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GENERAL ARTICLE

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Hay versus haylage: Forage type influences the equine urinary metabonome and faecal microbiota

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Abstract

Background: Gut 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 among 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 were 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 mo 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 suggest 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

forage, hay, haylage, horse, metabonomics, microbiota

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1 | 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 and equine metabolic syndrome (EMS)[2], which have economic and welfare implications. Equine metabolic syndrome 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, this 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].

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[11,12]. The majority of the bacteria that reside in the intestine are obligate anaerobes and, therefore, cannot always be analysed using culture techniques[13]. 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[14-18].

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[17], in faecal water in relation to impact of age and obesity on the microbiome[19] and in the lipid composition of horse blood following induction of laminitis using oligofructose[20]. 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 5 y 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 and EMS[4,21,22]. 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).

2 | MATERIALS AND METHODS

2.1 | Animals and husbandry

Twenty Welsh Mountain ponies (aged 7-9 y 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 y 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 allowed adequate space for free movement, modest exercise and social interactions. From the time of group establishment (5 y 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 h 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[23]) were noted. This information, alongside any medication administered, age and group assignment of each study pony is listed in Table S1.

2.2 | Equine biofluid sample acquisition

Once a month, over the duration of the study (July 2016 to July 2017), urine, faeces and blood samples were collected from all ponies. Mid-stream urine was collected between 08.00 am and 14.00 pm and stored at −80°C in 2 ml aliquots. Fresh faeces were collected between 08.00 am and 12.00 PM, no more than 5 min after evacuation from multiple sites in the faecal ball. Blood samples were collected between 08.00 am and 09.00 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.

2.3 | 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 min. The homogenate was centrifuged for 30 min 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.

2.4 | 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[24] and Chenomx (NMR suite 8.2). Metabolic time-series plots were generated in R using the SANTA-R package.

2.5 | 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 as this target have previously been shown to recognise a larger number of OTUs[25]. The primers used were U515F (GTGYCAGCMGCCGCGGTA) and U927R (CCCGYCAATTCMTTTRAGT) and these produced a DNA fragment 300 base pairs in length[26]. Amplification was performed using the following conditions: 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 35 s and 72°C for one minute, followed by 72°C for 8 min. 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 s, 55°C for 30 s, 72°C for 30 s and followed by 72°C for 5 min. 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).

2.6 | 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 used 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].

3 | RESULTS

3.1 | 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 than 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 were no significant differences between the bacterial diversity of the hay and haylage groups in any of the 13 m of the study ($P > .05$, 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 ($P > .05$, Figure S1A and B). When samples were grouped by pony, significant differences were observed between several ponies ($P < .05$, see asterisks in Figure S1C). Bacterial diversity of faecal samples taken from all ponies was significantly higher in month 6 (December 2016, $P < .05$) when all samples from this month were grouped together (Figure S1D).

3.2 | 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 ($P < .05$), whereas there was no significant difference for *Bacteroidia* reads ($P > .05$). When the number of reads for *Clostridia* and *Bacteroidia* were compared between hay- and haylage-fed ponies for each month of the study, no significant differences were observed ($P > .05$). Overall, there were significantly more reads identified belonging to the bacterial classes *Alphaproteobacteria*, *Planctomycetia* and *Mollicutes* in the ponies fed haylage compared with those fed hay. In addition, significantly more reads were identified as belonging to *Verruco5* in the ponies fed hay ($P < .05$) compared with 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- 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 with the hay-fed group in the same month (< 1%), but this was not significant ($P > .05$, 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

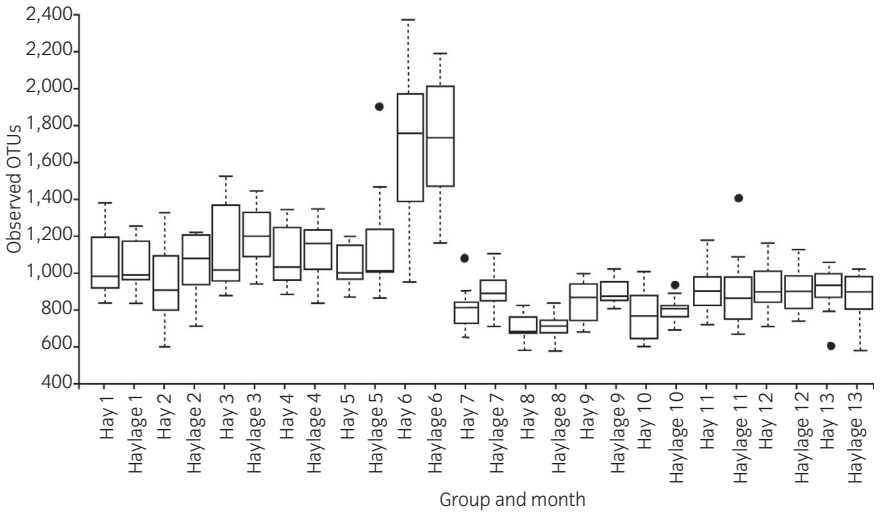

FIGURE 1 Alpha diversity as boxplots showing samples by group (hay/haylage) and the month the sample was collected. The number of observed OTUs per sample was taken at 30,000 reads per sample. Differences in bacterial diversity between hay and haylage groups when comparing observed OTUs per month were not significant ($P > .05$)

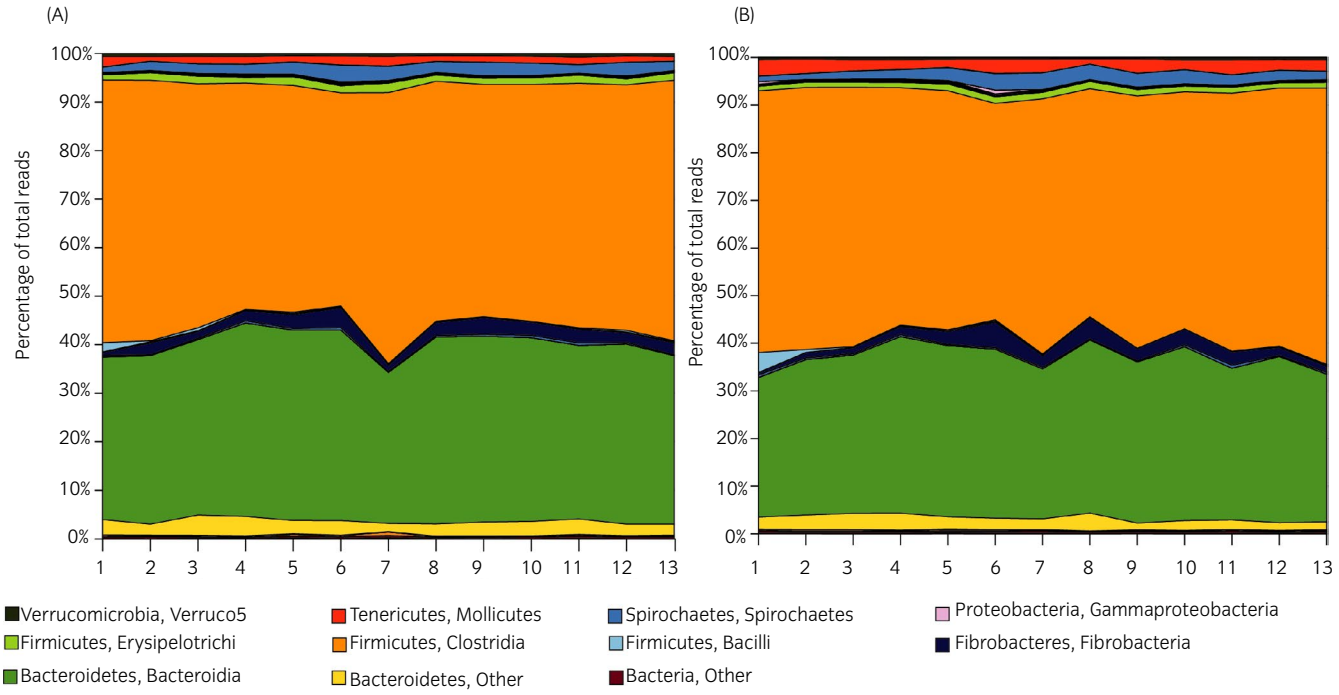

FIGURE 2 Mean bacterial community profiles at class level over the 13 mo of the study. (A) As means for the hay-fed ponies and (B) as means for the haylage-fed ponies

at family level and this was associated with the higher abundance of *Planococcaceae* in Month 7 (January 2016) in haylage-fed ponies (3%) compared with hay-fed ponies (< 1%), but this difference was not significant ($P > .05$).

3.3 | 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 ha-y or haylage-fed ponies is visualised in Figure 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.

3.4 | 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 sulphate, 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

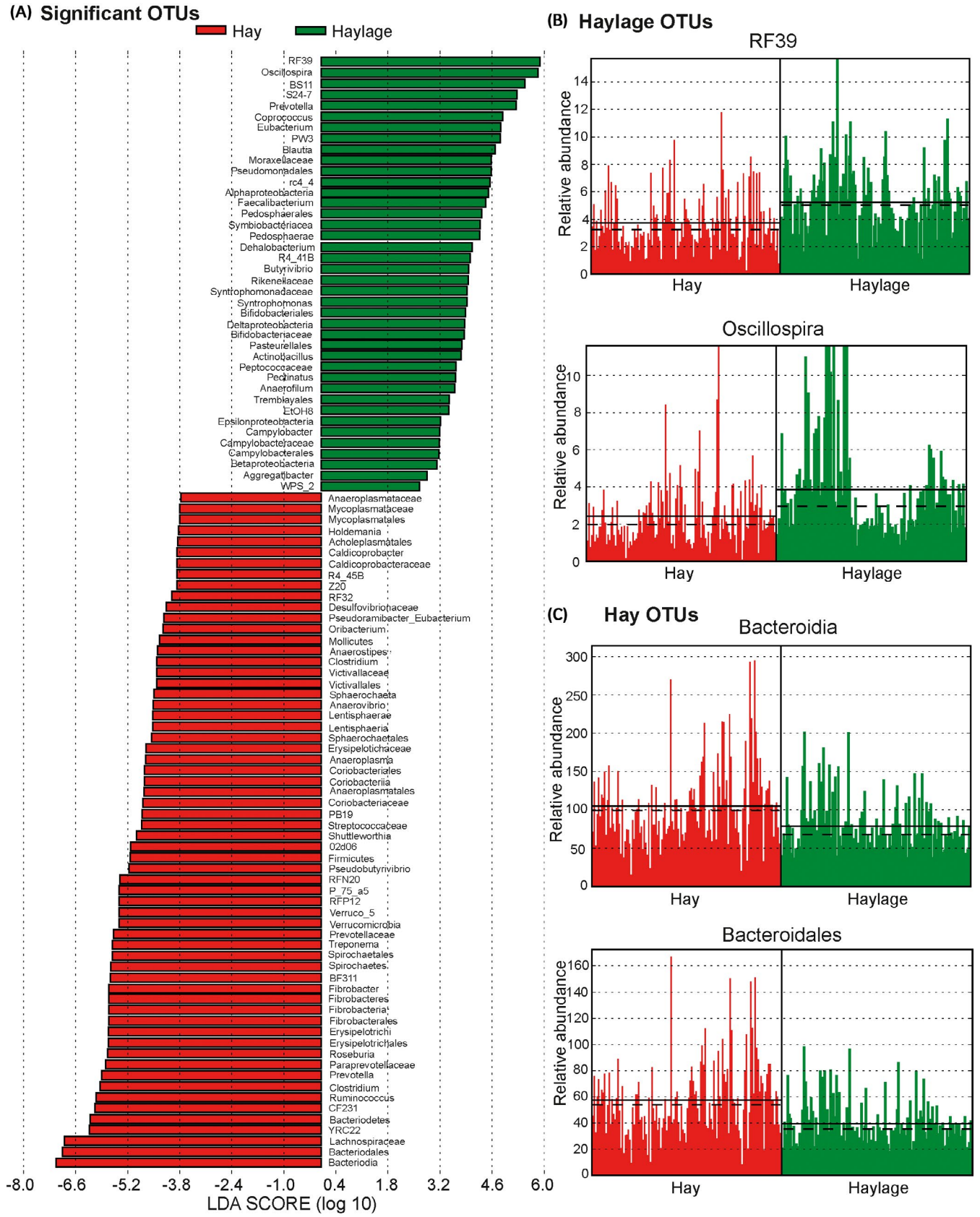

FIGURE 3 OTUs that were identified by LEfSe analysis as significantly different when comparing the faecal microbiota of the ponies fed on hay or haylage. (A) LDA scores plot indicating the strength of the association of an OTU with the two groups, (B) relative abundance in all samples for the two OTUs with the strongest association with the haylage group and (C) the two OTUs with the strongest association to the hay-fed ponies

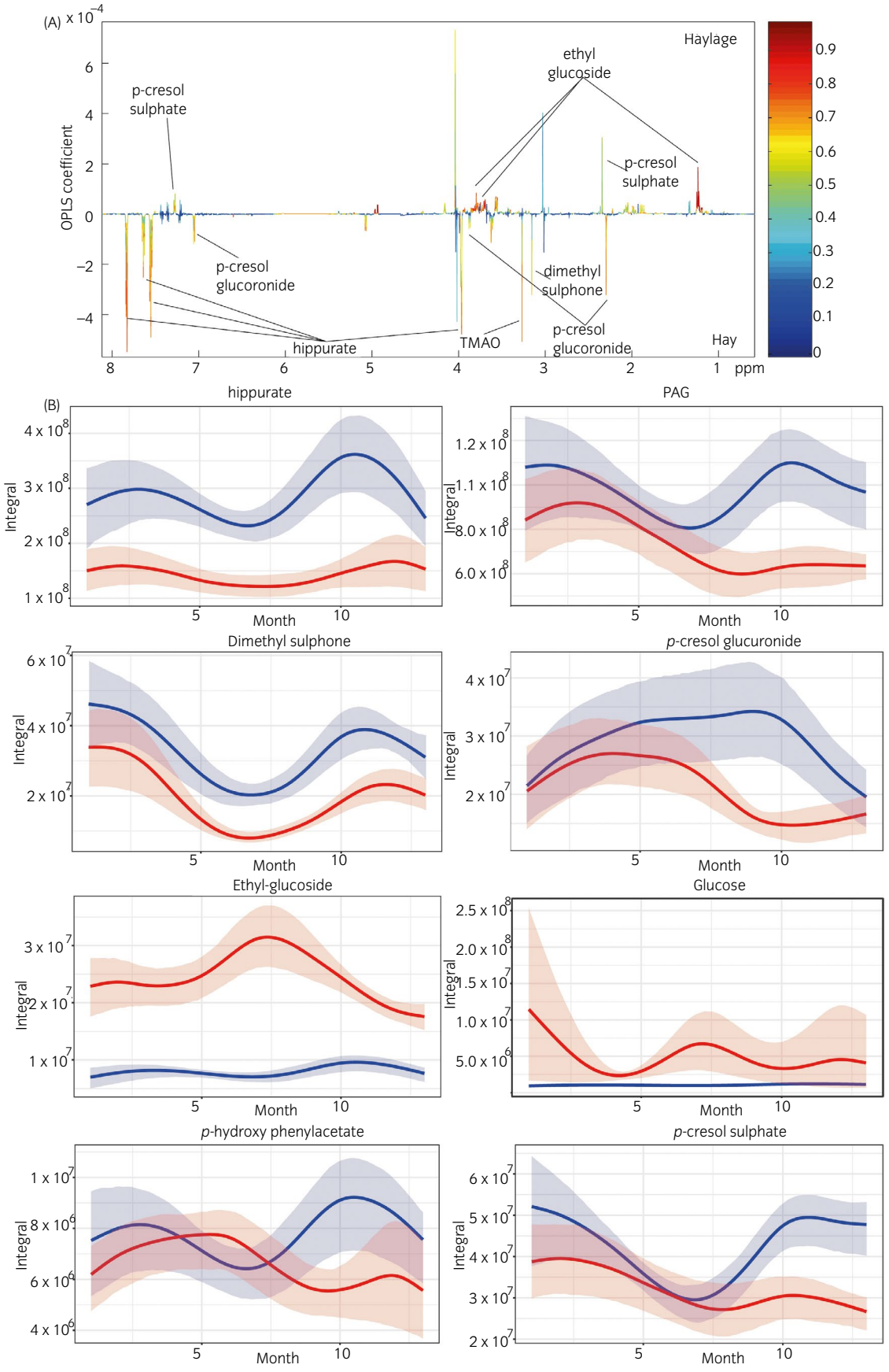

FIGURE 4 (A) Example of an OPLS-DA model built with urinary spectra from each month; this model was built with the samples taken from hay-fed and haylage-fed ponies in Month 9 (March 2017). (B) The integrals for each metabolite found to be different between the ponies in the hay- and haylage-fed groups 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 ($P < .05$), except those for glucose, *p*-hydroxy phenylacetate and *p*-cresol sulphate ($P > .05$)

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 with haylage for every month of the study (13 mo 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 mo 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 mo 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). Ethylglucoside was higher in the urine of the haylage=fed ponies at all months compared with 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).

3.5 | Differences in faecal metabolome between ponies fed on hay or haylage were only observed in 3 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 contain 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.

3.6 | Correlations present between bacterial groups and biofluid metabolites

A correlogram was constructed using the number of counts for the 10 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.

3.7 | Laminitis was diagnosed in three of the study ponies

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

4 | DISCUSSION

This study identified no statistically significant differences in bacterial community profile (at class level) or bacterial diversity of equine faeces from ponies fed on hay vs those fed on haylage. However, taxonomic resolution to the level of bacterial order revealed an increased abundance of *Bacteroidales* (*Lachnospiraceae*) in the faeces of hay-fed ponies and an increased abundance of *Clostridiales* (*Rumminococcaceae)* in the faeces of haylage-fed ponies. Distinct urinary metabolic phenotypes were associated with each of the two forage types; hay-fed ponies had consistently

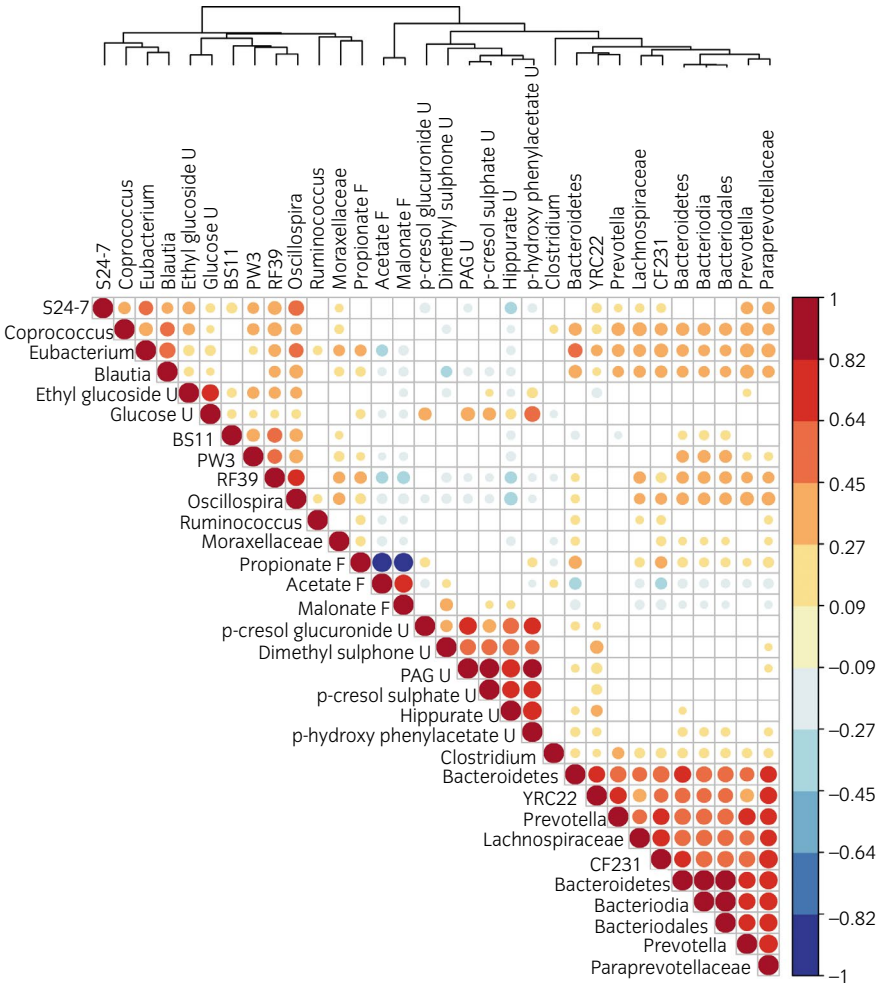

FIGURE 5 Correlogram identifying correlations ($P < .05$) between the bacterial counts for the 10 most strongly correlated OTUs with the two groups of ponies from LEfSe analysis and integrals of urinary and faecal metabolites found to be different between the two groups. The dendrogram at the top of the figure shows how closely related the quantity of a metabolite or number of reads were for a specific bacterium

higher abundance of urinary hippurate and haylage-fed ponies had consistently higher abundance of urinary ethyl glucoside. These data indicate significant differences in host-microbial co-metabolism associated with feeding the two different types of forage (hay vs haylage).

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

Faecal bacterial diversity and community profiles of the two groups of ponies oscillated over the 13-month duration of the study. There was a significant increase in the faecal bacterial diversity of the ponies in Month 6; 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[19]. 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 mo 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 with healthy matched controls[17]. The current study identified reduced hippurate excretion in the urine of haylage-fed ponies compared with 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 13-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- 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[24]. However, the negative correlation between acetate/malonate and propionate does suggest functional variation in SCFA production by hindgut bacteria.

The results presented here, although 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 in 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 provide 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.

5 | 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. Metabolites (hippurate and ethyl-glucoside) produced as a result of host-microbiota co-metabolism were consistently found to differ in concentration in the urine of the hay- and haylage-maintained ponies. This suggests that forage choice has the potential to affect the metabolism of the bacteria that reside within the gut of horses.

AUTHOR CONTRIBUTIONS

S. McNally, C. Proudman, R. La Ragione, C. Argo and R. Eustace contributed to study design. S. McNally, C. Argo, S. Emery and R. Eustace contributed to sample collection. S. McNally, G. Walton and J. Swann contributed to sample analysis. J. Leng, S. McNally, J. Swann and R. La Ragione contributed to data analysis. J. Leng, G. Walton, J. Swann, S. McNally, C. Proudman, C. Argo, R. Eustace and R. La Ragione contributed to data interpretation. All authors contributed to the manuscript preparation and approved the final manuscript.

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CONFLICT OF INTERESTS

Robert Eustace is the owner of Equi Life Ltd and Sue Emery is an employee of Equi Life Ltd.

ETHICAL ANIMAL RESEARCH

This study was approved by the AWERB and conducted under the jurisdiction of the ASPA (1986), Home Office licence number 30/3370.

INFORMED CONSENT

Not applicable.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13456.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are openly available in MetaboLights at [https://www.ebi.ac.uk/metabolights/MTBLS2479], reference number [MTBLS2479] and the European Nucleotide Archive database at [https://www.ebi.ac.uk/ena/browser/view/PRJEB43205] project PRJEB43205.

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REFERENCES

1. Stephenson HM, Green MJ, Freeman SL. Prevalence of obesity in a population of horses in the UK. Rec: Vet; 2010. p. 6281.
2. Johnson PJ, Wiedmeyer CE, Messer NT, Ganjam VK. Medical implications of obesity in horses—lessons for human obesity. J Diabetes Sci Technol. 2009;3:163–74.
3. Johnson PJ. The equine metabolic syndrome peripheral cushing's syndrome. Vet Clin North Am - Equine Pract. 2002;18:271–93.
4. Bamford NJ, Potter SJ, Harris PA, Bailey SR. Domestic animal endocrinology breed differences in insulin sensitivity and insulinemic responses to oral glucose in horses and ponies of moderate body condition score. Domest Anim Endocrinol. 2014;47:101–7.
5. Frank N, Walsh DM. Repeatability of oral sugar test results, glucagon-like peptide-1 measurements, and serum high-molecular-weight adiponectin concentrations in horses. J Vet Intern Med Intern Med. 2017;31:1178–87.
6. Cantarelli C, Dau SL, Stefanello S, Azevedo MS, Bastiani GRD, Palma HE, et al Domestic Animal Endocrinology Evaluation of oral sugar test response for detection of equine metabolic syndrome in obese Crioulo horses. Domest Anim Endocrinol. 2018;63:31–7.
7. Johnson PJ, Wiedmeyer CE, LaCarrubba A, Ganjam VK, Messer NT IV. Diabetes, insulin resistance, and metabolic syndrome in horses. J Diabetes Sci Technol. 2012;6:534–40.
8. Paniagua JA. Nutrition, insulin resistance and dysfunctional adipose tissue determine the different components of metabolic syndrome. World J Diabetes. 2016;7:483.
9. Di Daniele ND, Noce A, Vidiri MF, Moriconi E, Marrone G, Annicchiarico-Petruzzelli M, et al Impact of Mediterranean diet on metabolic syndrome, cancer and longevity. Oncotarget. 2017;8:8947–79.
10. Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Newbold CJ. Identification of a Core Bacterial Community within the Large Intestine of the Horse. PLoS One. 2013;8:1–12.
11. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505:559–63.
12. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334:105–8.
13. Daly K, Stewart CS, Flint HJ, Shirazi-Beechey SP. Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. FEMS Micro Ecolo. 2001;38:141–51.
14. Costa MC, Arroyo LG, Allen-Vercoe E, Stämpfli HR, Kim PT, Sturgeon A, et al Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3–V5 region of the 16s rRNA gene. PLoS One. 2012;7:e41484.
15. Dougal K, Harris PA, Edwards A, Pachebat JA, Blackmore TM, Worgan HJ, et al A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS Microbiol Ecol. 2012;82:642–52.
16. Ericsson AC, Johnson PJ, Lopes MA, Perry SC, Lanter R. A Microbiological map of the healthy equine gastrointestinal tract. PLoS One. 2016;11:1–17.
17. Leng J, Proudman C, Darby A, Blow F, Townsend N, Miller A, et al Exploration of the fecal microbiota and biomarker discovery in equine grass sickness. J Proteome Res. 2018;17:1120–8.
18. Stewart HL, Pitta D, Indugu N, Vecchiarelli B, Engiles JB, Southwood LL. Characterization of the fecal microbiota of healthy horses. Am J Vet Res. 2018;79:811–9.
19. Morrison PK, Newbold CJ, Jones E, Worgan HJ, Grove-white DH, Dugdale AH, et al et al The equine gastrointestinal microbiome: impacts of age and obesity. Front Microbiol. 2018;9:1–13.
20. Keller MD, Pollitt CC, Marx UC. Nuclear magnetic resonance-based metabonomic study of early time point laminitis in an oligofructose-overload model. Equine Vet J. 2011;43:737–43.
21. Alford P, Geller S, Richrdson B, Slater M, Honnas C. A multicenter, matched case-control study of risk factors for equine laminitis. Prev Vet Med. 2001;49:209–22.
22. Karikoski NP, Horn I, McGowan TW, McGowan CM. The prevalence of endocrinopathic laminitis among horses presented for laminitis at a first-opinion/referral equine hospital. Domest Anim Endocrinol. 2011;41:111–7.
23. Orsini JA, Parsons CS, Capewell L, Smith G. Prognostic indicators of poor outcome in horses with laminitis at a tertiary care hospital. Can Vet J. 2010;51:623–8.
24. Escalona EE, Leng J, Dona AC, Merrifield CA, Holmes E, Proudman CJ, et al Dominant components of the thoroughbred metabolome characterised by 1H-nuclear magnetic resonance spectroscopy: a metabolite atlas of common biofluids. Equine Vet J. 2015;47:721–30.
25. Rintala A, Pietilä S, Munukka E, Eerola E, Pursiheimo JP, Laiho A, et al Gut microbiota analysis results are highly dependent on the 16s rRNA gene target region, whereas the impact of DNA extraction is minor. J Biomol Tech. 2017;28:19–30.
26. Ellis RJ, Bruce KD, Jenkins C, Stothard JR, Ajarova L, Mugisha L, et al Comparison of the distal gut microbiota from people and animals in Africa. PLoS One. 2013;8:e54783.
27. Bolyen E, Rideout J, Dillon M, Bokulich N, Abnet C, Al-Ghalith G, et al QIIME 2: reproducible, interactive, scalable, and extensible microbiome data science. PeerJ Prepr. 2018;6:e27295v2.
28. Callahan BJ, Mcmurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.
29. Desantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–72.
30. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60. http://genomebiology.com/2011/12/6/R60
31. Muhonen S, Julliand V, Lindberg JE, Bertilsson J, Jansson A. Effects on the equine colon ecosystem of grass silage and

haylage diets after an abrupt change from hay. J Anim Sci. 2009;87:2291–8.
32. Salem SE, Maddox TW, Berg A, Antczak P, Ketley JM, Williams NJ, et al Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. Sci Rep. 2018;8:1–10.
33. Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Geor RJ, et al Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PLoS One. 2014;9(2):e87424.
34. Shepherd ML, Jr, Swecker WS, Jensen, RV, Ponder, MA, Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS Microbiol Lett. 2012;326:62–8.
35. Walshe N, Duggan V, Cabrera-rubio R, Crispie F, Cotter P, Feehan O, et al Removal of adult cyathostomins alters faecal microbiota and promotes an inflammatory phenotype in horses. Int J Parasitol. 2019;49:489–500.
36. Weese JS, Holcombe SJ, Embertson RM, Kurtz KA, Roessner HA, Jalali M, et al Changes in the faecal microbiota of mares precede the development of post partum colic. Equine Vet J. 2015;47:641–9.
37. Pallister T, Jackson MA, Martin TC, Zierer J, Jennings A, Mohney RP, et al Hippurate as a metabolomic marker of gut microbiome diversity: modulation by diet and relationship to metabolic syndrome. Sci Rep. 2017;7:1–9.
38. Teague C, Holmes E, Maibaum E, Nicholson J, Tang H, Chan Q, et al Ethyl glucoside in human urine following dietary exposure: detection by 1H NMR spectroscopy as a result of metabonomic screening of humans. Analyst. 2004;129:259–64.
39. Morgan R, Keen J, McGowan C. Equine metabolic syndrome. Vet Rec. 2015;177:173–9.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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