

Forage chemical composition drives early metabolic shifts in ponies fed different haylages

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Abstract

Background: Rapid changes between feeds can cause dysbiosis and colic in horses. The metabolic consequences of transitioning between haylages (HY) are currently unknown.

Objectives: To characterise urinary and faecal metabonomics in ponies transitioned between HY varying in botanical composition and physiological age and to evaluate the speed of metabolic adaptation.

Study Design: In vivo experiments repeated Latin Square.

Methods: Eight Welsh Mountain ponies were fed four HY (R1, R2, M1, M2) across 4 × 15-day periods; a structured 3-day (Days 13–15) changeover was observed: old:new 70:30, 50:50 and 30:70. Day 1 was 100% new haylage. Apparent digestibility was determined (multivariate mixed-effects model) from total faecal collections on Days 10–12. Urinary and faecal collections were performed on Days 0, 5, 12 and 14. Metabolic profiling of urine and faeces was performed using ¹H NMR spectroscopy with principal component analysis and orthogonal projections to latent-structures-discriminate analysis to identify metabolic shifts. Alignment and normalisation (total quotient method) were carried out in Mathworks Matlab R2014a. Significance: $p \leq 0.05$.

Results: Ponies achieved metabolic adaptation within 5 days. Metabolites were functionally stable at mid-changeover (Day 14). Forage chemical composition drove changes. Most significant differences were between physiologically young ryegrass haylage (R1) and the other three HY, for example, D5 urinary glycine R1 992 ± 504.4 vs. R2 222 ± 130 . Faecal differences included altered faecal short-chain fatty acid plus amino-acid metabolites, for example, from Day 12 full adaptation to 5 days post-

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change for acetate: $R1 14 \pm 5.5$, $R2 19 \pm 5.0$; *m*-hydroxyphenol-acetate: $R1 0.9 \pm 0.22$, $M2 1.2 \pm 0.63$; indole-3-acetate: $R1 1 \pm 0.3$, $M2 2 \pm 1.2$.

Main Limitations: Ponies were only fed forage. Results may not reflect more abrupt changes between HY or between different forages.

Conclusions: Metabolic adaptation to new HY occurred within the first 5 days and was shaped by forage composition. Three-day structured introduction supported metabolic stability. Metabonomics provides a sensitive, non-invasive tool and helps discriminate between forages for nutrient availability and hindgut function. Vigilance is still recommended in the first 5 days of introducing a new haylage.

KEYWORDS

changeover, faeces, haylage, metabolomics, pony, urine

1 | INTRODUCTION

Colic remains the leading cause of emergency veterinary intervention, financial loss and mortality in horses across the globe.^{1–3} Although changes in complementary feeds are often cited as a major colic risk in horses,⁴ forage change itself is also recognised as a risk factor for colic.⁵ Rapid transitions between forage type, nutritional profile or even between batches of the same forage are established contributors to spasmodic colic.^{2,4,6–8} Forage is inherently variable due to species composition, grass physiological age and harvest conditions. While ‘haylage’ is used as a generic term for wrapped conserved forage, chemical composition and the preservation processes of exclusion of air or lactic acid fermentation can cause substantial variations, with early-cut material being typically lower dry matter (DM), energy-dense and low-fibre, whereas later cuts are generally higher DM, more fibrous and less nutrient-rich.⁹

Muhonen⁷ and Harris⁹ highlight the variability between hays and recommended gradual transitions over 1–3 weeks to minimise colic risk. However, this can be impractical when feeding haylage, as aerobic spoilage can occur post-opening, with bacteria and fungi increasing 4 days post-opening¹⁰ and the latter particularly occurring in higher DM-wrapped forages.^{11,12} Most haylage in the United Kingdom is not accompanied by a nutritional analysis, and although Moore-Colyer¹³ reported that while 5.4% of owners changed between haylages (HY) over 2–3 days, the majority changed over immediately, potentially exposing metabolically sensitive or gut-vulnerable horses to pathologies due to large fluctuations in nutrient supply.

The microbiota of grazing or forage-fed horses has been reported to shift with season and forage botanical composition.^{14,15} This suggests that (1) horses have resilience resulting in low perturbation and fast re-establishment of the initial microbiome occurs, albeit with different diversity and abundance profiles or (2) the microbiome moves into the ‘redundancy state’, whereby dietary changes shift the microbiome in a direction away from ‘normal’ for that animal, but these new taxa possess genes and proteins that are functionally redundant, thus do not negatively influence the gut ecosystem function.^{16,17} High

microbial diversity¹⁸ generally supports resilience; however, the fact that inter-animal variation can be 52% on the same diet^{18–20} suggests that functional redundancy in the gut ecosystem is a phenomenon in horses as it is in humans.¹⁷ Metabonomics, which is a systematic study of small-molecule metabolites produced from cellular processes delivering a real-time snapshot of metabolic activity, may therefore provide a more sensitive indicator of gut physiological change than microbiome profiling alone, with urinary metabolites capturing systemic metabolic outputs, and faecal metabolites reflecting hindgut activity.²¹

This study aimed to determine whether switching between different HY over a 3-day period altered gut physiology as represented by changes in urinary and faecal metabonomics. Metabonomics was selected over microbiome profiling because metabolites represent the final outputs of host-microbial activity and provide a robust point-in-time indicator of gut function. Urinary profiles were chosen to capture digestive end-products, while faecal profiles would reflect diet-driven shifts in hindgut microbial activity. This approach suited our focus on digestion and fermentation, particularly given the likelihood of functional redundancy in the equine gut microbiome, where taxonomic shifts can be poor predictors of metabolic outputs.

The hypothesis for this study was that HY with contrasting botanical and chemical compositions would produce measurable shifts in urinary and faecal metabonomic profiles in ponies. This work provides the first integrated characterisation of faecal and urinary metabonomes during a forage changeover, offering novel evidence on the impact of forage changes, possible dysbiosis risk and practical forage-management strategies.

2 | MATERIALS AND METHODS

The four HY chosen for the trial were representative of the different categories of HY fed to horses across the United Kingdom. Thus, two monoculture temporary rye grassland pastures cut at different physiological ages (R1 and R2) and two permanent mixed species pastures, also cut at two physiological ages (M1 and M2) were used. The R1

was a single-species rye grass (*Lolium multiflorum*) in a vegetative state conserved on 24 May 2021; M1 was a mixed species pasture consisting of *Lolium perenne*, *Festuca rubra*, *Poa trivialis* and *Holcus lanatus*, with some anthesis (full flowering) evident, conserved on 2 June 2021; M2 was grown in the field adjacent to M1 and was similar in pasture composition and management to M1 but just conserved in a wholly reproductive state on 8 July 2021; R2 was a single-species *L. perenne* pasture conserved on 28 July 2021.

The eight Welsh Mountain ponies, which have been kept on grassland and managed as a group for more than 6 years, were all deemed to be in good health following a pre-trial veterinary dental and hoof assessment. None of the ponies, aged 9 (± 2.1) years, had a history of metabolic disorders, and their average bodyweight (BW) was 264 kg (range 217–305 kg). The trial was a replicated Latin square of 4 $\times$ 15-day periods, with all ponies following the same order of diets, that is, M2 to R1 to R2 to M1 to M2, which was chosen to replicate the biggest difference between HY that owners frequently encounter, for example, the change from a more ‘hay-like’ (M2) mature mixed-species forage to a more vegetative monoculture nutrient-dense R1 haylage. The transition from R1 to R2 and from M1 to M2 was intentionally designed to be moderate, focusing on physiological ageing effects on metabonomic output rather than dramatic shifts in forage composition. Nonetheless, the potential influence of differing monoculture species (*L. multiflorum* [R1] vs. *L. perenne* [R2]) cannot be excluded, nor can any changes in species proportions that may have occurred during the maturation process between the M1 and M2 HY.

Ten days prior to the commencement of the trial, ponies were individually housed in 12.2 m² loose boxes to acclimatise them to the trial conditions. Ponies were exercised twice daily for 30 min per session in a dry-lot area. Boxes were covered with rubber mat/comfort stall flooring (Haygain Ltd) with $\frac{1}{4}$ of the area covered with shavings to absorb urine. Water was provided ad libitum from 15-L plastic tubs. During the acclimatisation period (Period 0), ponies were fed a diet of meadow grass HY (Country haylage, Cherry Orchard Farm, Youngwood Ln, Nailsea, Bristol BS48 4NP) that had been harvested and conserved from a different area of the farm to that used in the trial. This HY was harvested on 8 July 2021 and had a chemical composition, determined by Association of Official Analytical Chemists²² methods detailed below, of DM 848 g/kg, pH 5.91, crude protein (CP) 51.1 g/kg DM, neutral detergent fibre (NDF) 547 g/kg DM, acid detergent fibre (ADF) 329 g/kg DM.

Before commencement of the trial and at the beginning of each new period, ponies were weighed on an equine appropriately calibrated weighbridge and the diet adjusted so they received 1.75% BW in DM/day. The HY was given in four equal meals at 08.00, 12.00, 16.00 and 20.00, in small (4.4 cm) holed hay nets. Each 15-day period was divided into 12 days where ponies were fed 100% of their allocated HY and then 3 days changeover, that is, Day 13, old:new 70:30; Day 14 old:new 50:50 and Day 15 old:new at 30:70. Faecal samples for apparent digestibility determination were pooled from collections made on Days 10–12. Faecal samples were dried in a force-draught oven at 55°C until constant weight and then DM digestibility

determined as per the method detailed in Murray.²³ During collection periods, ponies were given observed exercise in pairs in a dry lot area and any faecal outputs collected and recorded. During non-collection days, exercise was provided twice daily in the form of ‘free group play’ for 30 min in an outdoor arena.

The four HY were all made by Country Haylage (Cherry Orchard Farm, Youngwood Ln, Nailsea, Bristol BS48 4NP, UK) and were originally made into large 600 kg square bales wrapped in black plastic before being stored in large stacks on their premises. The R1 and M1 were stored for approx. 4 months and the later cut M2 and R2 for 3 months before being processed into small bales of approximately 20 kg each on 5 October 21. The small 20 kg bales were made by opening each large square bale and feeding it through a large commercial small baler on the Country Haylage premises. As part of the process, the small bales were vacuum packed into thick-gauge white plastic bags and clearly labelled with type, big bale number and date. Bales were then transported by road to the study site and stored on wooden pallets in a covered barn until they were opened and fed to the ponies.

Samples of HY for chemical composition were collected from freshly opened individual 20-kg bales from five different areas and pooled for analysis. Samples were dried at 60°C in a force-draught oven until they reached constant weight to establish DM and then milled in a Fritsch P19 cutting mill and standard cyclone system with a 2-mm sieve. Milled samples were then analysed according to the Association of Official Analytical Chemists,²² for organic matter, ADF, and NDF. CP was determined using the Kjeldahl process; gross energy via bomb calorimetry in a Parr 6100 calorimeter, and pH was read using a Thermo Scientific Orion Star A211 meter after 25 g of chopped HY had been soaked at room temperature in 100 mL of de-ionised water. Water-soluble carbohydrate (WSC) content was determined using the phenol-sulfuric acid method.²⁴

2.1 | Urinary and faecal sample collection

Urine and faecal samples for metabonomics were collected at multiple time points to capture variation in the metabonome due to diet adaptation and changes. Sampling points during changeovers were guided by modest changes in faecal pH, thus samples were collected on Day 0, capturing baseline metabonomic data before ponies were transitioned to their first study diets. During each period, urine and faecal samples were collected on the morning of Day 6 after five full days of adaptation (called Day 5, D5), on Day 12 (D12) at full adaptation and the mid-changeover Day 14 (D14) when ponies were on a 50:50 mix of two HY. Urine was collected free catch during evacuation into deionised, washed, sterile collection beakers attached to a handle and transferred in triplicate into 5-mL sterile cryovials and placed immediately on ice. A sample of freshly spontaneously voided faeces for metabonomic profiling was placed on ice before being transported to the laboratory. Faecal water samples were collected in triplicate from squeezed freshly voided faeces into 2.0-mL sterile

tubes for pH analyses. All samples were frozen at -80°C within 3 h of collection.

2.2 | Metabolic profiling of samples

Untargeted metabolic profiles were measured by ^1H NMR spectroscopy in urine and faecal water extracted via centrifugation from the collected faecal samples. Samples were analysed as described by Escalona.²¹

For metabolite identification, 2D spectra were acquired, consisting of a Total Correlation Spectroscopy, using the Bruker pulse sequence 'dipsi2gpphzs', slightly modified to include presaturation with 8 scans, 256 t_1 increments, a spectral width of 13.7 ppm in both dimensions and a relaxation delay of 2 s, along with a Heteronuclear Single-Quantum Correlation spectrum, using the Bruker pulse sequence 'hsqcetgpsisp2.2', with 16 scans, 256 t_1 increments, a spectral width of 210 ppm in the ^{13}C dimension and 20.8 ppm in the ^1H dimension and a relaxation time of 1.75 s. Alignment and normalisation (total quotient method) of spectra was carried out in Mathworks Matlab R2014a.

2.3 | Data analyses

Metabonomic data were analysed initially by unsupervised principal component analysis (PCA) models. Where spatial clustering between groups occurred, supervised models were constructed using orthogonal projections to latent-structures-discriminate analysis (OPLS-DA). OPLS-DA models were used to identify group-specific metabolic changes. Within OPLS-DA models, goodness of fit is measured by $R^2\text{Y}$ and prediction ability is measured by $Q^2\text{Y}$. Only OPLS-DA models with $Q^2\text{Y}$ values >0.4 and individual metabolite abundance differences ($R^2 > 0.4$) are reported. To ensure models were not over-fitting, OPLS-DA models underwent permutation testing, to 1000 permutations, to ascertain the significance by comparing the permuted model to the original OPLS-DA model. Only models with good predictive ability and no over-fitting ($p < 0.01$) were kept. Where models were kept, and differing metabolites were observed, concentrations were predicted by measuring the peak area and number of protons against that and the concentration of the trimethylsilyl propionate singlet. All models used normal distribution with identity link and were estimated using Schall (1991) interaction algorithm. Variance components, dispersion parameters and denominator degrees of freedom were estimated using the average-information algorithm.

Apparent digestibility data were initially subjected to normal distribution analyses via the Shapiro-Wilk test. As digestibility parameters are biologically correlated and measured repeatedly within animals and diets, a multivariate mixed-effects model analysis was done in residual maximum likelihood (Genstat 22nd edition) to capture the nested structure of the data, reduce bias and improve power. False discovery rate (Benjamini-Hochberg) post hoc pairwise testing and finally individual analysis of variances were performed with differences between means determined using standard error of differences.

3 | RESULTS

3.1 | Haylage chemistry, faecal pH and digestibility

Physiological age together with grass species caused notable differences in HY chemical composition, and this was reflected in the in vivo apparent dry matter digestibility (DMD) between the HY (Table 1) with $R1 > M1 > M2$ and R2. Faecal pH on Day 12 was not significantly different between diets (Table S1). The higher WSC content and lower ADF content of the R1 haylage resulted in a mean of 66 g/kg higher DMD than the next physiologically youngest haylage, M1. M1 had a mean DMD of 60 g/kg and was more digestible than R2. R2 and M2, conserved only 3 weeks apart, had similar DMD, although different in species composition.

3.2 | Urinary metabonomic profiles

Metabonomic profiles for the pre-trial diet, which was a mature meadow haylage, showed that all ponies started the trial with similar urinary metabonomic profiles with low inter-pony variability, so any differences found were due to the different diets fed. Differences in urinary metabolite output associated with HY changeovers and time on diet are summarised in Table 2. Across all transitions, only five $Q^2\text{Y}$ robust models showed meaningful differences between metabolic profiles, and these were between maximum dietary adaptation (D12) and the early phase of adaptation to the subsequent new diet (D5). These shifts were not reflected in individual metabolite changes, as most metabolite outputs were similar, with the exception of glycine, which was higher in the R1 D12 to R2 D5 transition 992 ± 504.4 versus 222 ± 130.6 respectively.

No metabolic differences were detected between maximum time on diet (D12) and the mid-changeover period (D14; 50:50 diet). The only observed difference during this changeover was the M1:M2 transition, where the D14 profile differed from the early exposure to M2 (D5). This difference was in the overall metabolic profile but was not mirrored by any specific differences in individual metabolite concentrations.

3.3 | Faecal metabolic profiles

No differentiating clusters were observed in the faecal metabonomic profiles between D5 and D12 while ponies were consuming the same haylage (Table S1).

Minimal changes were noted between most of the changeovers with only some loose clustering noted for R1 D12 compared with the dispersed pattern for R1:R2 D14 (Figures S1–S4).

Table 3 shows overall model and individual faecal metabolite differences across different time points. Maximum adaptation to a diet occurred in the first 5 days, not during the changeover between diets on D14. Most differences were seen between maximum adaptation to a diet (D12) and up to 5 days (D5) on the new diet. Several

TABLE 1 Mean $\pm$ SD chemical, energy and pH contents of four haylages: Meadow haylage 1 (M1), Meadow haylage 2 (M2), *Lolium multiflorum* haylage 1 (R1) and *Lolium perenne* haylage 2 (R2) fed to eight ponies and their apparent digestibilities.

Parameter	M1	M2	R1	R2
Conservation date	2 June 2021	8 July 2021	24 May 2021	28 July 2021
pH	5.8 $\pm$ 0.09	5.9 $\pm$ 0.19	5.7 $\pm$ 0.10	5.9 $\pm$ 0.09
Dry matter (g/kg)	768 $\pm$ 42.9	800 $\pm$ 54.8	597 $\pm$ 38.8	822 $\pm$ 15.3
Neutral detergent fibre (g/kg DM)	583 $\pm$ 37.03	589 $\pm$ 25.10	402 $\pm$ 122.6	562 $\pm$ 16.53
Acid detergent (g/kg DM) Fibre	306 $\pm$ 27.00	347 $\pm$ 28.60	193 $\pm$ 7.60	347 $\pm$ 19.64
Crude protein (g/kg DM)	75 $\pm$ 4.49	47 $\pm$ 5.90	67 $\pm$ 5.75	63 $\pm$ 5.36
Water-soluble carbohydrates (g/kg DM)	156 $\pm$ 53.54	169 $\pm$ 14.89	411 $\pm$ 16.30	214 $\pm$ 14.39
Gross energy (MJ/kg DM)	17.6 $\pm$ 0.09	17.7 $\pm$ 0.20	16.5 $\pm$ 0.33	17.3 $\pm$ 0.36
Digestible energy (MJ/kg DM)	13.5 $\pm$ 1.64	11.5 $\pm$ 1.28	15.3 $\pm$ 1.84	12.3 $\pm$ 1.41
Faecal pH	6.06 $\pm$ 0.14	6.13 $\pm$ 0.10	6.02 $\pm$ 0.11	6.41 $\pm$ 0.12
Dry matter digestibility ^a (g/kg)	647 ^B $\pm$ 9.4	577 ^C $\pm$ 12.9	713 ^A $\pm$ 30.5	587 ^C $\pm$ 22.9

Note: Values sharing the same letters (A, B, C) are similar ($p < 0.05$).

^aSED = 21.4, $p < 0.00$.

TABLE 2 Urinary metabolite differences between Day 12 (maximum time on diet) and Day 5 of consuming a diet and the mid-point of changeover at Day 14 when ponies were given a 50:50 diet of the old haylage to the new haylage.

Haylage change	Q ² Y and p	Metabolites	R ²	Conc. of metabolites	p -value
R1 D12 to R2 D5	0.66 and <0.001	Glycine	0.65	R1 992 $\pm$ 504.4 R2 222 $\pm$ 130.6	0.034
R2 D12 to M1 D5	0.76 and <0.001	Carnitine	0.7	R2 36 $\pm$ 33.3 M1 51 $\pm$ 69.7	0.450
	0.76 and <0.001	Propionyl carnitine	0.7	R2 15 $\pm$ 19.6 M1 36 $\pm$ 59.9	0.206
	0.76 and <0.001	Acetoacetate	0.6	R2 133 $\pm$ 133.6 M1 327 $\pm$ 442.4	0.113
M1 D12 to M2 D5	0.69 and <0.001	Malonate	0.45	M1 371 $\pm$ 446.2 M2 337 $\pm$ 6921.2	0.104
M1:M2 50:50 (D14) to M2 D5	0.63 and <0.001	Propionyl carnitine	0.6	M1 32.7 $\pm$ 38.9 M2 39 $\pm$ 37.58	0.690

TABLE 3 Faecal metabolite differences between Day 12 full adaptation to HY and Day 5 of adaptation to a haylage and to the mid-point 50:50 diet at Day 14 of the changeover from one haylage to another.

Haylage and time	Q ² Y and p	Metabolites	R ²	Concs	p -value
R1 D12 to R2 D5	0.76 and <0.001	Acetate	0.55	R1 14 $\pm$ 5.5 R2 19 $\pm$ 5.0	0.006
M2 D12 to R1 D5	0.82 and <0.001	<i>m</i> -Hydroxyphenol-acetate	0.7	M2 1.2 $\pm$ 0.63 R1 0.9 $\pm$ 0.22	0.028
	0.82 and <0.001	Indole-3-acetate	0.65	M2 2 $\pm$ 1.2 R1 1 $\pm$ 0.3	0.006
R1 D12 to R1: R2 50: 50	0.57 and <0.011	Propionate	0.4	R1 18 $\pm$ 8.0 R1: R223 $\pm$ 16.4	0.01
	0.57 <0.011	Acetate	0.4	R1 14 $\pm$ 4.2 R1 R2 18 $\pm$ 10.2	0.01
M1 D12 to M2 D5	0.72 <0.001	<i>p</i> -Hydroxyphenylpyruvate	0.6	M1 2 $\pm$ 0.4 M2 1 $\pm$ 0.4	0.003
	0.72 <0.001	3-Hydroxykynurenine	0.65	M1 1 $\pm$ 0.3 M2 0.8 $\pm$ 0.28	0.001

Note: The p -values are the level of significance (in bold).

individual faecal metabolites showed significant shifts, and three out of the five differences involved R1.

Individual metabolite differences confirmed this showing that when horses moved from R1 haylage (D12) to R2 (D5), acetate concentrations increased markedly (14 ± 5.5) to 19.0 ± 5.0 $\mu\text{mol/g}$; $p = 0.006$, aligning with enhanced fibre fermentation of the more fibrous R2 forage. In contrast, during the transition from M2 (D12) to early adaptation on R1 (D5), metabolites derived from microbial aromatic amino acid (AA) degradation decreased, with both *m*-hydroxyphenyl-acetate (1.2 ± 0.63 to 0.9 ± 0.22 $\mu\text{mol/g}$; $p = 0.028$) and indole-3-acetate (2 ± 1.2 to 1 ± 0.22 $\mu\text{mol/g}$; $p = 0.006$) reduced. At the midpoint of the R1:R2 50:50 diet (Day 14), horses exhibited higher propionate (18 ± 8.0 to 23 ± 16.4 $\mu\text{mol/g}$; $p = 0.01$) and acetate (14 ± 4.2 to 18 ± 10.2 $\mu\text{mol/g}$; $p = 0.01$) relative to the fully adapted R1 state. Finally, during the M1 to M2 transition, metabolites associated with tyrosine and tryptophan catabolism declined, with *p*-hydroxyphenylpyruvate (2 ± 0.4 to 1 ± 0.4 $\mu\text{mol/g}$; $p = 0.003$) and 3-hydroxykynurenine (1 ± 0.3 to 0.8 ± 0.28 $\mu\text{mol/g}$; $p = 0.001$) both reduced.

4 | DISCUSSION

This study provides the first integrated characterisation of urinary and faecal metabonomic responses in ponies undergoing transitions between HY of differing botanical and physiological composition, revealing a rapid 5-day pattern of digestive adaptation. Metabonomic differences were noted between HY of different chemical composition with the physiologically young, low-fibre, high-WSC R1 haylage producing different metabonomic profiles compared with the other three HY. Elevated urinary glycine during the R1 $\rightarrow$ R2 transition indicated greater small-intestinal protein availability from R1, challenging assumptions that conserved forages provide uniformly low bioavailable protein. Across all forage transitions, hindgut functional profiles remained largely unchanged during the mid-transition (50:50) phase, indicating that structured 3-day changeovers did not cause microbial perturbation in this group of ponies. Faecal metabolite changes, particularly shifts in short-chain fatty acids (SCFAs) and aromatic AA derivatives, reflected rapid microbial reconfiguration aligned with fibre and carbohydrate supply, but without evidence of acidosis or dysbiosis.

4.1 | Urinary and faecal metabonomes

Analysis of the urinary and faecal metabonomic profiles revealed three main findings. Firstly, ponies had adapted to the new HY by D5, indicating a rapid stabilisation of hindgut metabolic activity, with no further major shifts with continued feeding. Secondly, the ponies maintained functional gut stability throughout dietary changeovers, with little evidence of disruption of metabolic output during transitioning between forages using this short 3-day changeover. Thirdly, metabolic responses were driven by the chemical composition of the forages rather than by haylage category per se. The two ryegrass HY

differed in both grass species (*L. multiflorum* vs. *L. perenne*) and physiological age at harvest, and although the meadow HY were conserved from adjacent, identically managed fields and harvested at different times, some botanical variability was inevitable. Consequently, the patterns observed reflect the combined influence of species and maturity as they occur in commercially relevant HY. The pattern of changes also suggests that adaptation was not caused by large shifts in individual metabolites but by small, adjustments. These small adjustments were most obvious between R1 and the other three HY and were sufficient to reflect the dietary differences between the HY but did not drop faecal pH, nor cause an increase in the metabolite lactic acid, which are common indicators of acidosis.²⁵

The ability of this group of ponies to generally transition smoothly from forages of similar chemical composition, that is, R2, M1 and R2, to the lower NDF, ADF and higher WSC containing R1 haylage, may be attributed to their microbiomes. Forages have consistently been noted to stimulate richer, more diverse more stable microbiomes, with larger core communities,^{19,26–28} than diets containing cereals, all of which promote gut resilience.^{29–31} As the microbiome profile was not determined in this study, it is not possible to confirm whether the microbial populations per se were stable. However, the ponies had been maintained on all-forage diets for 5+ years which, as documented above, confers a resilience to dietary changes such as those that occurred when transitioning between R1 and the other three HY. This agrees with the findings of Julliard²⁵ and Kristoffersen³² who noted that there was greater stability in caecal microbiota during diet changes when horses were fed hay solus, compared with those fed forage plus cereals. Although the ponies showed no large differences in metabolite output between diets, there was considerable individual variation in both metabolite profiles and the DMD of the four HY (Table S2). This inter-individual variability did not prevent clear differentiation in digestibility between diets, but it may have contributed to the absence of distinct clustering in the urinary and faecal PCA plots.

Although the metabolic differences observed between D12 and D5 during transitions between R1 and the other three HY were subtle and not accompanied by dysbiosis-associated change, such as increased lactic acid or reduced faecal pH in this group of ponies, this early adaptation window represented the period in which most metabonomic shifts occurred. For horses with heightened gut sensitivity or underlying metabolic vulnerabilities, this phase may therefore constitute a potential risk point. Rapidly fermentable forages, particularly those with high WSC content such as the R1 haylage, have previously been associated with caecal microbial imbalances (dysbiosis), a recognised risk factor for colic.^{33–36} Accordingly, vigilance is warranted during the first 5 days following a forage change, even when transitions are managed over a structured 5-day period, in horses predisposed to gastrointestinal disturbance.

4.2 | Individual urinary and faecal metabolite differences

Glycine was the only significantly elevated urinary metabolite when comparing R1 at D12 with R2 at D5 suggesting that forage

physiological age when harvested can have a major impact on the extent of AA digestion and absorption in the foregut of the horse. These results show that the R1 haylage provided more AA than was metabolically required by the horse. Glycine plays a central role in hepatic deamination processes, converting excess nitrogen to urea for excretion. When nitrogen load is high, the liver increases glycine synthesis and turnover increases, resulting in higher circulating and urinary glycine.³⁷

This study demonstrates that urinary metabonomics has advanced and refined how we can measure the site and extent of protein digestion in the horse. Transitioning away from invasive gut partition techniques³⁸ or isotope labelling and serial blood sampling^{39,40} is a more ethically responsible and cost-effective approach. Further, unpublished data from our group indicate that, *in vitro*, the R1 haylage undergoes substantial dry-matter loss during simulated foregut digestion prior to *in vitro* fermentation. Thus, coupled with the metabonomic data the results strongly suggest that protein within R1 haylage was available to the horse in the small intestine compared to the other three HY. Being able to measure protein bioavailability is highly relevant for diet formulation for horses,^{40,41} and the data from this study suggest that metabonomics may be a useful tool for doing this. Although DeBoer et al.⁴² found that plasma AA increased in horses grazing pasture, specifically Teff, alfalfa and cool season grasses, the correlation between forage AA and plasma AA was weak. This implies that factors other than AA content alone influence digestion and absorption. Traditionally conserved forages have been regarded as poor sources of bioavailable protein,⁴³ yet the present study demonstrates that a physiologically young haylage can indeed provide adequate protein for ponies on maintenance diets. The changes in SCFAs noted in the individual faecal metabonomes mostly between R1 and the other three HY indicate rapid microbial reconfiguration, perhaps even an up-regulation of microbes already present but not dominant when consuming the more mature, less fermentable HY. The effect of transitioning between HY reflected our predicted profiles in terms of acetate and propionate production and mirrored the NDF, ADF and WSC contents in the different HY. The lower aromatic AA metabolites, indole-3-acetate and hydroxyphenyl derivatives (reflecting microbial metabolism of tryptophan and tyrosine/phenylalanine), noted in R1 compared with the other HY, likely reflects the loss of the soluble protein fractions in the small intestine. Grasses in their vegetative state are less lignified, so a greater proportion of their protein is in the cell contents, where it is more accessible to enzymatic digestion in the small intestine.⁴⁴ The undigested fibre fraction passing through to the hindgut from the R1 haylage would therefore have been closer in chemical composition to the more fibrous M1, R2 and M2 HY, thus producing similar metabolic profiles.

4.3 | Practical implications

The findings from this study suggest that while 3-day transitions between HY of broadly similar composition are unlikely to disrupt hindgut metabolic output, larger differences in WSC, fibre fractions or

maturity do precipitate measurable metabolic shifts within the first 5 days post-change. Therefore, this is the time when equid health (faecal output, consistency, appetite and comfort) must be monitored carefully.

As haylage chemistry matters more than category, the value of routine nutritional analyses (even just WSC, NDF and ADF) must be reinforced. UK forage producers should be encouraged to adopt the Swedish approach, where 84% of survey respondents reported having access to forage nutritional analyses⁴⁵ which informed their ration formulation. Such information will also enable owners to anticipate the effects on gut health of potential dietary shifts when buying haylage or switching between different batches.

In the absence of formal analyses, information on forage type (meadow vs. monocultures vs. mixed species) and stage of maturity at harvest could at least provide owners with information on the degree of difference between HY. However, it should be emphasised that this information will be of limited value in terms of assessing the nutrient content.

When changing between similar HY (type, physiological maturity, harvesting technique and produced on the same farm) a 3-day transition is likely to minimise shifts in metabolic output. However, individual variation in sensitivity means that changes may have some impact.

This study did not evaluate more substantial forage changes, such as transitions from haylage to hay or between different hays, and the 3-day changeover used here should not be interpreted as a recommendation for those scenarios.

5 | LIMITATIONS

This study addressed animals that had been maintained on all forage diets for many years and were changed between HY over 3 days. Therefore, these results might not reflect responses of animals consuming forage plus complementary feeds or more abrupt changes from one haylage to another nor between different forage types. Species and maturity effects could not be fully separated, as the two ryegrass HY differed in both characteristics, and the meadow HY, although grown in adjacent, identically managed fields, naturally contained variable grass species.

6 | CONCLUSION

This study shows that ponies adapt rapidly to changes between HY, with both urinary and faecal metabonomic profiles stabilising within 5 days of exposure to a new haylage. Metabonomic changes were observed between HY of differing chemical composition, with the low fibre, high WSC R1 haylage differing from the other three higher fibre forages. No evidence of dysbiosis was detected in these forage-adapted healthy ponies, but the results highlight that the short early-adaptation 5-day window represented a period of physiological responsiveness that may cause microbial perturbation, especially in horses with gastrointestinal or metabolic vulnerabilities. The rise in

urinary glycine on R1 demonstrated that physiologically young haylage provided a greater surplus of pre-caecal AAs than the more mature HY, challenging the assumption that all conserved forages are low in bioavailable protein. Although most conserved grass forages will supply some foregut available protein, there will still be a need for routine protein supplementation in most instances, especially when the more physiologically mature forages are fed. This study has established that urinary and faecal metabolomics are robust, ethically sound tools for helping to assess the site of digestion and absorption, hindgut functional stability and protein availability from forage, producing implications for evidence-based ration formulation and management of haylage-fed horses.

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CONFLICT OF INTEREST STATEMENT

Meriel Moore-Colyer and Simon Daniels declare no conflicts of interest. Pat Harris is an employee of the study funder.

AUTHOR CONTRIBUTIONS

M. J. S. Moore-Colyer: Conceptualization; investigation; writing – original draft; methodology; formal analysis; data curation; project administration; resources. **S. Daniels:** Conceptualization; investigation; methodology; writing – review and editing; formal analysis; project administration; data curation; resources; software. **P. Harris:** Conceptualization; funding acquisition; writing – review and editing; supervision.

DATA INTEGRITY STATEMENT

Meriel Moore-Colyer and Simon Daniels have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of data analysis.

ETHICAL ANIMAL RESEARCH

The experimental protocol was approved by the Royal Agricultural University Ethics Review (20204603-Daniels).

PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1001/evj.70340>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in MetaboLights at <https://www.ebi.ac.uk/metabolights/MTBLS15865>, reference MTBLS15865.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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