The effect of diet change and insulin dysregulation on the fecal microbiome of ponies

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Key words

16S rRNA, hindgut, horse, glucagon-like peptide-1, equine metabolic syndrome, endocrine

Summary statement

Examining the fecal microbiome of ponies introduced to pasture showed that metabolically healthy ponies have greater microbial stability when challenged with this dietary change, compared to ponies with insulin dysregulation.

Abstract

The equine microbiome can change in response to dietary alteration and might play a role in insulin dysregulation. The aim of this study was to determine the effect of adding pasture to a hay diet on the fecal bacterial microbiome of both healthy and insulin-dysregulated ponies. Fecal samples were collected from sixteen ponies before and after dietary change to enable bacterial 16S rRNA sequencing of the V3-V4 region. The dominant phyla in all samples were the Firmicutes and Bacteroidetes. The evenness of the bacterial populations decreased after grazing pasture, and when a pony was moderately insulin-dysregulated (P = 0.001). Evenness scores negatively correlated with post-prandial glucagon-like peptide-1 concentrations after a hay-only diet (r^2 = -0.7, P = 0.001). A change in diet explained 3% of fecal microbiome variability. We conclude that metabolically healthy ponies have greater microbial stability when challenged with a subtle dietary change, compared to moderately insulin-dysregulated ponies.

List of abbreviations

aGLP-1, active glucagon-like peptide-1; BW, bodyweight; EMS, equine metabolic syndrome; MID, moderately insulin-dysregulated; NIR, normally insulin-regulated; NMDS, Non-metric multidimensional scaling; OGT, oral glucose test; OTU, Operational taxonomic unit; rRNA. ribosomal RNA; SID, severely insulin-dysregulated

Introduction

As with other mammalian species, the equine gastrointestinal microbiome comprises a large and complex assortment of bacteria, viruses, archaea, protozoa and fungi, that is integral to the overall health of the horse (Dicks et al., 2014). The gastrointestinal microbiome influences metabolism, endocrine signalling and the immune system in humans (Nicholson et al., 2012), and in horses has been implicated in several diseases including laminitis (Milinovich et al., 2010) and equine metabolic syndrome (EMS) (Elzinga et al., 2016).

The core microbiota of both the fore- and hindgut have been described in horses. The rapid transit time of ingesta and exposure to environmental bacteria results in a fluctuating foregut microbiome often dominated by Proteobacteria (Ericsson et al., 2016). In comparison, the hindgut microbiome appears to be more stable, and is dominated by the phyla Bacteroidetes and Firmicutes, with Fibrobacter, Spirochaetes, and Verrucomicrobia also abundant (Fliegerova et al., 2016, St-Pierre et al., 2013, Desrousseaux et al., 2012, Stewart et al., 2018, Shepherd et al., 2012, O' Donnell et al., 2013, Proudman et al., 2015). The fecal microbiome has been shown to represent the populations of the distal hindgut (Dougal et al., 2012, Costa et al., 2015) and fecal collection is a valid, non-invasive sampling technique provided the samples are collected immediately after defecation and stored appropriately (Beckers et al., 2017, Fliegerova et al., 2016).

The hindgut fermentation capacity of horses is utilised to extract short-chain volatile fatty acids from previously undigested dietary components, such as fibre (Al Jassim and Andrews, 2009). The hindgut microbiota of horses can respond relatively quickly to changes in diet (Fernandes et al., 2014), and is highly variable, both within and between individuals (Blackmore et al., 2013). For example, increasing the starch in a horse's diet changes the composition of the fecal microbiome, with increases in amylolytic bacteria, gram positive cocci, and lactobacilli (Harlow et al., 2016). Altering the diet from a hand-fed forage (alfalfa) and grain mix, to a pasture-based diet, also changed (within 4 days) the composition of the fecal microbiome (Fernandes et al., 2014). Horses kept solely on pasture also experienced fluctuations in their fecal microbiome, which was possibly related to changes in the environment, season or pasture composition (Salem et al., 2018). A common dietary change for horses is the transition from a hay-only diet to a combination of hay and pasture, when animals are allowed to graze. However, the effect of the addition of pasture to a horse's diet on the fecal microbiome has not been examined.

In humans and mice, perturbations of the fecal microbiome have also been associated with insulin secretion and sensitivity (Naderpoor et al., 2019), incretin action (Lee et al., 2018, Hwang et al., 2015, Xu et al., 2019), and obesity (Ley et al., 2005, Beaumont et al., 2016). It has been reported that horses with EMS have lower microbial diversity than healthy horses, but with greater abundance of Verrucomicrobiota subdivision 5 (now classified as the separate phyla Kiritimatiellaeota) (Elzinga et al., 2016, Spring et al., 2016). However, other reports suggest that obese horses have higher microbial diversity (Biddle et al., 2018, Morrison et al., 2018), which is difficult to reconcile, as many horses with EMS are also obese. The impact of dietary change on the fecal microbiome of horses with EMS is currently unknown. Identifying the relative proportions of key commensal bacteria in the hindgut may provide an insight into the efficiency of dietary energy extraction by horses and ponies with EMS.

This study aimed to describe the effect of a common dietary change (the addition of pasture) on the fecal bacterial microbiome of healthy ponies, and ponies with EMS. Under this aim, a secondary objective was to describe differences in the bacterial fecal microbiome between ponies with different degrees of insulin regulation, ranging from normal to severe dysregulation. To further investigate this secondary objective, we determined whether any metabolic parameters could be associated with differences in the microbiome.

Materials and methods

Subjects and study design

Sixteen mixed-breed ponies (6 x Shetland/Shetland cross-breed, 3 x Welsh/Welsh cross-breed, 7 x other breeds) were included in this study which was performed in South East Queensland, Australia. Following a veterinary examination, which included haematological and biochemical analyses, each pony was considered healthy, except for the presence of clinical signs of EMS in 11 individuals. The diagnosis of EMS was based on evidence of regional adiposity and insulin dysregulation. The presence of insulin dysregulation was confirmed with a standard oral glucose test (OGT) using 1 g/kg bodyweight (BW) dextrose powder added to a small meal (Bertin and de Laat, 2017). Ethical approval for the study was granted by the University of Queensland (QUT/SVS/316/16) and Queensland University of Technology (1600000877), and the study was conducted according to the relevant national guidelines and state regulations which govern these committees.

The ponies were classified by their (2 hour) post-prandial serum insulin response to the OGT as normally insulin-regulated ([insulin] < 60 μIU/mL; NIR), moderately insulin-dysregulated ([insulin] 60-279 μIU/mL; MID), or severely insulin-dysregulated ([insulin] ≥ 280 μIU/mL; SID), as previously described (Fitzgerald et al., 2019b). The basal adrenocorticotrophic hormone concentration for each pony was measured to exclude pituitary *pars intermedia* dysfunction, using a seasonally-adjusted diagnostic cut-off value, as previously described (Secombe et al., 2017, Fitzgerald et al., 2019b). Serum aspartate aminotransferase, alkaline phosphatase, gamma glutamyltransferase and total bilirubin were measured in a commercial laboratory, to assess liver function in each pony one week prior to the study commencing, as liver disease can result in gut microbial dysbiosis (Tripathi et al., 2018).

During the study, the ponies were housed individually in pasture-free (dirt) yards for an initial 10-day period and fed at 2% BW (as fed) with prime alfalfa hay, plus a commercial low-sugar vitamin and mineral pellet (Kentucky Equine Research, Mulgrave, Victoria, Australia). In the next phase, the ponies were allowed to graze pasture in individual, adjacent strips (4.2 m x 21 m) for 4 h each day (8 am-12 pm), for 5 consecutive days. In the evening, the ponies received a meal of the same alfalfa hay, at 0.7% BW (as fed). It was estimated that the ponies would consume up to 1.3% of their BW during the morning grazing period (Longland et al., 2016), thus the total amount of feed available was judged to be similar for the two study periods. To estimate the total dietary intake, the total fecal output was measured, with all feces collected for the initial three days of each period. The daily fecal mass was weighed, subsampled, dried, and re-weighed for each 24 h period. Total fecal output was calculated by determining: total wet weight x dry subsample weight/wet subsample weight.

Samples

On the last day of each diet period, a whole freshly-passed uncontaminated fecal ball was collected from each pony. The fecal ball was collected with a gloved hand from the top of the fecal pile to minimise skin and environmental contamination. The samples were placed in sterile 50 mL tubes, and immediately stored at -20°C.

The prime alfalfa hay fed during the study was sourced from a single batch. Hay samples were collected from 12 randomly sampled bales using a bale corer and pooled prior to analysis. The paddock grazed during the study consisted of couch, rye grass and clover. Pasture samples were collected at 10 am on the day prior to grazing. Six sampling locations

were selected at random within a diagonal pattern across the whole paddock, by tossing a 0.25 m^2 square frame. The grass was cut 1 cm from the ground, pooled and immediately microwaved to prevent further metabolism of plant carbohydrates, and to facilitate dry matter measurements. Fodder samples were sent to a commercial laboratory (DPI, New South Wales, Australia), accredited by the National Association of Testing Authorities, for further analysis.

Post-prandial blood samples (6 mL; for association of the microbiome with metabolic parameters) were collected by jugular venepuncture at 10 am (ponies were fed at 8 am) on the same morning as fecal samples were collected. The blood was divided equally between a clot activator vacutainer tube and an EDTA tube (Becton Dickinson, New Jersey). The clot activator tube was allowed to stand for 30 min at ambient temperature prior to centrifugation (10 min at $1500 \times g$) and the separation of serum. The EDTA tube was placed on ice for 10 min prior to centrifugation (10 min at $1500 \times g$) and the separation of plasma. Samples were immediately frozen at -20°C, then transferred to -80°C for storage within 72 h. Serum insulin concentrations were measured at a commercial laboratory (Vetpath, Western Australia, Australia) using an Immulite 2000 XPi (Siemens Healthcare, Brisbane, Queensland, Australia). Plasma aGLP-1 was measured using a commercially-available ELISA (Millipore, Abacus ALS, Meadowbrook, Australia) previously validated for use in horses (de Laat et al., 2016).

DNA extraction and 16S amplicon sequencing

Genomic DNA was extracted from all fecal samples. A 0.25 g sub-sample was taken from the centre of the fecal ball with sterile forceps and the DNA extracted using the Power Fecal Kit (Qiagen, Victoria, Australia) according to the manufacturer's protocol. Extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher, Victoria, Australia), and diluted to 5 ng/μL. PCR was used to amplify the V3-V4 region of the bacterial 16S rRNA gene following the 16S metagenomics sequencing library preparation protocol (Illumina, Victoria, Australia). Briefly, 25 μL PCR reactions were performed, consisting of 5 ng/μL template, 5 μL of 1 μM forward primer, 5 μL of 1 μM reverse primer, and 12.5 μL of $2 \times$ KAPA HiFi HotStart Ready Mix (Sigma-Aldrich, Sydney, Australia). The primer sequences used in this study were published previously (Klindworth et al., 2013): 16S rRNA Amplicon PCR Forward Primer S-D-Bact-0341-b-S-17 = 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'

16S Amplicon PCR Reverse Primer S-D-Bact-0785-a-A-21 = 5'-
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC
C-3'

The PCR protocol consisted of an initial denaturation step at 95°C for 3 min, 25 cycles of
denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30
sec, followed by a final extension at 72°C for 5 min and held at 4°C. PCR clean-up was
performed using JetSeq Clean beads (20 μL per sample; Bioline, New South Wales,
Australia), and 80% ethanol. The PCR product was then resuspended in 52.5 μL of 10 mM
Tris (pH 8.5). Samples were indexed using the Nextera XT Index primers (Illumina, Victoria,
Australia). A 50 μL reaction consisted of 5 μL PCR product, 5 μL primer 1 (Illumina,
Victoria, Australia), 5 μL primer 2 (Illumina, Victoria, Australia), 25 μL 2 × KAPA HiFi
HotStart Ready Mix (Sigma-Aldrich, Sydney, Australia), and 10 μL PCR-grade water. The
PCR protocol consisted of an initial denaturation of 95°C for 3 min, 8 cycles of denaturation
at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, followed
by a final extension at 72°C for 5 min and held at 4°C. Another clean-up step was performed
using the JetSeq Clean beads (Bioline, New South Wales, Australia), as previously described.
Libraries were quantified and normalised to 45 nM, then pooled for sequencing on a MiSeq
(Illumina, Victoria, Australia) using 600 cycle sequencing chemistry.

Data Analysis

The pony morphometric, fecal and hormone data analyses were performed in SigmaPlot v.13
with a significance level of $P < 0.05$ accepted. Sample size was determined using *a priori*
power analyses ($α < 0.05$, $β$ 0.8) to enable comparisons between metabolic groups using
previous data. Normality of the data distribution was tested using the Shapiro-Wilk test. The
fecal data were analysed using the Wilcoxon Signed Rank Test. The ponies' signalment and
blood measurements were analysed using a one-way ANOVA test when the data were
normally distributed, and the Kruskal-Wallis one-way ANOVA on Ranks, when the data
were not.

Quality control via read trimming (forward reads were trimmed at 0-20 bp and truncated at
280bp, reverse reads were trimmed at 0-20 bp and truncated at 260 bp) and chimaera
identification were performed on the 16S rRNA sequences using the Quantitative Insights
Into Microbial Ecology (QIIME2) pipeline and DADA2 (Callahan et al., 2016). Sequence
alignments and phylogenetic trees were constructed using MAFFT and FastTree,

respectively. Operational taxonomic units (OTUs) were defined at 97% sequence identity. Alpha rarefaction plots were used to determine sequence sampling depth, while maintaining maximum representation of OTUs in the samples. Rarefied data were used to estimate alpha and beta diversity only. Phylogenetic assignment of the OTUs was performed against the SILVA database, release 132.

Alpha diversity measures (measurements of within sample population diversity) included Shannon's diversity index (Shannon, 1948), observed OTUs, Faith's phylogenetic diversity (Faith, 1992), and Pielou's Evenness (Pielou, 1966). Alpha diversity measures were analysed using a two-way repeated measures ANOVA, where pony was the repeated factor, and diet and insulin regulation group were fixed factors. Correlations were assessed using Pearson's Correlation Coefficient. Non-metric multidimensional scaling (NMDS) and hierarchical clustering of bacterial phyla relative abundance values was based upon Bray-Curtis dissimilarity and were performed using the VEGAN package (Dixon, 2003) within R 3.2.2. Beta diversity measures (measurements of between sample diversity and stability) included Jaccard distance, Bray-Curtis distance, unweighted UniFrac distance, and weighted UniFrac distance. The dissimilarity distance between diets (within pony) were analysed using a one-way ANOVA to assess differences between insulin regulation groups.

Gneiss differential abundance modelling was applied via QIIME2 by creating a hierarchical correlation clustering tree (de Laat et al., 2019), which clusters co-occurring OTUs together into niche groups. These clusters were compared between groups of samples (such as insulin groups) and ratios of OTU counts (balances) were calculated. A difference in a balance between clusters was used to show that OTUs within the cluster were differentially abundant between the sample groups. Calculated balances transformed the microbial abundance data and were normally distributed (Morton et al., 2017). A linear regression model was applied to the balances, incorporating individual pony identification, diet and insulin regulation group. OTUs present at counts below 10 were filtered out prior to model construction. Additional differential abundance analysis was performed using Phyloseq (Callahan et al., 2016, McMurdie and Holmes, 2013). OTU counts were transformed to proportions of reads within the sample group.

Results

i.   Samples and animals

The percent dry matter of the hay was more than double that of the pasture (Table S1), and so total fecal dry matter was greater when ponies were fed hay only, compared to when they were grazing pasture with an evening hay meal (Fig. 1; $Z = 3.5$, $P = 0.001$). All ponies maintained a normal appetite throughout the study period, and there was no evidence of gastrointestinal upset as a consequence of the dietary change. Liver function was normal in all individuals, with the only biochemical change of note being that basal total bilirubin concentrations were lower in the SID group compared to the NIR group, although still within the normal reference range supplied by the laboratory (Table 1). The post-prandial concentration of the incretin hormone active glucagon-like peptide-1 (aGLP-1) was elevated in MID ponies compared to NIR ponies when on the hay diet. On the pasture diet, both the MID and SID groups had a GLP-1 concentration greater than NIR ponies (Table 1).

ii.   Characterisation of the microbiome in the cohort

Overall, 24,133 reads met our quality threshold. The mean 16S ribosomal RNA (rRNA) sequence count was 141,247, with a range of 52,936 to 886,568 reads per sample. Rarefaction curves based on observed OTUs and Shannon's and Faith's diversity were used to establish that 44,000 reads per sample resulted in no loss of diversity (Fig. S1) so this number was used for diversity measurements. The DADA2 algorithm identified 16,492 OTUs with just 37 conserved in all 32 samples.

The fecal microbiome from all ponies was dominated by the Phyla Firmicutes (35.5-55.7%) and Bacteroidetes (29.1 - 40.6%; Fig. 2). Phyla that were less abundant but present in all samples included Spirochaetes (2-15.8%), Kiritimatiellaeota (0.7-7.2%) and Fibrobacteres (0.2-7.6%). Thirty-seven bacterial classes were identified. Those present at >1% in at least one sample included Clostridia, Bacteroidia, Spirochaetia, Kiritimatiellae, Fibrobacteria, Negativicutes, Erysipelotrichia, Saccharimonidie, Melainabacteria, Verrucomicrobiae, Mollicutes, Synergista, Alphaproteobacteria, Planctomycetacia, and Bacilli. The majority of OTUs were present in low abundance, with a median of 19, mean of 274, and maximum of 82,519 occurrences over all 32 samples.

iii. Diversity

a. Alpha diversity

The diversity of the fecal microbiome decreased when ponies were fed the combined pasture plus hay diet (Shannon's Index, Holm-Sidak post-hoc, t = 3.5, P = 0.004; Table 2), compared to hay only. In particular, the proportion and diversity of the order Fibrobacterales decreased with pasture consumption (Fig. S2). The evenness of the bacterial populations decreased when ponies were on the combined pasture plus hay diet. This observation was most striking in the MID pony group (Evenness, t > 2.9, P < 0.001; Table 2). The number of OTUs and phylogenetic diversity were not different between diets or pony groups, indicating that similar taxa were found in both dietary treatments (Table 2).

Due to the significant effect of both diet and insulin regulation on bacterial evenness, we tested for a relationship between Pielou's evenness score with two known markers of equine insulin dysregulation; post-prandial serum insulin and aGLP-1 concentration (Bamford et al., 2015). Evenness was negatively correlated with post-prandial aGLP-1 concentration on the hay diet, but no significant relationship was found on the pasture diet (Fig. 3). No association was evident between post-prandial insulin concentrations and population evenness on either diet (hay, r^2 = -0.2, P = 0.4; pasture/hay, r^2 = -0.1, P = 0.7).

b. Beta diversity

None of the beta diversity indices that measured the dissimilarity between the hay diet and the combined pasture/hay diet differed between pony groups (Table 3; Fig. 4A). The microbial population appeared to be more dissimilar after a pasture/hay diet, whereas the population clustered closer after a diet of hay only (Fig. 4B).

iv. Differential Abundance: Gneiss Clustering and Regression

Multivariate linear regression showed that the insulin regulation status of each pony accounted for more variation within the faecal microbiome than the variation typically observed between hosts (Table S2). A subtle change in diet contributed relatively little to fecal microbiome variation (3%). When pony, diet and insulin regulation status were defined within the regression model, 18.1% of variation within the fecal microbiome was explained (Table S2).

Two niche groups (y2 and y23) were found to separate samples by diet due to a decrease in abundance of taxa on pasture relative to hay (Fig. 5). Y2 taxa were also increased in abundance in the MID cohort relative to NIR and SID. The y2 balance was near the root of the hierarchical clustering tree (de Laat et al., 2019) and reflected relative abundance changes to 7,714 OTUs (Fig. 6).

Several bacterial taxa were more abundant in the MID group relative to SID and NIR groups (P = 0.0006; Fig. 6A,B). The greatest relative abundance increase was phylum Firmicutes (to 3,548 unique taxa). Niche y10 exhibited decreased abundance in taxa in both MID and SID groups relative to ponies with normal insulin regulation (P = 0.006; Fig. 6C). Samples from MID ponies were separated by niche y12, in which taxa were more abundant relative to the other groups (P = 0.007; Fig. 5).

Discussion

This study demonstrated that changes to the fecal microbiome occur in ponies following a subtle change in diet, and that ponies with different insulin regulatory capabilities have different fecal microbial composition and respond differently to diet change. Associations have been made between the gastrointestinal microbiome and endocrine function in both humans (Naderpoor et al., 2019) and mouse models of disease (Hwang et al., 2015, Raza et al., 2017), and this microbiome-endocrine axis also appears to be evident in the horse. A few studies have begun to explore the relationship between metabolic derangements and the gastrointestinal microbiome in horses, although the small number of studies undertaken and the use of different methodologies has prevented a clear consensus (Milinovich et al., 2008, Steelman et al., 2012, Elzinga et al., 2016, Biddle et al., 2018). The current study used a cohort of ponies housed on the same property and maintained under controlled conditions, in an effort to minimise variability arising from non-standardised feeding practices. The results showed that whereas minimal changes to the overall microbial populations occurred between diets (beta diversity), the abundance of certain taxa was higher when ponies were fed a hay-only diet. Further, a difference associated with insulin regulation status was evident, with MID ponies' possessing bacterial populations that were less even than both the SID and NIR pony groups on a hay-only diet.

Equine microbiomes are dominated by key commensal bacteria, and broad taxonomic groups are resilient in the face of dietary change in healthy horses. Abundance differences are usually observed at the genus or species level (Steelman et al., 2012, Dougal et al., 2017).

The dominant phyla of the equine fecal microbiome are the Firmicutes and Bacteroidetes (Stewart et al., 2018, Salem et al., 2018, O' Donnell et al., 2013, Proudman et al., 2015), and this was further confirmed in this study. While the fecal microbial population remains stable in the short term when there are no disturbances to a pony's diet or routine (Blackmore et al., 2013), variation associated with seasonality and change in forage type occurred over a 12 month period (Salem et al., 2018). In addition, changing from a forage-concentrate to a pasture-based diet altered fecal microbiome composition within 4 days (Fernandes et al., 2014). The diet change in the current study was subtle, with ponies still receiving an evening meal of the same hay that was fed during the hay-only period in addition to the incorporation of pasture during a 4 h grazing period over 5 days. Despite this, diet was predicted to account for 3% of variation, and changes were observed in the diversity (Shannon's index) and evenness of the fecal microbial populations due to the change in diet over a similar timeframe to the Fernandes et al. (2014) study, as well as a decrease in fecal output when grazing. The greater DM of the hay could have contributed to reduced fecal output on pasture, as well as changes in the microbiome, such as the increase in Fibrobacteres, which also occurs in ruminants when fed greater DM content (Zhang et al., 2017). As the field component of the study was completed in less than one month, season was unlikely to be a major contributing factor to microbial variation (Salem et al., 2018).

Access to fresh grass resulted in a reduction in the diversity and uniformity of the bacterial populations, which confirms that the introduction of new forage types affects fecal microbial composition. A reduction in uniformity could be associated with the consumption of environmental microbes, such as those in soil, or the selection for and against niche groups in response to the new dietary component. Two niche groups were observed to decrease in abundance on the combined pasture plus hay diet relative to the hay-only diet. A previous study demonstrated that the introduction of haylage increased Fibrobacter populations (Salem et al., 2018), while the current study found a decreased proportion and diversity of some taxa in order Fibrobacterales, and fewer Verrucomicrobia, during pasture consumption. A reduction in bacterial diversity might be an adaptive response during periods of dietary change and relate to characteristics of the forage, such as DM content as discussed above, but studies that specifically investigate the reasons underlying adaptation of the microbiota to forage type would be valuable.

With higher Pielou's evenness scores, the NIR group exhibited greater stability of taxa and metabolically healthy ponies may exhibit stable microbiomes that vary considerably from each other and maintain stability. It also appears that with more severe insulin dysregulation (SID group) the taxa can regain stability although potentially with differences in diversity from metabolically healthy ponies. Reasons for this recovery in the evenness of taxa in more severely insulin-dysregulated animals are not known, and studies that specifically examine this phenomenon are warranted. Although Bray-Curtis dissimilarity did not indicate changes in the abundances of taxa within the groups, the marginal P value of 0.07 may indicate that further investigation of the variation in taxa abundance between metabolically healthy and dysregulated animals is justified. These data might be relevant to the selection of donors for fecal microbiota transplantation when transfaunation is being considered as an option for treating chronic diarrhoea in horses (Mullen et al., 2018). Host selection might influence the success of transfaunation, and the current findings suggest that the use of metabolically healthy donors is preferable.

A lower Pielou's evenness score in the MID group suggests less even counts of OTUs, and microbiomes of moderately insulin-dysregulated ponies may be prone to shifts in rare taxa after diet changes. The abundance of both the Bacteroidetes and Firmicutes was greater in the MID ponies, who also displayed higher aGLP-1 concentration and lower community evenness. These measures strongly correlated throughout the whole cohort, indicating that greater abundance of key taxa in these phyla in the fecal microbiome may be involved in insulin dysregulation in a subset of ponies with EMS. We have found that the positive linear relationship between aGLP-1 (which augments insulin secretion) and insulin is lost during severe insulin dysregulation in ponies (Fitzgerald et al., 2019a), which may help to explain the lack of an association between aGLP-1 and community evenness in the ponies with SID in this study. The current results agree with a recent study which also reported that a number of organisms from the phylum Firmicutes were overrepresented in horses with EMS (Elzinga et al., 2016). In addition, the abundance of Clostridiaceae (phylum Firmicutes) increases linearly with plasma insulin concentration in mice (Kreznar et al., 2017). Taken together these data support the existence of a relationship between Firmicutes and insulin regulation.

It is not known why moderate insulin dysregulation is correlated with changes in the abundance of key commensal bacteria. The abundance of bacteria associated with insulin resistance differs in the gut microbiome between strains of mice and has been shown to correlate with their responsiveness to high fat/sugar diets, indicating that microbial

communities can predispose to metabolic dysfunction and that this may be driven by genetic variation (Kreznar et al., 2017). Specific Bacteroidetes genera have been found to increase in diabetic mice when they were fed a high-fat diet, compared to mice that did not develop diabetes when fed the same diet (Serino et al., 2012). Further, the phyla Bacteroidetes and Firmicutes can be highly location-specific, and express glycans on their cell surface in order to recognise and interact with specific host cells (Coyne and Comstock, 2008, Ormerod et al., 2016). Depletion of these phyla in the mouse caecum following antibiotic treatment promoted the secretion of GLP-1 from intestinal L-cells (Hwang et al., 2015). Currently, GLP-1 secretion from the small intestine has been confirmed in horses (Kheder et al., 2018), but the presence and function of enteroendeocrine cells in the equine large intestine requires clarification. However, colonic L-cells can secrete GLP-1 after stimulation by short-chain fatty acids in other species (Tolhurst et al., 2012). As the fecal microbiome only represents microbial populations from the distal hindgut (Costa et al., 2015, Fliegerova et al., 2016), it is not known whether the changes in microbial evenness in the current study are a consequence of incretin signalling. Studies that determine whether the differentially abundant niche groups are involved in cross-talk with enteroendocrine cells in the small and/or large intestine, and play a role in incretin signalling and metabolic dysfunction, would be very informative for our understanding of equine insulin dysregulation.

Organisms from the phylum Kiritimatiellaeota (previously subdivision 5 of the Verrucomicriobia phylum) are present in the equine gastrointestinal microbiome and increased in abundance in ponies with EMS (Elzinga et al., 2016, Steelman et al., 2012). In this study, it was the fourth most abundant phylum, which is consistent with the known role of these bacteria in anaerobic environments (Spring et al., 2016). Taxa within Kiritimatiellaeota contributed to niche group changes that were evident between the two diets, and also separated insulin-dysregulated from metabolically healthy ponies. There are very little data about this phylum which has only been recently recognised as a separate taxonomic group from Verrucomicrobiae and its location in mucus layers and biofilms may be relevant to intestinal health, which may impact the gut-endocrine axis (Spring et al., 2016). Recognition of these two distinct phyla will improve our ability to describe the microbial composition of the equine fecal microbiome, and indeed Verrucomicrobiae were less abundant than Kiritimatiellaeota in this study. Improved identification of organisms highlights the evolving nature of gastrointestinal microbiome research and indicates that analysis of the equine fecal microbiome needs to remain flexible. Previously, many of the

microorganisms present in the GIT were not able to be identified as they could not be cultured outside of the gastrointestinal tract (Delgado et al., 2006, Deshmukh et al., 2019). The use of 16S rRNA sequencing technology in current studies has enhanced the identification of bacterial species. However, assigning function to the microorganisms identified is not possible without shotgun metagenomic technologies, and ongoing refinement of genome sequencing will greatly enhance our understanding of the microbiome.

Of 16,492 OTUs identified, just 37 were observed in all 32 samples. This indicates there is extensive functional redundancy within the microbiome, wherein many taxa can fulfil a niche and niches overlap. Shifts in functional capacity within a microbiome cannot be reliably detected by 16S rRNA sequencing. Grouping co-occurring taxa into balance trees as done here may approximate niche groups and avoid challenges in comparing microbiome samples which consist predominantly of unique OTU's (Morton et al., 2017). Balances can be highly discriminatory and list the same taxon on both sides. Related taxa are more likely to co-occur due to taxa of the same species sharing similar traits (Silverman et al., 2017). The balances shown in this study reflect relative abundance differences in closely-related taxa, and suggest that niche changes were occurring. The taxa that decreased in relative abundance on pasture, have been associated with fibre intake and complex polysaccharide fermentation in humans (Kovatcheva-Datchary et al., 2015, Rampelli et al., 2015), healthy gut function and a decrease in inflammation (Punzalan and Qamar, 2017, Stärkel et al., 2016, Verdu et al., 2016), and the production of short chain fatty acids (Flint, 2004). The abundance of two niche groups was influenced by pasture and one was further influenced in the MID cohort. Thus, some niches may be impacted differently in MID ponies by a diet change.

A change in the relative abundance of key taxa with respect to insulin regulation has been linked to metabolic and immune health in other species. For example, the Christensenellaceae R-7 group, which was decreased in abundance in insulin-dysregulated ponies in this study, was associated with having a lower BMI in healthy humans (Tamura et al., 2017) and was also decreased in abundance in patients with colorectal cancer (Stebegg et al., 2019). The relative abundance of Rikenellaceae was increased in insulin-dysregulated ponies in this study, is positively-correlated with insulin concentrations in mice (Kreznar et al., 2017), and is also increased in human patients with cardiovascular disease and type 2 diabetes mellitus relative to paired healthy controls (Sanchez-Alcoholado et al., 2017). These data suggest an association between Rikenellaceae and insulin regulation in multiple species, including horses, and investigations into whether these changes precede the onset of metabolic

dysfunction would help to determine whether microbiome dysbiosis is a cause or an effect of insulin dysregulation. Lastly, Erysipelotrichaceae UCG-004 taxa decrease in abundance in obese humans, with or without metabolic syndrome (Chávez-Carbajal et al., 2019). Obesity commonly co-occurs with insulin dysregulation in ponies with EMS but was not evident in all of the dysregulated ponies in this cohort, which may have influenced why Erysipelotrichaceae were more abundant in MID, relative to SID ponies. Such shifts in functional capacity may partly explain why the insulin response in the MID group was attenuated compared to the SID group.

Bilirubin enters the GIT via the liver and in rats is metabolised by members of the Bacteroidetes phylum to urobilinoids (Vitek et al., 2005). In the horse, Bacteroidetes may fulfil a similar function. However, the differences in serum bilirubin that occurred between ponies of different metabolic states was not accompanied by a difference in the proportion of Bacteroidetes in their fecal microbiome. Thus, any association between bilirubin and metabolic status may not be associated with the microbiome. This is an unexplored area of equine medicine.

Conclusions

This study has shown that metabolically healthy ponies have greater microbial stability when challenged with a subtle dietary change compared to moderately insulin-dysregulated ponies. Taxa within the phyla Bacteroidetes, Firmicutes, Kiritimatiellaeota and Spirochaetes have been identified as differentially abundant when compared between both the metabolic status of the pony and the diet. Based on the data from this study we recommend further studies into the presence of a microbe-gut-endocrine axis in the horse and its potential role in insulin dysregulation.

Acknowledgements

Competing interests

No competing interests declared.

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Data availability

All data referred to in and underpinning this publication are openly available in QUT Research Data Finder (doi.org/10.25912/5d8448c72c4c4) and can be found at: https://data.researchdatafinder.qut.edu.au/dataset/the-effect-of

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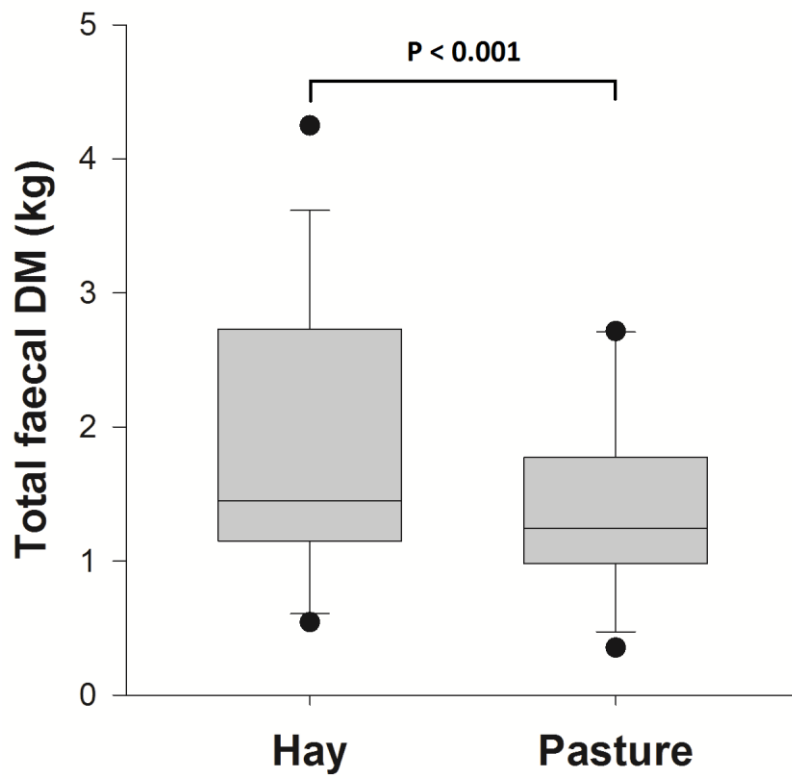
Figures

Figure 1: The total fecal output from 16 ponies on two different diets. Median (± IQR) total daily fecal output over a 3-day period was greater on a hay only diet, compared to a pasture/hay diet (P < 0.001).
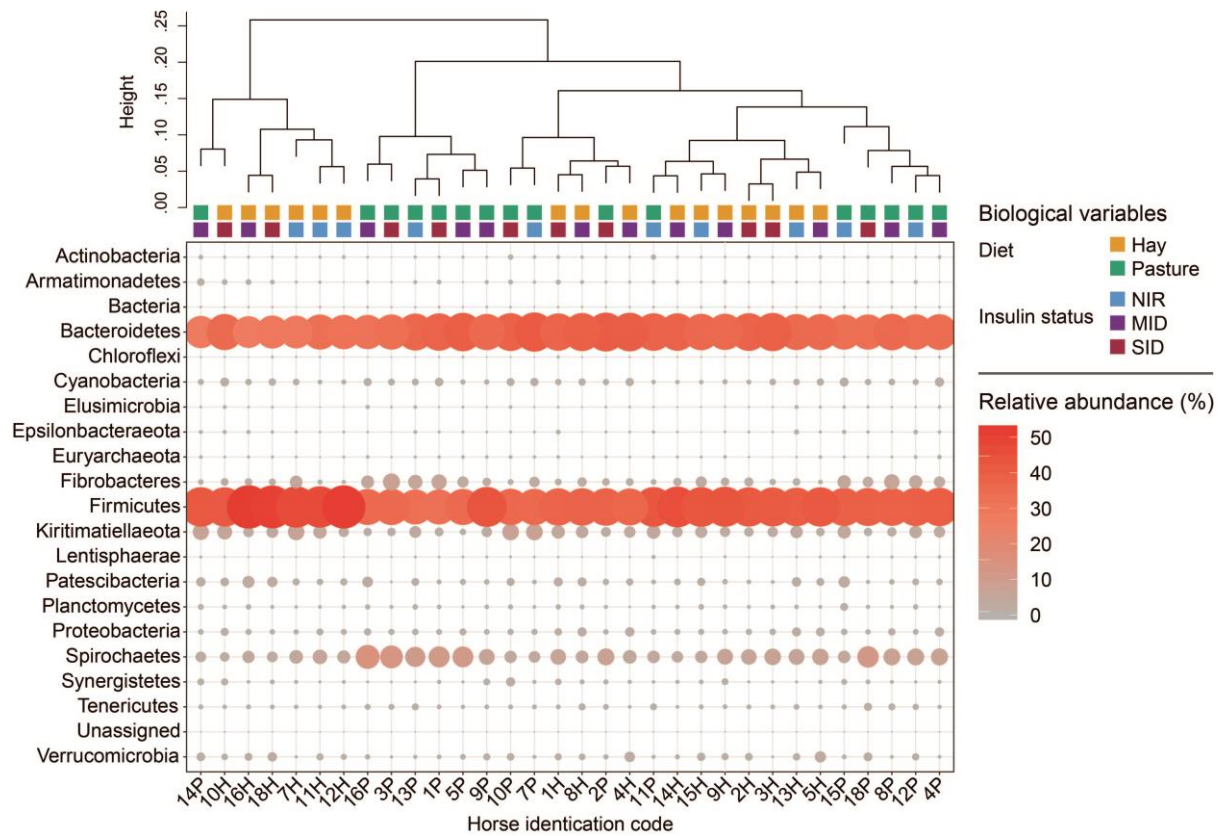
Figure 2: Relative abundance estimates of phyla in fecal samples from 16 ponies. The ponies received two diets (H, hay; P, pasture plus hay) and were classified according to their level of insulin regulation (NIR, normal insulin regulation; MID, moderately insulin dysregulated; SID, severely insulin dysregulated). Clustering shows broad relationships between samples. Diet influences clusters only at the tips. Circle size and color show the relative abundance of phyla in each sample.
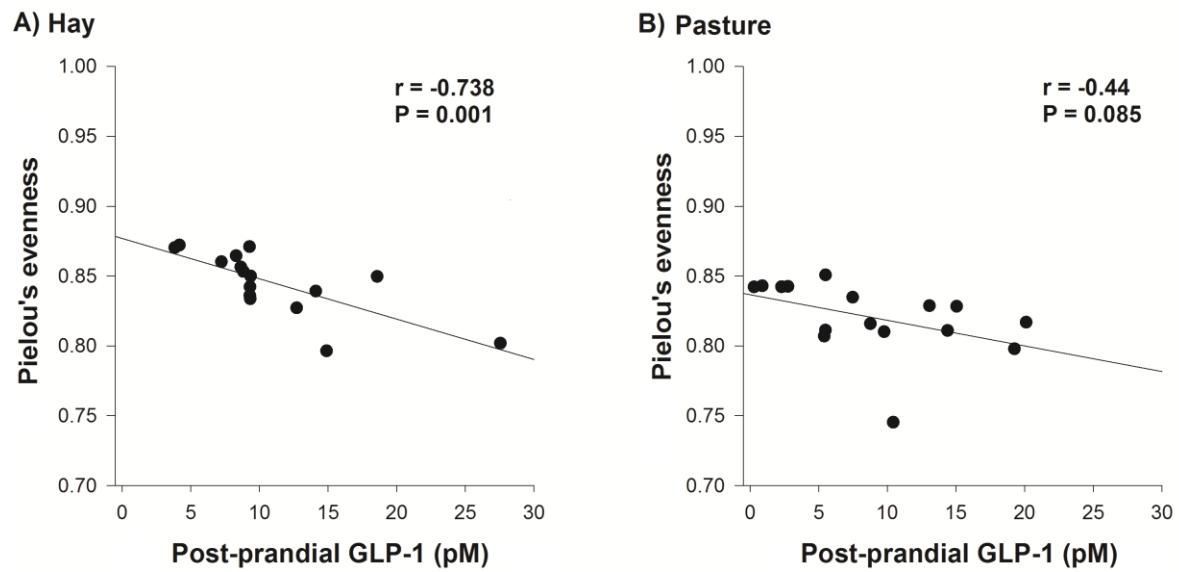
Figure 3: The association between Pielou's evenness alpha diversity measure and the post-prandial aGLP-1 concentration. Evenness was negatively correlated with post-prandial aGLP-1 concentration on a hay diet in ponies (A), but not when ponies ate a combination of pasture and hay (B).
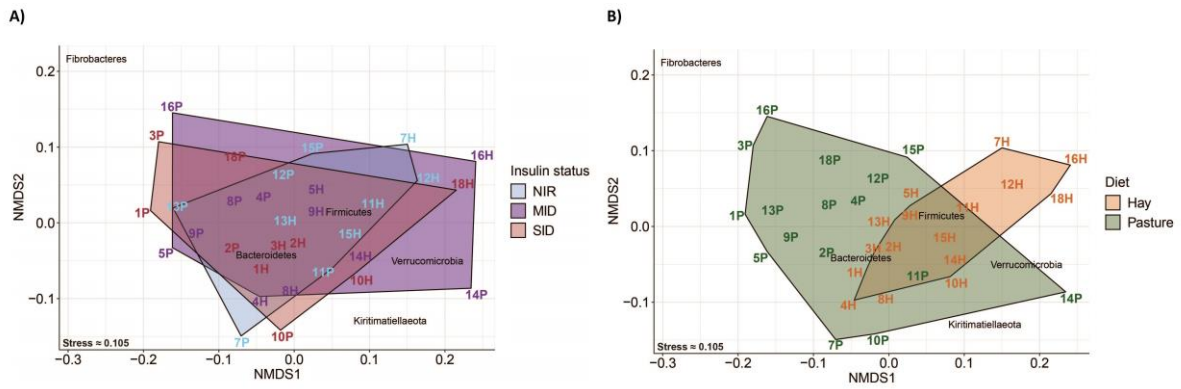
Figure 4: Non-metric multidimensional scaling (NMDS) plots calculated from the Bray-Curtis dissimilarity data. The clusters show the effect of insulin regulation (A; NIR, normal insulin regulation; MID, moderately insulin dysregulated; SID, severely insulin dysregulated) and diet (B).
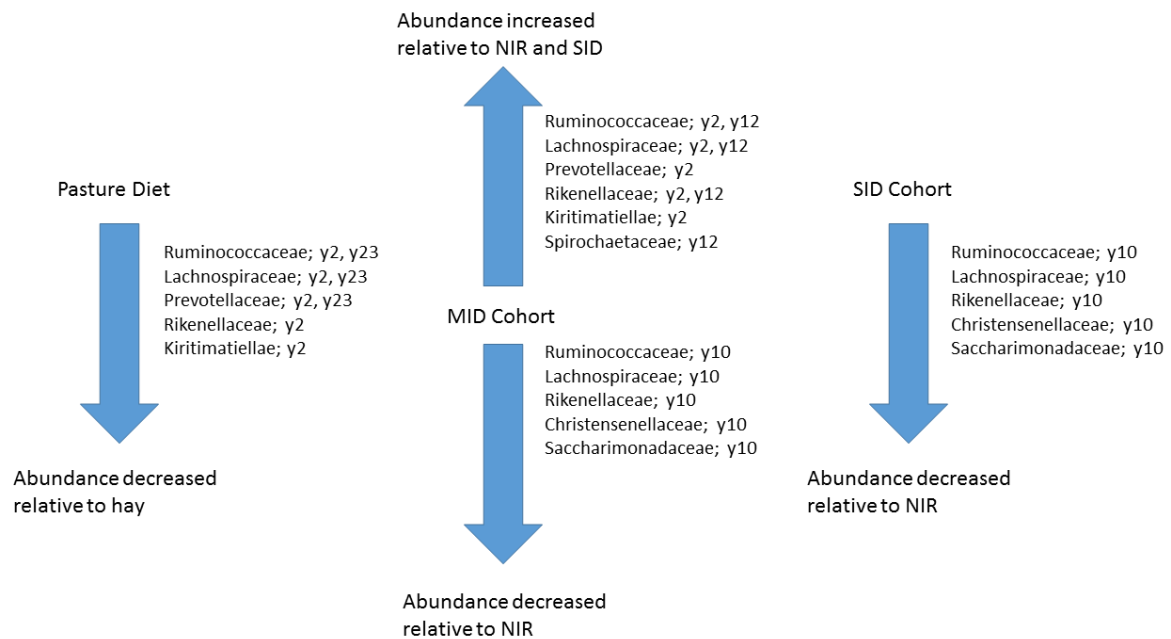
Figure 5: **The differential abundance of niche groups in the equine fecal microbiome affected by diet and insulin regulation status of the pony.** The addition of pasture to a hay diet reduced the abundance of four niches. Insulin-dysregulation either increased or decreased niche abundance relative to ponies with normal insulin-regulation. Niche groups y2 (P = 0.0006), y10 (P = 0.006), y12 (P = 0.007) and y23 (P = 2.97 x 10^{-7}) refer to balances in the hierarchical clustering tree (available in the online data repository). A smaller number (y2) was closer to the root of the tree and represents a larger number of taxa. Taxonomies are shown to family level.
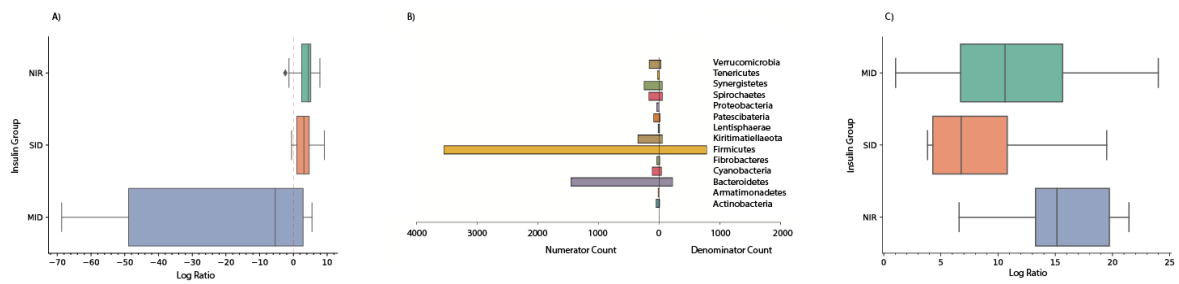
Figure 6: Differential abundance of bacterial taxa in 16 ponies with differing insulin regulation. A) The major taxa were, on average, more abundant in the moderately insulin-dysregulated (MID) group relative to severely insulin-dysregulated (SID) and normally insulin-dysregulated (NIR) groups (y2, P = 0.0006). B) Phyla comprising the niche compared in A. The numerator count represents the MID group. C) The average abundance of taxa within niche y 10 (P = 0.006) changed, on average, in the SID and MID ponies relative to NIR ponies. In MID ponies the range of taxa abundance was greater than the other groups.

Table 1: Enteroinsular and liver function parameters of 16 ponies undergoing dietary change.

Variable	Unit of measure	NIR	MID	SID	P Value
n	Count	5	6	5	-
Age	Years	15.2 (7.6)	11.5 (6)	11.8 (5)	0.6
Baseline AST	U/L	275 [156]	331 [131]	306 [73]	0.7
Baseline Alkaline Phosphatase	U/L	243 (76)	213 (71)	185 (78)	0.5
Baseline GGT	U/L	21.8 (11)	17.3 (6)	21 (7)	0.6
Baseline Total Bilirubin	μ.mol/L	24 (6.6)[a]	18.7 (4)[ab]	14.5 (4)[b]	**0.04**
Post-prandial aGLP-1 hay	pM	8.3 [3.8][a]	14.5 [9.0][b]	8.8 [2.9][ab]	**0.01**
Post-prandial aGLP-1 pasture	pM	2.3 (2)[a]	13.1 (5.9)[b]	10.1 (4.0)[b]	**0.004**
Post-prandial insulin hay	μIU/mL	111 [157][a]	210 [128][a]	430 [154][b]	**0.001**
Post-prandial insulin pasture	μIU/mL	33 [20][a]	137 [162][b]	444 [160][b]	**0.001**

Key: Data are reported as mean (± s.d.) or median [IQR]. The ponies were grouped by their insulin response to an oral glucose test as normally insulin regulated (NIR), moderately insulin-dysregulated (MID), or severely insulin-dysregulated (SID). aGLP-1, active glucagon-like peptide-1; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase.

Table 2 Mean (± s.d.) alpha diversity in normally insulin-regulated (NIR), moderately insulin-dysregulated (MID), and severely insulin-dysregulated (SID) ponies when consuming a hay only diet or a combined pasture/hay (P/H) diet.

		Means			P Values		
Alpha	Diet	NIR	MID	SID	SI	Diet	Diet × SI
Shannons	Hay	9.0 (0.3)[a]	8.7 (0.3)[a]	8.9 (0.3)[a]	0.232	0.004	0.243
Index	P/H	8.8 (0.1)[b]	8.5 (0.5)[b]	8.4 (0.3)[b]			
Observed	Hay	1404 (336)	1468 (1678)	1500 (345)	0.652	0.629	0.41
OTU's	P/H	1382 (186)	1575 (511)	1260 (396)			
Faith's	Hay	88.2 (18.8)	94.3 (7.1)	87.0 (9.5)	0.594	0.493	0.772
PD	P/H	98.0 (16.7)	97.5 (27.9)	86.2 (23.5)			
Evenness	Hay	0.863 (0.009)[a]	0.825 (0.02)[b]	0.852 (0.01)[a]	**<0.001**	**<0.001**	0.737
	P/H	0.844 (0.004)[c]	0.800 (0.03)[d]	0.822 (0.01)[c]			

Key: SI, insulin responsiveness group; NIR, normal insulin regulation; MID, moderately insulin dysregulated; SID, severely insulin dysregulated; OTU, operational taxonomic unit; PD, phylogenetic diversity; P/H, combined pasture/hay diet.

Table 3 Mean (± s.d.) beta diversity measurements of the dissimilarity between a hay diet and a combined pasture/hay diet when fed to ponies with normal insulin regulation (NIR) and ponies with moderate (MID) or severe (SID) insulin dysregulation.

Beta	NIR	MID	SID	P Value
Jaccard	0.713 (0.03)	0.664 (0.03)	0.671 (0.06)	0.16
Bray-Curtis	0.619 (0.04)	0.541 (0.04)	0.574 (0.07)	0.07
Unweighted Uni-Frac	0.525 (0.04)	0.49 (0.07)	0.462 (0.06)	0.25
Weighted Uni-Frac	0.226 (0.01)	0.236 (0.03)	0.237 (0.05)	0.88

Table S1: Forage analyses. All results are reported on a dry matter basis unless otherwise stated. All units of % are g/100g equivalent.

	Units	LOD	Hay	Pasture
DM	%	0.5	91.2	41.7
Sward height	cm		-	7.6
Sward density	$g/10cm^2$		-	43
NDF	%	4.0	44	68
ADF	%	4.0	30	37.5
DMD	%	39	63	57.5
Crude protein	%	2.0	19.3	11.75
WSC	%	4.0	4.3	4.45
Starch	%	2	3	2.5
NSC	%		7.3	6.95

Key: LOD, limit of detection; DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; DMD, dry matter digestibility; WSC, water soluble carbohydrate; NSC, non-structural carbohydrate calculated as WSC + starch

Table S2: Summary of the simplical linear regression. The model R^2 value was 0.18.

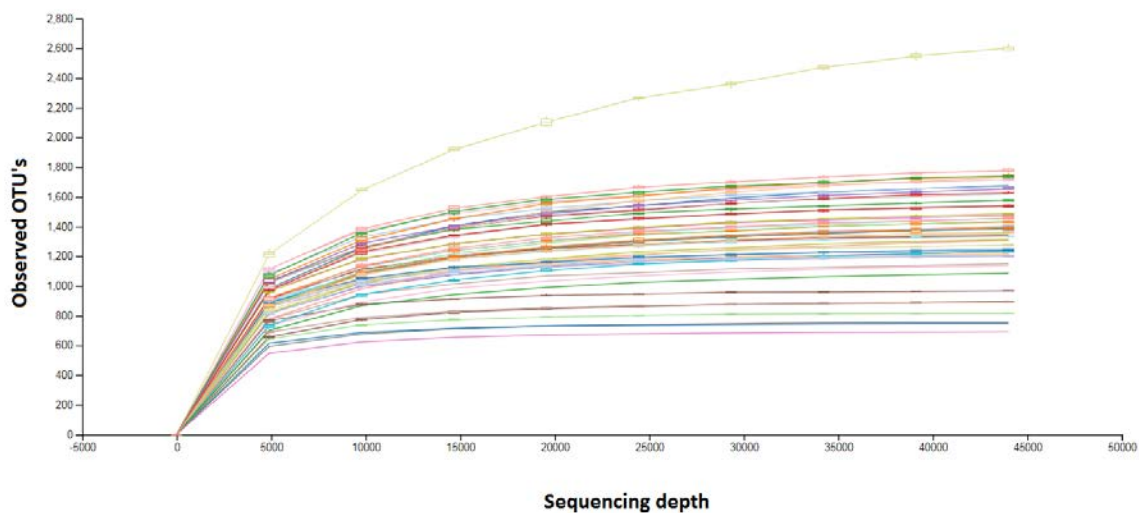| Treatment	R squared	% explanatory power
Intercept	0.09	8.74
Hay Diet	0.15	3.03
NIR	0.13	5.49
SID	0.12	5.19
Pony ID	0.13	4.85

Fig. S1: Rarefaction curve of the OTU's to a sampling depth of 44,000 reads indicated greater than 94% coverage of OTUs except for one pony with 86% coverage.
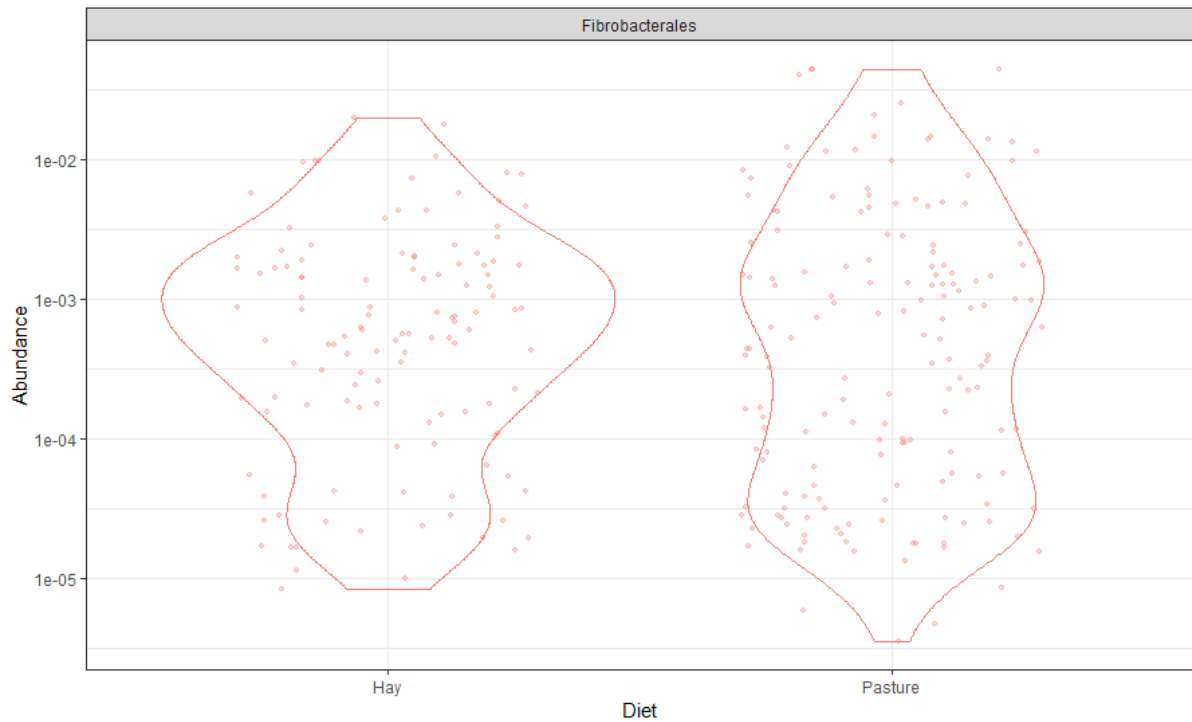
Fig. S2: Violin plot of the transformed relative abundances of order Fibrobacterales influenced by diet. On pasture, Fibrobacterales exhibit a bimodal distribution, in which two taxa predominate; one at higher and one at lower abundance. The hay diet is correlated with a shift in the population; a reduction of the taxa at lower abundance and an increase in the other.

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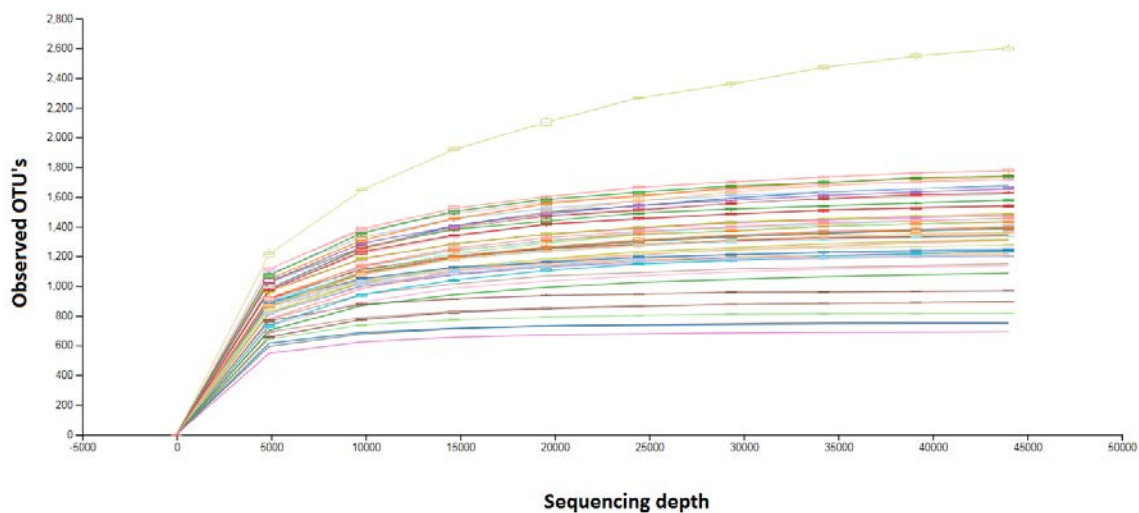| Treatment	R squared	% explanatory power
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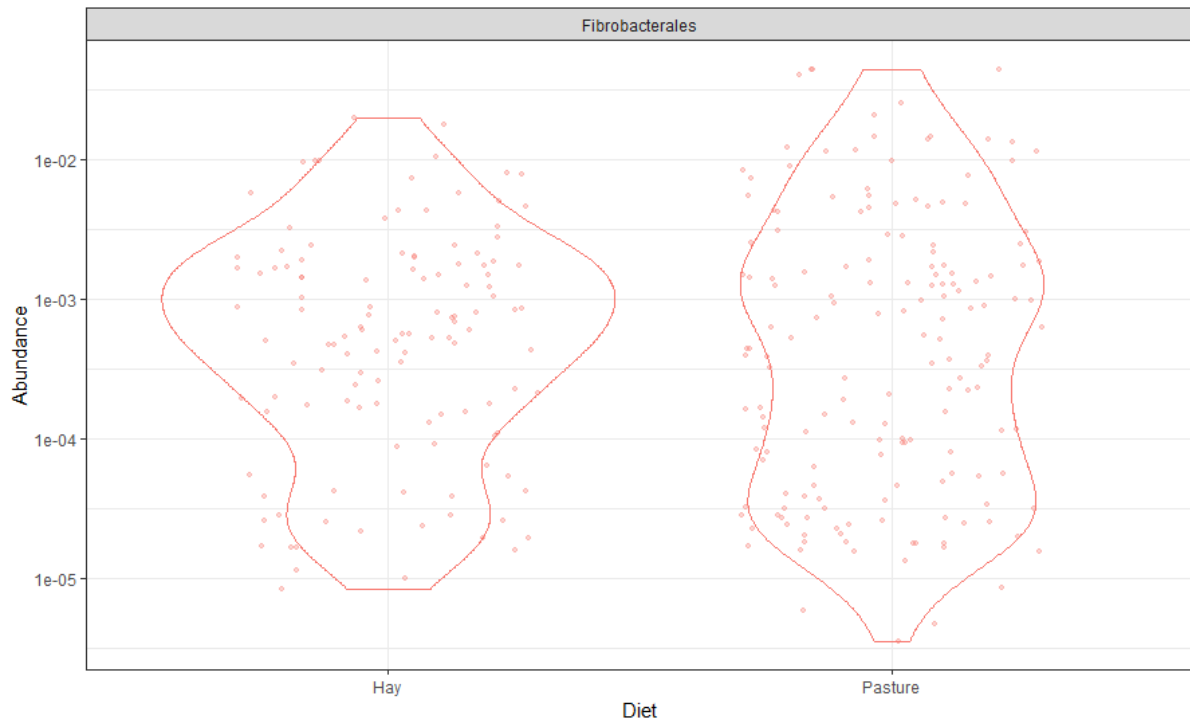
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