Journal of proteome research

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J. Proteome Res., **Just Accepted Manuscript** • DOI: 10.1021/acs.jproteome.7b00784 • Publication Date (Web): 24 Jan 2018

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Exploration of the fecal microbiota and biomarker discovery in equine grass sickness.

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Keywords: Equine dysautonomia, metabonomics, metabolites, nuclear magnetic resonance spectroscopy, DNA sequencing, horse.

ABSTRACT

Equine grass sickness (EGS) is a frequently fatal disease of horses, responsible for the death of 1-2% of the UK horse population annually. The etiology of this disease is currently uncharacterized although there is evidence it is associated with *Clostridium botulinum* neurotoxin in the gut. Prevention is currently not possible and ileal biopsy diagnosis is invasive. The aim of this study was to characterize the fecal microbiota and biofluid metabolic profiles of EGS horses, to further understand the mechanisms underlying this disease and identify metabolic biomarkers to aid in diagnosis. Urine, plasma and feces were collected from horses with EGS, matched controls (MC), and hospital controls (HC). Sequencing the16S rRNA gene of the fecal bacterial population of the study horses found a severe dysbiosis in EGS horses, with an increase in *Bacteroidetes* and a decrease in *Firmicutes* bacteria. Metabolic profiling by ^{1}H nuclear magnetic resonance (NMR) spectroscopy found EGS to be associated with the lower urinary excretion of hippurate and 4-cresyl sulfate and higher excretion of *O*-acetyl carnitine and trimethylamine-*N*-oxide (TMAO). The predictive ability of the complete urinary metabolic signature and using the four discriminatory urinary metabolites to classify horses by disease status was assessed using a second (test) set of horses. The urinary metabolome and a combination of the four candidate biomarkers showed promise in aiding the identification of horses with EGS. Characterization of the metabolic shifts associated with EGS offers the potential of a non-invasive test to aid pre-mortem diagnosis.

INTRODUCTION

Equine grass sickness (EGS) has been recognized for over 100 years, but its etiology remains unknown and no experimental model for the disease exists. EGS is associated with neuronal damage throughout the autonomic nervous system with changes seen most consistently in the wall of the ileum. Clinical signs observed in horses with EGS are: loss of appetite, ileus, inability to pass feces, large colon impaction, tachycardia and ptosis of the eyes. The etiological hypothesis best supported by evidence is that of toxicoinfection by *Clostridium botulinum*[1,2]. Both the organism and associated neurotoxins have been identified in the ingesta of diseased horses[3]. *C. botulinum* neurotoxins have been demonstrated to result in neuronal pathology *in vitro*, although recent evidence suggests that this pathology is not consistent with that observed in clinical disease cases[4]. It is unknown whether *C. botulinum* and/or its associated neurotoxins are present in the gastrointestinal tract of diseased horses due to ingestion or due to the *in situ* production of botulinum neurotoxin by resident, commensal bacteria. The composition of gut microbial communities and host metabolome have not been characterized in horses affected by EGS.

This study applied fecal bacterial and metabolic profiling approaches in parallel to determine the gut microbial and metabolic variation associated with EGS. The intestinal microbiota of the healthy horse and that of horses suffering from colitis has recently been defined using bacterial DNA sequencing [5–7]. Metabonomic analysis of biofluids allows for the capturing of products arising from the horse genome and are those derived from environmental sources such as the gut microbiota, diet and interactions between these and the host. Combining sequencing approaches to bacterial community profiling and metabonomics has been widely used to study the effects of disease in both human and mice, however their use in equine research is rare[8–10]. In this study we have used an integrated metabolic and bacterial community profiling approach, to assess the systemic effect of EGS on the horse. Our aim is to identity potential diagnostic markers for EGS and to inform hypotheses on disease aetiology.

EXPERIMENTAL SECTION

Study populations and sample collection

Urine, plasma and feces samples were collected over a two year period (2012-2013) from a total of 40 horses: 19 with EGS, 15 co-grazing, matched controls (MC) and 6 hospital control (HC) horses. Cases of EGS were sampled at the Philip Leverhulme Equine hospital, pre-mortem, before laparotomy and 16 were confirmed with histological analysis of an ileal biopsy[11] (Table S1). All urine was sampled free flowing, feces were collected during rectal examination during clinical investigation in ill horses and after defecation from healthy control horses. Selection criteria for matched control (MC) horses were: grazing in the same field at the same time as affected horses and matched on sex and age where possible. As EGS is highly seasonal in prevalence (most cases occur between March and June[2,12–17]) samples were acquired from MC horses as soon as possible after presentation of the EGS case; typically within two days. HC horses presented with clinical signs consistent with equine grass sickness but subsequent investigation, including histology of an ileal biopsy in some cases, led to an alternative diagnosis. This group was included to identify microbial and metabolic variation unique to EGS that was distinct from differences caused by gastrointestinal illness. Extra information on how all biofluids were handled can be found in Supplementary Methods S1.

To validate the statistical model created with data from EGS, MC and HC samples (calibration set), a comparison test set of new samples was collected during 2014. This comprised urine, blood and fecal samples from five histologically confirmed cases of acute grass sickness and ten healthy Thoroughbred racehorses (five in race training and fed on concentrates, five out of training and fed grass only). All samples were stored at -80°C until defrosted for analysis. (Information on all horses sampled can be found in Table S1 in the supporting information.)

Fecal bacteria DNA sequencing

Bacterial DNA was extracted from 31 faecal samples (13 EGS, 5 HC and 13 MC from the calibration set) amplified using PCR of the V4 region of the 16S rRNA gene, before being analyzed on the MiSeq Illumina platform at the Centre for Genomic Research, Liverpool. Detailed methodology for the preparation of feces for bacterial DNA sequencing is provided in supporting information (Supplementary Methods S2).

Filtering and processing of the raw reads was performed using QIIME version 1.6.0[18]. Sequences were clustered into OTUs at the level of 96% similarity by reference-based picking with the USEARCH programme in QIIME version 1.8.0. OTUs were classified using the Ribosomal Database Project (RDP) classifier 2.0[19]. Sequences from each OTU were aligned using PyNast[18] and a phylogenetic tree was built using 'Fasttree'[20]. Alpha rarefaction analysis was performed using the script 'multiple_rarefactions.py' and beta diversity analysis using 'jackknifed_beta_diversity.py'. The difference between the alpha rarefaction values of EGS horses compared to control horses was assessed using a Kruskal-Wallis test. Taxa summary plots were generated using 'summarize_taxa_through_plots.py' at phyla and class levels of taxonomic classification. Linear discriminant analysis on effect size (LEfSe)[21] was used to identify bacterial taxa that were significantly different between the faeces of the three groups of horses sampled.

Sequencing files were submitted the European Nucleotide Archive and can be found open access in study PRJEV11642.

^1H NMR spectroscopic analysis of biofluids

All urine samples were prepared for ^{1}H NMR analysis by adding 200 µl phosphate buffer (pH 7.4; 100% D_2O) containing 1 mM of the internal standard 3-trimethylsilyl-1-[2,2,3,3-2H4] propionate (TSP) to 400 µl of each sample. All plasma samples were prepared by adding 450 µl saline solution (100% D_2O) to 100 µl of each sample. Each fecal sample (30 mg) was combined with 1.2 ml phosphate buffer prior to lysis by bead beating for 10 minutes. All fecal samples were then centrifuged for 10 minutes at 16,000 *g*, the pellet discarded and the supernatant transferred. This was repeated on two further occasions to ensure the removal of all solid particles in the samples. All biofluid samples were transferred to 5 mm NMR tubes before ^{1}H NMR analysis. Spectroscopic analysis of all samples was carried out on a 700 MHz Bruker NMR spectrometer equipped with a cryo-probe. Standard one-dimensional ^{1}H NMR spectra were acquired for all urine and fecal samples with water peak suppression using a standard pulse sequence. Water suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin echo spectra were acquired for plasma samples. CPMG experiment was used to measure plasma metabolic profiles as this attenuates broad signals arising from macromolecules found in plasma, which may mask peaks from lower weight molecules. For all samples, 8 dummy scans were followed by 256 scans and collected in 64K data points. Chemical shifts in the urine and fecal spectra were referenced to the TSP singlet at δ 0.0. A spectral width of 20 ppm and an acquisition time per scan of 3.12 seconds was used.

^{1}H NMR spectra were phased and baseline corrected in Topspin 3.0 before alignment and normalization (total intensity method) was performed in the Matlab environment (R2014a, Mathworks) with in-house scripts. Multivariate statistical analysis was applied to compare profiles of the same biofluid between study groups in the calibration set of samples. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) models were constructed for pair-wise comparisons of the study groups for each biofluid to identify metabolic variation between the classes. The predictive performance (Q^2Y) of the OPLS-DA models were measured and only those considered significant after permutation testing (1000 permutations; $p < 0.01$) were used. Metabolites were assigned to peaks using the database of equine metabolites found in Escalona e*t al.* 2015[22] and Chenomx (NMR suite 8.2).

Predictive model for potential biomarkers

To assess the diagnostic potential of the metabolites identified from the calibration set, these discriminatory metabolic features were used to predict the class membership of samples from the test set. NMR profiles from the test set were centred to the mean of the calibration set and divided by the standard deviation of the calibration set to mirror the spectra processing used to construct the original model. Using the prediction function in the OPLS-DA model class membership (where 1 = disease and 0 = control) was predicted for each test set sample based upon both the complete urinary metabolic profile and the profile created using four candidate urinary metabolite biomarkers (4-cresyl sulfate, *O*-acetyl carnitine, hippurate and TMAO) for EGS. The concentration of a single urinary metabolite to predict disease state was assessed by building linear regression models with the concentration of the metabolite in the calibration set and using these to predict disease state for the test set with the concentrations of that metabolite in the test set.

RESULTS AND DISCUSSION

EGS is associated with reduced diversity of fecal microbiota.

Miseq analysis of bacterial DNA extracted from equine feces resulted in a total read count number across all samples of 10,085,808 (minimum of 173,469 and maximum of 595,973). The mean number of counts per samples run was 373,548 and the median was 386,902, with a standard deviation of 92,907. To explore the differences in fecal bacterial community diversity between groups, within-sample bacterial diversity (alpha diversity, measured as observed species) was compared. Figure 1 shows the range of diversity measures for each group of horses at a sampling depth of 200,000 sequences per sample. Fecal samples from EGS horses had the lowest diversity (1,695 ± 720 observed species), with MC horses being highest (3,361 ± 474) and HC horses having an intermediate bacterial diversity (3,148 ± 504). A Kruskal-Wallis test showed that the difference in the number of observed species between EGS horses and the two control groups was statistically significant ($p < 0.01$). A second measure of alpha diversity, Chao1, was calculated showing significantly lower diversity in EGS horses compared to the two control groups (Kruskall Wallis test; $p < 0.01$). Beta diversity analysis showed that the bacterial diversity within the feces of horses with grass sickness is different and has greater variation than the two control groups (Figure S1). As the fecal microbiota has been shown to be representative of the colonic microbiota[6,23] this indicates that EGS is associated with a significant reduction in the diversity of colonic microbiota of the colon. Reduced bacterial diversity is generally associated with poor health and has been observed previously with equine colitis and recurrent *Clostridium difficile* infection in humans[5,24]. Reduced diversity can impair the competitive exclusion capacity of the gut microbiota and could allow potential bacterial pathogens to establish, such as *C. botulinum*.

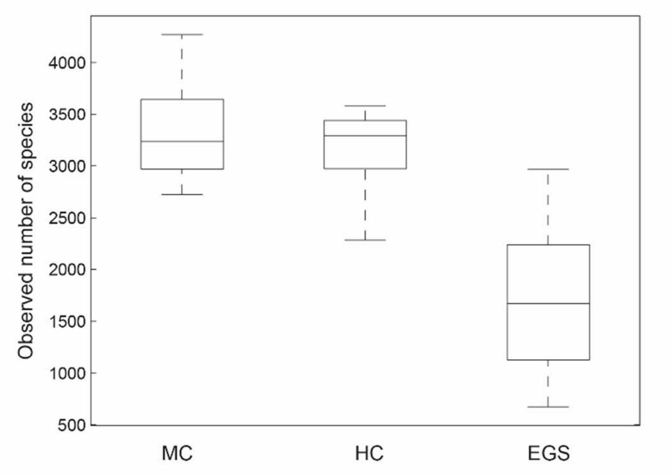

Figure 1. Alpha diversity levels, measured as observed number of species, for the fecal bacterial communities of horses with grass sickness (EGS), hospital controls (HC) and matched controls (MC) at 200,000 sequences per sample.

EGS is associated with bacterial dysbiosis.

To explore the differences in fecal bacterial communities the relative abundance of bacterial orders in each sample was visualized as percentage bars for the total reads. Figure 2A shows phylum level average proportion; a higher relative abundance of *Bacteroidetes* and *Proteobacteria* was observed in EGS horses compared to the control horses, while the relative abundance of *Firmicutes* and *Verrucomicrobia* was lower. A similar pattern can be seen in the percentage reads plots at class level; an increase in the relative abundance of *Bacteroidia* and *Gammaproteobacteria* and a decrease in the relative abundance of *Clostridia* and *Verruco-5* in the faeces of EGS horses compared to the two control groups (see Figure S2). Differences were also observed at order-level (Figure 2B); EGS horses had a higher relative abundance of *Bacteroidales, Fusobacteriales* and *Enterobacteriales* compared to the fecal microbiota of MC and HC horses. Conversely, *Clostridiales*, *Spirochaetales* and *Fibrobacterales* were lower in relative abundance. These changes were consistent with those reported in the faecal microbiota of horses with colitis; where an increase in the relative abundance of *Bacteroidetes* and a decrease in *Firmicutes* have previously been reported[5]. These bacterial modulations have been observed in humans with Parkinson's disease, inflammatory bowel disease and irritable bowel syndrome[25–28]. Interestingly, biomarkers of systemic inflammation have also been seen in EGS[29]. A chemical trigger or genetic predisposition is hypothesized to initiate gut microbial-associated inflammation in humans[30]. EGS often occurs after a change in pasture/diet and only to specific horses, suggesting that both genetic and environmental factors may contribute to the disease. Inflammation in EGS horses may be an immunological response to a compositional shift in the gut microbiota towards an inflammatory population. However, it is also plausible that bacterial alterations could result from changes in the gastrointestinal environment following the onset of EGS[31].

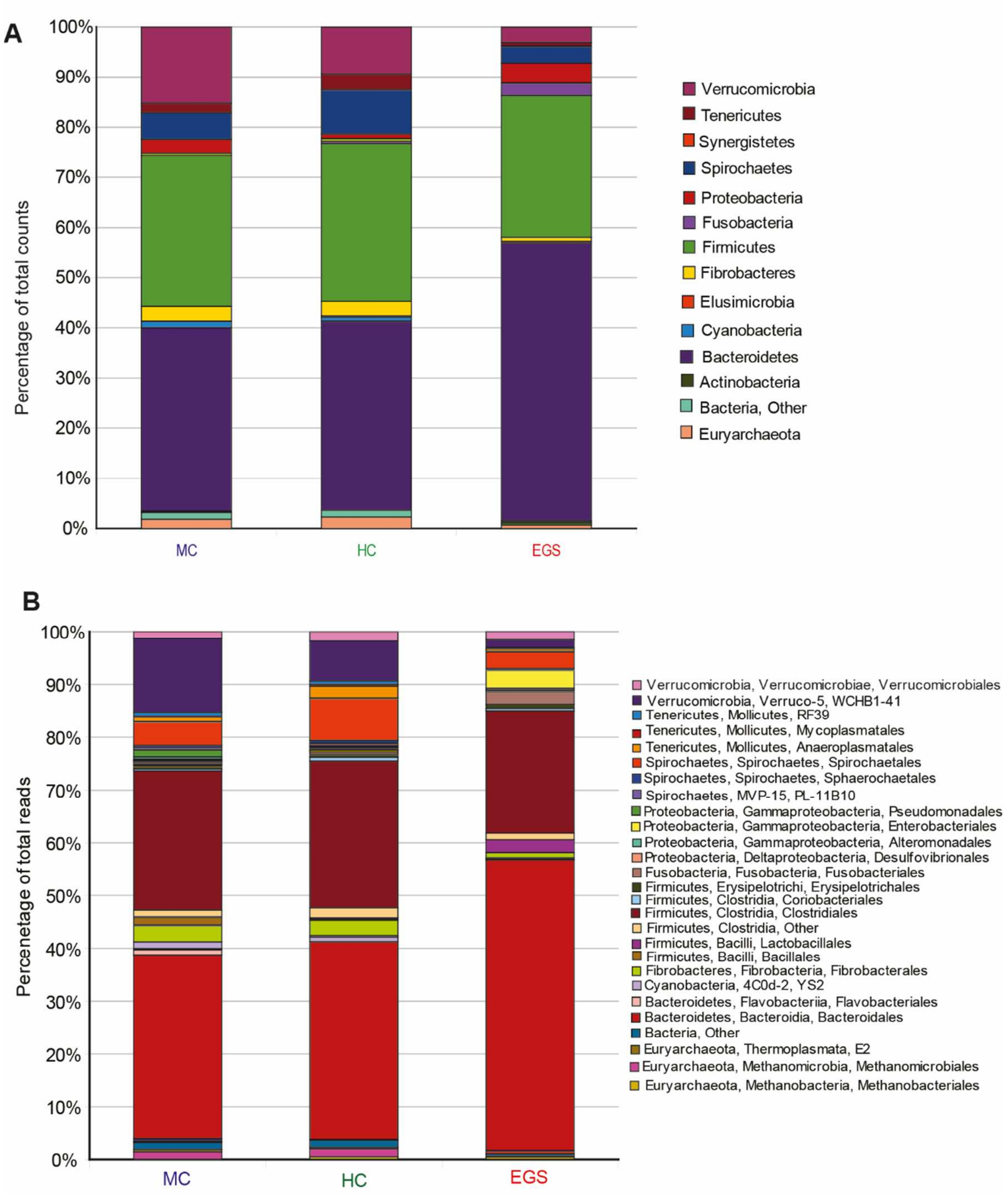

Figure 2. Percentages of total identifiable reads for prominent bacterial (A) phyla and (B) orders identified following sequencing of fecal bacterial DNA. Each bar is an average for the three groups of horses sampled. The key for (A) notes the dominant bacterial phyla represented by each coloured bar and for (B) depicts the dominant bacterial class, preceded by this order's class and phyla. MC, matched control horses; HC, hospital control horses; EGS, equine grass sickness horses.

LEfSe analysis was performed to identify significant differences between bacterial taxa abundance in each group (Figure 3). 82 operational taxonomic units (OTUs) were identified as significantly different between the three groups, 20 of which were potential bacterial markers for EGS. These candidate markers belong to five bacterial classes: *Clostridia* (45% of the differential OTUs belong to this order), *Gammaproteobacteria* (15%), *Fusobacteria* (15%), *Bacteroidia* (15%)*,* and *Deltaproteobacteria* (10%). Within these bacterial groups there were several that belong to the phyla *Fusobacteria*, the order *Enterobacteriales*, the family *Enterobacteriaceae* and the genus *Veillonella. Fusobacteria*, a phylum of Gram negative bacilli were increased in abundance in the feces of EGS horses. As these bacterial groups are present at multiple taxonomic levels; the association between these groups and EGS is more robust. These bacteria are present in horses with diarrhea[32] and are higher in abundance in horses with colitis[5] and humans with Crohn's disease[33]. An increase in *Enterobacteriales* and its bacterial family *Enterobacteriaceae*, lactate producing bacteria, may contribute to the increase in lactic acid which is often associated with horses suffering from intestinal disease[34]. *Enterobacteriaceae* have not been previously associated with equine disease but have been linked with Crohn's disease and chronic HIV infection in humans[35,36].

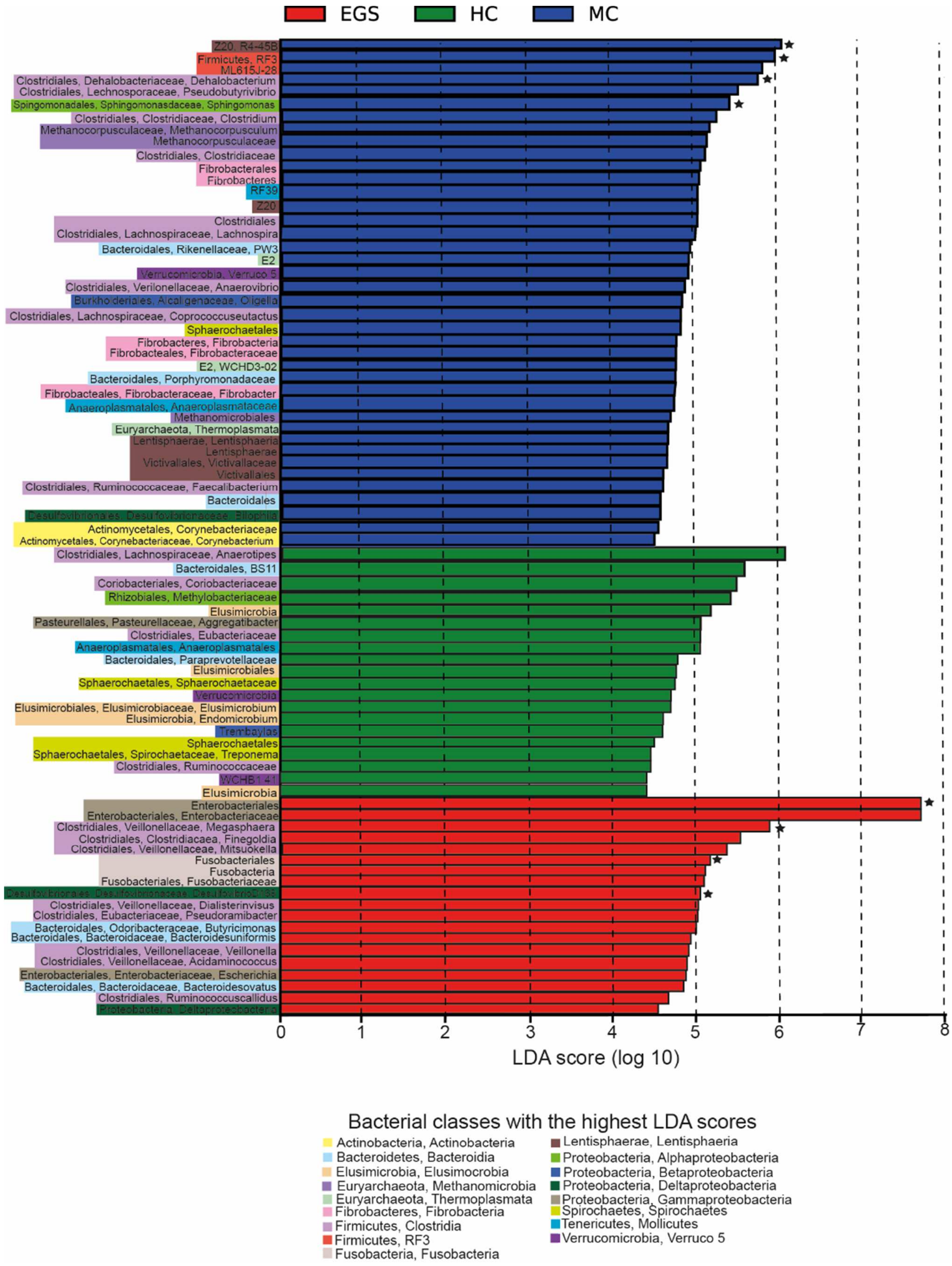
EGS
HC
MC
Z20, R4-45B
Firmicutes, RF3
ML615J-28
Clostridiales, Dehalobacteriaceae, Dehalobacterium
Clostridiales, Lechnosporaceae, Pseudobutyrivibrio
Spingomonadales, Sphingomonasdaceae, Sphingomonas
Clostridiales, Clostridiaceae, Clostridium
Methanocorpusculaceae, Methanocorpusculum
Methanocorpusculaceae
Clostridiales, Clostridiaceae
Fibrobacterales
Fibrobacteres
RF39
Z20
Clostridiales
Clostridiales, Lachnospiraceae, Lachnospira
Bacteroidales, Rikenellaceae, PW3
E2
Verrucomicrobia, Verruco 5
Clostridiales, Verilonellaceae, Anaerovibrio
Burkholderiales, Alcaligenaceae, Oligella
Clostridiales, Lachnospiraceae, Coprococcuseutactus
Sphaerochaetales
Fibrobacteres, Fibrobacteria
Fibrobacteales, Fibrobacteraceae
E2, WCHD3-02
Bacteroidales, Porphyromonadaceae
Fibrobacteales, Fibrobacteraceae, Fibrobacter
Anaeroplasmatales, Anaeroplasmataceae
Methanomicrobiales
Euryarchaeota, Thermoplasmata
Lentisphaerae, Lentisphaeria
Lentisphaerae
Victivallales, Victivallaceae
Victivallales
Clostridiales, Ruminococcaceae, Faecalibacterium
Bacteroidales
Desulfovibrionales, Desulfovibrionaceae, Bilophila
Actinomycetales, Corynebacteriaceae
Actinomycetales, Corynebacteriaceae, Corynebacterium
Clostridiales, Lachnospiraceae, Anaerotipes
Bacteroidales, BS11
Coriobacteriales, Coriobacteriaceae
Rhizobiales, Methylobacteriaceae
Elusimicrobia
Pasteurellales, Pasteurellaceae, Aggregatibacter
Clostridiales, Eubacteriaceae
Anaeroplasmatales, Anaeroplasmatales
Bacteroidales, Paraprevotellaceae
Elusimicrobiales
Sphaerochaetales, Sphaerochaetaceae
Verrucomicrobia
Elusimicrobiales, Elusimicrobiaceae, Elusimicrobium
Elusimicrobia, Endomicrobium
Trembaylas
Sphaerochaetales
Sphaerochaetales, Spirochaetaceae, Treponema
Clostridiales, Ruminococcaceae
WCHB1 41
Elusimicrobia
Enterobacteriales
Enterobacteriales, Enterobacteriaceae
Clostridiales, Veillonellaceae, Megasphaera
Clostridiales, Clostridiacaea, Finegoldia
Clostridiales, Veillonellaceae, Mitsuokella
Fusobacteriales
Fusobacteria
Fusobacteriales, Fusobacteriaceae
Desulfovibrionales, Desulfovibrionaceae, DesulfovibrioD168
Clostridiales, Veillonellaceae, Dialisterinvisus
Clostridiales, Eubacteriaceae, Pseudoramibacter
Bacteroidales, Odoribacteraceae, Butyricimonas
Bacteroidales, Bacteroidaceae, Bacteroidesuniformis
Clostridiales, Veillonellaceae, Veillonella
Clostridiales, Veillonellaceae, Acidaminococcus
Enterobacteriales, Enterobacteriaceae, Escherichia
Bacteroidales, Bacteroidaceae, Bacteroidesovatus
Clostridiales, Ruminococcuscallidus
Proteobacteria, Deltaproteobacteria
0
1
2
3
4
5
6
7
8
LDA score (log 10)
Bacterial classes with the highest LDA scores
Actinobacteria, Actinobacteria
Bacteroidetes, Bacteroidia
Elusimicrobia, Elusimocrobia
Euryarchaeota, Methanomicrobia
Euryarchaeota, Thermoplasmata
Fibrobacteres, Fibrobacteria
Firmicutes, Clostridia
Firmicutes, RF3
Fusobacteria, Fusobacteria
Lentisphaerae, Lentisphaeria
Proteobacteria, Alphaproteobacteria
Proteobacteria, Betaproteobacteria
Proteobacteria, Deltaproteobacteria
Proteobacteria, Gammaproteobacteria
Spirochaetes, Spirochaetes
Tenericutes, Mollicutes
Verrucomicrobia, Verruco 5

Figure 3. OTUs that differ between the fecal samples of equine grass sickness (EGS), hospital control (HC) and matched control (MC) horses from LEfSe analysis. Bars on the graph indicate linear discriminant analysis (LDA) scores and asterisks indicate those OTUs whose relative abundance is shown in Fig. 4.

Four representative OTUs were selected from those enriched in EGS horses and four that were enriched in MC horses. *Enterobacteriales*, *Megashpaera*, *Fusobacteriales* and *DesulfovibrioD168* were chose to represent OTUs with higher counts in EGS horses and *RF4-45B*, *RF3*, *Dehalobacterium* and *Sphingomonas* represented OTUs with higher counts in MC horses. The relative abundance of these representative OTUs are presented in Figure 4. The four OTUs increased in relative abundance in MC horses were not seen to increase in all individuals, and could also be found at a high relative abundance in some EGS and HC horses (Figure 4A). The four OTUs identified as significantly higher in relative abundance in EGS horses were not ubiquitously increased in all EGS horses sampled (Figure 4B). This suggests that EGS may be associated with a variable bacterial signature capable of evoking a host response. Notably, OTUs with a higher relative abundance in EGS horses were universally absent from all MC horses.

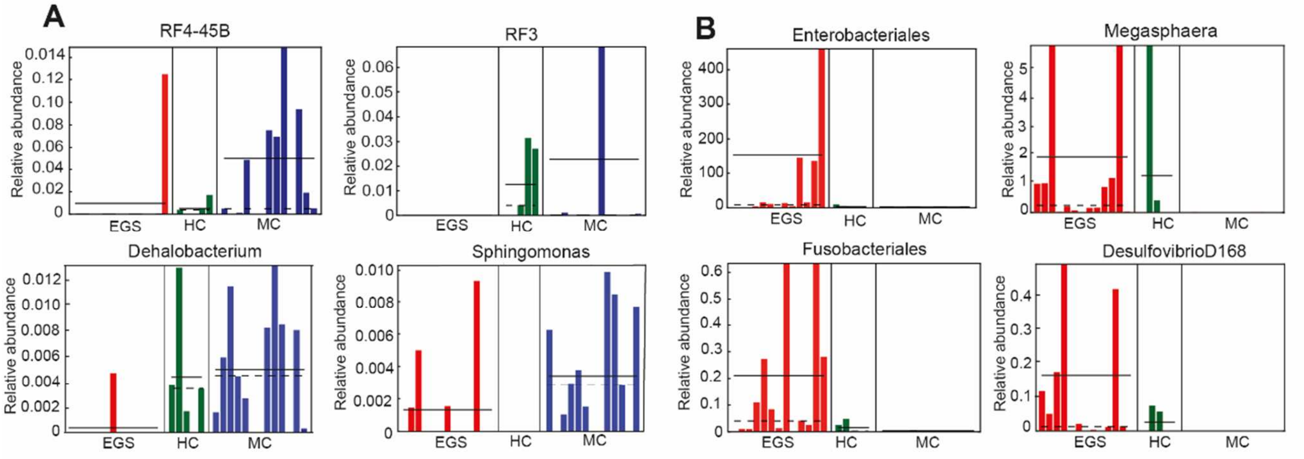

Figure 4. (A) The relative abundance of four representative OTUs with high LDA scores in the fecal microbiota of matched control horses (n = 13) and (B) the relative abundance four representative OTUs with high LDA scores in horses with equine grass sickness (n = 12). OTUs chosen from Fig. 3 to represent differences within the two groups of horses on the basis of high LDA scores and belonging to different taxonomic groups. Bars in each plot of (A) and (B)

correspond to individual horses and colours indicate the group the horses belong to. The solid line corresponds to the mean of each group and the dashed line is the median.

Epidemiological data suggests an association between *C. botulinum* and EGS[1,2]. Our analysis identified a lower relative abundance of the bacterial family *Clostridia*, in which *C. botulinum* resides, in the fecal microbiota of EGS compared to MC. As such, this study provides no evidence to support the *C. botulinum* hypothesis but nor does it preclude its involvement. It is possible that an unknown environmental trigger could initiate autochthonous *C. botulinum* to produce the neurotoxin or that horizontal gene transfer could occur[37], leading to botulinum neurotoxicosis.

Gut microbial-mammalian co-metabolism is perturbed by EGS.

Metabolic profiles were captured from urine, plasma and feces obtained from all horses sampled. An example of ^{1}H NMR spectra obtain from each type of equine biofluids can be seen in Figure S3. A multivariate OPLS-DA model with strong discriminative ability ($Q^2Y = 0.82$) was obtained comparing the urinary metabolic profiles of EGS and MC horses (Figure 5). From this analysis, EGS horses were found to excrete lower amounts hippurate and 4-cresol, both known gut microbial-host co-metabolites compared to MC horses. Hippurate is a carboxylic acid formed from the gut bacterial metabolism of benzyl alcohol to benzoate, which is absorbed from the gut and subsequently metabolized by the horse to form hippurate. This metabolite has previously been detected in horse urine[22] and reduced excretion reflects changes in the metabolic output of the gut microbiota. A reduced hippurate excretion been associated with human inflammatory bowel diseases[38–41] and blood hippurate has recently been associated with bacterial community diversity within human faeces[42]. 4-cresyl sulfate excretion was lower in EGS horses compared to

MC. This metabolite is formed from the bacterial metabolism of tyrosine to 4-cresol, which is absorbed and sulfated in the host to produce 4-cresyl sulfate. As the Clostridium genus are known producers of cresol[43], the lower excretion of 4-cresyl sulfate is consistent with the lower abundance of bacteria from this genus in EGS horses.

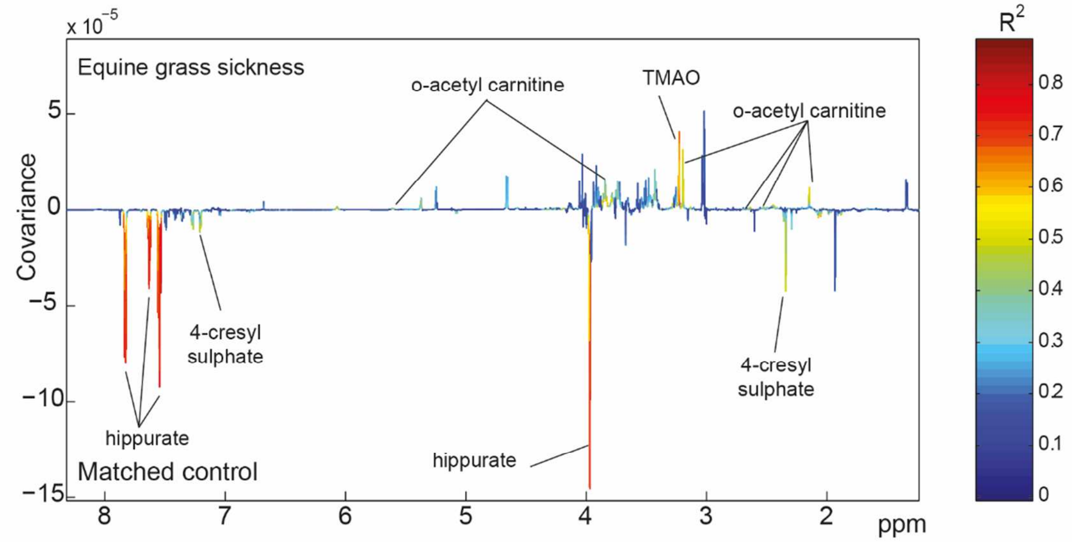

Figure 5. Coefficients plot for the OPLS-DA model comparing the urinary metabolic profiles of horses with grass sickness and their matched controls ($Q^2Y = 0.82$). The model was constructed with one orthogonal component.

Horses with EGS were found to excrete higher amounts of *O*-acetyl carnitine and trimethylamine-*N*-oxide (TMAO) in EGS urine compared with MC urine. TMAO is produced when choline is metabolized by bacteria to trimethylamine and subsequently oxidised in the host's liver to TMAO[44]. *Proteobacteria* and *Verrucomicrobia* were more abundant in the feces of EGS horses compared to MC and are known to metabolize choline. *O*-acetyl carnitine excretion is synthesized from acyl-CoA and carnitine and is known to have neuroprotective properties in small and large animal models[45,46]. *O*-acetyl carnitine is previously unreported in the urine of horses, so the significance of its association with EGS is unknown.

Perturbations to the plasma and fecal metabolome associated with EGS can be found in supporting information (Figures S4 and S5), however the predictive ability of these models was lower than that of urine (Q^2Y = 0.40 and 0.62 respectively). Glucose and glycerol were observed to be increased in the plasma of EGS horses and 1-methyl histidine, *p*-hydroxy-phenylacetate, valine, citrate, glutamine, acetate and β-aminoisobutyrate were decreased in the plasma of EGS horses when compared to MC horses. A reduction in amino acids in the plasma of horses with EGS has previously been reported[47]. This may be linked to a reduction in gut motility caused by EGS which could potentially prevent dietary amino acids from reaching the colon and being absorbed. Increased plasma glucose is consistent with previous reports[48] and is a non-specific finding in horses with intestinal disease[49–52]. Elevations in both plasma glucose and glycerol is associated with an increase in the sympathetic output of horses with intestinal disease[51], which is consistent with stimulation of the sympathetic nervous system seen in horses with EGS[53]. Fecal samples from EGS horses contained higher amounts of phenylacetylglycine (PAG) and glycerol than controls, whereas methanol concentration was lower. OPLS-DA pairwise comparisons between EGS and HC were also carried out but all models built had poor predictive ability (Q^2Y < 0.1). This may be due to the wide range of other intestinal diseases included in the HC group, and therefore a wider range of metabonomic differences in biofluids. EGS predominately affects the gastrointestinal system and potentially the gastrointestinal intestinal bacterial environment; this may explain why the most significant changes identified in the urinary metabolic profile are associated with bacteria-horse co-metabolites.

Biomarker signature of EGS is able to predict class membership in an independent study population.

The OPLS-DA model constructed on the urinary metabolic profiles of the horses (calibration set) was used to predict the class membership (control vs. EGS) of urine samples collected from an independent set of horses (test set; *n* = 15, Figure 6). The complete urinary metabolic phenotype was a good discriminator for horses with EGS compared to healthy horses (Figure 6A) and the ROC curve for this model showed good sensitivity and specificity (Figure S6A). The predicted class value of all samples from test set EGS samples were greater than 0 (0.61 ± 0.39), where the true value was 1, and the predicted class value for all but one of the healthy horses was less than 0 (-0.63 ± 0.46) for all but one of the healthy control horses (true value was 1). The use of single urinary metabolites was assessed using simple linear regression models, however there were large overlaps between boxplots of predicted class values, due to the inter-individual variation of the metabolite concentrations between horses (Figure S7). The use of single metabolite concentrations was deemed not to be useful. Plasma and fecal metabolic profiles did not correctly predict disease class of the test set horses and separation between the predicted class values for the two groups was not seen (Figure S8).

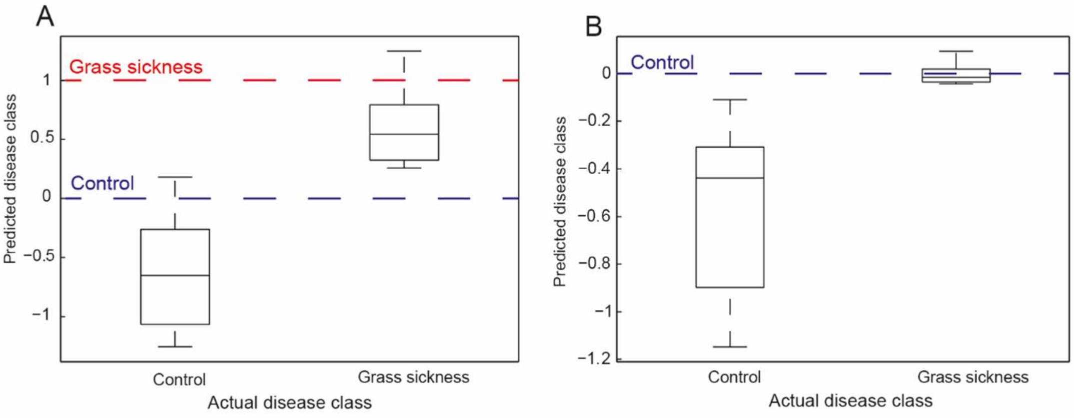

Figure 6. Box plots showing the predicted class values for the independent test set of urine samples calculated by the predictive OPLS-DA model built using the calibration set of urine samples. These were constructed using (A) urinary metabolic profiles and (B) concentrations of urinary hippurate, 4-cresyl sulfate, *O*-acetyl carnitine and TMAO. The dashed lines indicate the precise class values for the two groups, where 0 represents controls and 1 represents grass sickness.

The whole urinary metabolome was good at discriminating between EGS and control horses, but to use the complete equine metabolome diagnostically would be impractical in the field. As such, the model based upon the urinary metabolic profiles was refined to utilize the four metabolites that were found to be significantly different between EGS and MC horses ($p < 0.05$, $R^2 = 0.164$); hippurate, 4-cresyl sulfate, TMAO and *O*-acetyl carnitine. The predictive ability of this panel was weaker than that of the complete urinary metabolic profile. The boxplot in Figure 6B shows that based on this refined model the two groups could be discriminated, with no overlap between the predicted values of the two groups. The predicted class values for all control horses were less than 0, while the predicted class values for the EGS horses fell between -0.04 and 0.09. This model had good specificity and sensitivity (Figure S6B) which suggests that the refined model could distinguish between healthy and EGS horses (control < -0.1 and EGS > -0.05). We acknowledge the small size of the test set used for the predictive model. The use of Thoroughbred racehorses for the control group, rather than co-grazing, matched controls may influence the predictive performance of the test. However, it is not possible to state whether this difference in control selection improved or reduced the predictive performance of the model. This is particularly true given that the test set control horses were fed a variety of diets compared to the calibration set. Such dietary variation is more reflective of “real-world” situations. Nonetheless, this small test set demonstrates the potential utility of urinary biomarkers in a rapid, point-of-care diagnostic test for use in the field.

CONCLUSIONS

EGS was found to have a profound impact on the community structure and function of the gut microbiota with downstream effects on the urinary metabolic signatures of horses. Our study demonstrates that EGS is associated with a reduction in the diversity of the fecal microbiota, alterations to its community structure and bacterial-host metabolic interactions. We have demonstrated that collectively urinary 4-cresyl-sulfate, hippurate, TMAO and *O*-acetyl carnitine have the potential to discriminate EGS horses from others. Given the lack of non-invasive diagnostic tests for EGS this work offers promising diagnostic targets for future validation.

ASSOCIATED CONTENT

Supporting information

The following files are available free of charge at ACS website http://pubs.acs.org:

Table S1. Information on all horses sampled.

Supplementary Methods S1. Detailed methodology of horse biofluid sample acquisition.

Supplementary Methods S2. Detailed methodology for the preparation of feces for bacterial DNA sequencing

Figure S1. PCoA plots showing unweighted and weighted beta diversity.

Figure S2. Percentage of total reads belonging to phyla, class, order and family.

Figure S3. Example urinary metabolic spectra from one horse from each of the three study groups.

Figure S4. Coefficients plot for the OPLS-DA model comparing the plasma metabolic profiles of horses with grass sickness and their matched controls.

Figure S5. Coefficients plot for the OPLS-DA model comparing the fecal metabolic profiles of horses with grass sickness and their matched controls.

Figure S6. ROC curves plotting the sentivity and specificity of the urinary metabolic spectra and the concentration of the four canditate biomarkers.

Figure S7. Boxplots showing predicted disease class using linear regression models of the individual concentrations of the four urine metabolites to discriminate EGS horses from healthy controls.

Figure S8. Box plots showing the predicted class values for the independent test set of plasma and feces by the predictive OPLS-DA model.

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Notes

The authors declare no competing financial interest.

Author Contributions

J. Leng contributed to all sections, C. Proudman contributed to study design, study execution and preparation of manuscript. A. Darby and F. Blow contributed to data analysis and interpretation and preparation of manuscript. N. Townsend and A. Miller contributed to study execution. J Swann contributed to study design, data analysis and interpretation and preparation of manuscript. All authors gave their final approval on the manuscript.

ACKNOWLEDGEMENTS

We acknowledge help from the staff at Philip Leverhulme Equine hospital whom helped collect samples from some of the horses in this study. Additional samples were also supplied by Dr Tim Mair from Bell Equine Veterinary Clinic and Mr Seamus Miller from Rufford vets. This work was funded by The Equine Grass Sickness Fund and the University of Reading.

ABBREVIATIONS

EGS, Equine grass sickness; MC, Matched control; HC, Hospital control; rRNA, Ribosomal ribonucleic acid; ^{1}H NMR, Proton nuclear magnetic resonance; TMAO, Trimethylamine-*N*-oxide; DNA, Deoxyribonucleic acid; PCR, Polymerase chain reaction; OTU, Operational taxonomic unit; QIIME, Quantitative insights into microbial ecology; LEfSe, Linear discriminate analysis effect size; TSP, 3-trimethylsilyl-1-[2,2,3,3-2H4] propionate; PAG, Phenylacetylglycine

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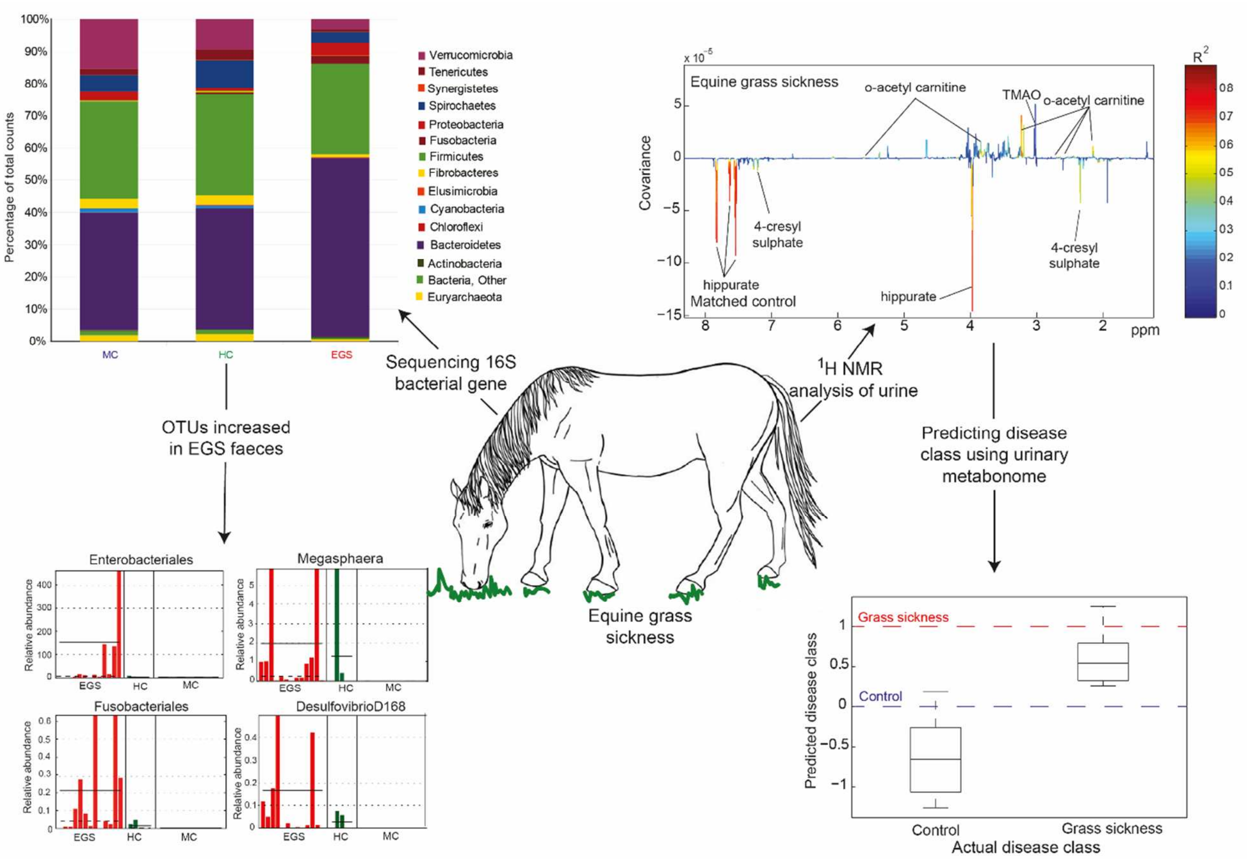
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