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ORIGINAL ARTICLE

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The faecal microbiome of Exmoor ponies shows step-wise compositional changes with increasing levels of management by humans

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Abstract

Background: Horses can suffer from gastrointestinal (GI) disease in domestic environments, often precipitated by human-led changes in management. Understanding the consequences of these changes on equine gut microbiota is key to the prevention of such disease episodes.

Objective: Profile the faecal microbiota of adult female Exmoor ponies under three management conditions, representing increasing levels of management by humans, encompassing different diets; whilst controlling for age, breed and sex.

Study design: Cross-sectional descriptive.

Methods: Faecal samples were collected from three populations of Exmoor ponies kept under contrasting management conditions: 29 adult female ponies in groups with low management (LM) ($n = 10$), medium management (MM) ($n = 10$) and high management (HM) ($n = 9$) levels, based on diet, drug use, handling and exercise. Faecal microbial composition was profiled via high-throughput sequencing of the bacterial 16S rRNA gene, and functional metagenome predictions.

Results: We observed profound step-wise changes in microbiome structure in the transition from LM to MM to HM. A relatively high abundance of Proteobacteria and Tenericutes was associated with the HM group; higher abundance of Methanobacteria was observed in the LM group. The MM group had intermediate levels of these taxa and exhibited high 'within group' variation in alpha diversity. Functional predictions revealed increased amino acid and lipid metabolism in HM; energy metabolism in LM and carbohydrate metabolism and immune/metabolic disease pathways in MM.

Main limitations: Low group sizes, incomplete knowledge of bacterial genomes in equine gut microbiota and it was not possible to assess the relative impact of diet, drug use, handling and exercise on the microbiome as variables were confounded.

Conclusions: Human-led management factors had profound step-wise effects on faecal microbial composition. Based on functional metagenome predictions, we hypothesise that dietary differences between groups were the major driver of observed differences.

KEYWORDS
16S rRNA sequencing, diet, horse, management, microbiome

1 | INTRODUCTION

Domestication is associated with changes to an animal's natural environment, diet, breeding and behaviour,[1–4] often with a negative impact on animal health.[5–8] In particular, captive animals commonly suffer from gastrointestinal (GI) pathology,[9–11] indicating that factors such as stress, and diet changes associated with captive management can result in changes to the GI microenvironment which compromise animal health. Horses constitute a prime example of a species which suffers from a high incidence of (often life-threatening) GI disease in captivity[12–14]; prevalence of analogous diseases in wild populations are unknown due to lack of surveillance. Unsurprisingly, the diet and feeding methods of domestic horses often do not mimic those of wild equids and, furthermore, changes in feeding, environment and management can precede episodes of GI disease.[14–18] The impact of management change on the microbiome has been implicated in the pathogenesis of such disease episodes. Notably, increased abundance of Proteobacteria of the equine gut has been intrinsically linked to GI diseases such as colic and colitis[19–22]; furthermore, obesity and metabolic disease are also associated with alterations to the equine GI microbiota.[23–25] As the causality of these relationships is not yet established, it is important to further our understanding of how human-led differences in equine lifestyle impact the gut microbiota in healthy horses, so that we can begin to hypothesise how the microbiome may be linked with an increased risk of disease.

Attempting to address this, a handful of studies have compared the faecal microbiota of domesticated horses with wild horses, donkeys and zebra.[4,26–28] However, the majority of these studies were confounded by one or more variable which are known to affect the microbiome (e.g. species, breed, age, sex[29–34]), and unsurprisingly showed varying results. For example, one study found that domesticated horses versus Przewalski's horses had reduced microbial alpha diversity[3]; whilst others showed no difference between groups.[26–28] There was also little agreement in the taxonomic changes observed between these studies. Therefore, whilst these data are a useful indication of the variation that we can expect to see in the gut microbiota within the genus *Equus* and in different environments, it is difficult to draw conclusions as to the specific impact of human-led management factors.

Therefore, in this study, we aimed to describe similarities and differences between the faecal microbiota composition of horses under three different types of management – representing step-wise increases in the level of human intervention, in particular in relation diet.

2 | MATERIALS AND METHODS

2.1 | Animals

Freshly voided faecal samples from 29 adult female Exmoor ponies were collected in May 2019 from three populations in Somerset and Devon, categorised at different levels of human intervention in management whilst matching each sub-population for breed, sex, approximate age and time of sampling, and recording differences in diet and recent/historical drug treatments. A low management (LM) group ($n = 10$) was identified, in which animals were either born on site or transported to it from Exmoor 15 years prior to this study. They grazed 344 acres of rough moorland dominated by purple moor grass, brown, bent and deer grass, bracken, heather and other native plant species and had no supplementary rations. These animals received no handling, nor had they ever received veterinary medications, such as anthelmintics or antibiotics. A medium management (MM) group ($n = 10$) was identified in which animals were also born wild on Exmoor but were rotated several times a year between grazing on managed moorland and rye grass mix pasture of approximately 60 acres (stocking density of 2 acres/horse) on Exmoor. This group were supplemented with hay sporadically throughout the year, administered a yearly anthelmintic in winter, and were minimally handled for health monitoring, anthelmintic administration, and hoof trimming. Finally, a highly managed (HM) group ($n = 9$) was identified in which the animals were born in captivity and grazed on ryegrass pasture but stabled at night and fed supplementary rations (hay and concentrate) during winter. The HM group were regularly handled for grooming, riding and activities such as hoof trimming and veterinary treatment when necessary. They were given anthelmintics three times a year to control gastrointestinal helminth burdens.

At the time of sampling none of the animals in the study were stabled or receiving supplementary rations on a regular basis; hence, the main dietary difference between the three groups was the level of access to ryegrass versus rough moorland grazing. Animals in HM group were not exercised/ridden during the study period. All animals in the study were selected to have a body condition score of between 3 and 4 out of 5[35]; however, this was done

visually for the LM group without palpation of the body. None of the animals in the study were known to be pregnant or lactating (NB. There were no males in the LM herd and hence pregnancy was not possible).

2.2 | Sample collection and processing

Freshly voided faecal samples were collected from study participants in May 2019. Samples were collected from the ground with a gloved hand after waiting for the individual to defecate, thereby minimising sampling stress for the animals. A subsample was placed in a 5 mL cryovial tube using sterile forceps and immediately snap frozen in liquid nitrogen before storing at −80°C until further processing. For the LM and MM groups, the first 10 individuals that defecated and met body condition score criteria were sampled. The HM group had only nine individuals, so all were sampled.

2.3 | 16S rRNA sequencing

As described by Peachey et al.[36] genomic DNA was extracted from individual faecal samples, as well as from three negative 'blank' (= no DNA) controls, using the PowerSoil DNA Isolation Kit (Qiagen), according to the manufacturers' instructions. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was PCR-amplified using forward (TACGGGAGGCAGCAG) and reverse primers (CCAGGGTATCTAATCC). For PCR amplification the following thermocycling protocol was used: 98°C /2 min, 20 cycles of 98°C/15 s, 63°C/30 s, and 72°C/30 s, and 72°C/5 min. Amplicons were purified using AMPure XP PCR Purification beads (Beckman Coulter). The index PCR was performed using the NEBNext hot start high-fidelity DNA polymerase and Nextera XT index primers (Illumina) according to the following thermocycling protocol: 98°C/30 s, 8 cycles of 98°C/10 s, 65°C/75 s and at 65°C/5 min. The indexed samples were purified using AMPure XP beads and quantified using the Qubit Quant-iTTM dsDNA High-Spec Assay Kit (Life Technologies). Then, equal quantities of DNA from each sample were pooled and the resulting library was quantified using the NEBNextLibrary Quant Kit for Illumina (New England Biolabs Inc.). Sequencing was performed on an Illumina MiSeq platform using V3 chemistry (301 bp paired-end reads). Samples from all three groups were processed in the same batch and no samples had to be excluded from analysis.

2.4 | Bioinformatics

Raw paired-end Illumina reads were processed using the Quantitative Insights into Microbial Ecology 2 (QIIME2-2018.4; https://qiime2.org) software suite.[37] Successfully joined sequences were quality filtered (Read cut-off: 17; 286 and 17; 255 for forward and reverse, respectively), dereplicated, chimeras identified, and paired-end reads merged in QIIME2 using DADA2.[38] A phylogenetic tree was generated for diversity analysis, followed by calculation of alpha and beta diversity metrics using the 'core-metrics-phylogenetic command' in QIIME2. Sequences were assigned to taxonomy using the feature classifier: SILVA 99% OTUs full-length sequences.[39] A feature table with the assigned taxonomy was exported from QIIME2 alongside a weighted UniFrac distance matrix for downstream biostatistical analysis. Statistical analyses were executed using the Calypso software[40] (cgenome.net/calypso/); total sum scaling (TSS) normalisation was applied, followed by square route transformation to account for the non-normal distribution of taxonomic counts data. Beta diversity of microbial communities was calculated using weighted UniFrac distances and samples were clustered according to management group using principle coordinates analysis (PCoA) and supervised Canonical Correspondence Analysis (CCA). Differences in bacterial alpha diversity (Shannon diversity) between groups were evaluated using an ANOVA. Differences in the relative abundances of individual microbial species between groups were assessed using the Linear Discriminant Analysis Effect Size (LEfSe) workflow[41] by assigning group as the comparison class. Relative abundance was visualised using ANOVA plots. Networks of Pearsons correlation were created to identify correlations between bacteria associated with each of the groups. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST)[42] was used to predict the functional pathways associated with the bacterial taxa identified by 16S rRNA sequencing. LEfSe analysis was used to compare the output of PICRUSt between groups, in the form of KEGG Orthology (KO's).[43] These data were inputted into a network analysis to demonstrate links between functional pathways associated with the three respective groups.

3 | RESULTS

3.1 | Microbiota profiling

From the 29 faecal samples 1 946 910 (range per sample: 30 244–275 624) raw paired-end reads were generated. Following primer trimming, joining of paired-end reads, filtering of low-quality sequences, denoising and chimera removal, a total of 844 229 (range per sample: 10 077-128 378) high-quality sequences were retained for further bioinformatics analyses. The rarefaction curves generated following in silico subtraction of low-quality and contaminant sequences indicated that all samples were sequenced at adequate read depth and quality, thus allowing us to undertake further analyses. Raw data and metadata were uploaded to the European Nucleotide Archive (ENA) (accession number: PRJEB39336). The OTU were classified into 23 phyla with Firmicutes (36.71%) and Bacteroidetes (30.99%) were predominant in all samples (Figure 1); 0.09% of OTUs could not be assigned to any bacterial group.

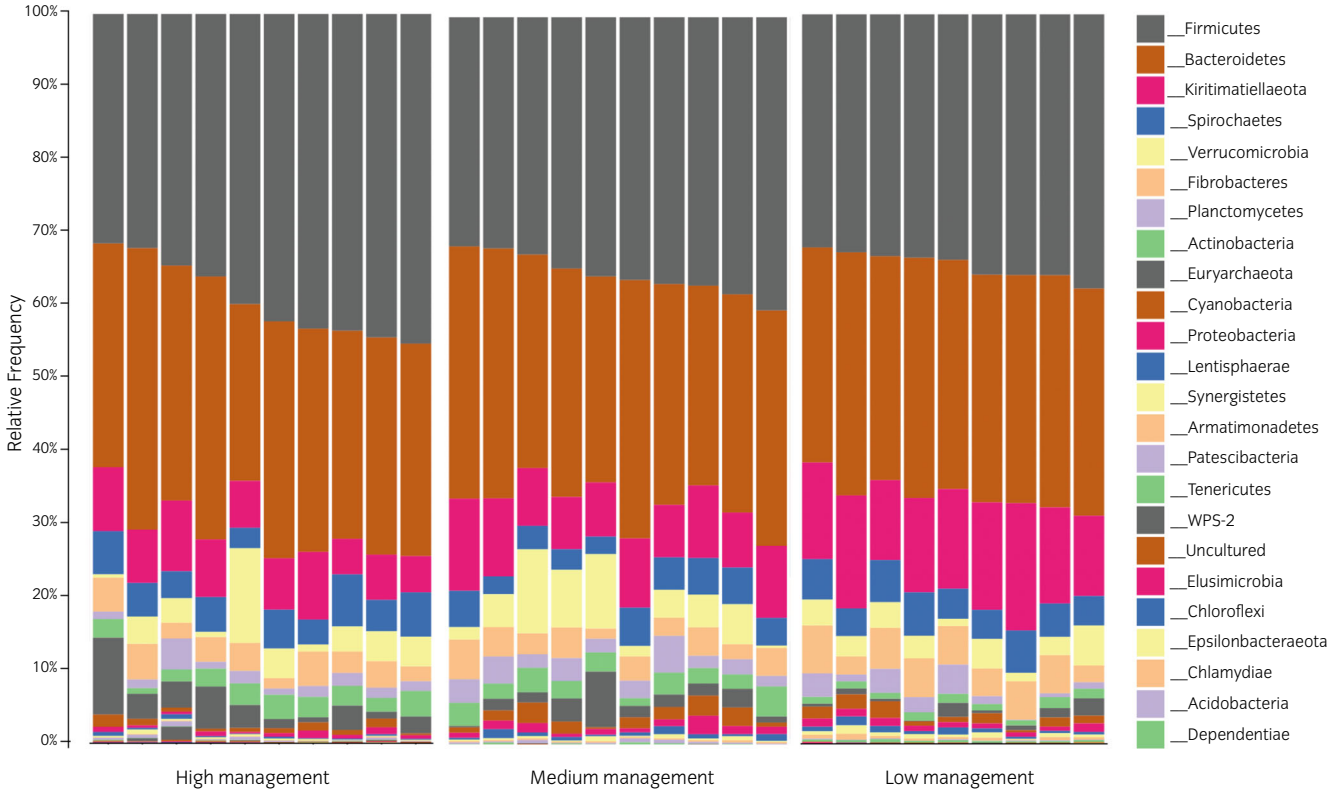

FIGURE 1 Bar charts depicting the relative abundances of faecal bacterial phyla of the low, medium and high management Exmoor pony groups.

3.2 | Clear step-wise differences in beta diversity were observed with increasing levels of human intervention

Principle Coordinate Analysis (PCoA) demonstrated clear clustering by population with the greatest difference seen between the HM AND LM groups with the MM group spanning between the two (Figure 2A). CCA showed a highly significant difference between the three groups ($p = 0.001$, $F = 4.59$) (Figure 2B).

There were no significant differences in alpha diversity between groups (evenness $p = 0.35$; Shannon $p = 0.2$; Richness $p = 0.33$). However, greater inter-individual variation in alpha diversity was seen between animals in the MM group (Figure S1).

3.3 | Many bacterial taxa showed clear step-wise differences in relative abundance according to human intervention level

Differences in the abundance of specific bacterial taxa between the groups were evaluated using LEfSe analysis[41] (Table 1 and Table S1). Examples of taxa which altered most significantly between groups are represented graphically in ANOVA plots (Figure 3). Many observed differences were characterised by step-wise alterations between management groups from HM, to MM, to LM; with MM representing an intermediate between the two other groups. The highest disparity (with regards to number of significant differences observed) between study groups was seen at the family level, and these differences were represented visually in a network analysis for clarity (Figure 4). As for the PCoA, the network analysis showed clear clustering by group, with the least correlation between taxa in the HM and LM groups, and the HM group showing a profile spanning that of the other two groups (Figure 4). In summary, the taxa Kirimatiellaeota (Lowest significant taxonomic level (LTL): Kiritimatiellae), Tenericutes (LTL: Anaeroplasma, Mycoplasma), Alphaproteobacteria (LTL: Rhizobales), Deltproteobacteria (LTL: Desulfovibrio), Synergistes (LTL: Pyramidobacter), Actinomycetales (LTL: Mycobacterium), Negativicutes (LTL: *Acidaminococcaceae*), Optituales and Lentispherae (LRL: *Victivallaceae*) showed step-wise reductions in relative abundance from HM > MM > LM (Figure 3). Whereas the taxa Chlorflexi (LTL: *Anaerolineaceae*), Actinobacteria (LTL: *Eggerthellaceae*, *Atopobiaceae*, *Coriobacteriaceae*), Euryarchaeota (LTL: Methanobrevibacter) and Clostridiales (LTL: *Lachnospiraceae*, *Shuttlesworthia*) showed step-wise reductions in relative abundance from LM > MM > LM (Figure 3). The abundance of taxa in the MM group often represented an intermediate state between the two other groups, as seen in the network analysis. However, there were some taxa which were increased in abundance compared to the other two groups; e.g. increases in relative abundance were observed for Actinobacteria (LTL: Micrococcales (*Arthrobacter*)) and Propionibacteriales (LTL: *Aeromicrobium*), Gammaproteobacteria (LTL: Pseudomonales and *Succinovibrionaceae*), Bacillales (LTL:

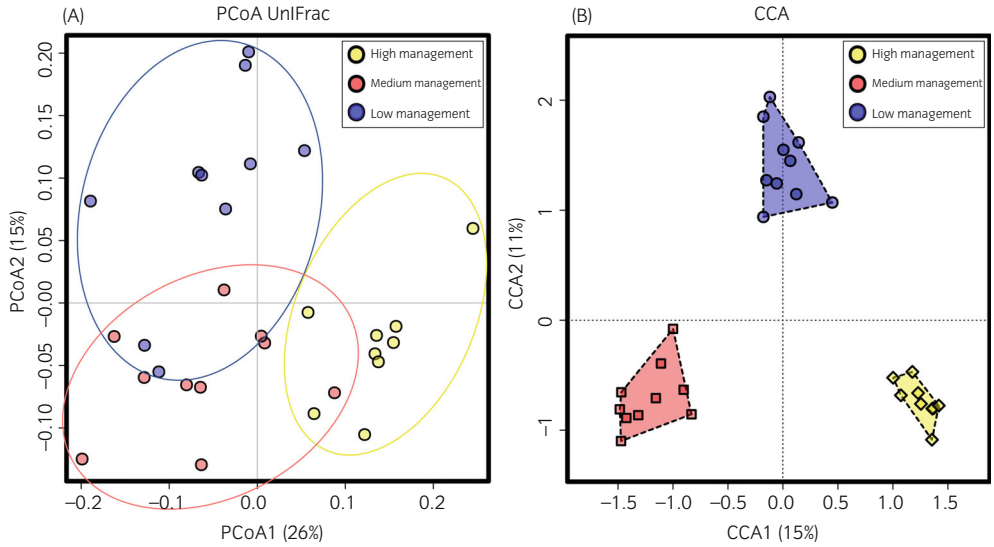

FIGURE 2 Multivariate analysis of weighted unifrac distance beta diversity measures between samples from low, medium and high management Exmoor groups showed clear clustering according to domestication level. (A) Unsupervised principle coordinates analysis (PCoA) plots comparing the faecal microbial beta diversity of low, medium and high management groups. (B) The faecal microbial beta diversity of faecal samples ordered by low, medium and high management groups using supervised Canonical Correspondence Analysis (CCA) ($p = 0.001$).

TABLE 1 Bacterial taxa with a significantly different relative abundance between high (HM), medium (MM) and low management (LM) groups according to Linear Discriminant Analysis Effect Size (LEfSe) analysis. Rows under group headings are highlighted grey when the taxa were relatively *increased* in that group. LEfSe values are included in the columns (N.B. only those taxa which changed in a step-wise modus from HM, MM to LM groups are shown here; for full LEfSe results see Table S1.

Phylum	Class	Order	Family	Genus	High management	Low management
Euryarchaeota						
	Methanobacteria					
		Methanobacteriales				
			Methanobacteriaceae			
				Methanobrevibacter		
Chloroflexi						
	Anaerolineae					
		Anaerolineales				
			Anaerolineaceae			
Firmicutes	Clostridia					
		Clostridiales				
			Lachnospiraceae			
				Shuttleworthia		
Actinobacteria						
	Coriobacteriia					
		Coriobacteriales				
			Eggerthellaceae			
			Atopobiaceae			
			Coriobacteriaceae	*Parvibacter*		
	Actinobacteria	Actinomycetales	*Mycobacteriaceae*			
				Mycobacterium		

(Continues)

TABLE 1 (Continued)

Phylum	Class	Order	Family	Genus	High management	Low management
Tenericutes						
	Mollicutes					
		Anaeroplasmatales				
			Anaeroplasmataceae			
				Anaeroplasma		
		Mycoplasmatales	*Mycoplasmataceae*	*Mycoplasma*		
Kiritimatiellaeota						
	Kiritimatiellae					
Lentisphaerae						
	Lentisphaeria					
		Victivallales				
			Victivallaceae			
Synergistetes						
	Synergistia					
		Synergistales				
			Synergistaceae			
				Pyramidobacter		
	Thermoplasmata					
Proteobacteria	Deltaproteobacteria					
		Desulfovibrionales				
			Desulfovibrionaceae			
				Desulfovibrio		
		Myxococcales				
	Alphaproteobacteria					
		Rhizobiales				
Firmicutes	Negativicutes					
		Acidaminococcales	*Acidaminococcaceae*			
Verrucomicrobia	Opitutae	Opitutales				

LDA score	Colour
2.5–3	
3–3.5	
3.5–4	

Bacillus), Selenomondales (LTL: *Anaerovibrio*), *Prevotellaceae* UCG001, *Saccharomonas* (*synonyms* – *Zymomonas*, *Pseudomonas*), *Methanocorpusculum* and *Clostridium butyricum* (Table S1).

3.4 | Predicted functional analysis from 16S sequencing data

Network analysis with Pearson's Correlation was used to demonstrate functional differences between the groups; the clearest differences between groups were seen at KEGG level 2 (Figure 5). As seen with beta diversity and taxonomic analyses, functional pathways in the LM AND HM groups were poorly correlated with each other, whereas pathways upregulated in the MM group spanned between them. LEfSe analysis of predicted functional data supported the network analysis (Figure S2); glycan, amino acid, lipid, polyketides and xenobiotics metabolism were increased in the HM group; carbohydrate metabolism, cancers, neurogenerative diseases, excretory system, immune system diseases and metabolic diseases were increased in the MM group; and energy metabolism, transcription, membrane transporters and genetic information processing were increased in the LM group.

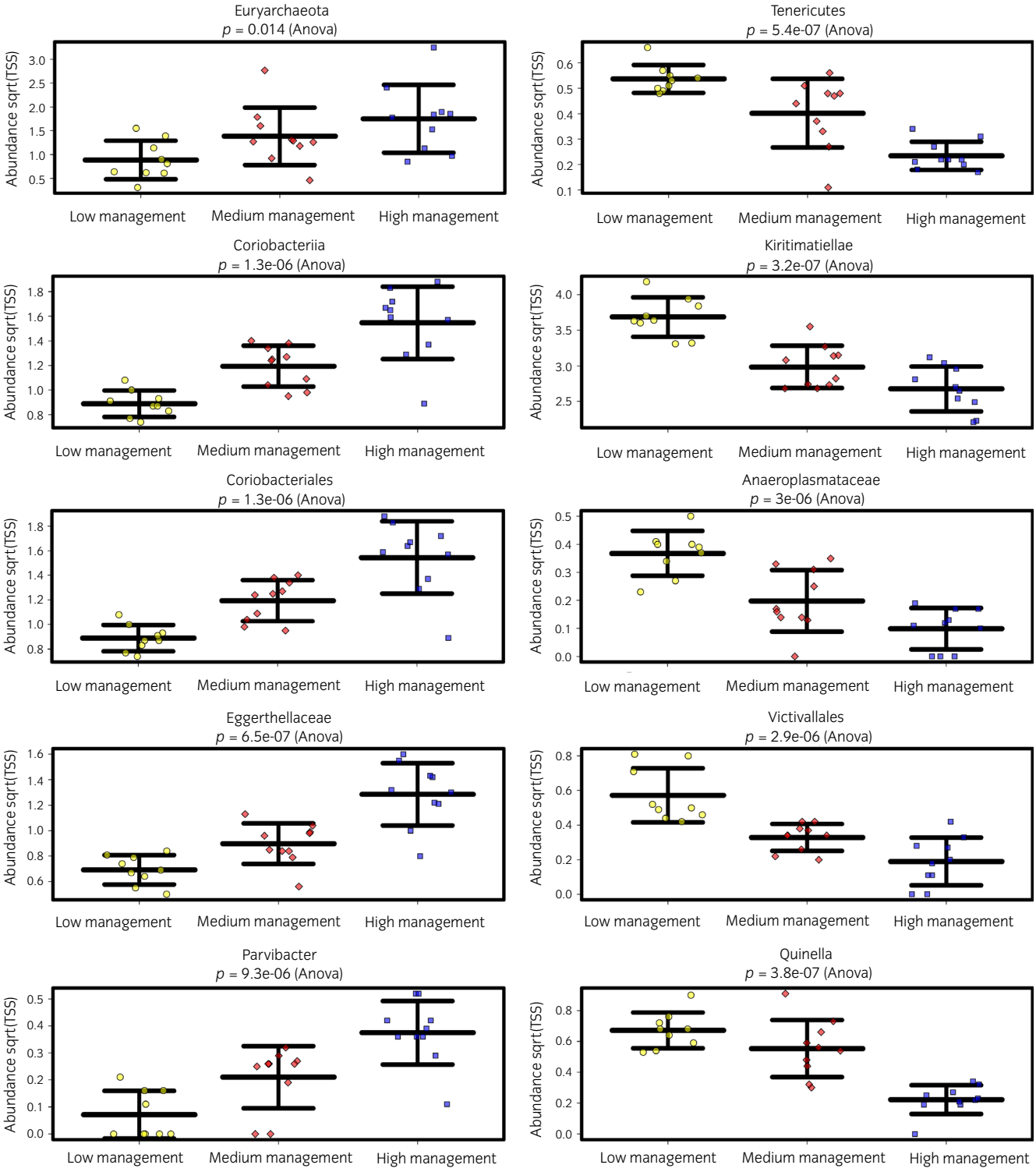

FIGURE 3 Examples of bacterial taxa showing significant step-wise changes in relative abundance between low, medium and high management group at each taxonomic level. Represented by ANOVA plots (those which were most statistically significant were selected for presentation).

4 | DISCUSSION

In this descriptive study, we report a clear difference in faecal microbial composition and predicted function between adult horses of the same breed and sex, but different levels of management by humans; ranging from no human intervention to multi-factorial human intervention. The overall composition of the faecal microbiota in the study participants was similar to that previously described for

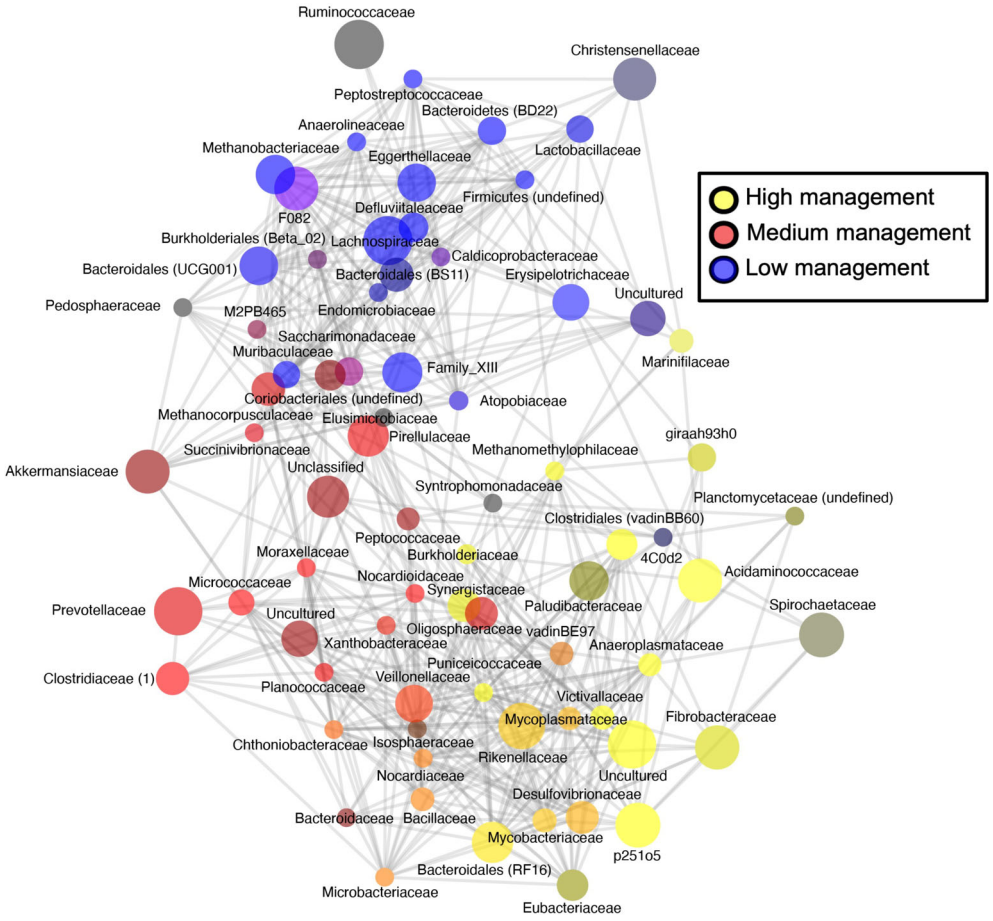

FIGURE 4 Network analysis with Pearson Correlation at family level. Showing correlations between bacterial families and their relative association with low, medium and high management groups. P values are above each graph.

horses, with the most abundant phyla being Firmicutes and Bacteroidetes.[21,24,44–46] There were clear differences in global composition between the groups according to unsupervised beta diversity analysis. This was supported by supervised multivariate analysis using CCA, in which the differences between the three groups were significant ($p = 0.001$). Clustering of individuals according to PCoA (Figure 2B) showed that the greatest difference in microbial beta diversity was between LM and HM groups, and the MM animals spanned between them, indicating step-wise differences in the composition of the faecal microbiota that could be in response to increasing levels of management by humans, including differences in diet. The predicted functional changes mirrored those in the microbiome data, with a network analysis revealing the least correlation between the LM and HM groups. To our knowledge, this is the first time a step-wise pattern in composition between unrelated groups has been described in the equine GI microbiota; we hypothesise that this effect was likely due to human intervention, with diet being a major driver.

Overall, taxonomic analysis using a combination of LEfSe and network analysis corroborated the findings of multivariate analyses, demonstrating clear step-wise changes in the abundance of many bacterial taxa between the HM, MM and LM groups (Figures 2–4). Notably, members of the Classes Alpha- and Delta-proteobacteria were significantly higher in the, apparently healthy, HM ponies. Higher levels of the Class Alphaproteobacteria were largely attributable to increases in the order Rhizobales; whereas an increase in Class Deltaproteobacteria was attributable to an increased abundance of genus *Desulfovibrio*. In general, elevated levels of Proteobacteria in horses have been linked to increased starch intake.[20,47,48] The HM group had the highest level of access to ryegrass pasture, which has a higher starch level than rough moorland grasses,[49–51] hence this observation may be linked to dietary starch levels. Similar increases in Proteobacteria in horses have also been associated with intestinal inflammation, colic and equine grass sickness.[21,52,53] In particular, Weese et al.[52] showed that Proteobacteria increased in horses in the days prior to presenting with colon torsion, suggesting that bacteria belonging to this phylum could be playing a role in precipitating disease. It is plausible that increases in Proteobacteria associated with dietary starch intake, such as seen here, could be a risk factor for the development of GI disease; although, as we had no health data from animals in this study we cannot prove or disprove this hypothesis here.

In the LM group, many of the relatively abundant taxa also had strong links to diet. In particular, Euryarchaeota (genus *Methanobrevibacter*) was the bacterial taxa most strongly associated with this group. *Methanobrevibacter* spp facilitate the fermentation rate and colonic energy production in the form of short chain fatty acids by removing H2 from bacterial fermentation,[54] which is important to utilise energy from fibrous diets. High levels of Euryarchaeota are associated with high-fibre diets in horses[55,56]; and have also been linked to

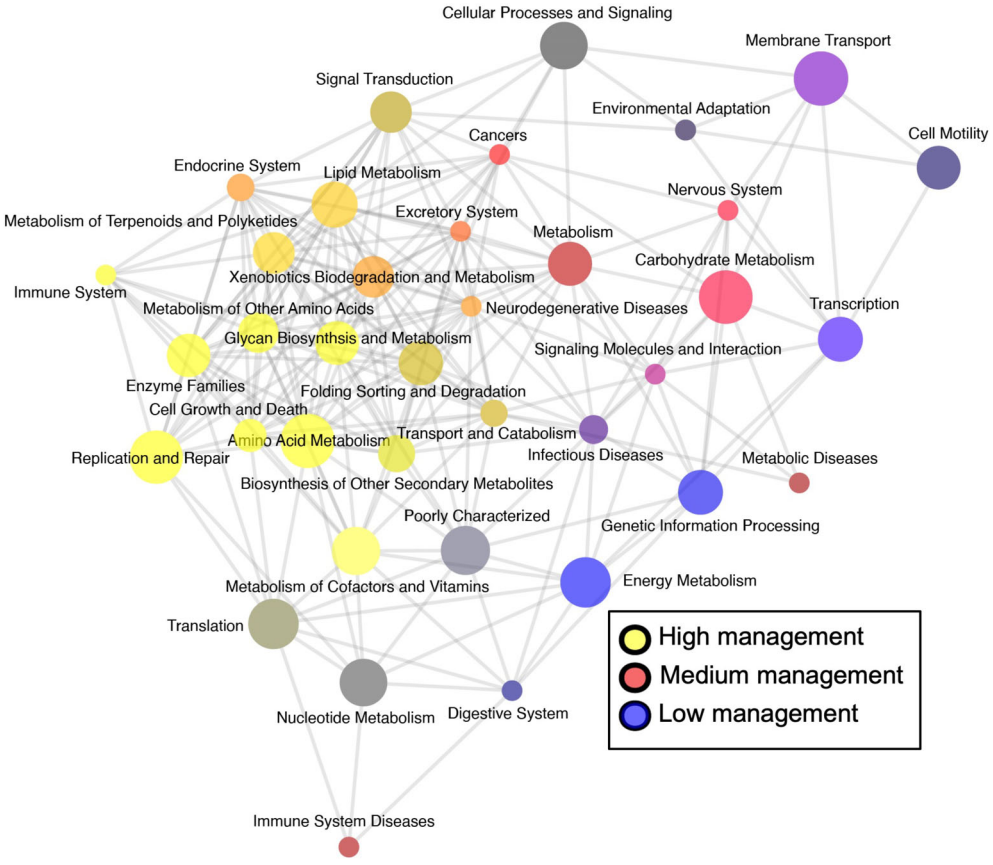

FIGURE 5 Network analysis with Pearson Correlation of predicted functional pathways at KEGG level 2 based on taxonomic data. Showing correlations between metabolic pathways, and their relative association with the low, medium and high management groups.

wild horses in studies comparing wild and captive populations.[27] Therefore, the association of these taxa with the LM group in this study most likely reflects the relative high fibre content of the rough moorland diet of the LM population. Complementary to the increase in Methanobrevibacter was a similar increase in *Anaerolinaceae*, *Coriobacteriaceae* and *Lachnospiraceae* in the LM group. *Anaerolinaceae* are anaerobic fermenters producing methanogens, and are synergistically linked with members of Euryarcheota,[57] such as *Methanobrevobacter*. Furthermore, *Coriobacteriaceae* have been shown to increase in abundance proportionately to increasing concentration of cellobiose supplementation in horses[58] (cellobiose forms when cellulose is partially hydrolysed by the enzyme cellulase[59]), suggesting that an increase in these bacteria could be a downstream effect of the high level of fibre in a rough moorland diet; likewise, *Lachnospiraceae* are also associated with the breakdown of cellulose and hemicellulose.[57,60] An increased abundance of *Lachnospiraceae* and this family have been reported in the faecal microbiome of healthy horses when compared with animals with colitis[21] and diarrhoea.[61] Overall, the predicted functional analysis performed in this study supports a hypothesis that the gut microbial composition of the LM group was geared towards fibre digestion, with KEGG pathway for energy metabolism being significantly increased, compared with the other two groups.

The MM group had intermediate levels of many taxa, but there were some taxa which were relatively increased in this group in comparison to both HM and LM groups. These included two orders belonging to Actinobacteria (Micrococcales (*Arthrobacter*) and Propionibacteriales (*Aeromicrobium*)) and Gammaproteobacteria (Pseudomonales and Succinovibrionaceae). Actinobacteria have a diverse range of functions, however, members of the orders Micrococcales and Proprionibacteriales have previously been identified as opportunistic pathogens, for example Aeromicorbium is in the same family as the well-known equine opportunistic respiratory pathogens Rhodococcus equi and Nocardia.[62,63] Members of Pseudomonales (gammaproteobacteria) are also considered to be pathobionts. In contrast to the relatively stable management of the other groups, over the course of a year the diet of the MM herd would be expected to fluctuate more frequently, as they were grazed on different pasture types and had sporadic supplementation with hay. Dietary changes have been shown to cause alterations to microbial composition and alpha diversity in horses[20,26,46,64–66] and, interestingly, there was also a much higher variation in the microbial alpha diversity in the MM group when compared to the other groups (Figure S2). Furthermore, the predicted functional analysis for the MM group included increases in KEGG pathways for cancers, metabolic diseases and immune system diseases, all of which appear to represent an unhealthier phenotype. Currently, the KEGG pathway analysis is designed to identify microbial gene pathways which are associated with human disease states, and so we cannot know how this relates to equine diseases.[43] At this point in time, we do not understand the equine microbiome well enough to be sure that functional predictions are completely accurate, however, if they are, these observations could be an indication that regular changes

in diet on a small holding are more disruptive to gut microbiota than either a low or high intensivity system with a relatively stable management. This, of course, is analogous to the observation that changes in diet have been found to precede GI disease in horses.[14–18]

Based on previous studies of the equine microbiome,[67] and given that all animals were apparently clinically healthy at the time of sampling, the differences in management between groups which were likely to be responsible for the observed differences were diet,[20,27,65,66] drug use[45,68,69] and, potentially, handling stress[70] however due to the descriptive nature of the study we cannot evaluate the relative impact of these factors on differences in microbiome composition between groups. The functional predictions we performed indicated that dietary differences were the main driver of differences, at least between the LM and HM groups. The main difference in diet between groups was the level of access to managed ryegrass pasture versus rough moorland grazing; although the MM and HM groups had also received supplementary hay and concentrate (in the case of the latter) over the winter. None of the animals in the study had been treated with an antibiotic, nor anthelmintic in the last 8 weeks, and previous studies have shown that anthelmintic use alone has small and transient effects on the GI microbiota[45,67–69]; however, interestingly, the predicted increase in Xenobiotic metabolism in the HM group suggests that frequent drug use may have long lasting effects on equine gut microbiota.

4.1 | Study limitations

When considering the observed gut microbial differences between groups we have drawn upon previous literature describing the proposed function of bacterial taxa; however, given the sparsity of equine microbiome data in general, and the level of variation between studies, it was not always possible to find a clear explanation for our findings. Furthermore, the majority of bacterial genome data available are from human gut microbiota and, therefore, this may reduce the accuracy of our functional predictions. We have used faecal microbiota as a proxy for the gut microbiota; faecal samples have been shown to broadly represent the distal colonic microbiota of horses[71,72] but will not be representative of the gut content further proximal in the GI tract. As this study was descriptive and not done under experimental conditions the drivers (and mechanisms thereof) of differences between groups cannot be proven. Taking into account all of the above, mechanistic studies are required to validate the functional hypotheses generated by this work.

5 | CONCLUSION

We have identified clear step-wise changes to the faecal microbiome correlating with the level of human intervention in management of Exmoor ponies. The patterns in microbial abundance and functional predictions support the hypothesis that differences in diet were responsible for majority of these changes. Based on our data, we could potentially suggest faecal biomarkers indicating relative dietary starch levels in Exmoor ponies; for example, higher levels of *Methanobrevibacter* in the faecal microbiota associate with high fibre, and higher levels of proteobacteria associate with higher starch. However, validation of these biomarkers in the faecal microbiome would be needed, and surveys should be extended to other systems and breeds of horses, as it is likely that each breed will have its own microbiome variation and different benchmarks for what is considered optimal.

AUTHOR CONTRIBUTIONS

Laura Peachey and Katie Bull designed the research. Laura Peachey, Katie Bull and Gareth Davies carried out the research and performed data processing and analyses. Katie Bull drafted the manuscript text, with input from Laura Peachey. The figures were prepared by Katie Bull and Timothy Jenkins, with input from Laura Peachey. The manuscript was reviewed and edited by Timothy Jenkins, Katie Bull and Laura Peachey prior to submission. All authors reviewed and approved the manuscript prior to submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.13961.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ENA at https://www.ebi.ac.uk/ena/browser/view/PRJEB39336 reference number PRJEB39336.

ETHICAL ANIMAL RESEARCH

This study was approved by the Institutional Ethical Review Committee, Department of Veterinary Medicine, University of Bristol. UIN Number: UB/19/025.

INFORMED CONSENT

Written informed consent was obtained from the owner of each of the groups of ponies from which study samples were collected.

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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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