The Horse Gut Microbiome Responds in a Highly Individualized Manner to Forage Lignification

Andres Gomez (gomeza@umn.edu)
University of Minnesota https://orcid.org/0000-0002-1174-0368
Ashok Kumar Sharma
University of Minnesota
Amanda Grev
University of Minnesota
Craig Sheaffer
University of Minnesota
Krishona Martinson
University of Minnesota

Research Article

Keywords: Equine, Alfalfa, Reduced lignin, fecal microbiome

Posted Date: May 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-27270/v1

Version of Record: A version of this preprint was published at Journal of Equine Veterinary Science on January 1st, 2021. See the published version at https://doi.org/10.1016/j.jevs.2020.103306.

Abstract

Background: Although contributions of the equine gut microbiome to forage utilization are well recognized, the impact of alfalfa lignification on the equine gut microbiome remains unknown. Here, we characterized microbial community dynamics in the equine distal gut when feeding reduced lignin (RL) and reference alfalfa hays (CON-control) (Medicago sativa L.) to adult stock-type horses. Hay from RL and CON cultivars were similar in crude protein, neutral detergent fiber, and equine digestible energy, but differed in acid detergent lignin content (RL:74 g kg -1 vs. CON: 81 g kg -1). Dietary treatments were fed to six horses in a crossover study. Experimental periods consisted of a 9-d dietary adaptation phase followed by a 5-d total fecal collection phase, during which horses were housed in individual box stalls and manure was removed on a continuous 24-h basis. At 12-h intervals, feces were thoroughly mixed, frozen, and used for bacterial community composition analyses via V4, 16S rRNA amplicon MiSeq sequencing.

Results: RL alfalfa did not result in specific fecal microbiome composition across all horses. However, upon incorporating individual horse in the model, it was shown that the microbiome of each subject did respond to hay lignin content in an individualized manner over time, in terms of alpha and beta diversity. Closer inspection of specific taxonomic changes upon feeding the two diets also revealed horse-specific trends, with unique amplicon sequence variants classified as Akkermansia , Fibrobacter succinogenes , Treponema, and Paludibacter fluctuating significantly in abundance when RL alfalfa was fed, depending on horse. Along these lines, horse-specific associations between individual gut microbiome traits and characteristics of the digested CON or RL alfalfa were observed, mainly in regards to dry matter digestibility and mean feed particle size.

Conclusions: These results indicate that the horse gut microbiome responds in an individualized manner to small changes in the amount of acid detergent lignin in alfalfa hay, potentially impacting several feed digestibility characteristics. The implications of horse-specific responses to forage quality in regards to metabolic health and performance remain to be elucidated.

Background

Horses (*Equus caballus* L.) are hindgut fermenters with a small stomach and larger cecum and colon harboring up to 10^8 microorganisms/g, which has allowed them to evolve as grazers on lands producing marginal forages [1]. The enzymatic machinery encoded by this microbiome is the sole contributor to the degradation and fermentation of forage cell wall components, including pectin, hemicellulose, and cellulose [2,3]. Indeed, volatile fatty acid production from bacterial fermentation in the equine distal gut can account for up to 30% of a horse's digestible energy (DE) intake [4]. However, it is thought to be much greater when considering microbe fermentation outside of the cecum, which underscores the critical importance of colonic microbes for energy production in equines (Costa et al., 2015).

Forage, mostly in the form of pasture and hay, should make up at least half of an adult horse's ration, and can usually meet the DE requirements of adult horses up to moderate exercise [5,6]. A forage commonly fed to horses is alfalfa (*Medicago sativa* L.); however, the digestibility and utilization of alfalfa by horses and other livestock can be hampered by its lignin content [7,8]. Lignin is the second most abundant component of secondary plant cell walls [9] and can reduce feeding value by negatively affecting microbial degradation and the digestion of feed by microbiome-encoded carbohydrate active enzymes [10].

Cultivars of reduced lignin (**RL**) alfalfa are now commercially available, and field research evaluating RL alfalfa under different harvest frequencies has demonstrated a reduction in total herbage acid detergent lignin (ADL) and an increase in 48-h neutral detergent fiber digestibility (NDFD48) and relative forage quality (RFQ) compared to reference (or **CON**ventional) cultivars [11]. When feeding RL alfalfa hay to lambs, researchers found that dry matter digestibility (DMD) and NDFD48 was greater for the RL hay [12]. Similarly, when RL alfalfa was included as 50% of the ration for lactating dairy cows, NDFD48 was increased and resulted in 1.3 kg more milk production per head per day [13]. Indeed, Grev et al. (2019) reported a 4% increase in apparent DMD when horses consumed RL alfalfa hay compared to CON alfalfa hay. While RL alfalfa has been shown to improve forage digestibility for sheep and cattle, it is unknown if changes in lignin will impact the equine microbiome. Therefore, the objective of this study was to evaluate the equine fecal microbiome when feeding **RL** or **CON** alfalfa hay to adult horses.

Materials And Methods

All experimental procedures were conducted according to those approved by the University of Minnesota Institutional Animal Care and Use Committee (1710-35228A).

Horses

Dietary treatments consisted in two commercially available alfalfa cultivars, including RL alfalfa ('54HVX41', Forage Genetics, Napa, ID) and CON alfalfa ('WL355.RR', W-L Alfalfa, Ozark, MO). Both alfalfa types were harvested into small square bales prior to the start of the study. The experiment was completed using a crossover design with two treatments (alfalfa cultivar) and two periods. Six adult (20 ± 4 years), stock-type horses with an average body weight (BW) of 544 kg (SE ± 36 kg) and body condition score (BCS; Henneke et al., 1983) of 5.7 (SE ± 1.0) were blocked by BW and divided into two similar herds with three horses each. Prior to the start of the study, horses were acclimated to their herd and paddock and given free-choice access to legume-grass mixed hay and water. Herds remained together for the duration of the study. Each experimental period consisted of a 9-d dietary adaptation phase (d 1 to 9) followed by a 5-d total fecal collection phase (d 10 to 14; [14]). A graphical representation of the experimental design can be seen in **Fig. 1**.

At the beginning of each adaptation phase (d 1), horses were weighed using a livestock platform scale and BCS was assessed (Henneke et al., 1983). For the duration of the adaptation phase, horse herds were

housed in dry lots with access to shelter and water. Horses received their experimental diet on an *ad libitum basis, with* hay fed twice daily at 0800 and 1900 h. With each morning feeding, horses were also given 0.9 kg of a commercially prepared ration balancer (vitamin and mineral mix) to ensure that all nutritional requirements were met for adult horses at maintenance (NRC, 2007). At the beginning of each fecal collection phase (d 10), horses were moved to individual rubber-matted boxstalls (3.6 × 3.6 m), where they were housed for the duration of the fecal collection phase (Fig. 1). Each morning of the fecal collection phase, representative forage samples were obtained by randomly sampling hay bales from each dietary treatment using a core-sampler (Penn State Forage Sampler, University Park, PA). Hay cores for each dietary treatment were combined by day and stored at -20 °C for later analysis. Hay was offered at 2% BW from hay nets (Half Bale Net, Hay Chix, Taylor Falls, MN) and was fed in two equal portions at 0800 and 1900 h. Horses had free-choice access to water throughout the fecal collection phase.

Samples

Manure was removed from the stalls on a continuous 24-h basis to allow for determination of total daily fecal output and to reduce any possible contamination with hay or urine. Feces for each horse were collected individually into large plastic containers lined with plastic bags that remained closed throughout the day to retain moisture. Cumulative feces weight was recorded every 12 h, at which time the collected feces was thoroughly mixed and subsampled in duplicate. Subsamples were placed in sealed collection bags and stored at -20 °C until DNA extraction. DNA from each fecal sample was extracted using the Qiagen power soil extraction kit, following manufacturer's instructions. Extracted DNA underwent MiSeq sequencing of the 16S rRNA V4 variable region (dual index approach; [15]) at the University of Minnesota Genomic Center (St. Paul, MN, USA).

Feed digesta analyses

Hay samples were thawed, dried in a forced-air oven at 60 °C for 48 h, and ground to pass through a 1-mm screen in a Cyclotec (Foss, Hillerod, Denmark). Dry matter was determined by dividing the weight of the sample after drying by the wet weight of the sample. Ground samples were analyzed for forage nutritive value via wet chemistry by a commercial forage testing laboratory (Equi-Analytical, Ithaca, NY) using the following methods: crude protein was calculated as the percentage of nitrogen multiplied by 6.25 [16] neutral and acid detergent fibers and acid detergent lignin were measured using filter bag techniques [17,18], and equine DE was calculated using an equation developed by Pagan [19].

Bioinformatics and data analyses of amplicon sequence data

High quality (Q > 30, using fastx_toolkit) paired-end reads were considered for downstream analysis using Dada2, mafft, and fasttree plugins of Qiime2 to obtain unique amplicon sequence variants (ASVs), align

representative sequences, and generate rooted and unrooted phylogenetic trees [20–23]. Greengenes reference sequences (clustered at 99% sequence identity) were downloaded and trained using naïve Bayes classifier, and this trained classifier was further used for taxonomic assignment of representative ASVs using the feature-classifier classify-sklearn plugin within Qime2 [24]. All downstream microbial community ecology analyses were performed in the R statistical interface [25]. Relative abundances of each ASV and phylogenetic tree (rooted) were used for calculating Bray-Curtis and UniFrac distance matrices, principal coordinate and alpha diversity analysis using the vegan, ape, and phyloseq packages in R [26–28]. Permutational Multivariate Analysis of Variance (PERMANOVA) was calculated using the Adonis function of the vegan package. Selection of significantly discriminating taxonomic markers was carried out using indicator species analysis on the labdsv package, Wilcoxon rank sum tests and fold changes of each discriminating ASV between CON or RL alfalfa for a given horse [29]. Generalized linear models were constructed using the *glm* function in R to mine for associations between different feed digestibility and microbiome traits[30]. All graphs were plotted using boxplot, ggplots, and EnhancedVolcano functions within the R [31,32].

Results

Nutrient composition of RL and CON alfalfa hays

With the exception of ADL, nutrient content was similar between alfalfa cultivars, and concentrations for all nutrients fell within the normal range for alfalfa hay (**Table 1**; [11,33–35]). The nutrient profiles for both alfalfa hays in the present study were sufficient to meet the daily DE and CP requirements for adult horses at maintenance (NRC, 2007). In the current study, the RL alfalfa hay demonstrated a 9% reduction in ADL compared to CON alfalfa hay. Previous research comparing RL and CON alfalfa hays has also reported a reduction in ADL with little to no change in other nutrient components (Mertens and McCaslin, 2008). Similarly, RL alfalfa cultivars have shown a 6 to 24% decrease in total herbage ADL compared to CON alfalfa cultivars [11,36].

Table 1
Nutritional composition of RL and CON diets. DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; ADL, acid detergent lignin; DE, equine digestible energy of reduced lignin-RL ('54HVX41') and reference-CON ('WL355.RR') alfalfa hay fed to adult horses at maintenance.

Nutrient	Reduced Lignin	Reference	SE
	Nutrient Composition		
DM, g kg^{-1}	892	892	1.68
CP, g kg^{-1}	196	202	3.53
ADF, g kg^{-1}	343	348	11.70
NDF, g kg^{-1}	430	435	8.06
ADL, g kg^{-1}	74[b]	81[a]	4.73
Equine DE, Mcal kg^{-1}	2.4	2.4	0.02
[ab]Within row, means without a common letter differ based on a Tukey's HSD test (P ≤ 0.05)			

Shifting between RL or CON alfalfa triggered unique microbiome changes in each individual horse

After bioinformatic processing for amplicon quality control and taxonomic assignment of unique sequence variants, an average of 21,836 ± 6,052 reads per fecal sample were obtained. Feeding RL alfalfa resulted in only minor effects on fecal microbiome composition across all horses (Bray-Curtis distance PERMANOVA, R2 = 0.01, $P = 0.002$; **Fig. 2a**). However, upon closer inspection, the data showed that each horse's microbiome was unique, and that each horse's microbiome responded to dietary lignin content in an individualized manner (horse effect: R2 = 0.11, $P < 0.001$; sampling date effect: R2 = 0.10, $P < 0.001$; interaction horse*diet: R2 = 0.04, $P < 0.001$; interaction diet*sampling time: R2 = 0.07, $P = 0.02$; interaction horse*sampling time: R2 = 0.3, $P < 0.001$; Fig. 2**b**). That is, feeding CON vs. RL alfalfa significantly shifted each horse's fecal microbiome composition in a unique fashion over time (Fig. 2**c-h**).

For instance, the fecal microbiome of horse *An*, who started the feeding trial on CON alfalfa, drastically changed when fed the RL alfalfa (treatment effect: R2 = 0.09, $P < 0.001$; sampling date effect: R2 = 0.53, P = 0.009; **Fig. 2c**). Within each horse, this microbiome composition shift showed unique dynamic trends across the 5 sampling days, indicating that the distal microbiome of the sampled horses not only changed according to forage lignin content but also across each day and even within a day depending on time of sample collection (morning or afternoon). For instance, horse microbiome variation within sampling days was also unique to each horse, with some individuals showing greater variation between AM and PM profiles in the same day (e.g. *Be* and *Zi*, **Fig. 3a-f**).

Similar trends were observed when evaluating microbiome diversity and richness. For example, feeding CON or RL alfalfa did not have an effect on mean gut microbiome diversity (Shannon index) when horses were grouped, but diversity seemed to be unique to specific horses (ANOVA, treatment effect: F = 0.5, *P* = 0.5; horse effect: F = 2.7, *P* = 0.03; date affect: F = 2.3, *P* = 0.02; interaction horse*sampling time: F = 1.9, *P* = 0.01; **Fig. 4a)**. The number of observed amplicon sequence variants (ASV richness) also followed that trend (Fig. 4**b**). Interestingly, however, patterns of alpha diversity across each horse were not consistent; that is, diversity increased upon feeding RL alfalfa in only one horse (*An*), while in others, the effect was opposite and/or not supported by the data (Figs. 4 **a and b**).

To further investigate factors that explaining the individual patterns observed, we applied an indicator species analysis and identified bacterial taxa that increased or diminished in abundance when feeding CON or RL alfalfa. As expected, the taxa that dominated the gut microbiome when feeding each alfalfa hay were unique in each individual horse (**Additional file 1: Table S1-S6**). For instance, in horse *An*, feeding RL alfalfa mainly resulted in significant increases in the abundance of several ASVs affiliated to *Akkermansia*, while feeding CON alfalfa was associated with increased abundance of unknown ASVs from the Bacteroidales order (Indicator value > 0.7, Wilcoxon test's *P* < 0.001, **Additional file 1: Table S1**). In contrast, these specific taxa did not fluctuate in the same way in other horses, which showed different abundance dynamics of other indicator taxa (e.g. horse *Ba*, **Fig. 5a and b, Table S2-S6)**. Furthermore, feeding CON or RL alfalfa also resulted in changes at very specific taxonomic levels within the same broad taxonomic group. For instance, other ASVs affiliated to the genus *Akkermansia* were also observed in higher abundance in horse *An* when feeding CON alfalfa, and other Bacteroidales were abundant when feeding RL alfalfa, but with a lower magnitude change (**Fig. 5b**).

This phenomenon was seen with many of the indicator taxa identified as enriched in either the CON or RL diet, indicating that the microbiome changes observed upon feeding the two diets operate beyond the genus level in the horse gut microbiome (e.g. different species and strains within the same genera were observed to fluctuate; **Fig. 5 c-h**). To test this hypothesis, we looked for compositional changes when feeding CON or RL alfalfa at the genus level in one of the horses (*An*). Although differences were still detectable and significant, the magnitude of the change was smaller and microbiome distinctions between the two diets were less clear compared with the ASV (species/strain) level analysis (treatment effect: R2 = 0.08, *P* = 0.04; sampling date effect: R2 = 0.63, *P* = 0.02; **Additional file 2: Figure S1a, Additional file 1: Table S7)**. Of note, the main genera discriminating between the RL and CON diets in horse *An* were *Lachnospira* and genera affiliated to the Paraprevorellaceae family, respectively (Indicator value = 0.7, Wilcoxon test's *P* < 0.01, **Additional file 2: Figure S1b)**, which did not come out as markers of the CON or RL diet at the strain level analyses. Furthermore, we used a second distance matrix that considers phylogenetic distances between taxa (weighted UniFrac on ASV relative abundances), and also observed less distinction in the microbiomes of CON- and RL-fed horses, with no significant differences in half of the cases (**Additional file 3: Figure S2 c-h**). However, individual horse was still the main driver of the microbiome patterns observed (**Additional file 3: Figure S2a**).

Despite the strain level and horse specific heterogeneity detected, most of the taxa that fluctuated in response to the CON or RL alfalfa have been typically associated with fibrolytic or glycolytic functions in mammals that primarily depend on forage for subsistence. For example, *Akkermansia* ASVs, which mostly increased in horse An under the RL alfalfa diet, were close in identity to *A. glycaniphila* (Basic Local Alignment Search Tool-BLAST: % identity = 93.5%, coverage 97%, E-value = 5e-109). Furthermore, the *Akkermansia* sequences identified are closer to 16S rRNA clones isolated from the feces of black rhinos (BLAST: % identity = 99.6%, coverage 97%, E-value = 8e-132). Other ASVs fluctuating the most in the gut microbiome of each horse (indicator value > 0.7) under RL alfalfa diets were affiliated to unclassified *Paludibacter*, Ruminococcaceae, Bacteroidales, *Treponema*, and *Fibrobacter succinogenes*. Different taxa that increased under the CON alfalfa diet included *Solibacillus*, *Acinetobacter lwoffii*, and *Methanocorpusculum* (Fig. 5**c-h**). However, these patterns were not uniform across all horses.

Forage digestibility traits are associated with individual microbiome profiles

Next, we sought to investigate if the microbiome patterns seen in each horse were associated with data on forage digestibility for the CON and RL alfalfa hay diets. Specific digesta characteristics did co-vary with particular microbiome traits, but with covariation patterns unique to each individual horse. For example, in horse *An*, apparent dry matter digestibility (DMD) was significantly associated with principal coordinate scores along axis 1 (PCo.1), increasing under the RL alfalfa diet (generalized linear model-GLM: t=2.5, P= 0.02, **Figure 6a**). We also observed that mean FPS in horse *An* and *Fa* positively correlated with scores along PCo.1 (GLM: t=3.78-4.38, P< 0.01, **Figure 6b,e)** and abundances of *Akkermansia and Treponema* ASVs (GLM: t=2.89-5.6, P< 0.01, **Figure 6c,f),** both indicator taxa in these two horses. No associations between apparent DMD and microbial traits were observed in horse *Fa* (GLM: t=1, P= 0.3, **Figure 6d)**; In other horses, some of these trends were also observed, although in an opposite trend. For instance particularly in horses *Da* and *Ba* lower mean feed particle size (FPS) was observed under the RL treatment, and in association with PCo.1 scores. The same horses also showed lower FPS associated with the RL hay, along with increased abundances of *Paludibacter* (GLM, P<0.05, **Figure 6g-l**). No associations were found between alpha diversity (Shannon's H or ASV richness) metrics and individual feed digestibility traits.

Discussion

Herein, we report changes in the equine distal gut microbiome upon feeding alfalfa hay with a reduced lignin concentration (9% reduction). The compositional changes observed were not uniform across all horses, but were significant and unique to each individual, involved different groups of bacteria at very specific taxonomic levels (species or strains), and showed different dynamism over time. The data also showed horse-specific associations between microbiome composition and specific digestibility traits, including apparent DMD and mean FPS.

Individualized responses to alfalfa lignification

One of the most intriguing findings reported here is the high individualization of the equine distal gut microbiome upon the CON or RL feeding intervention. This level of individualization in response to a feeding intervention or baseline diets over time is a common characteristic of the human gut microbiome [37]. For example, evidence has shown that the introduction of diverse dietary polysaccharides that aim at stimulating gut microbial communities (prebiotic fibers) triggers unique compositional changes in specific individuals [38,39]. Microbiome patterns at a very specific taxonomic level (e.g. strains), as reported here, have also been shown in response to single antibiotics or antibiotic cocktails [40,41]. Likewise, interventions with specific probiotic strains in humans are reported to yield highly individualized engraftment responses, likely pre-determined by individual microbial repertoires previous to intervention [42,43].

Thus, this level of individualization in the equine microbiome may be expected, except for the fact that such individuality to a feeding intervention is not commonly reported in domestic or wild animals. In other domesticated species, dogs have shown individual responses to antibiotics [44,45], and there have been reports of individualized responses of bovines to rumen transfaunation, with rumen microbial communities returning to pre-transfaunation states in an individual manner [46]. Detection of intra-individual variation is facilitated by profiling serial samples from the same individual, which has previously revealed horse-specific microbiome profiles at the stomach mucosal level in adults [47] and individual-specific fecal microbiome composition in ponies [48]. However, individualized microbiome responses to a particular intervention in equines have only been reported for antibiotics [49]. To the best of our knowledge, this is the first report that emphasizes individual equine microbiome profiles in response to a forage feeding intervention, which is remarkable considering that this change consisted of a small reduction (9%) in the concentration of lignin in alfalfa hay. Moreover, the responses to each hay type were also highly individualized day to day and even within a single sampling day (Fig. 3).

Along these lines, the observation that taxonomic changes associated with hay lignification were associated specific ASVs within the same core taxa (*Akkermansia*, *Treponema, Paludibacter, Bacteroidales, Ruminococcaceae*, and *Fibrobacter*, among others) is remarkable. This observation not only underscores the specificity and heterogeneity of fiber metabolism in the gut microbiome of herbivores [50], but also may indicate that efficiency of hay degradation may differ across individual horses. It is possible that such heterogeneity may be linked to individual genetic backgrounds. For example, host genetic control on the mammalian microbiome should operate on deeper branches of the bacterial phylogenetic tree across mammals (species and strains), as opposed to on broader taxonomic groups [51], with bacterial communities further modulated by genetic diversity and individual life history (e.g. physiological changes associated with individual growth, development, and health status) [52]. Indeed, the modern American Quarter Horse exhibits high genetic diversity, which is the result of increased gene flow during domestication and wide geographical spread due to its historical use in transportation [53,54]. Thus, just as in humans, diverse genetic variants in the American Quarter Horse may be linked to significant variation of gut microbiome profiles between individuals at very specific taxonomic groups

[55,56]. This linked variation between host genetics and gut microbiome may determine subject-specific immune and metabolic responses to diets or disease challenges [57].

Individual microbiome profiles and feed efficency

However, from the data available, it is not possible to determine what these horse-specific microbiome responses to minor differences in alfalfa lignification mean, from a functional or physiological perspective. All ASVs that fluctuated in response to hay lignification are affiliated to core taxa in the equine gut and have been associated with hay consumption [58–60]. These taxa were differently associated with markers of RL or CON alfalfa for each of the six horses (**Figure 5**), with different ASVs from the same genera or species (e.g. *Akkermansia, Treponema, Paludibacter*) arising in response to RL alfalfa consumption in some horses and blooming when consuming CON alfalfa in others. Thus, it could be assumed that these unique taxonomic responses are overridden by functional redundancy, as previously shown for the gut microbiome of heterogeneous human populations [55].

Nonetheless, discriminant microbiome profiles were uniquely associated with apparent DMD and mean FPS for both types of hay. This observation may indicate that not all horses were equally efficient at metabolizing each hay type, and that their gut microbiome may be an important determinant of this individuality. For instance, less lignified hay would allow for increased substrate availability for degrading/fermenting microbes [12], resulting in blooms of specialized fibrolytic or fermentative bacteria such as *F. succiniogenes* and *Akkermansia,* as seen in a some individuals. But while in some horses microbiome changes associated with RL alfalfa were associated with higher DMD, in others, unique microbiome profiles were correlated with lower DMD values (**Figure 5**). The same phenomenon was seen with mean FPS, whose lower values are associated with increased digestible energy [61]. Thus, the data show that the effect of feeding less lignified hay with the intent of improving the efficiency of forage utilization in horses might have to be considered on an individual basis.

Thus, these results also open a debate on individualized nutritional interventions in equines that can target the gut microbiome to improve performance and health. In humans, the individualized nutrition concept considers that each individual digests and metabolizes identical foods in a different way, depending on genomic and microbiome traits unique to each subject [37,62]. The individualized nutrition framework in humans considers information about an individual's lifestyle, dietary habits, genetics, functional genomic landscape, metabolome, and microbiome as critical data to understand mechanisms and treatments behind a myriad of diseases, with promising results for ameliorating symptoms associated with metabolic disease [63,64]. Given the high rates of metabolic disorders in domestic animals, including companion, work, and endurance horses [65–67], and the economic losses linked to these health issues, an individualized nutrition approach for metabolic and other diseases that considers individualized microbiome profiles and specific metabolic responses to particular diets may prove to be promising.

Conclusion

In summary, we report highly individualized gut microbiome responses in horses to feeding alfalfa hay with reduced lignin concentrations, and show that horse-specific patterns are also associated with feed digestibility traits. The findings underscore the importance of considering individual and temporal factors when designing or evaluating feeding interventions in equines and other domestic animals, and highlight the value of fecal microbiome profiling as a relatively inexpensive way to account for such individuality. Future research should focus on uncovering what these individualized microbiome patterns represent from a functional perspective and on elucidating individual physiological responses of horses to feed beyond assessing feed digestibility traits. Examples include assessing the functional potential of the gut microbiome of each individual through shotgun metagenomics sequencing, profiling microbiome contributions to systemic metabolism using non-targeted and targeted metabolomic approaches, and evaluating the gene regulatory landscape at the gastrointestinal level in each individual when considering specific feeding interventions.

Abbreviations

RL: Reduced lignin

CON: Reference

NDFD48: 48-h neutral detergent fiber digestibility

RFQ: Relative forage quality

DMD: Dry matter digestibility

BW: Body weight

BCS: Body condition score

DM: Dry matter

CP: Crude protein

DE: Digestible energy

ADF: Acid detergent fiber

NDF: Neutral detergent fiber

ADL: Acid detergent lignin

ASVs: Amplicon sequence variants

PERMANOVA: Permutational Multivariate Analysis of Variance

Declarations

Ethics approval and consent to participate

The experimental procedure of the present study was reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee (1710-35228A).

Consent for publication

Not Applicable

Availability of data and material

Sequencing data will be deposited at NCBI SRA.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by funds from the USDA-NIFA Agricultural Experimental station (MIN-16-122) conferred to AGo and KM, and startup funds conferred to AG by the Agricultural Research, Education, Extension and Technology Transfer (AGREET) initiative, University of Minnesota.

Author’s contributions:

KM, AGr and CS made substantial contributions to the conception and design of the study. KM, and AGr performed the experiment. AGo and AKS performed the bioinformatics and statistical analyses on microbiome data. AGo, KM, and AKS drafted the manuscript and prepared all tables and figures. AGo, AKS, AGr, CS, and KM revised the manuscript critically for important intellectual content.

Acknowledgements

AG thanks Laura Mayer for her assistance with DNA extractions and Dr. Milena Saqui-Salces for providing lab space and equipment access. The Minnesota Supercomputing Institute (MSI) at The

University of Minnesota provided high performance computational resources for all data analyses carried out in this research.

References

1. Hume ID. Fermentation in the Hindgut of Mammals. In: Mackie RI, White BA, editors. Gastrointestinal Microbiology: Volume 1 Gastrointestinal Ecosystems and Fermentations. Boston, MA: Springer US; 1997. p. 84–115.
2. Biddle A, Stewart L, Blanchard J, Leschine S. Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. Diversity . Multidisciplinary Digital Publishing Institute; 2013;5:627–40.
3. Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol. 2008;6:121–31.
4. Glinsky MJ, Smith RM, Spires HR, Davis CL. Measurement of volatile fatty acid production rates in the cecum of the pony. J Anim Sci. 1976;42:1465–70.
5. Council NR, Others. Nutrient requirements of horses 6th ed National Academy Press. Washington, DC, USA. 2007;
6. Becvarova I, Scott Pleasant R, Thatcher CD. Clinical Assessment of Nutritional Status and Feeding Programs in Horses [Internet]. Veterinary Clinics of North America: Equine Practice. 2009. p. 1–21. Available from: http://dx.doi.org/10.1016/j.cveq.2009.01.001
7. Sewalt VJH, Ni W, Jung HG, Dixon RA. Lignin Impact on Fiber Degradation: Increased Enzymatic Digestibility of Genetically Engineered Tobacco (Nicotiana tabacum) Stems Reduced in Lignin Content. J Agric Food Chem. American Chemical Society; 1997;45:1977–83.
8. Casler MD, Buxton DR, Vogel KP. Genetic modification of lignin concentration affects fitness of perennial herbaceous plants. Theor Appl Genet. 2002;104:127–31.
9. Li X, Zhang Y, Hannoufa A, Yu P. Transformation with TT8 and HB12 RNAi constructs in model forage (Medicago sativa, alfalfa) affects carbohydrate structure and metabolic characteristics in ruminant livestock systems. J Agric Food Chem. ACS Publications; 2015;63:9590–600.
10. Liu N, Yu P. Molecular clustering, interrelationships and carbohydrate conformation in hull and seeds among barley cultivars. J Cereal Sci. 2011;53:379–83.
11. Grev AM, Scott Wells M, Samac DA, Martinson KL, Sheaffer CC. Forage Accumulation and Nutritive Value of Reduced Lignin and Reference Alfalfa Cultivars [Internet]. Agronomy Journal. 2017. p. 2749–61. Available from: http://dx.doi.org/10.2134/agronj2017.04.0237
12. Mertens DR, McCaslin M. Evaluation of alfalfa hays with down-regulated lignin biosynthesis. J Dairy Sci. 2008;91.
13. Weakley D, Mertens DR, McCaslin M. Lactating cow responses to alfalfa hays with down-regulated lignin biosynthesis. J Dairy Sci. 2008;91.
14. Staniar WB, Bussard JR, Repard NM, Hall MH, Burk AO. Voluntary intake and digestibility of teff hay fed to horses1 [Internet]. Journal of Animal Science. 2010. p. 3296–303. Available from: http://dx.doi.org/10.2527/jas.2009-2668

15. Gohl DM, Vangay P, Garbe J, MacLean A, Hauge A, Becker A, et al. Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. Nat Biotechnol. 2016;34:942–9.
16. AOAC International, Latimer GW. Official Methods of Analysis of AOAC International. AOAC International; 2012.
17. Ankom Technology. Method_5_ADF_A200.pdf [Internet]. 2017. Available from: https://www.ankom.com/sites/default/files/document-files/Method_5_ADF_A200.pdf
18. Ankom Technology. Method_6_NDF_A200.pdf [Internet]. 2017. Available from: https://www.ankom.com/sites/default/files/document-files/Method_6_NDF_A200.pdf
19. Pagan JD. Measuring the digestible energy content of horse feeds. Advances in Equine Nutrition Nottingham University Press, Nottingham. 1998;71–6.
20. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. nature.com; 2016;13:581–3.
21. Katoh K, Kuma K-I, Toh H, Miyata T. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. academic.oup.com; 2005;33:511–8.
22. Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol. academic.oup.com; 2009;26:1641–50.
23. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Author Correction: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. peerj.com; 2019;37:1091.
24. 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.
25. Core Team R, Others. R: A language and environment for statistical computing. R Foundation for statistical computing, Vienna. 2013;
26. Oksanen J, Kindt R, Legendre P, O'Hara B, Stevens MHH, Oksanen MJ, et al. The vegan package. Community ecology package. researchgate.net; 2007;10:631–7.
27. Paradis E, Blomberg S, Bolker B, Brown J, Claude J, Cuong HS, et al. Package "ape." Analyses of phylogenetics and evolution, version [Internet]. cran.stat.unipd.it; 2019;2. Available from: https://cran.stat.unipd.it/web/packages/ape/ape.pdf
28. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8:e61217.
29. Roberts DW, Roberts MDW. Package "labdsv." Ordination and Multivariate [Internet]. r-project.org; 2016; Available from: ftp://www.r-project.org/pub/R/web/packages/labdsv/labdsv.pdf
30. Zeileis A, Kleiber C, Jackman S. Regression Models for Count Data in R. J Stat Softw. Foundation for Open Access Statistics; 2008;27:1–25.
31. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer; 2016.
32. Blighe K. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version 1.2. 0. 2019.
33. Potts L, Hinkson J, Graham B, Löest C, Turner J. Nitrogen Retention and Nutrient Digestibility in

Geldings Fed Grass Hay, Alfalfa Hay, or Alfalfa Cubes [Internet]. Journal of Equine Veterinary Science. 2010. p. 330–3. Available from: http://dx.doi.org/10.1016/j.jevs.2010.04.007
34. Earing JE, Cassill BD, Hayes SH, Vanzant ES, Lawrence LM. Comparison of in vitro digestibility estimates using the DaisyII incubator with in vivo digestibility estimates in horses1 [Internet]. Journal of Animal Science. 2010. p. 3954–63. Available from: http://dx.doi.org/10.2527/jas.2010-2989
35. Woodward AD, Nielsen BD, Liesman J, Lavin T, Trottier NL. Protein quality and utilization of timothy, oat-supplemented timothy, and alfalfa at differing harvest maturities in exercised Arabian horses. J Anim Sci. 2011;89:4081–92.
36. Getachew G, Ibáñez AM, Pittroff W, Dandekar AM, McCaslin M, Goyal S, et al. A comparative study between lignin down regulated alfalfa lines and their respective unmodified controls on the nutritional characteristics of hay. Anim Feed Sci Technol. 2011;170:192–200.
37. Johnson AJ, Vangay P, Al-Ghalith GA, Hillmann BM, Ward TL, Shields-Cutler RR, et al. Daily Sampling Reveals Personalized Diet-Microbiome Associations in Humans. Cell Host Microbe. 2019;25:789–802.e5.
38. Carlson JL, Erickson JM, Hess JM, Gould TJ, Slavin JL. Prebiotic Dietary Fiber and Gut Health: Comparing the in Vitro Fermentations of Beta-Glucan, Inulin and Xylooligosaccharide. Nutrients [Internet]. 2017;9. Available from: http://dx.doi.org/10.3390/nu9121361
39. Smits SA, Marcobal A, Higginbottom S, Sonnenburg JL, Kashyap PC. Individualized Responses of Gut Microbiota to Dietary Intervention Modeled in Humanized Mice. mSystems [Internet]. 2016;1. Available from: http://dx.doi.org/10.1128/mSystems.00098-16
40. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4554–61.
41. Koo H, Hakim JA, Crossman DK, Kumar R, Lefkowitz EJ, Morrow CD. Individualized recovery of gut microbial strains post antibiotics. NPJ Biofilms Microbiomes. 2019;5:30.
42. Maldonado-Gómez MX, Martínez I, Bottacini F, O'Callaghan A, Ventura M, van Sinderen D, et al. Stable Engraftment of Bifidobacterium longum AH1206 in the Human Gut Depends on Individualized Features of the Resident Microbiome. Cell Host Microbe. 2016;20:515–26.
43. Berry D. Making It Stick: A Compelling Case for Precision Microbiome Reconstitution. Cell Host Microbe. 2016. p. 415–7.
44. Igarashi H, Maeda S, Ohno K, Horigome A, Odamaki T, Tsujimoto H. Effect of oral administration of metronidazole or prednisolone on fecal microbiota in dogs. PLoS One. 2014;9:e107909.
45. Suchodolski JS, Dowd SE, Westermarck E, Steiner JM, Wolcott RD, Spillmann T, et al. The effect of the macrolide antibiotic tylosin on microbial diversity in the canine small intestine as demonstrated by massive parallel 16S rRNA gene sequencing [Internet]. BMC Microbiology. 2009. p. 210. Available from: http://dx.doi.org/10.1186/1471-2180-9-210
46. Zhou M, Peng Y-J, Chen Y, Klinger CM, Oba M, Liu J-X, et al. Assessment of microbiome changes after rumen transfaunation: implications on improving feed efficiency in beef cattle. Microbiome. 2018;6:62.
47. Perkins GA, den Bakker HC, Burton AJ, Erb HN, McDonough SP, McDonough PL, et al. Equine stomachs harbor an abundant and diverse mucosal microbiota. Appl Environ Microbiol. 2012;78:2522–32.
48. Blackmore TM, Dugdale A, Argo CM, Curtis G, Pinloche E, Harris PA, et al. Strong stability and host

specific bacterial community in faeces of ponies. PLoS One. 2013;8:e75079.
49. Costa MC, Stämpfli HR, Arroyo LG, Allen-Vercoe E, Gomes RG, Weese JS. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. BMC Vet Res. 2015;11:19.
50. Iakiviak M, Devendran S, Skorupski A, Moon YH, Mackie RI, Cann I. Functional and modular analyses of diverse endoglucanases from Ruminococcus albus 8, a specialist plant cell wall degrading bacterium. Sci Rep. 2016;6:29979.
51. Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol. 2008;6:776–88.
52. Phillips CD, Phelan G, Dowd SE, McDonough MM, Ferguson AW, Delton Hanson J, et al. Microbiome analysis among bats describes influences of host phylogeny, life history, physiology and geography. Mol Ecol. 2012;21:2617–27.
53. Petersen JL, Mickelson JR, Cothran EG, Andersson LS, Axelsson J, Bailey E, et al. Genetic diversity in the modern horse illustrated from genome-wide SNP data. PLoS One. 2013;8:e54997.
54. Petersen JL, Mickelson JR, Cleary KD, McCue ME. The American Quarter Horse: population structure and relationship to the thoroughbred. J Hered. 2014;105:148–62.
55. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486:207–14.
56. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell. 2014;159:789–99.
57. Hall AB, Tolonen AC, Xavier RJ. Human genetic variation and the gut microbiome in disease. Nat Rev Genet. 2017;18:690–9.
58. Costa MC, Silva G, Ramos RV, Staempfli HR, Arroyo LG, Kim P, et al. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet J. 2015;205:74–80.
59. 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;8:8510.
60. Daly K, Proudman CJ, Duncan SH, Flint HJ, Dyer J, Shirazi-Beechey SP. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. Br J Nutr. 2012;107:989–95.
61. Fan Y, Guo P, Yang Y, Xia T, Liu L, Ma Y. Effects of particle size and adaptation duration on the digestible and metabolizable energy contents and digestibility of various chemical constituents in wheat for finishing pigs determined by the direct or indirect method [Internet]. Asian-Australasian Journal of Animal Sciences. 2016. p. 554–61. Available from: http://dx.doi.org/10.5713/ajas.16.0324
62. Adalsteinsdottir SA, Magnusdottir OK, Halldorsson TI, Birgisdottir BE. Towards an Individualized Nutrition Treatment: Role of the Gastrointestinal Microbiome in the Interplay Between Diet and Obesity. Curr Obes Rep. 2018;7:289–93.
63. de Toro-Martín J, Arsenault BJ, Després J-P, Vohl M-C. Precision Nutrition: A Review of Personalized Nutritional Approaches for the Prevention and Management of Metabolic Syndrome. Nutrients [Internet]. 2017;9. Available from: http://dx.doi.org/10.3390/nu9080913
64. Dorner B, Friedrich EK. Position of the Academy of Nutrition and Dietetics: Individualized Nutrition

Approaches for Older Adults: Long-Term Care, Post-Acute Care, and Other Settings. J Acad Nutr Diet. 2018;118:724–35.
65. Adamu L, Rasedee AFN b., Mohd Adzahan N, Rasedee A, Ahmad B. The Use of a Metabolic Disorder Index as a Predictor for Metabolic Eliminations in Endurance Horses. J Equine Vet Sci. 2017;51:113–21.
66. Zak A, Siwinska N, Elzinga S, Barker VD, Stefaniak T, Schanbacher BJ, et al. Effects of equine metabolic syndrome on inflammation and acute-phase markers in horses. Domest Anim Endocrinol. 2020;72:106448.
67. Tumpa A, Barić Rafaj R. Metabolic disorders and inflammation in obese dogs, cats, horses and cattle. Veterinarska stanica. Hrvatski veterinarski institut; 2019;50:481–94.

Figures

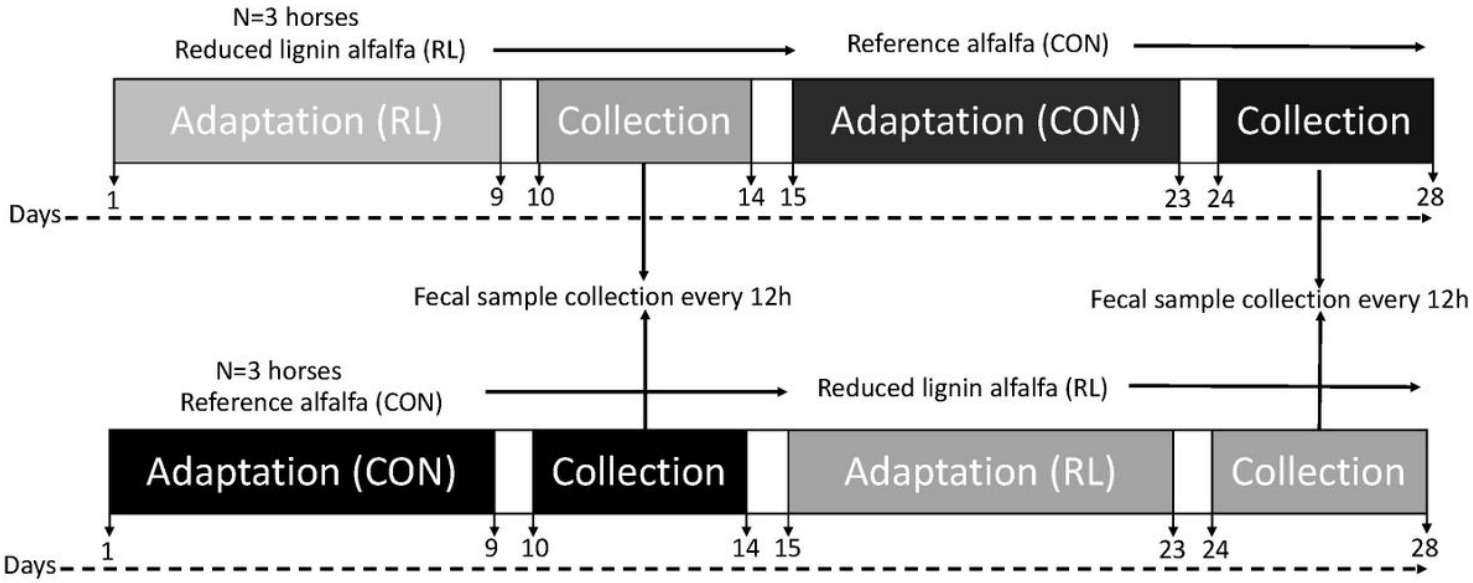

Figure 1

Schematic sampling procedure describing the RL (reduced lignin) and CON (reference) alfalfa feeding trials, for 6 horses along 28 experimental days.

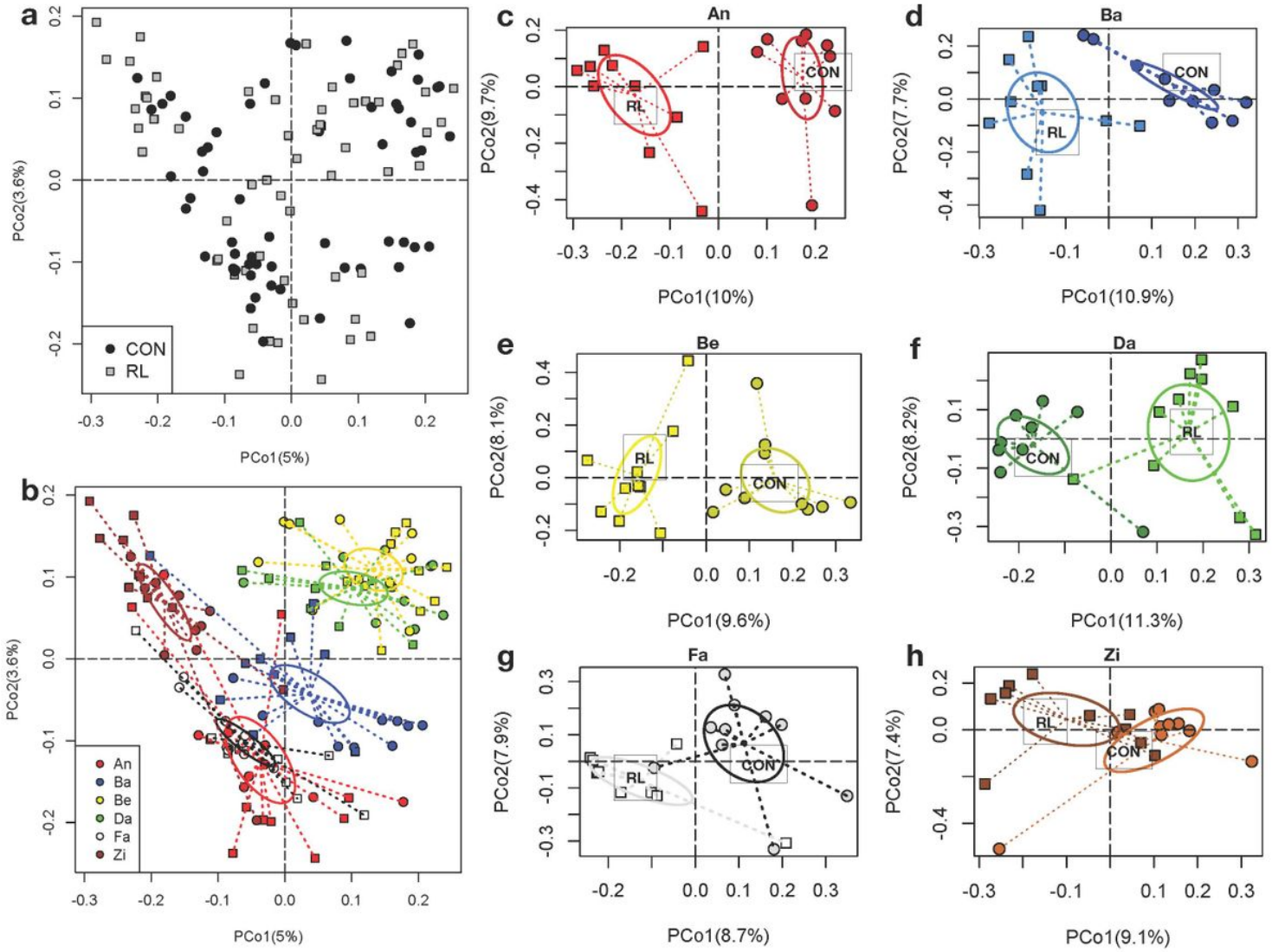

Figure 2

Bacterial community composition shifts when feeding RL or CON alfalfa, depending on individual horse. (a) PCoA based on Bray-Curtis distance matrices show no apparent fecal microbiome differences when feeding RL or CON. However, clustering of samples seems to be driven mainly by individual horses: An, Ba, Be, Da, Fa and Zi (b). Thus, an analysis of microbiome changes when feeding RL or CON alfalfa was conducted within each horse, revealing that lignification of forage significantly shits the equine microbiome in an individual manner (c-h)

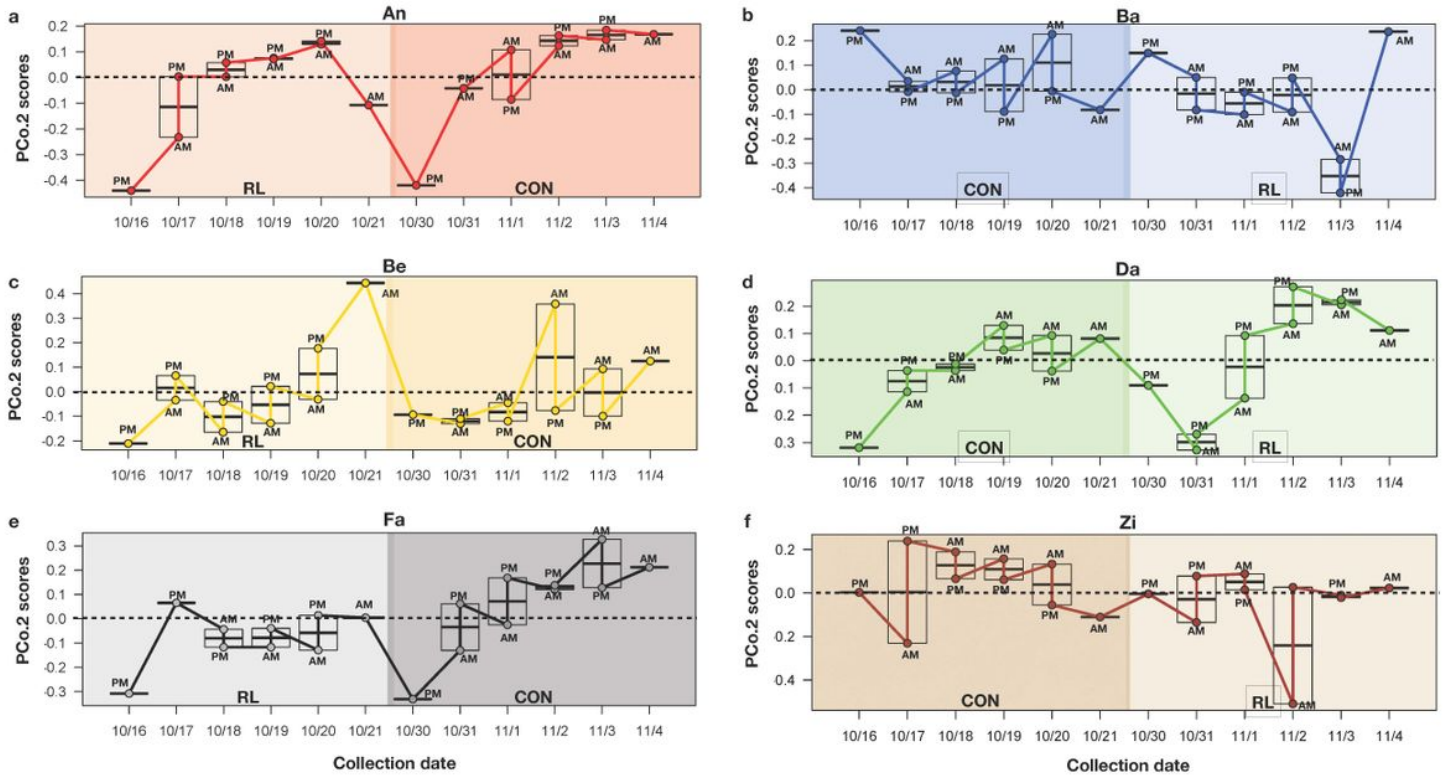

Figure 3

The fecal microbiome of individual horses changes in a unique manner over time, when fed RL or CN alfalfa. Panels a-f show the dynamics of gut microbiome composition (ordination scores along PCo.2 as seen in figure 2 c-h) in the context of sampling date and time in each horse sampled. The lines connect samples taken at each day in the morning (AM) or afternoon (PM)

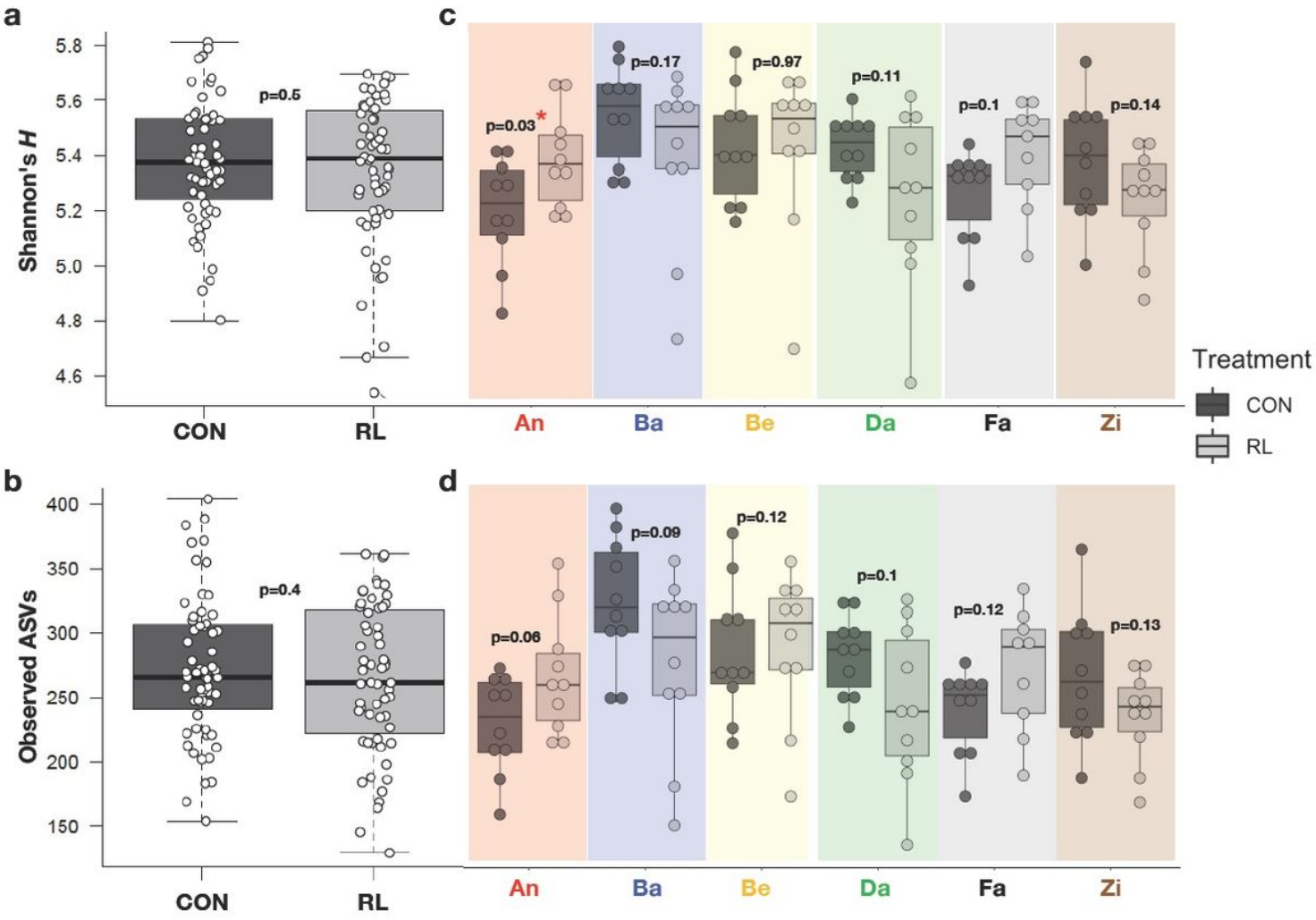

Figure 4

Bacterial (alpha) diversity does not change significantly when RL or CON alfalfa are fed to the horses sampled. (a) Shannon's H metrics of bacterial alpha diversity in all 6 horses shows no significant differences. However, the same metrics show either increasing or decreasing diversity associated with RL on each individual horse (An, Ba, Be, Da, Fa, Zi), with significant differences only detected in horse An (b). Panels b-c show the same trend, based on the observed ASV richness metrics.

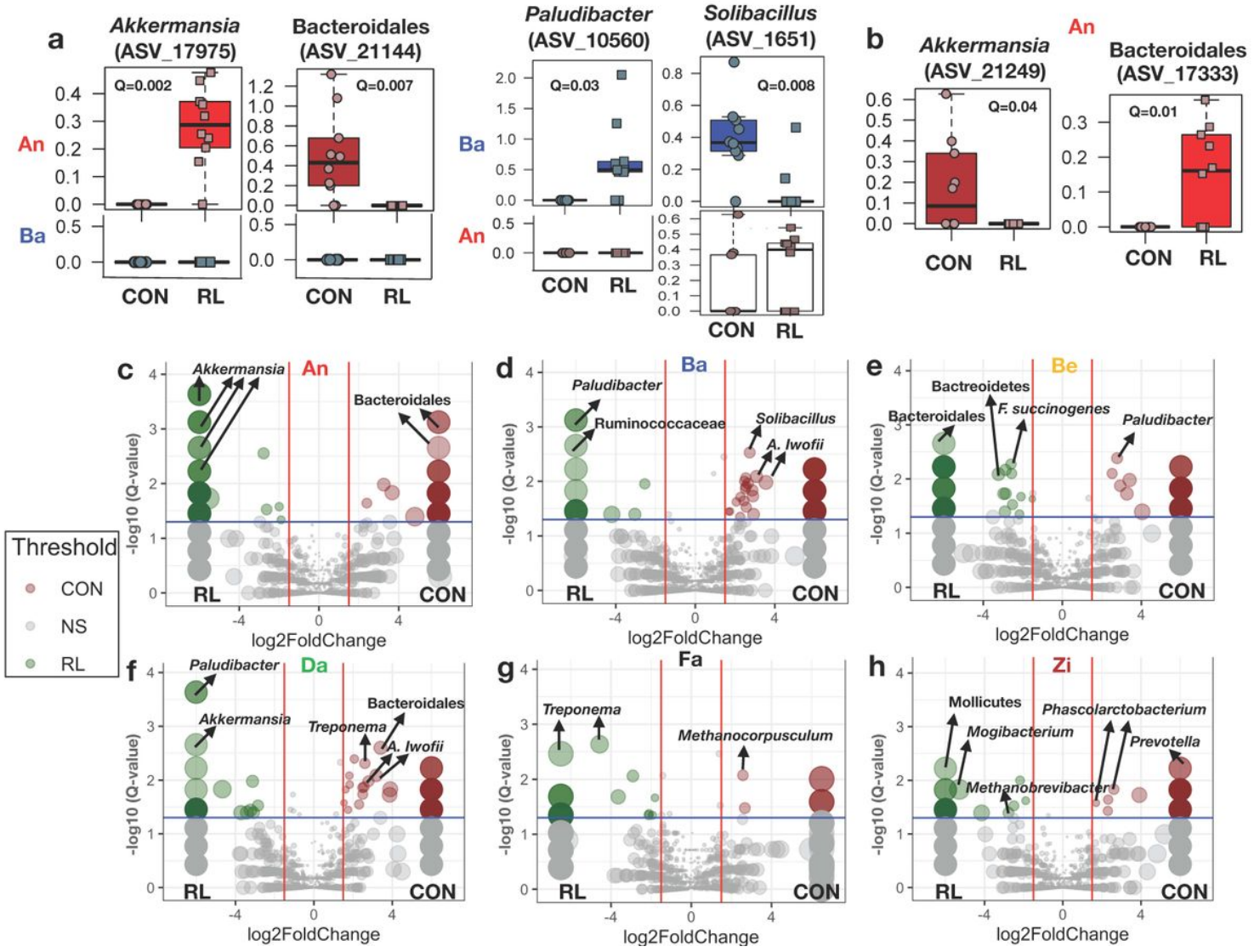

Figure 5

Discriminant taxonomic profiles when feeding RL or CON alfalfa are different for each horse sampled. (a) Main indicator ASVs in horse An under RL or CON, unknown Akkermansia and Bacteroidales respectively, have different abundant dynamics in horse Ba. Conversely, unknown Paludibacter and Solibacillus, the main discriminant taxa of RL and CON respectively in horse Ba, show different abundance patterns in horse An. (b) Different ASVs affiliated to unknown Akkermansia and Bacteroidales in horse An, have an opposite abundance pattern as shown in a. All Q-values reflect Wilcoxon rank sum tests adjusted for false discovery rate (FDR). (c-h). Volcano plots show that discriminant taxa affiliated with feeding RL or CON are different in each individual horse sampled. Every symbol in the volcano plots represents an ASV, red or green colors represent significantly discriminant ASVs in CON and RL treatments respectively[Indicator value (IV) > 0.7 in all horses except Zi (IV>0.65), Q-value <0.05, shown as -log10]. Some symbols look darker due to superimposition in the plot. Size of the symbol is associated with the fold change in abundance of that specific ASV (log2).

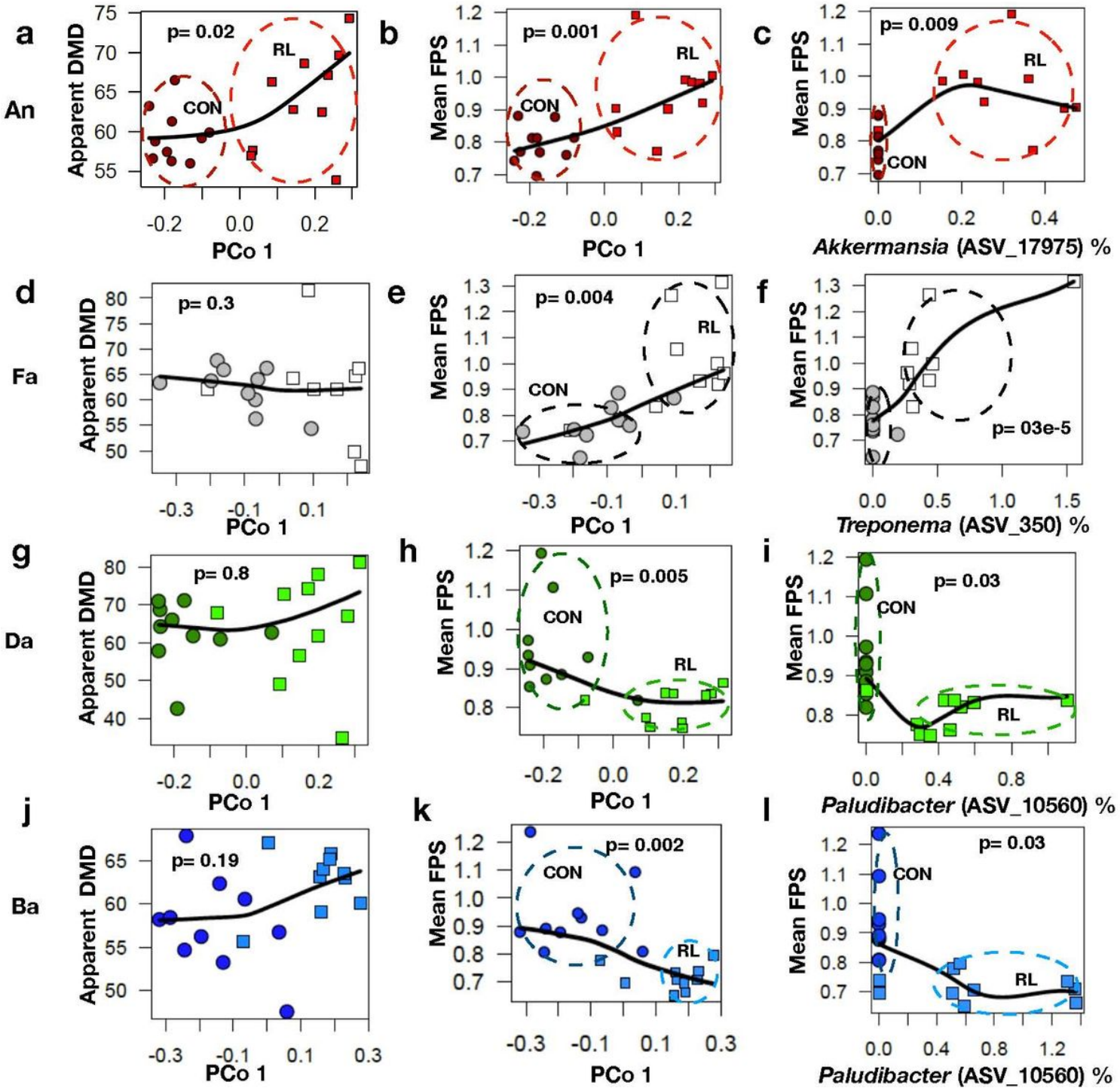

Figure 6

Associations between specific feed digestibility traits and microbiome features in selected horses. The panels (a-l) show how different compositional microbiome traits in horses An, Da and Ba (Scores along PCo.1 as shown in figure 2c-h, and abundance of main indicator taxa as shown in figure 5), associate in a different manner with individual digestibility traits: Apparent dry matter digestibility (DMD), and mean particle size in feces (FPS). No other significant associations were found between other microbiome or feed digestibility traits in these or other horses (Be, Zi or Fa). The P-values were calculated based on a generalized linear model-GLM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile3.png
- Additionalfile1.xlsx
- Additionalfile2.png