Journal of proteome research

Article

Cite This: J. Proteome Res. 2018, 17, 1120−1128

pubs.acs.org/jpr

Exploration of the Fecal Microbiota and Biomarker Discovery in Equine Grass Sickness

Joy Leng,*[,†] Chris Proudman,[†] Alistair Darby,[‡] Frances Blow,[‡] Neil Townsend,[§] Andrew Miller,[§] and Jonathan Swann[∥]

[†]School of Veterinary Medicine, Vet School Main Building, Daphne Jackson Road, University of Surrey, Guildford, Surrey GU2 7AL, United Kingdom

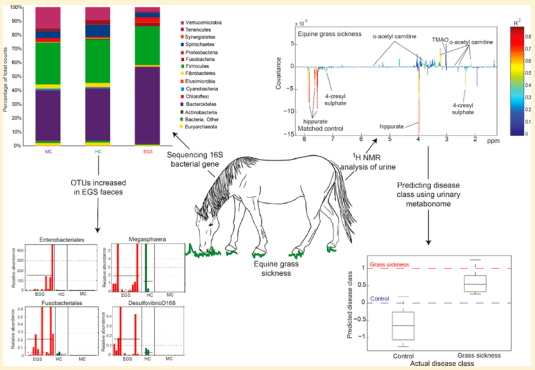
[‡]School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, United Kingdom

[§]Philip Leverhulme Equine Hospital, University of Liverpool, Leahurst Campus, Chester High Road, Wirral CH64 7TE, United Kingdom

[∥]Division of Computational and Systems Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London SW7 2AZ, United Kingdom

Supporting Information

ABSTRACT: Equine grass sickness (EGS) is a frequently fatal disease of horses, responsible for the death of 1 to 2% of the U.K. 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 to identify metabolic biomarkers to aid in diagnosis. Urine, plasma, and feces were collected from horses with EGS, matched controls, and hospital controls. 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 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. 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 noninvasive test to aid premortem diagnosis.



KEYWORDS: *equine dysautonomia, metabonomics, metabolites, nuclear magnetic resonance spectroscopy, DNA sequencing, horse*

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* 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 those derived from environmental sources such as the gut microbiota, diet, and interactions between these and the host. Combining

Received: October 31, 2017
Published: January 24, 2018

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

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 etiology.

EXPERIMENTAL SECTION

Study Populations and Sample Collection

Urine, plasma, and feces samples were collected over a 2 year period (2012−2013) from a total of 40 horses: 19 with EGS; 15 cograzing, matched controls (MC); and 6 hospital control (HC) horses. Cases of EGS were sampled at the Philip Leverhulme Equine hospital, premortem, before laparotomy, and 16 were confirmed with histological analysis of an ileal biopsy[11] (Table S1). All urine was sampled free-flowing, and 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. Because 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 2 days. HC horses presented with clinical signs consistent with EGS, 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 fecal 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 the 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 program 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 with 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 feces of the three groups of horses sampled. Sequencing files were submitted the European Nucleotide Archive and can be found open access in study PRJEV11642.

^{1}H NMR Spectroscopic Analysis of Biofluids

All urine samples were prepared for ^{1}H NMR analysis by adding 200 μL of phosphate buffer (pH 7.4; 100% D_2O) containing 1 mM of the internal standard 3-trimethylsilyl-1-[2,2,3,3-$^2H_4$] propionate (TSP) to 400 μL of each sample. All plasma samples were prepared by adding 450 μL of saline solution (100% D_2O) to 100 μL of each sample. Each fecal sample (30 mg) was combined with 1.2 mL of phosphate buffer prior to lysis by bead beating for 10 min. All fecal samples were then centrifuged for 10 min at 16 000g, 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 1D ^{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 s 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 pairwise 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 was 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 et 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 centered 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 =

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

disease and 0 = control) was predicted for each test set sample based on 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

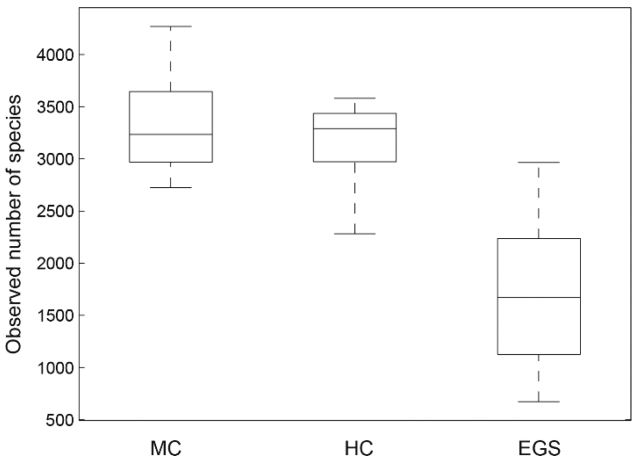


Figure 1. Alpha diversity levels, measured as observed number of species, for the fecal bacterial communities of horses with grass sickness (EGS), hospital controls (HC), and matched controls (MC) at 200 000 sequences per sample.

of horses at a sampling depth of 200 000 sequences per sample. Fecal samples from EGS horses had the lowest diversity (1695 ± 720 observed species), with MC horses being highest (3361 ± 474) and HC horses having an intermediate bacterial diversity (3148 ± 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 with 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). Because 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 previously observed 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*.

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 with 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 feces of EGS horses compared with 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 with 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 fecal 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 toward 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]

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. Because 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

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

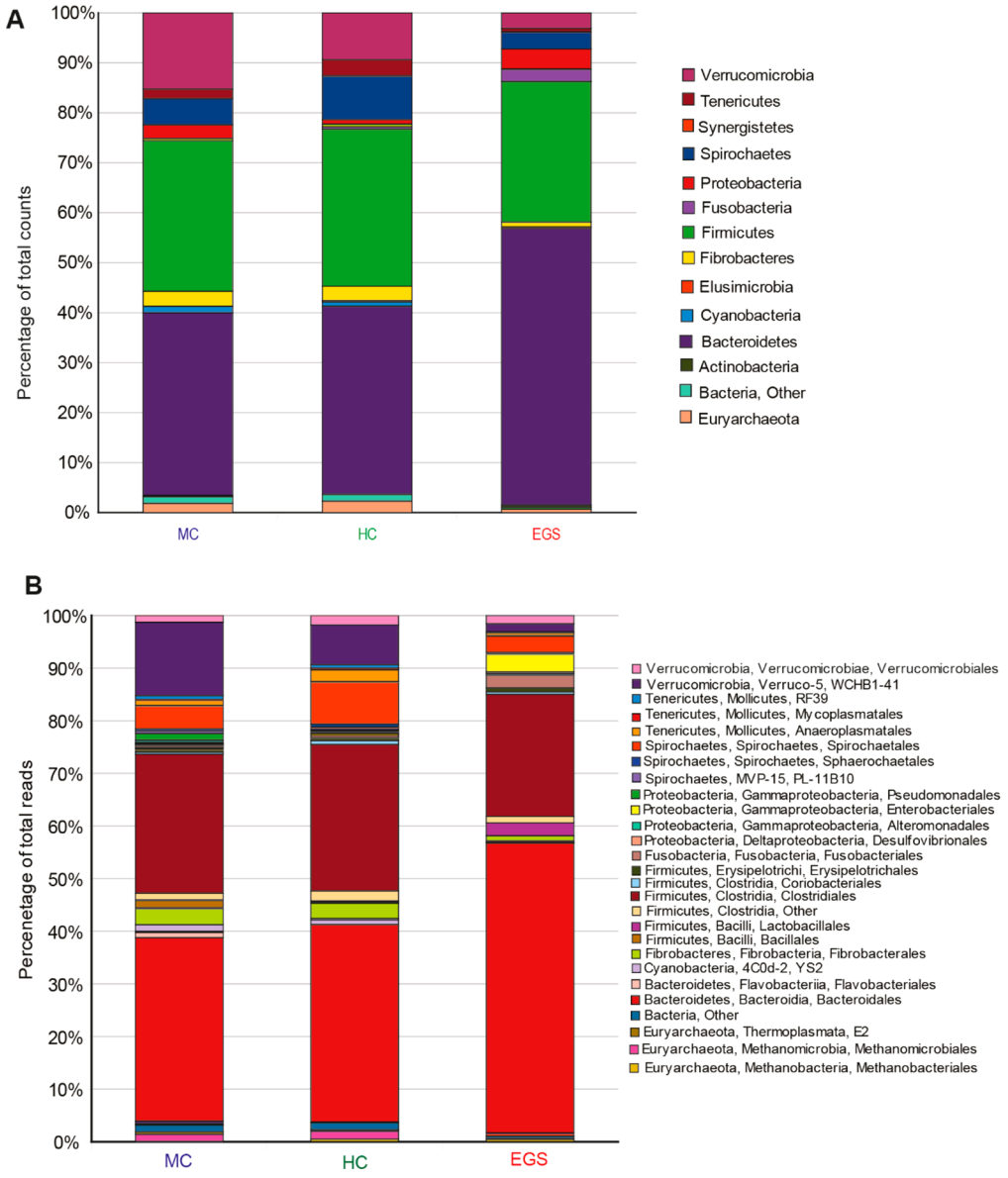


Figure 2. Percentages of total identifiable reads for prominent bacterial (A) phyla and (B) orders identified following sequencing of fecal bacterial DNA. Each bar is an average for the three groups of horses sampled. The key for panel A notes the dominant bacterial phyla represented by each colored bar and the for panel 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.

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]

Four representative OTUs were selected from those enriched in EGS horses and four that were enriched in MC horses. *Enterobacteriales*, *Megasphaera*, *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 is 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.

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 with MC. As such, this study provides no evidence to support the *C. botulinum* hypothesis, 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.

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

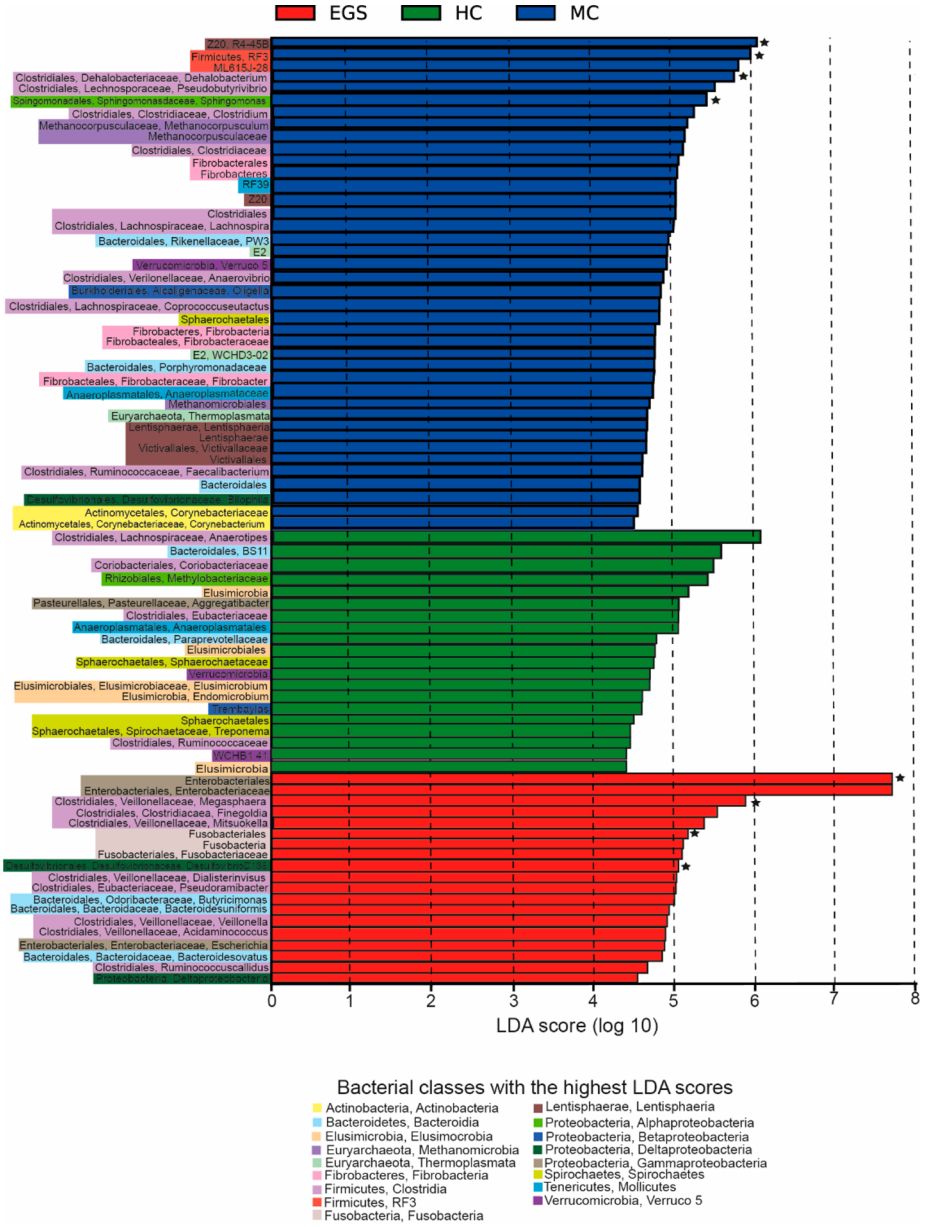


Figure 3. OTUs that differ between the fecal samples of equine grass sickness (EGS), hospital control (HC), and matched control (MC) horses from LEfSe analysis. Bars on the graph indicate linear discriminant analysis (LDA) scores, and asterisks indicate those OTUs whose relative abundance is shown in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

Gut Microbial–Mammalian Cometabolism 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 cometabolites compared with 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 has been associated with human inflammatory bowel diseases[38−41] and blood hippurate has recently been associated with bacterial community diversity within human feces.[42] 4-Cresyl sulfate excretion was lower in EGS horses compared with 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. Because the *Clostridium* genus is a known producer of cresol,[43] the lower excretion of 4-cresyl sulfate is consistent with the lower abundance of bacteria from this genus in EGS horses.

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 oxidized in the host's liver to TMAO.[44] *Proteobacteria* and *Verrucomicrobia* were more abundant in the

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

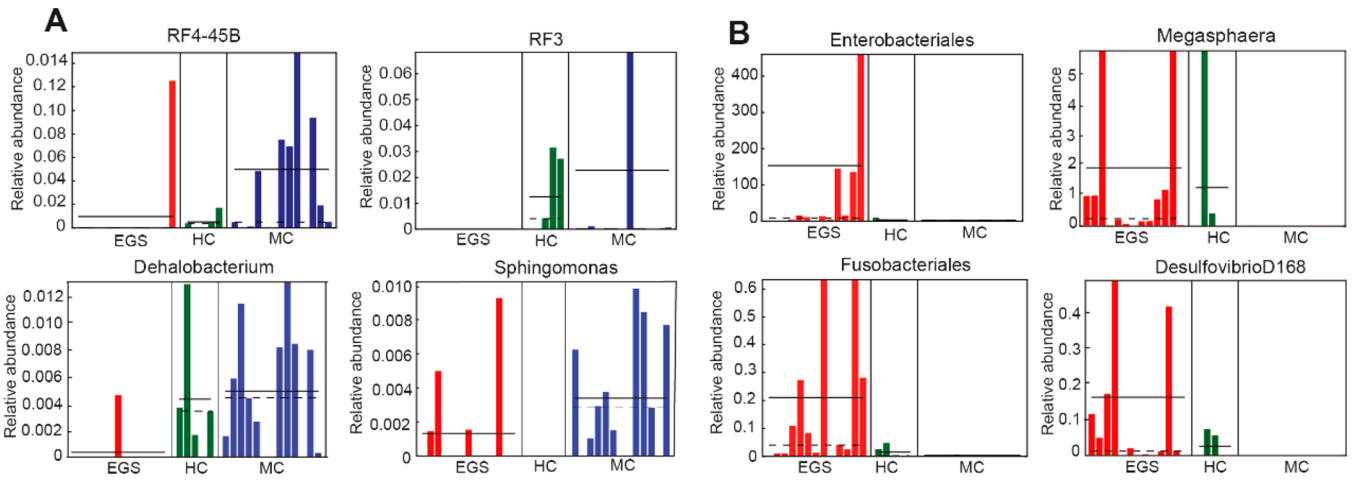


Figure 4. (A) Relative abundance of four representative OTUs with high LDA scores in the fecal microbiota of matched control horses (n = 13) and (B) the relative abundance four representative OTUs with high LDA scores in horses with equine grass sickness (n = 12). OTUs chosen from Figure 3 to represent 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 panels A and B correspond to individual horses and colors indicate the group the horses belong to. The solid line corresponds to the mean of each group and the dashed line is the median.

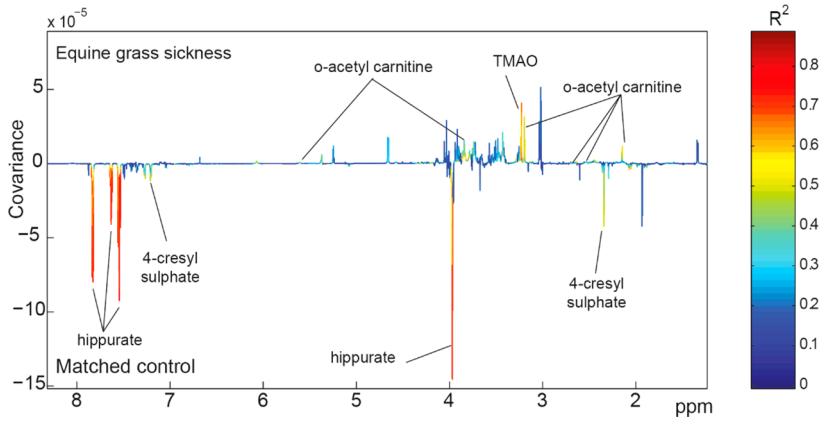


Figure 5. Coefficients plot for the OPLS-DA model comparing the urinary metabolic profiles of horses with grass sickness and their matched controls (Q^2Y = 0.82). The model was constructed with one orthogonal component.

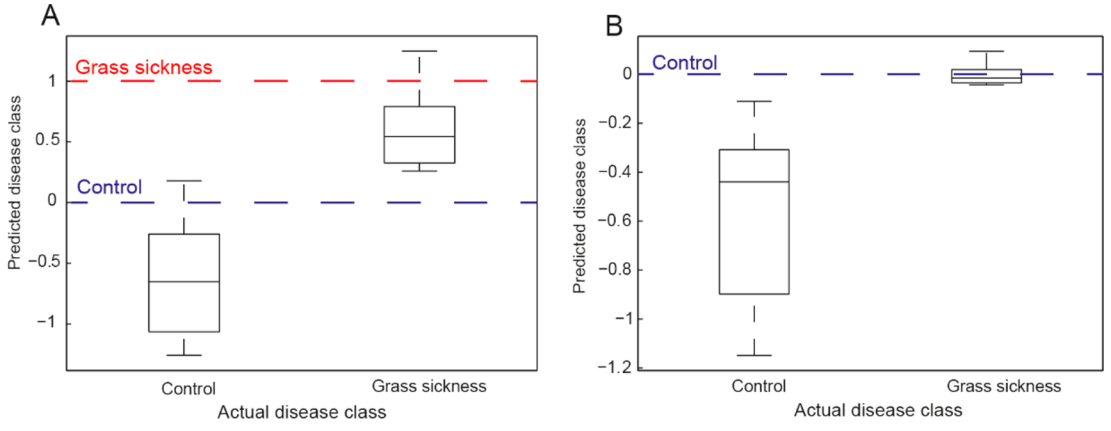


Figure 6. Box plots showing the predicted class values for the independent test set of urine samples calculated by the predictive OPLS-DA model built using the calibration set of urine samples. These were constructed using (A) urinary metabolic profiles and (B) concentrations of urinary hippurate, 4-cresyl 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.

feces of EGS horses compared with 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.

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

Perturbations to the plasma and fecal metabolome associated with EGS can be found in the 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*-hydroxyphenylacetate, valine, citrate, glutamine, acetate, and *β*-aminoisobutyrate were decreased in the plasma of EGS horses when compared with 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 nonspecific finding in horses with intestinal disease.[49−52] Elevations in both plasma glucose and glycerol are 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 cometabolites.

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 with 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 was 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 interindividual 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 the disease class of the test set horses, and separation between the predicted class values for the two groups was not seen (Figure S8).

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 on 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 cograzing, 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 with 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 noninvasive diagnostic tests for EGS, this work offers promising diagnostic targets for future validation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00784.

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 sensitivity and specificity of the urinary metabolic spectra and the concentration of the four candidate biomarkers. Figure S7. Boxplots showing predicted disease class using linear regression models of the individual concentrations of the four urine metabolites

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

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. (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: j.leng@surrey.ac.uk.

ORCID

Joy Leng: 0000-0001-8347-0339

Author Contributions

J.L. contributed to all sections, C.P. contributed to study design, study execution, and preparation of manuscript. A.D. and F.B. contributed to data analysis and interpretation and preparation of manuscript. N.T. and A.M. contributed to study execution. J.S. contributed to study design, data analysis, and interpretation and preparation of manuscript. All authors gave their final approval on the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge the staff at Philip Leverhulme Equine Hospital, who 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-$^{2}H_4$] propionate; PAG, phenylacetylglycine

REFERENCES

(1) Tocher, J. F.; Tocher, J. W.; Brown, W.; Buxton, J. B. Grass sickness investigative report. *Vet. Rec.* **1923**, *3* (37−45), 75−89.

(2) Gilmour, J. Grass Sickness. *In Pract.* **1988**, *10*, 165−167.

(3) Hunter, L. C.; Miller, J. K.; Poxton, I. R. The association of Clostridium botulinum type C with equine grass sickness: a toxicoinfection? *Equine Vet. J.* **1999**, *31* (6), 492−499.

(4) Mcgorum, B. C.; Scholes, S.; Milne, E. M.; Eaton, S. L.; Wishart, T. M.; Poxton, I. R.; Moss, S.; Wernery, U.; Davey, T.; Harris, J. B.; et al. Equine grass sickness, but not botulism, causes autonomic and enteric neurodegeneration and increases soluble N-ethylmaleimide-sensitive factor attachment receptor protein expression within neuronal perikarya. *Equine Vet. J.* **2016**, *48* (6), 786−791.

(5) Costa, M. C.; Arroyo, L. G.; Allen-Vercoe, E.; Stämpfli, H. R.; Kim, P. T.; Sturgeon, A.; Weese, J. S. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16s rRNA gene. *PLoS One* **2012**, *7* (7), e41484.

(6) Dougal, K.; Harris, P. A.; Edwards, A.; Pachebat, J. A.; Blackmore, T. M.; Worgan, H. J.; Newbold, C. J. A comparison of the microbiome and the metabolome of different regions of the equine hindgut. *FEMS Microbiol. Ecol.* **2012**, *82* (3), 642−652.

(7) Dougal, K.; de la Fuente, G.; Harris, P. A.; Girdwood, S. E.; Pinloche, E.; Newbold, C. J. Identification of a Core Bacterial Community within the Large Intestine of the Horse. *PLoS One* **2013**, *8* (10), e77660.

(8) Scarth, J. P.; Spencer, H. A.; Timbers, S. E.; Hudson, S. C.; Hillyer, L. L. The use of in vitro techonologies coupled with high resoultion accurate mass LC-MS for studying drug metabolism in equine drug surveillence. *Drug Test. Anal.* **2010**, *2* (1), 1−10.

(9) Keller, M. D.; Pollitt, C. C.; Marx, U. C. Nuclear magnetic resonance-based metabonomic study of early time point laminitis in an oligofructose-overload model. *Equine Vet. J.* **2011**, *43* (6), 737−743.

(10) Liu, Y.; Uboh, C. E.; Soma, L. R.; Li, X.; Guan, F.; You, Y.; Chen, J. W. Efficient use of retention time for the analysis of 302 drugs in equine plasma by liquid chromatography-ms/ms with scheduled multiple reaction monitoring and instant library searching for doping control. *Anal. Chem.* **2011**, *83* (17), 6834−6841.

(11) Scholes, S.; Vaillant, C.; Peacock, P.; Edwards, G.; Kelly, D. Diagnosis of grass sickness by ileal biopsy. *Vet. Rec.* **1993**, *133* (1), 7−10.

(12) Doxey, D. L.; Gilmour, J. S.; Milne, E. M. A comparative study of normal equine populations and those with grass sickness (dysautonomia) in eastern Scotland. *Equine Vet. J.* **1991**, *23* (5), 365−369.

(13) Wood, J. L. N.; Milne, E. M.; Doxey, D. L. A case-conytol study of grass sickness (equine dysautonomia) in the United Kingdom. *Vet. J.* **1998**, *156* (1), 7−14.

(14) McCarthy, H. E.; Proudman, C. J.; French, N. P. Epidemiology of equine grass sickness: a literature review (1909−1999). *Vet. Rec.* **2001**, *149* (12), 293−300.

(15) Lyle, C.; Pirie, R. S. Equine grass sickness. *In Pract.* **2009**, *31* (1), 26−32.

(16) Wylie, C. E.; Proudman, C. J.; Mcgorum, B. C.; Newton, J. R. A nationwide surveillance scheme for equine grass sickness in Great Britain: Results for the period 2000 − 2009. *Equine Vet. J.* **2011**, *43* (5), 571−579.

(17) Pirie, R. S.; Jago, R. C.; Hudson, N. P. H. Equine grass sickness. *Equine Vet. J.* **2014**, *46* (5), 545−553.

(18) Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F. D.; Costello, E. K.; Fierer, N.; Peña, A. G.; Goodrich, J. K.; Gordon, J. I.; et al. QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nat. Methods* **2010**, *7* (5), 335−336.

(19) Wang, Q.; Garrity, G. M.; Tiedje, J. M.; Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007**, *73* (16), 5261−5267.

(20) Price, M. N.; Dehal, P. S.; Arkin, A. P. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* **2009**, *26* (7), 1641−1650.

(21) Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W. S.; Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol.* **2011**, *12* (6), R60.

(22) Escalona, E. E.; Leng, J.; Dona, A. C.; Merrifield, C. A.; Holmes, E.; Proudman, C. J.; Swann, J. R. Dominant components of the Thoroughbred metabolome characterised by 1H-nuclear magnetic resonance spectroscopy: A metabolite atlas of common biofluids. *Equine Vet. J.* **2015**, *47* (6), 721−730.

(23) Costa, M. C.; Stämpfli, H. R.; Allen-Vercoe, E.; Weese, J. S. Development of the faecal microbiota in foals. *Equine Vet. J.* **2016**, *48* (6), 681−688.

(24) Rea, M. C.; O'Sullivan, O.; Shanahan, F.; O'Toole, P. W.; Stanton, C.; Ross, R. P.; Hill, C. Clostridium difficile carriage in elderly subjects and associated changes in the intestinal microbiota. *J. Clin. Microbiol.* **2012**, *50* (3), 867−875.

(25) Baumgart, M.; Dogan, B.; Rishniw, M.; Weitzman, G.; Bosworth, B.; Yantiss, R.; Orsi, R. H.; Wiedmann, M.; McDonough, P.; Kim, S. G.; et al. Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128

phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. *ISME J.* **2007**, *1* (5), 403−418.
(26) Frank, D. N.; St. Amand, A. L.; Feldman, R. A.; Boedeker, E. C.; Harpaz, N.; Pace, N. R. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (34), 13780−13785.
(27) Hildebrand, F.; Nguyen, T. L. A.; Brinkman, B.; Yunta, R. G.; Cauwe, B.; Vandenabeele, P.; Liston, A.; Raes, J. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol.* **2013**, *14* (1), R4.
(28) Mulak, A.; Bonaz, B. Brain-gut-microbiota axis in Parkinson's disease. *World J. Gastroenterol.* **2015**, *21* (37), 10609−10620.
(29) Copas, V. E. N.; Durham, a E.; Stratford, C. H.; McGorum, B. C.; Waggett, B.; Pirie, R. S. In equine grass sickness, serum amyloid A and fibrinogen are elevated, and can aid differential diagnosis from non-inflammatory causes of colic. *Vet. Rec.* **2013**, *172*, 395.
(30) Lupp, C.; Robertson, M. L.; Wickham, M. E.; Sekirov, I.; Champion, O. L.; Gaynor, E. C.; Finlay, B. B. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgorwth of Enterobacteriaceae. *Cell Host Microbe* **2007**, *2* (2), 119−129.
(31) Cottrell, D. F.; McGorum, B. C.; Pearson, G. T. The neurology and enterology of equine grass sickness: a review of basic mechanisms. *Neurogastroenterol. Motil.* **1999**, *11* (2), 79−92.
(32) Rodriguez, C.; Taminiau, B.; Brévers, B.; Avesani, V.; Van Broeck, J.; Leroux, A.; Gallot, M.; Bruwier, A.; Amory, H.; Delmée, M.; et al. Faecal microbiota characterisation of horses using 16 rdna barcoded pyrosequencing, and carriage rate of clostridium difficile at hospital admission. *BMC Microbiol.* **2015**, *15* (1), 181.
(33) Strauss, J.; Kaplan, G. G.; Beck, P. L.; Rioux, K.; Panaccione, R.; DeVinney, R.; Lynch, T.; Allen-vercoe, E. Invasive potential of gut mucosa-derived Fusobacterium buclearum positively correlated with IBD status of the host. *Inflamm. Bowel Dis.* **2011**, *17* (9), 1971−1978.
(34) Latson, K. M.; Nieto, J. E.; Beldomenico, P. M.; Snyder, J. R. Evaluation of peritoneal fluid lactate as a marker of intestinal ischaemia in equine colic. *Equine Vet. J.* **2005**, *37* (4), 342−346.
(35) Andoh, A.; Tsujikawa, T.; Sasaki, M.; Mitsuyama, K.; Suzuki, Y.; Matsui, T.; Matsumoto, T.; Benno, Y.; Fujiyama, Y. Faecal microbiota profile of Crohn's disease determined by terminal restriction fragment length polymorphism analysis. *Aliment. Pharmacol. Ther.* **2009**, *29* (1), 75−82.
(36) Dinh, D. M.; Volpe, G. E.; Duffalo, C.; Bhalchandra, S.; Tai, A. K.; Kane, A. V.; Wanke, C. A.; Ward, H. D. Intestinal Microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. *J. Infect. Dis.* **2015**, *211* (1), 19−27.
(37) Hauser, D.; Gibert, M.; Marvaud, J. C.; Eklund, M. W.; Popoff, M. R. Botulinal neurotoxin C1 complex genes, clostridial neurotoxin homology and genetic transfer in Clostridium botulinum. *Toxicon* **1995**, *33* (4), 515−526.
(38) Stephens, N. S.; Siffledeen, J.; Su, X.; Murdoch, T. B.; Fedorak, R. N.; Slupsky, C. M. Urinary NMR metabolomic profiles discriminate inflammatory bowel disease from healthy. *J. Crohns. Colitis* **2013**, *7* (2), e42−8.
(39) Dawiskiba, T.; Deja, S.; Mulak, A.; Zabek, A.; Jawień, E.; Pawełka, D.; Banasik, M.; Mastalerz-Migas, A.; Balcerzak, W.; Kaliszewski, K.; et al. Serum and urine metabolomic fngerprinting in diagnostics of inflammatory bowel diseases. *World J. Gastroenterol.* **2014**, *20* (1), 163−174.
(40) Schicho, R.; Shaykhutdinov, R.; Ngo, J.; Nazyrova, A.; Schneider, C.; Panaccione, R.; Kaplan, G. G.; Vogel, H. J.; Storr, M. Quantitative metabolomic profiling of serum, plasma, and urine by (1) H NMR spectroscopy discriminates between patients with inflammatory bowel disease and healthy individuals. *J. Proteome Res.* **2012**, *11* (6), 3344−3357.
(41) Williams, H. R. T.; Cox, I. J.; Walker, D. G.; North, B. V.; Patel, V. M.; Marshall, S. E.; Jewell, D. P.; Ghosh, S.; Thomas, H. J. W.; Teare, J. P.; et al. Characterization of inflammatory bowel disease with urinary metabolic profiling. *Am. J. Gastroenterol.* **2009**, *104* (6), 1435−1444.
(42) Pallister, T.; Jackson, M. A.; Martin, T. C.; Zierer, J.; Jennings, A.; Mohney, R. P.; Macgregor, A.; Steves, C. J.; Cassidy, A.; Spector, T. D.; et al. Hippurate as a metabolomic marker of gut microbiome diversity: Modulation by diet and relationship to metabolic syndrome. *Sci. Rep.* **2017**, *7*, 1−9.
(43) Lord, R. S.; Bralley, J. A. Clinical applications of urinary organic acids. Part 2. Dysbiosis markers. *Altern. Med. Rev.* **2008**, *13* (4), 292−306.
(44) Craciun, S.; Balskus, E. P. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (52), 21307−21312.
(45) Rosenthal, R. E.; Williams, R.; Bogaert, Y. E.; Getson, P. R.; Fiskum, G. Prevention of postischemic canine neurological injury through potentiation of brain energy metabolism by acetyl-L-carnitine. *Stroke* **1992**, *23* (9), 1312−1318.
(46) Lolic, M. M.; Fiskum, G.; Rosenthal, R. E. Neuroprotective Effects of Acetyl-L-Carnitine After Stroke in Rats. *Ainmals Emerg. Med.* **1997**, *29* (6), 758−765.
(47) McGorum, B. C.; Kirk, J. Equine dysautonomia (grass sickness) is associated with altered plasma amino acid levels and depletion of plasma sulphur amino acids. *Equine Vet. J.* **2001**, *33* (5), 473−477.
(48) Fintl, C.; Milne, E. M.; Mcgorum, B. C. Evaluation of urinalysis as an aid in the diagnosis of equine grass sickness. *Vet. Rec.* **2002**, *151* (24), 721−724.
(49) Moore, J. N.; Owen, R. R.; Lumsden, J. H. Clinical evaluation of bllod lactate levels in equine colic. *Equine Vet. J.* **1976**, *8* (2), 49−54.
(50) Milne, E. M.; Doxey, D. L.; Gilmour, J. S. Serum lipids and lipoproteins in equine colic and grass sickness. *Res. Vet. Sci.* **1990**, *48* (2), 170−174.
(51) Edner, H.; Nyman, G. C.; Essén-gustavsson, B. Metabolism before, during and after anaesthesia in colic and healthy horses. *Acta Vet. Scand.* **2007**, *49* (1), 34.
(52) Daly, K.; Proudman, C. J.; Duncan, S. H.; Flint, H. J.; Dyer, J.; Shirazi-beechey, S. P. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. *Br. J. Nutr.* **2012**, *107* (7), 989−995.
(53) Shotton, H. R.; Lincoln, J.; Mcgorum, B. C. Effects of Equine Grass Sickness on Sympathetic Neurons in Prevertebral and Paravertebral Ganglia. *J. Comp. Pathol.* **2011**, *145* (1), 35−44.

DOI: 10.1021/acs.jproteome.7b00784
J. Proteome Res. 2018, 17, 1120−1128