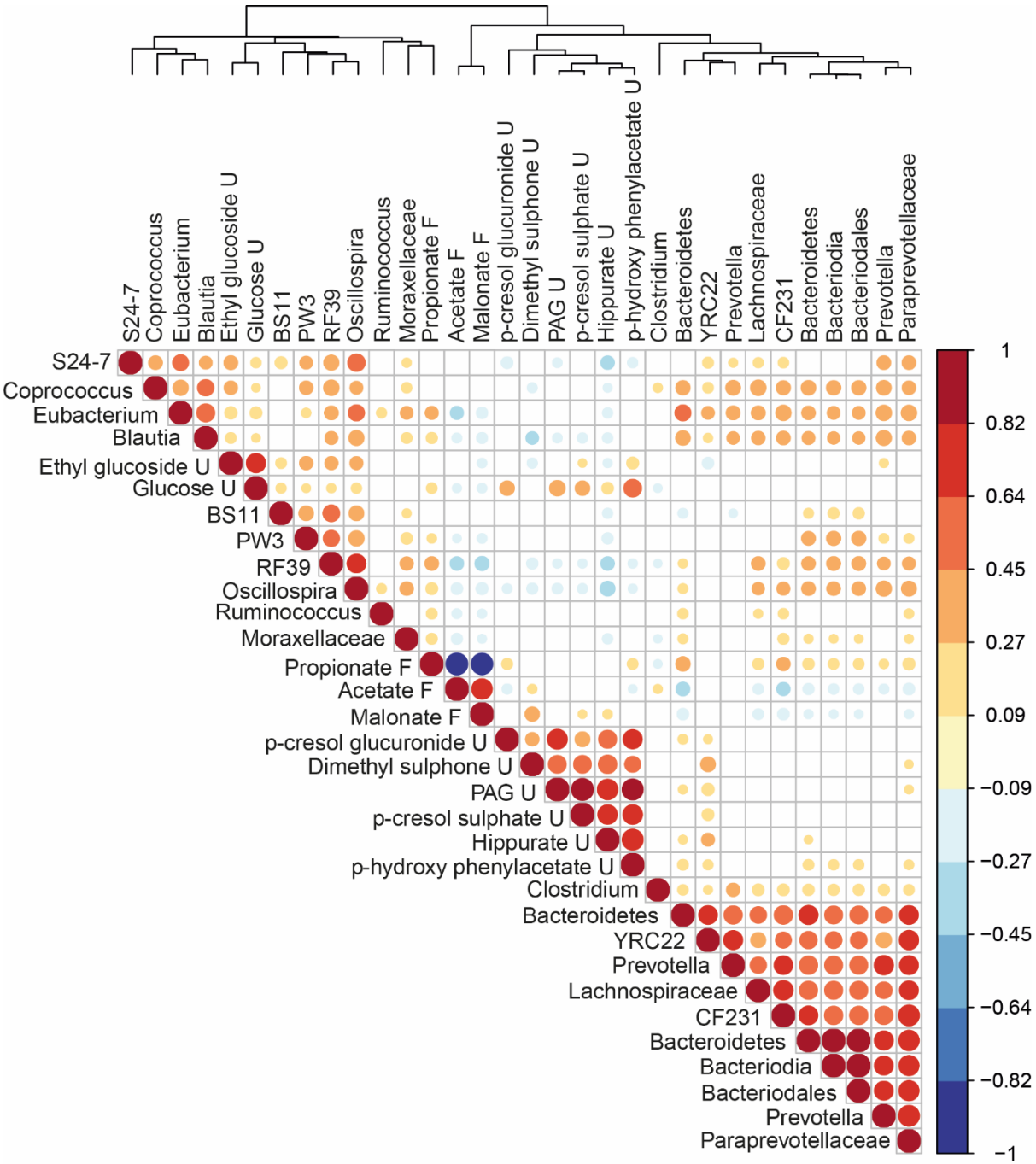


Figure 5: Correlogram identifying correlations ($p < 0.05$) between the bacterial counts for the ten most strongly correlated OTUs with the two groups of ponies from LEfSe analysis and integrals of urinary and faecal metabolites found to be different between the two groups. The dendrogram at the top of the figure shows how closely related the quantity of a metabolite or number of reads were for a specific bacterium.

SUPPLEMENTARY ITEMS

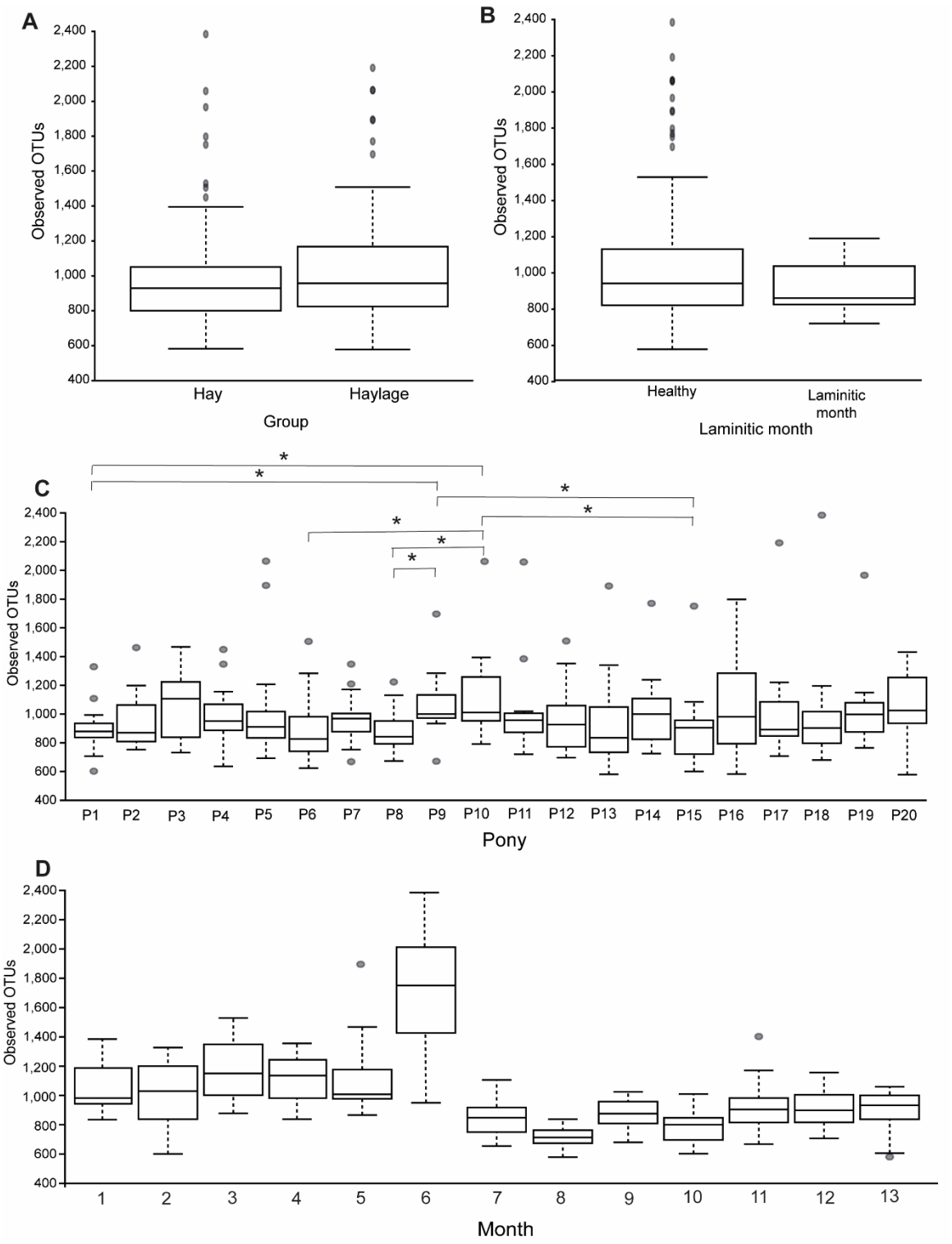


Figure S1: Boxplots illustrating the bacterial diversity (measured as observed OTUs) for all ponies when samples were grouped by A) hay or haylage group, B) the presence of laminitis, C) pony and D)

sample month. *Indicates boxplots that were found to be significantly different ($p < 0.05$) by pairwise Kruskal- Wallis tests.

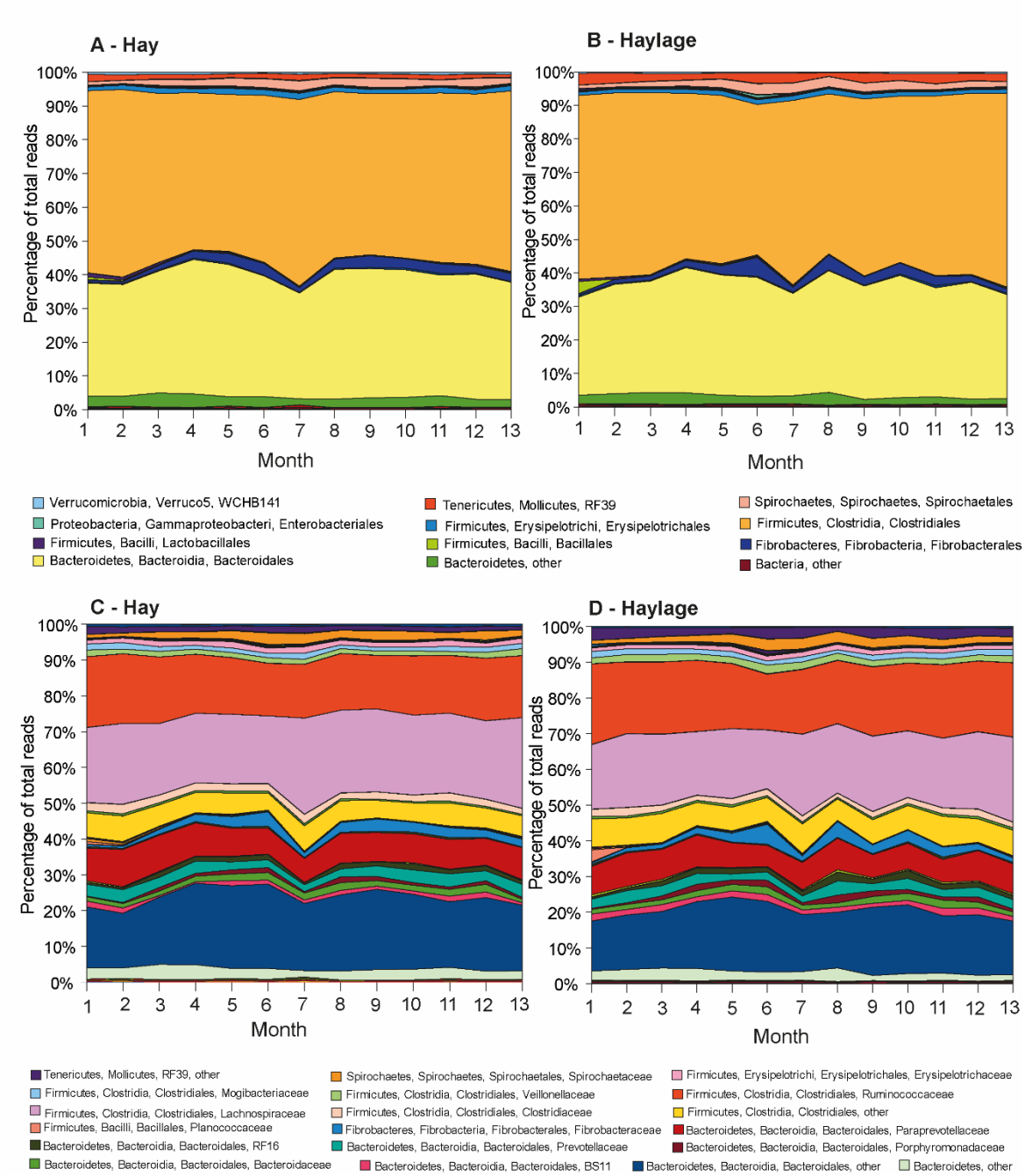


Figure S2: Mean bacterial community profiles for hay or haylage ponies over the 13 month period of the study. A) Means for each month for the hay fed ponies at the order level, B) haylage fed ponies at order level, C) hay fed ponies at the family level and D) haylage fed ponies at the family level.

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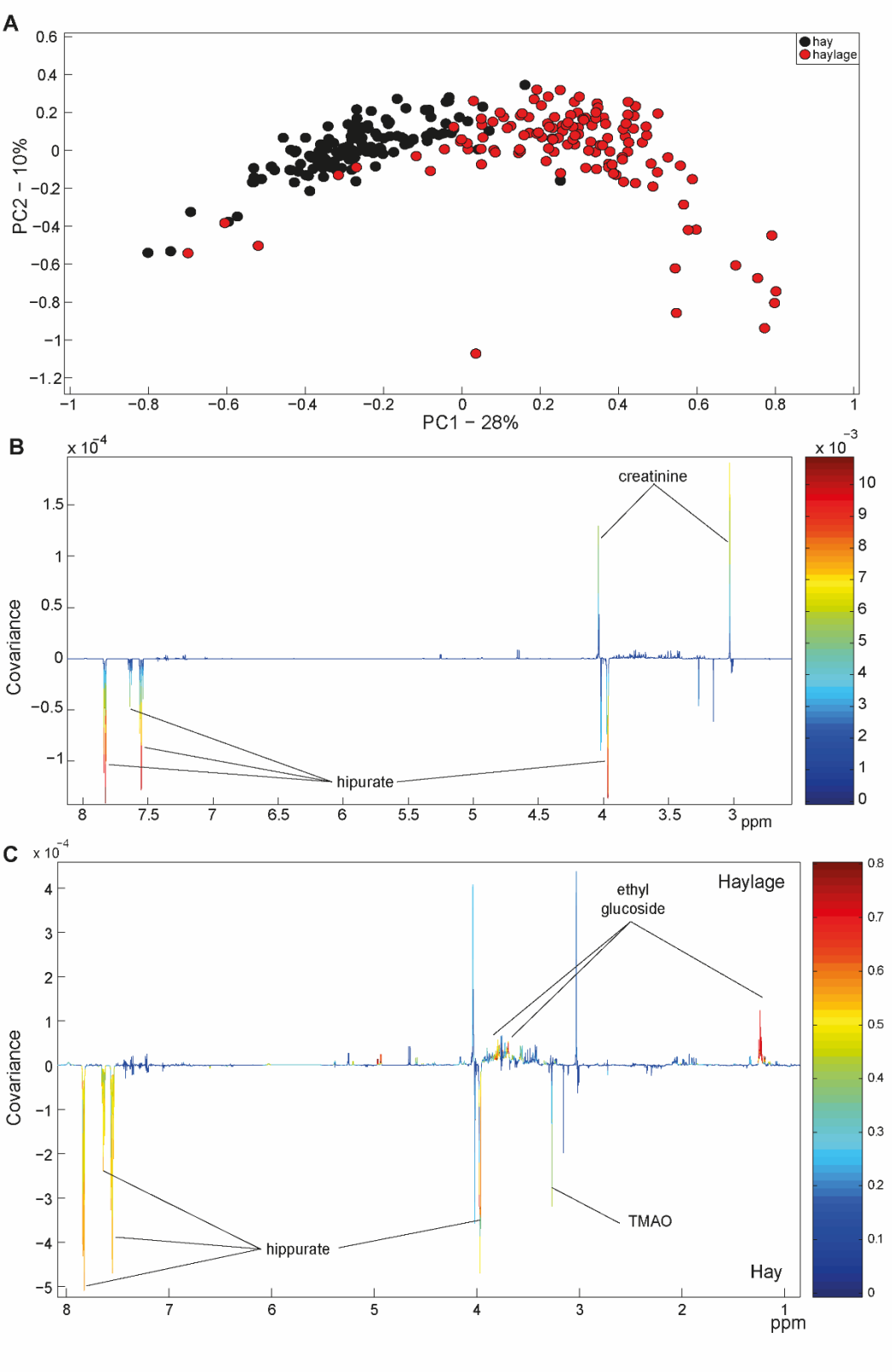
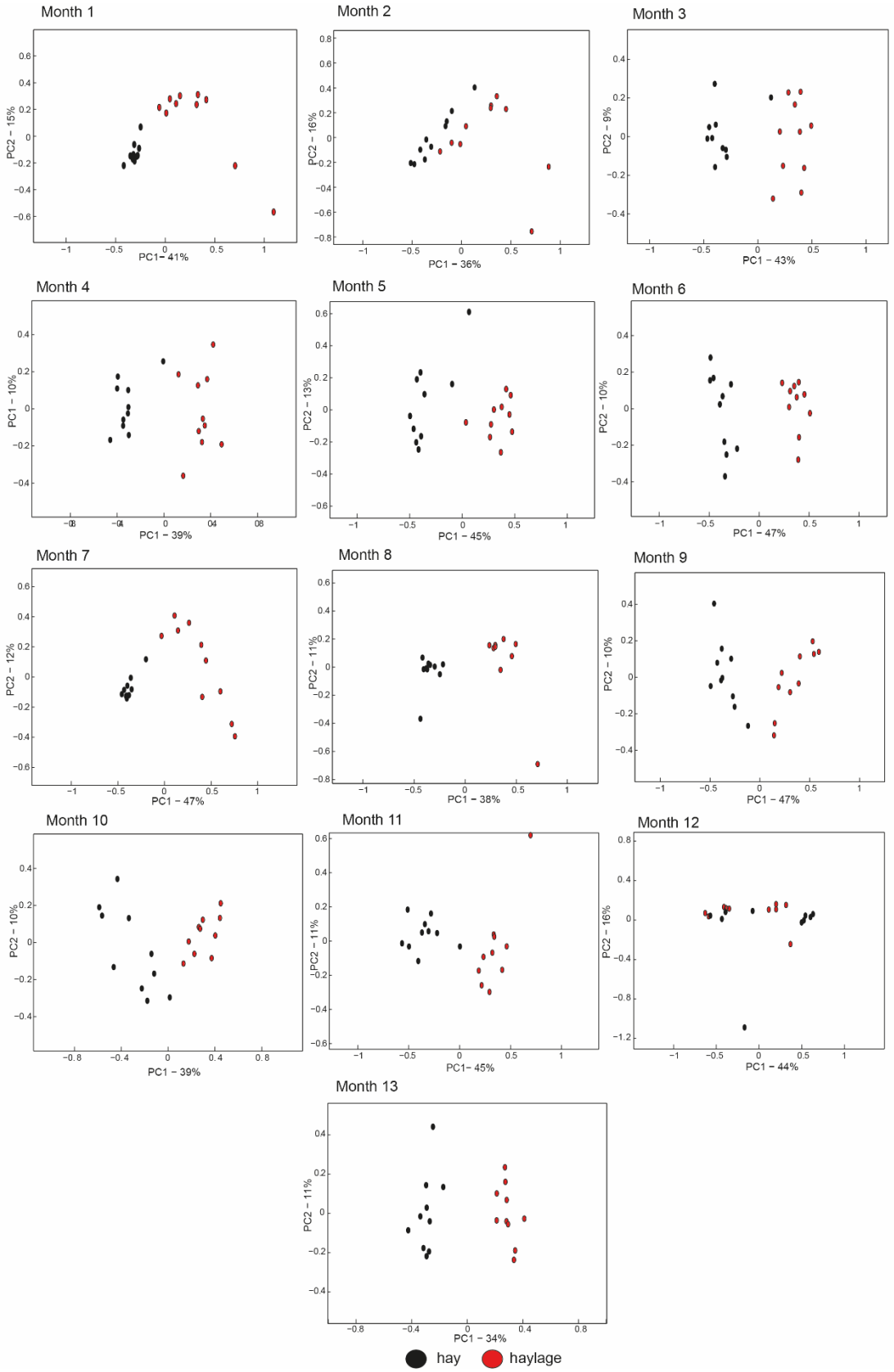


436 **Figure S3:** Multivariate models built with all the study urinary metabolic spectra. A) The scores plot of

437 a PCA model built with all the study urinary metabolic spectra (R^2 = 0.39), B) the loadings plot for PC1

438 of the PCA model and C) an OPLS-DA model built with all study urinary metabolic spectra (Q^2Y =
439 0.60). TMAO, trimethylamine-*N*-oxide.



440 **Figure S4:** Scores plots for the PCA models constructed with the urinary metabolic profiles from each

month. Points are coloured by whether the respective pony was fed hay or haylage. The R^2 values for these models are detailed in Table S2.

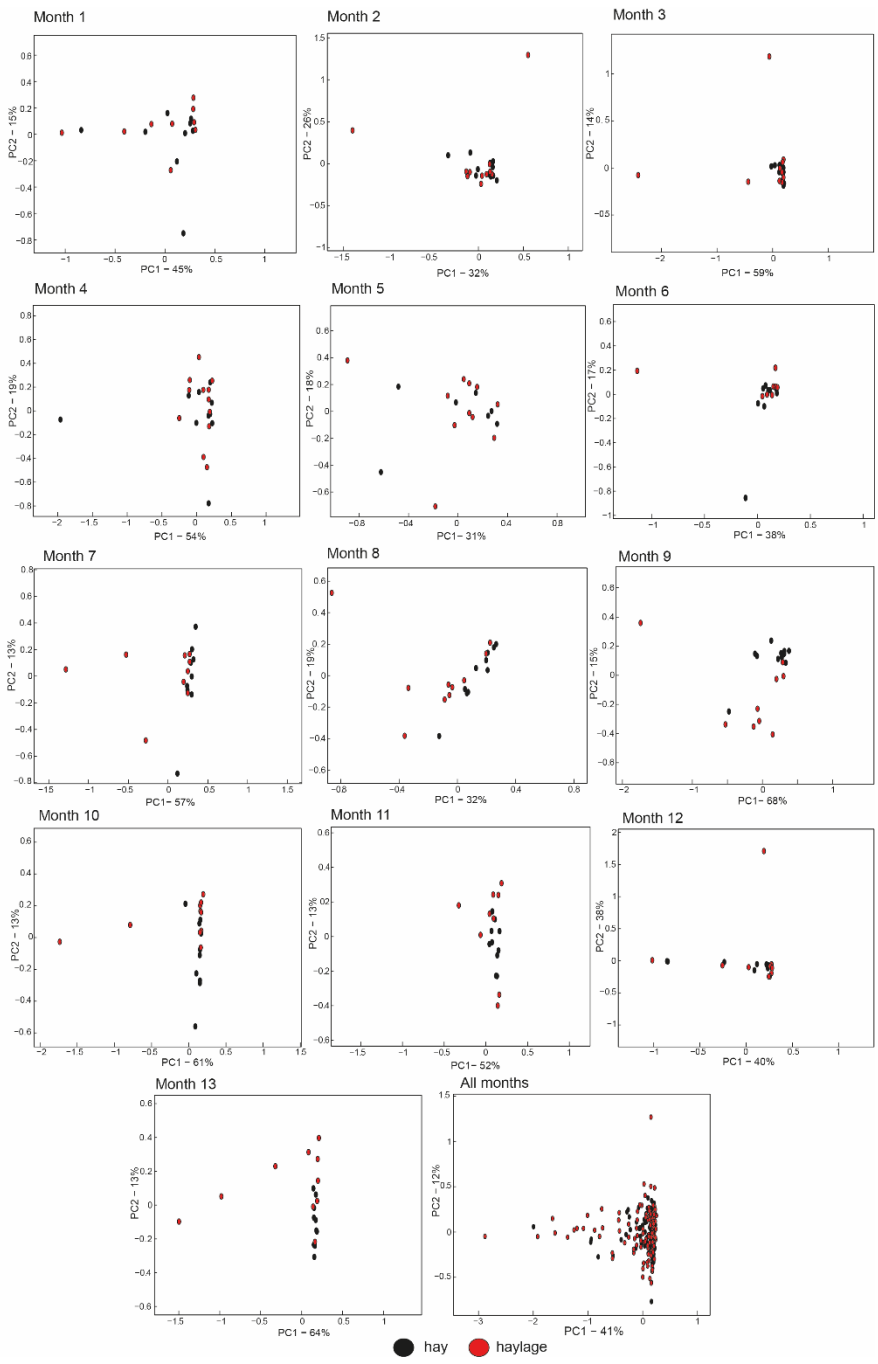


Figure S5: Scores plots for the PCA models constructed with the faecal metabolic profiles from each month and for all months together. Points are coloured by whether the respective pony was fed on hay or haylage. The R^2 values for these models are detailed in Table S2.

Table S1: Information on age, group, incidence of laminitis and general health status of the 20 study ponies.

Pony Name	Pony ID	Born	Group	Laminitic	Illness/drugs	Extra information
Blondie	P1	2009	Hay	No	Sedation, equipalazone + gentamyxin in month 2	Snotty nose month 1
Branston	P2	2008	Haylage	No		Reduced appetite month 8
Cedric	P3	2008	Haylage	No		
Clint	P4	2008	Hay	No		
Dalai	P5	2008	Haylage	No		Reduced appetite month 8
Dan	P6	2007	Hay	No		
De Niro	P7	2009	Haylage	No		
Dixie	P8	2008	Hay	No		
Gypsy	P9	2009	Haylage	No	Pyrexic + equipalazone month 8	
Jensen	P10	2008	Hay	No		Reduced appetite month 8
Kerry	P11	2009	Hay	No		
Lewis	P12	2009	Haylage	Yes - months 8 + 9		
Lippy	P13	2009	Haylage	Yes - months 2, 3 + 10		On box rest - month 1
Lizzie	P14	2009	Haylage	No		
Luna	P15	2009	Hay	No		Reduced appetite month 8
Mouse	P16	2009	Hay	No	Teeth problems and domosedan month 6	
Sally	P17	2009	Haylage	Yes - months 3 + 4		
Pip	P18	2009	Hay	No	Teeth problems month 2, 3, 4 +7. Gentamycin, crystapen + in month 4. Swollen jaw month 10. Impaction colic month 12	
Popeye	P19	2008	Hay	No		
Willow	P20	2007	Haylage	No		Feed reduced month 9

478 **Table S2:** PCA R^2 and OPLS-DA Q^2Y value for models built to compare the urinary metabolic profiles
479 of ponies fed on hay or haylage. The metabolites that were identified by OPLS-DA model to be
480 increased in the ponies maintained on hay or haylage.

Sample month	PCA R^2 value	OPLS-DA Q^2Y value	Metabolites increased in hay	Metabolites increased in haylage
M1 July 2016	0.56	0.67	hippurate	ethyl glucoside
M2 August 2016	0.53	0.41	hippurate	ethyl glucoside
M3 September 2016	0.52	0.85	hippurate, TMAO	PAG, glucose, ethyl glucoside
M4 October 2016	0.50	0.88	hippurate	glucose, creatinine, ethyl glucoside
M5 November 2016	0.58	0.76	hippurate	p-hydroxy-phenylacetate, glucose, creatinine, ethyl glucoside
M6 December 2016	0.57	0.94	hippurate	p-hydroxy-phenylacetate, glucose, ethyl glucoside
M7 January 2017	0.59	0.92	hippurate, PAG, TMAO, dimethyl sulphone	ethyl glucoside
M8 February 2017	0.49	0.90	hippurate, PAG	ethyl glucoside
M9 March 2017	0.56	0.83	hippurate, *p*-cresol glucuronide, TMAO, dimethyl sulphone	*p*-cresol sulphate, ethyl glucoside
M10 April 2017	0.49	0.85	hippurate, TMAO, dimethyl sulphone	quinate, ethyl glucoside
M11 May 2017	0.56	0.80	hippurate	glucose, quinate, ethyl glucoside
M12 June 2017	No differences - 0.60	0.22	N/A	N/A
M13 July 2017	0.45	0.91	hippurate	quinate, ethyl glucoside

481

482

Table S3: PCA R^2 and OPLS-DA Q^2Y value for models built to compare the faecal metabolic profiles of ponies fed on hay or haylage. A number of OPL-DA models with good predictive power (Q^2Y > 0.40) did not indicate any metabolites associated with the hay or haylage groups when the corresponding coefficients plot was drawn, indicated below by "no metabolites on OPLS-DA".

Sample month	PCA R^2 value	OPLS-DA Q^2Y value	Metabolites increased in hay	Metabolites increased in haylage
M1 July 2016	0.60	-0.60	-	-
M2 August 2016	0.58	-0.05	-	-
M3 September 2016	0.73	-0.31	-	-
M4 October 2016	0.73	0.05	-	-
M5 November 2016	0.49	-0.59	-	-
M6 December 2016	0.55	0.42	no metabolites on OPLS-DA	
M7 January 2017	0.70	-0.10	-	-
M8 February 2017	0.51	0.79	no metabolites on OPLS-DA	
M9 March 2017	0.83	0.63	malonate	acetate
M10 April 2017	0.74	0.74	malonate, propionate	-
M11 May 2017	0.66	0.64	no metabolites on OPLS-DA	
M12 June 2017	0.78	-0.84	-	-
M13 July 2017	0.76	0.51	acetate, propionate	-

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