Fecal microbiomics biomarkers for Chronic Wasting Disease

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Abstract

Chronic wasting disease (CWD) is a naturally occurring infectious, fatal, transmissible spongiform encephalopathy of cervids that has rapidly proliferated across North America over the past five decades. CWD belongs to a broader class of prion diseases, caused by accumulation of abnormally misfolded prion proteins. Healthy animals are believed to acquire this disease primarily by oral exposure to infected animal by-products or the environment containing misfolded prions. Thus, gastrointestinal tract represents a primary route of CWD infection. Recent studies propose a direct link between the gut microbiome and the pathogenesis of other prion diseases, however a direct link between CWD infection and gut microbiome has not been well established. Here we analyzed 50 fecal samples obtained from CWD-positive animals of different sexes from various regions in the US, compared to 50 healthy controls to identify CWD-dependent changes in the gut microbiome and the corresponding composition of key metabolites of host and microbial origin. Using high throughput sequencing of 16S ribosomal RNA gene, we identified promising trends in the gut microbiota that could potentially be CWD-dependent. While, in agreement with previously published studies, geographical origin exerts strong influence on the microbial composition of the gut, using CWD as a variable reveals 64 bacterial taxa that are differentially abundant between control and CWD-positive deer in our samples and can be explored as potential markers of CWD. Our results provide a potential tool for diagnostics and surveillance of CWD in the wild, as well as conceptual advances in our understanding of the disease.

Importance

This is a comprehensive study that tests the connection between the composition of the gut microbiome in deer in response to Chronic Wasting Disease (CWD). We analyzed 50 fecal samples obtained from CWD-positive animals compared to 50 healthy controls to identify CWD-dependent changes in the gut microbiome, matched with the analysis of fecal metabolites. Our results show promising trends suffesting that fecal microbial composition can directly correspond to CWD disease status. Furthermore, our data suggest that early stages of CWD that are outwardly asymptomatic exhibit the same changes as the later stage of the disease. These results point to microbial composition of the feces as a potential tool for diagnostics and surveillance of CWD in the wild, including non-invasive CWD detection in asymptomatic deer and deer habitats, and enable conceptual advances in our understanding of the disease.

Introduction

Chronic wasting disease (CWD) is a naturally occurring infectious, fatal, transmissible spongiform encephalopathy of cervids. Environmental contamination and excreta (e.g., saliva, urine, and feces) are thought to play a pivotal role in the rapid proliferation of CWD across North America over the past five decades (*1*).

CWD belongs to a broader class of prion diseases, caused by accumulation of abnormally misfolded prion proteins in the animals' tissues and organs (*2*). While the full spectrum of organismal effects of prion diseases is still being characterized, the most affected tissue is the brain, where misfolded prions cause neuronal loss that leads to progressive neuronal dysfunction and brain damage, which is eventually fatal. CWD is believed to be transmissible across species. Although CWD transmission to humans has not been directly demonstrated, such transmission has been found to occur in another prion disease (e.g., bovine spongiform encephalopathy or Mad Cow Disease), and thus it remains a concerning possibility. Importantly, ingestion of infectious prions represents an established route of infection, and therefore human consumption of CWD-infected meat is of strong concern. Thus, surveillance and diagnostics are very important to CWD prevention and control, and represents a global challenge for animal health.

Currently, disease confirmation in cervids relies largely on post-mortem detection of infectious prions in the medial retropharyngeal lymph nodes or obex in the brain via immunohistochemistry (IHC). Recently, a Real-Time Quaking-Induced Conversion (RT-QuIC) assay has been developed, which can detect CWD from deer ear punches with high sensitivity (*3*). Additional antemortem samples that can be tested by RT-QuIC include feces, urine, and rectoanal mucosa-associated lymphoid tissue (RAMALT) biopsies. However, despite the long-standing recognition of CWD and progress made in the understanding of the disease, there is no

non-invasive live-animal test with sensitivity greater than or equal to that of postmortem IHC or ELISA (*4-6*). While recent studies propose the use of RT-QuIC for CWD detection in fecal samples (*7*), no methods have currently been sufficiently optimized to enable disease surveillance using animal-derived materials and by-products in the wild.

While routes of CWD propagation are still being investigated, healthy animals are believed to acquire this disease by oral exposure to infected animal by-products containing misfolded prions, including feces, saliva, urine, and animal remains, which are accidentally ingested by the deer in the contaminated environment, and are then absorbed in the oral cavity and the digestive tract (*8-14*). Thus, gastrointestinal tract represents a major route of CWD infection and has a high potential of being affected by CWD.

Gastrointestinal health as well as normal animal physiology greatly depend on gut microbiota, a multi-species population of symbiotic and pathogenic microorganisms normally residing in the intestines of all animals. Gut microbiota respond to a wide variety of diseases and physiological changes in the body (see, e.g., (*15*)) and are essential for maintaining the normal gut health and barrier function (*16, 17*). Based on the available evidence, recent papers propose a direct link between the gut microbiota and the pathogenesis and pathology of prion diseases (*18-20*), but this highly promising field is still in its infancy.

Gut microbiota composition in the digestive tract (microbiome) can be analyzed using feces, which contain a representative sample of bacteria from each individual animal. Feces are commonly found in natural deer habitats and can be collected without disturbing the environment or the need to trap or handle animals. Thus, identification of potential feces-based diagnostic markers of CWD would provide a very useful tool for disease surveillance and control. Studies analyzing fecal samples from deer with CWD are emerging in the field (*7, 21-*

25), but currently no robust biomarkers that can inform disease surveillance, diagnostics, and its effects on normal animal physiology have been established.

Here we used fecal samples obtained from 50 CWD-positive farmed white-tailed deer (*Odocoileus virginianus*) of different sexes from various locations in the US, as well as 50 CWD negative farmed white-tailed deer controls, to identify CWD-dependent changes in the deer microbiome. Using high throughput sequencing of 16S ribosomal RNA gene, we identified promising trends in the gut microbiota that could potentially be CWD-dependent. While, in agreement with previously published studies, geographical origin exerts strong influence on the microbial composition of the gut, using CWD as a variable reveals 64 bacterial taxa that are differentially abundant between control and CWD-positive deer in our samples and can be explored as potential markers of CWD. Furthermore, we performed targeted metabolomics on the same fecal samples and cross-omics correlative analysis of these data with the microbial composition of the samples, to identify potential changes in host and microbial metabolites. Collectively, these changes represent potential microbial signatures that may prove to be specific to CWD-infected animals. Longer term, these results point to a possibility of non-invasive diagnostics and surveillance of CWD in wild and farmed white-tailed deer using fecal samples, as well as conceptual advances in our understanding of the disease.

Materials and Methods

Fecal Sample Collection and Preparation

Fecal samples were sourced from an existing United States Department of Agriculture APHIS sample repository. White-tailed deer (*Odocoileus virginianus*) for this study were

depopulated, farmed, naturally infected CWD positive and negative animals from the same herds that came from six different US states, coded and classified as Midwest, West, South, and East (Table S1). Feces were collected manually from the rectum of each animal using a new nitrile glove to prevent cross-contamination. Feces was then placed into 50 mL conical tubes and stored at -80° C until aliquots were removed for this study.

The CWD status of the samples was determined by immunohistochemistry (IHC) of the medial retropharyngeal lymph nodes and obex at the USDA National Veterinary Services Laboratory in Ames, Iowa as previously described (*26*). An example of the diagnostics result is shown in Fig. S1.

DNA Extraction

For DNA extraction, the automated KingFisher system was used with the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit (ThermoFisher Scientific, A42356). Sample amount used for DNA extraction: 50 mg.

Library Preparation

Libraries prepared by targeted amplification of the variable V3-V4 regions of the bacterial 16S rRNA gene and attachment of Nextera XT indexes (Illumina Catalog #FC-131-2001) with PrimeStar Taq DNA Polymerase (Takara, cat#R045A). Concentrations were measured using the Qubit dsDNA HS (Invitrogen, Cat# Q32851). Samples were pooled, then purified using GenElute PCR Clean-Up Kit (Sigma, SKU NA1020), and KAPA Pure Beads (Roche-07983298001). Final library concentration determined by Invitrogen Qubit, and final size determined by Agilent DNA 1000 Kit (Agilent, #5067-1504) on the Agilent Bioanalyzer.

Primer Design and Sequencing:

The gene-specific sequences used in this protocol target the 16S V3 and V4 regions. Sequencing primers were designed based on these gene-specific sequences with the addition of Illumina adapter overhang nucleotide sequences, to produce the following full-length primers:

16S Amplicon PCR Forward Primer:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

16S Amplicon PCR Reverse Primer:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

The library pools were sequenced on Illumina® MiSeq™ with a V2 reagent kit, 500 cycles, with a Nano Flowcell (Illumina, MS-103-1003). Paired-end sequencing (2X250 cycles) was applied.

Targeted metabolomics. Samples were lyophilized for 48 hours prior to metabolite extraction. Bile acids were extracted with 50% ACN spiked with the isotopically labeled standards cholic acid-d_5, lithocholic acid-d_5, sodium taurocholate-d_4 and sodium taurodeoxycholate-d_4 at concentrations of 2, 20, 1 and 1 µM, respectively. Bile acids were separated on a Waters ACQUITY UPLC BEH C_{18} column (2.1 mm × 150 mm, 1.7 µm, 130 Å) using a mobile phase of (A) 95% H2O 5% acetonitrile + 0.1% formic acid, and (B) 100% acetonitrile + 0.1% formic acid. The metabolites eluted with a linear gradient of 30% to 70% B over 16 min, at flow rate of 0.38 mL/min.

The short-chain fatty acids (SCFAs) were extracted with 70% IPA spiked with 50 µM of the isotopically labeled standards sodium acetate-$^{13}C_2$, sodium propionate-$^{13}C_3$ and sodium butyrate-$^{13}C_4$. The extracts were derivatized with 5 µL of 10 M pyridine, 10 µL of 250 mM *N*-(3-

dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) solution in 70% IPA and 10 µL of 250 mM 3-nitrophenylhydrazine (3-NPH) solution in 70% IPA. The mixture was reacted at 40 °C for 30 min. The reaction was quenched by adding 1.9 µL of formic acid. The SCFAs were separated with the same column and mobiles phases as the BAs, with the exception of a linear gradient of 10.0% to 47.5% B over 10.5 min, at flow rate of 0.38 mL/min.

The amino acids were extracted with water spiked with 50 µM of an isotopically labeled mix of 17 amino acids (Cambridge Isotope Laboratories). The amino acids were separated on a Imtakt Intrada Amino Acid column (3 mm × 150 mm, 3 µm) using a mobile phase of (A) acetonitrile + 0.3% formic acid, and (B) 20% acetonitrile 80% water + 80 mM ammonium formate. The amino acids eluted with a linear gradient of 20% B to 55% A over 12 min, at flow rate of 0.6 mL/min.

All of the LC-MS data was acquired on an Agilent 6460 Triple Quadrupole mass spectrometer with an electrospray ionization (ESI) source coupled with an Agilent 1290 Infinity II UPLC system. The LC-MS data was processed with Skyline v21.1 (MacCoss Lab, University of Washington).

Bioinformatics Analysis.

The 16S (V3-V4) paired-end amplicon sequences from 100 samples having read length of maximum 251 bases were quality assessed, followed by trimming and quality-based filtering using BBDuk (Bushnell, Brian. *BBMap: A Fast, Accurate, Splice-Aware Aligner. United States: N. p.*,2014; https://www.osti.gov/servlets/purl/1241166; http://jgi.doe.gov/data-and-tools/bb-tools/) to yield reads with average quality of Phred score(Q)>=30. The post trimming and filtering, quality report was assessed on reads level, base positions, length distribution etc.

The quality trimmed fastq files were further processed using M-CAMP™ web platform (*M-CAMP™: A cloud-based web platform with a novel approach for species-level classification of 16S rRNA microbiome sequences*. Schriefer et al. bioRxiv, 2021.08.25 456838; doi: https://doi.org/10.1101/2021.08.25.456838). The M-CAMP™ uses the hybrid approach of heuristic alignment and k-mer based classification using kraken2 software (*27*) (https://ccb.jhu.edu/software/kraken2/). Reads were mapped against the proprietary "Sigma-Aldrich-16S_V3-V4" reference database which contains the V3- V4 primer specific 16S gene sequence. The taxon relative abundance below the cut-off 0.01% were filtered out. Following this analysis, the data were independently reanalyzed using QIIME2 (https://qiime2.org/), and the results of the two analyses were broadly compared for alpha diversity, statistical differences between different sample groups (sex, origin, and disease status), and the relative abundance of the most abundant bacterial species. The results of both analyses are in agreement.

The clustered heatmap was generated on the prevalent taxa using CLR (centered log ratio) normalized abundance to study the covariates of interest, including sex, geographical region, and CWD status. Rarefaction curve was generated to study the species richness across the samples and the metadata groups. The community heterogeneity was studied using the different alpha diversity indices including Chao1 (a metric for species total richness), Shannon (a metric that incorporates both species richness and species evenness), Simpson, observed operational taxonomic units (OTUs) and Faith's phylogenetic diversity (pd). The alpha diversity indices-based group significance was tested in pairwise manner and all group simultaneously within the metadata category using the Kruskal-Wallis test. The association between the metadata groups and the microbial composition were studied using the beta diversity measure based upon the various similarity or dissimilarity metrices, including Bray Curtis, Jaccard, weighted Unifrac, un-

weighted Unifrac. 3D PCoA plots were generated to study the distances and closeness between samples and within groups based on the dissimilarity metrices. To identify sample outliers at rank level, the multivariate analysis is performed, and 3D view was plotted for the Principal Component Analysis (PCA) shown in Fig. 1. The PCA uses OTU frequency at the taxonomic rank level across samples to determine the principal axes of abundance variation. Transforming and plotting the OTU frequency data in principle component space allows us to separate the biome samples according to abundance variation.

Beta diversity metrices based group significance was performed using PERMANOVA method to test for significant divergence across and between the metadata categories. PERMANOVA method using 999 permutations is performed to assess the significance of pseudo-F statistics. The larger pseudo-F ratio value indicates greater group separation. Any separation between groups is not significant if a p-value was > 0.05. The diversity analysis was performed using the QIIME2, either directly or through the M-CAMP™ web platform (*28*, *29*).

Statistics in Table S3 were calculated in linear space of the normalized expression values (percentages) and p-value for each taxa was calculated by two-tailed Welch's t-test between CWD and control groups. Statistics in Table S4 were calculated in geometric space of the expression values (zero expression values were replaced with half of the non-zero minimum) and p-value for the ratio of each pair of taxa was calculated by one-tailed Welch's t-test between CWD and control groups. The non-parametric (Wilcoxon rank sum) tests were also performed for the identified significant taxa and pairs of taxa with the corresponding p-values included in Table S3 and Table S4.

R^2 PERMANOVA analysis of the sample variance in Table S5 is presented with the following fields of output: DF: Degree of Freedom; SumsOfSqs: The sum of squares represents a

measure of variation or deviation from the mean; MeanSqs : Mean squares, calculated by dividing the sum of squares by the degrees of freedom; F.Model : The F-statistic is a ratio of two variances; R^2: statistical measure of the effect size (e.g., R^2 of 0.25 means that 25% of the variation in distances is explained by the grouping being tested); Pr(>F): p value representation, deternining whether or not this result was likely a result of chance.

Data availability. The 16S rRNA sequencing data is available in the NCBI Sequence Read Archive with the BioProject ID: PRJNA936583.

Cross-omics analysis. The bacterial relative abundances were filtered to include enriched bacteria only (LEfSe FDR *p*-value < 0.05), and OTUs that were only abundant in more than 5% of samples. The metabolite ratios (normalized to isotopically labelled standards) were correlated to the filtered bacteria relative abundances using Spearman's rank correlation. The heatmap (Figure S10) was generated using the pheatmap R package (v 1.0.12).

Results

Analysis of deer feces microbial composition.

To compare the microbial composition of the fecal samples between healthy deer and deer with CWD, we isolated total DNA from fecal pellets of 50 CWD-negative and 50 CWD-positive depopulated farmed white-tailed deer. CWD-positive deer in this set were diagnosed by immunohistochemistry of paraffin-embedded sections of brain and lymph node, and the disease

status was classified as “negative” (neg) for those deer that showed no prion accumulation in these tissues, LN (lymph node) positive for the deer showing staining only in the lymph node but not the brain (LN pos), or brain and lymph node positive (BRLN) for the deer showing positive staining in both organs. LN positive and BRLN positive deer were then collectively classified into the CWD-positive (pos) groups. An example of brain diagnostics is shown in Fig. S1.

DNA extracted from individual fecal samples was amplified using targeted amplification of the variable V3-V4 regions of the bacterial 16S rRNA gene, and sequenced on the Illumina platform to identify their microbial composition. After stringent filtering for clean reads and discarding the data below 0.01% abundance in the sample, this analysis yielded 892 different microbial taxa present across the analyzed samples (Table S2).

We analyzed alpha diversity to determine potential overall differences in the diversity and richness of microbial communities in each individual sample. This analysis revealed no statistically significant differences in alpha diversity between samples from CWD positive and negative (Fig. S2). Thus, the overall richness of the microbial composition is not significantly affected by CWD.

Microbial composition of the deer feces is highly affected by CWD status.

Fecal samples used in this study differ from each other by several different parameters, including sex, geographic region of origin, and CWD status (Table S1). All of these parameters can potentially affect microbial composition. In particular, geographical origin is expected to strongly affect the repertoire of microorganisms found in the samples, given potentially large variability in microbial and plant ecology in different regions of the US. To determine whether the microbial composition in fecal samples significantly depends on any of these variables, we

performed principal component analysis (PCA), comparing beta diversity in samples from deer of different sex, different origin (Midwest, West, South, and East), and different disease status (CWD negative versus positive). We next compared p value of change between the groups of samples determined by PERMANOVA statistical test. The results of this analysis are shown in Fig. 1 and Table S5.

Samples sorted by CWD status as a variable showed an overall difference that was highly statistically significant (Fig. 1, top left and Table S6). In comparison, male versus female deer showed no differences in the overall microbial composition (Fig. 1, bottom right and Table S6). Geographic origin of the deer appeared to be a strong factor in differences between samples (Fig. 1, bottom left). However, analysis of the sample metadata (Table S1) indicated that this difference was potentially skewed by the fact that deer from some regions were almost exclusively CWD-positive, while in others most deer were CWD-negative (Table S1). Such "skewed" regions accounted for approximately 20% of the samples. When these regions were excluded from the analysis, geographical origin still emerged as a parameter driving a statistically significant difference between samples. Re-analysis of the samples limited to the regions where both CWD-positive and CWD-negative status was similarly represented (Midwest 5, Midwest 7, and South 1) showed less difference than the overall sample set (Fig. 1, top right). Thus, geographical origin and CWD status both are the likely drivers for the differences in the microbial composition in fecal samples. Notably, CWD status shows promising trends suggesting that deer from the same region, analyzed in sufficient numbers, may show differences in their gut microbiomes that are driven by the disease.

Microbial composition of the deer feces exhibits distinct CWD-dependent microbial signatures.

To identify specific microbial taxa that are differentially abundant between CWD positive and negative deer, we compiled the comprehensive and specific rank-wise (Level 1: Kingdom to Level 7: Species) abundance table summarizing the clade fragment (i.e., the taxa identified in each sample at different taxonomic levels) percentage of the total in each sample, the clade count and the taxon count for each specific taxonomical rank (Table S2). We next sorted these data by CWD status (positive versus negative) and calculated the p-value of difference in the abundance of each of the identified taxa between CWD and control, with the p-value cutoff of 0.05. This analysis revealed 64 hits, representing the taxa with abundances that were significantly ($p<0.05$) different between these two groups (Table S3).

We sorted these hits into different taxonomic levels to analyze specific changes in the CWD-dependent fecal microbiome. These hits were plotted separately by abundance, with hits at 1% or more average abundance in at least one group of samples shown in Fig 2 (a total of 23 taxa), and the lower abundance hits shown in Figs S3-S5. Notably, 8 taxa are 10% or more abundant in at least one condition (either CWD positive or CWD negative or both), and thus represent truly major components of the fecal microbiome.

To confirm this, we also performed LEfSe analysis and plotted LDA scores for differentially abundant taxa (Fig. 3, 4).

This analysis revealed several interesting trends. First, at each taxonomic level, microbial taxa grouped into distinct abundance patterns, with some taxa lower and others higher in either CWD or control (Fig. 2). These patterns could potentially be used to identify disease status. Furthermore, compiling all 64 differentially abundant taxa at all taxonomic levels into a single

pie chart reveals CWD-specific patterns, visually representing taxa with differential abundance in CWD and control (Figs. S3-S10). Such visual patterns, derived by scoring differential abundance of specific microbial taxa in each sample, could potentially represent a visual tool that may be useful as an output for future diagnostics.

Metabolic composition of the deer feces exhibits significant CWD-dependent changes

To see if the changes in microbiota are accompanied by changes in metabolic composition, we performed targeted metabolomics of short-chain fatty acids, amino acids, and bile acids in deer fecal samples and identified 54 metabolites (12, 31, and 11, respectively, see Table S7). Some of these metabolites showed significant differences between feces of deer with and without CWD (Fig. 5A).

Next, we quantified relative amounts of some of these metabolites in CWD-positive versus control feces. The ratio of GABA to the excitatory neurotransmitter glutamate and its precursor glutamine were significantly decreased in CWD-positive feces (Fig. 5B). DCA to CA and GCA ratios as well as LCA to CDCA and GCDCA ratios were also significantly decreased (Fig. 5C). These changes in metabolite levels, as well as their ratios, have a potential use as biomarkers for CWD diagnostics.

For this metabolite analysis, we used 69 of the original 100 samples used for the microbiomics described above, chosen based on the sample abundance. Using the 69 samples, we performed cross-omics analysis between the microbial taxa and the identified metabolites. The cross-omic analysis shows correlations between 63 enriched microbial taxa (LEfSe FDR $p < 0.05$) and 54 metabolites (Fig. 6). Among those, e.g., the relative abundance of the *Coriobacteriia* class had significant positive correlations (FDR $p<0.05$) with the secondary bile acids, DCA and LCA. Since secondary bile acids are produced exclusively by the gut

microbiome, such correlations are of special interest. In the *Coriobacteria* class, the *Eggerthellaceae* family (which was not enriched in healthy deer) also displayed a significant correlation to LCA. These correlations are in agreement with the literature as *Eggerthella* species are known to convert primary bile acids to secondary bile acids (*30*) and the relative abundance of *Bacillales* had significant negative correlation (< -0.5) with GABA and LCA.

Taxa ratios as potential diagnostic markers of CWD

The differences revealed by the individual taxa suggest that the microbiome changed in CWD. However, those analysis depends on the normalized expression values, i.e., the percentage of an expressed taxa. It would be more efficient to exploit the differences without measuring the entire microbiome of each sample.

Previous studies found that in some cases ratios of specific pairs of microbial taxa in each sample can be indicative of certain disease or physiological status. For example, Firmicutes to Bacteriodetes (F/B) ratio was previously proposed as a marker of obesity and type 2 diabetes (*31*), inflammatory bowel disease (*32*), and aging (*33*). In deer feces, this ratio is decreased in CWD positive animals at the Phylum level (one-tailed Welch's t-test p=0.04, Wilcoxon rank sum t-test p=0.01, see Table S8 for the full analysis). In humans, higher F/B ratios (>12) are found in healthy individuals, suggesting that reduced F/B ratio may be a potential clinical biomarker (*34*). In our study the F/B ratio in CWD-positive animals is lower than in control (21 in control versus 12 in CWD). However, another study reported an increase in the F/B ratio in CWD (*35*). Clearly, more studies are needed to evaluate F/B ratio as a potential diagnostic marker for CWD. However, the general idea of identifying ratios of specific pairs of taxa in CWD positive versus

negative samples, may be a promising approach that could potentially serve as a basis for a more rapid CWD scoring test compared to the full microbiomics signatures.

To test the potential of using ratios of comparably abundant taxa (>1 percent) and significantly changed taxa ($p<0.05$) in the fecal samples as CWD biomarkers, we calculated the ratios for each taxa pairs within each rank and found 41 ratios of taxa pairs with significantly higher ratios of control than CWD (fold-change>1.5 and p-value<0.05). These ratios represent potential CWD-dependent signatures in the fecal microbiome that could be explored for CWD surveillance and diagnostics (Fig.7 for $p<0.005$ and Figs S6-S9, Table S4 for $p<0.05$).

Discussion

Our study represents a comprehensive analysis of fecal microbiome from deer with and without CWD, with the goal of identifying potential biomarkers that could be utilized for novel types of ante mortem CWD surveillance and diagnostics. We identified a total of 64 microbial taxa that are differentially abundant between CWD and control, including 23 highly abundant taxa. While some of this variability could potentially be explained by contribution of other factors, such as geographical region, diet, seasonal changes, etc., our data proposes a potential use of abundant microbial taxa as a tool to detect “microbiomic signatures” of CWD. This approach could eventually lead to breakthroughs in our understanding and control of CWD.

A recent study that performed a similar type of analysis using feces from ~200 farmed deer, identified no robust changes in fecal microbiota associated with CWD, but did find differences related to the geographic origin of the deer, likely related to diet and partially correlated with sex (*35*). In our study geographical origin is also the main factor that drives fecal microbiome variability between the samples – perhaps not surprising, given that environmental microbial

composition differs greatly in different habitats. However, encouragingly, our data also points to potential changes in fecal microbiomes that may be directly linked to CWD. Evaluating these results in a larger sample set obtained from the same geographical region is essential for the identification of the global trends of the CWD-specific changes in fecal microbiomes.

Several of the microbial taxa and metabolites found altered between CWD-positive and control samples in our study have also been linked to aging and disease processes in humans and mice, and could be physiologically relevant to CWD. This is especially interesting and promising at the metabolite level. For instance, butyrate levels in human patients with diabetic nephropathy, which results in skeletal muscle atrophy, are significantly decreased (*36*) and a butyrate-containing diet has been shown to improve metabolism and reduce muscle atrophy in aging (*37*) and diabetic mice (*36*). This dovetails with the deceased butyrate levels in the CWD feces. GABA is a major inhibitory neurotransmitter in the brain. GABA transmission (*38*) and GABA and ornithine levels (*39*) in the cortex decrease in certain prion diseases. Our results show that the corresponding amino acid levels also decreased in the CWD feces. The secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA) that originate from the gut microbiome and their corresponding primary bile acids influence the neuropathology of Alzheimer's disease (AD) and the secondary to primary bile acid ratios change significantly in AD brains (*40*, *41*). While these changes have been previously reported in humans, it seems likely that changes in these metabolites also occur in deer in conjunction with CWD. While further studies are needed to determine which of these changes are driven by factors other than CWD, these metabolites seem promising to explore as potential biomarkers that could serve as basis for CWD diagnostics.

It is unclear at present what is the interdependence between CWD and changes in the fecal microbiome. The changes we report here are clearly heavily linked to the geopgraphical origin of samples, and may be influenced by such factors as diet, seasonal changes, etc. It is also possible that some of these changes reflect an overall effect on declining health in the prion-infected deer, e.g. changes in the body weight and muscle mass, wasting, or inflammation previously reported as symptoms of CWD and other prion diseases. Curiously, however, a subset of deer used in this study show no infectious prion staining in the brain, only in the lymph nodes (Table S1), suggesting that the disease status in these deer is milder than in those showing prion accumulation in both organs. In addition, none of the deer used in this study showed any obvious clinical symptoms of CWD. With this knowledge, it appears especially important to explore statistically significant changes in CWD-positive fecal microbiomes. Given CWD's relatively long incubation period, this approach may potentially be used for earlier detection of CWD and a substantial improvement in disease-managing strategies.

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References

1. N. J. Haley, E. A. Hoover, Chronic wasting disease of cervids: current knowledge and future perspectives. *Annu Rev Anim Biosci* **3**, 305-325 (2015).
2. Z. J. Lambert, J. J. Greenlee, E. D. Cassmann, M. H. West Greenlee, Differential Accumulation of Misfolded Prion Strains in Natural Hosts of Prion Diseases. *Viruses* **13**, (2021).
3. S. Vascellari, C. D. Orru, B. Caughey, Real-Time Quaking- Induced Conversion Assays for Prion Diseases, Synucleinopathies, and Tauopathies. *Front Aging Neurosci* **14**, 853050 (2022).
4. C. L. Holz *et al.*, Evaluation of Real-Time Quaking-Induced Conversion, ELISA, and Immunohistochemistry for Chronic Wasting Disease Diagnosis. *Front Vet Sci* **8**, 824815 (2021).
5. M. D. Schwabenlander *et al.*, Comparison of Chronic Wasting Disease Detection Methods and Procedures: Implications for Free-Ranging White-Tailed Deer (Odocoileus Virginianus) Surveillance and Management. *J Wildl Dis* **58**, 50-62 (2022).
6. D. Tewari, D. Steward, M. Fasnacht, J. Livengood, Detection by real-time quaking-induced conversion (RT-QuIC), ELISA, and IHC of chronic wasting disease prion in lymph nodes from Pennsylvania white-tailed deer with specific PRNP genotypes. *J Vet Diagn Invest* **33**, 943-948 (2021).
7. S. Hwang, J. J. Greenlee, E. M. Nicholson, Real-Time Quaking-Induced Conversion Detection of PrP(Sc) in Fecal Samples From Chronic Wasting Disease Infected White-Tailed Deer Using Bank Vole Substrate. *Front Vet Sci* **8**, 643754 (2021).
8. K. A. Davenport *et al.*, Assessment of Chronic Wasting Disease Prion Shedding in Deer Saliva with Occupancy Modeling. *J Clin Microbiol* **56**, (2018).
9. N. D. Denkers *et al.*, Very low oral exposure to prions of brain or saliva origin can transmit chronic wasting disease. *PLoS One* **15**, e0237410 (2020).
10. T. J. Kreeger, D. L. Montgomery, J. E. Jewell, W. Schultz, E. S. Williams, Oral transmission of chronic wasting disease in captive Shira's moose. *J Wildl Dis* **42**, 640-645 (2006).
11. C. K. Mathiason *et al.*, Infectious prions in the saliva and blood of deer with chronic wasting disease. *Science* **314**, 133-136 (2006).
12. I. H. Plummer, S. D. Wright, C. J. Johnson, J. A. Pedersen, M. D. Samuel, Temporal patterns of chronic wasting disease prion excretion in three cervid species. *J Gen Virol* **98**, 1932-1942 (2017).
13. C. J. Sigurdson *et al.*, Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (Odocoileus hemionus). *J Gen Virol* **80 (Pt 10)**, 2757-2764 (1999).
14. E. S. Williams, Chronic wasting disease. *Vet Pathol* **42**, 530-549 (2005).
15. S. Altmae, J. M. Franasiak, R. Mandar, The seminal microbiome in health and disease. *Nat Rev Urol* **16**, 703-721 (2019).
16. D. Cardoso-Silva *et al.*, Intestinal Barrier Function in Gluten-Related Disorders. *Nutrients* **11**, (2019).
17. S. Otani, C. M. Coopersmith, Gut integrity in critical illness. *J Intensive Care* **7**, 17 (2019).
18. B. M. Bradford, L. Tetlow, N. A. Mabbott, Prion disease pathogenesis in the absence of the commensal microbiota. *J Gen Virol* **98**, 1943-1952 (2017).
19. V. D'Argenio, D. Sarnataro, Microbiome Influence in the Pathogenesis of Prion and Alzheimer's Diseases. *Int J Mol Sci* **20**, (2019).
20. D. S. Donaldson, K. J. Else, N. A. Mabbott, The Gut-Associated Lymphoid Tissues in the Small Intestine, Not the Large Intestine, Play a Major Role in Oral Prion Disease Pathogenesis. *J Virol* **89**, 9532-9547 (2015).

21. Y. C. Cheng *et al.*, Early and Non-Invasive Detection of Chronic Wasting Disease Prions in Elk Feces by Real-Time Quaking Induced Conversion. *PLoS One* **11**, e0166187 (2016).
22. G. Di Guardo, G. Marruchella, Faecal CWD prion excretion and inflammation. *New Microbiol* **33**, 183-184 (2010).
23. C. K. Ellis, S. F. Volker, D. L. Griffin, K. C. VerCauteren, T. A. Nichols, Use of faecal volatile organic compound analysis for ante-mortem discrimination between CWD-positive, -negative exposed, and -known negative white-tailed deer (Odocoileus virginianus). *Prion* **13**, 94-105 (2019).
24. G. Tamguney *et al.*, Asymptomatic deer excrete infectious prions in faeces. *Nature* **461**, 529-532 (2009).
25. J. M. Tennant *et al.*, Shedding and stability of CWD prion seeding activity in cervid feces. *PLoS One* **15**, e0227094 (2020).
26. B. V. Thomsen *et al.*, Diagnostic accuracy of rectal mucosa biopsy testing for chronic wasting disease within white-tailed deer (Odocoileus virginianus) herds in North America: effects of age, sex, polymorphism at PRNP codon 96, and disease progression. *J Vet Diagn Invest* **24**, 878-887 (2012).
27. D. E. Wood, J. Lu, B. Langmead, Improved metagenomic analysis with Kraken 2. *Genome Biol* **20**, 257 (2019).
28. E. Bolyen *et al.*, Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**, 852-857 (2019).
29. E. Bolyen *et al.*, Author Correction: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**, 1091 (2019).
30. K. A. Krautkramer, J. Fan, F. Backhed, Gut microbial metabolites as multi-kingdom intermediates. *Nat Rev Microbiol* **19**, 77-94 (2021).
31. F. Magne *et al.*, The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? *Nutrients* **12**, (2020).
32. S. Stojanov, A. Berlec, B. Strukelj, The Influence of Probiotics on the Firmicutes/Bacteroidetes Ratio in the Treatment of Obesity and Inflammatory Bowel disease. *Microorganisms* **8**, (2020).
33. D. Mariat *et al.*, The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol* **9**, 123 (2009).
34. R. E. Ley, P. J. Turnbaugh, S. Klein, J. I. Gordon, Microbial ecology: human gut microbes associated with obesity. *Nature* **444**, 1022-1023 (2006).
35. D. Minich *et al.*, Alterations in gut microbiota linked to provenance, sex, and chronic wasting disease in white-tailed deer (Odocoileus virginianus). *Sci Rep* **11**, 13218 (2021).
36. G. Tang *et al.*, Butyrate ameliorates skeletal muscle atrophy in diabetic nephropathy by enhancing gut barrier function and FFA2-mediated PI3K/Akt/mTOR signals. *Br J Pharmacol* **179**, 159-178 (2022).
37. M. E. Walsh *et al.*, The histone deacetylase inhibitor butyrate improves metabolism and reduces muscle atrophy during aging. *Aging Cell* **14**, 957-970 (2015).
38. E. Bouzamondo-Bernstein *et al.*, The neurodegeneration sequence in prion diseases: evidence from functional, morphological and ultrastructural studies of the GABAergic system. *J Neuropathol Exp Neurol* **63**, 882-899 (2004).
39. J. M. Bourgognon *et al.*, Alterations in neuronal metabolism contribute to the pathogenesis of prion disease. *Cell Death Differ* **25**, 1408-1425 (2018).
40. P. Baloni *et al.*, Metabolic Network Analysis Reveals Altered Bile Acid Synthesis and Metabolism in Alzheimer's Disease. *Cell Rep Med* **1**, 100138 (2020).
41. J. Wang *et al.*, Peripheral serum metabolomic profiles inform central cognitive impairment. *Sci Rep* **10**, 14059 (2020).

Fig.1

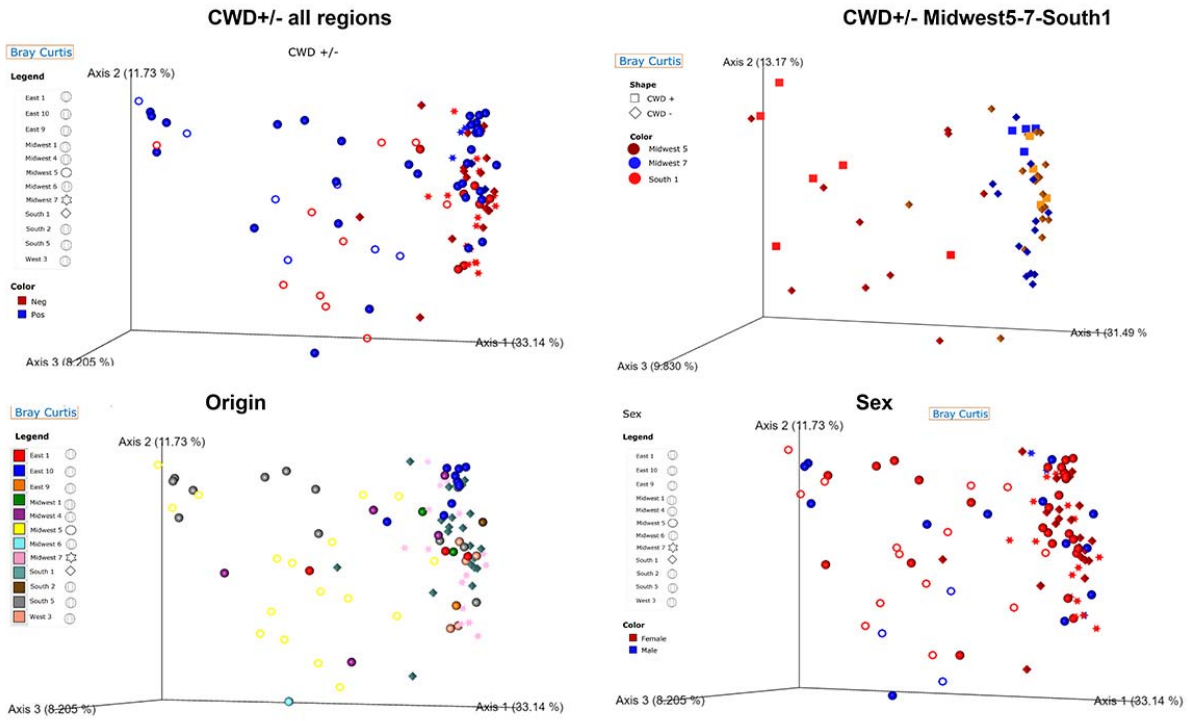

Figure 1. CWD status significantly affects fecal microbiome. PCoA Bray-Curtis plots of total microbiomic composition of the deer fecal samples grouped by different parameters including CWD status, geographical origin, and sex. The CWD status analysis was performed for the entire set of samples (top left) as well as separately for the regions that had good representation of both CWD positive and negative samples. See Dataset 1 for the Bray-Curtis box plot in different region combinations with different variables and Table S6 for statistical analysis.

Fig.2

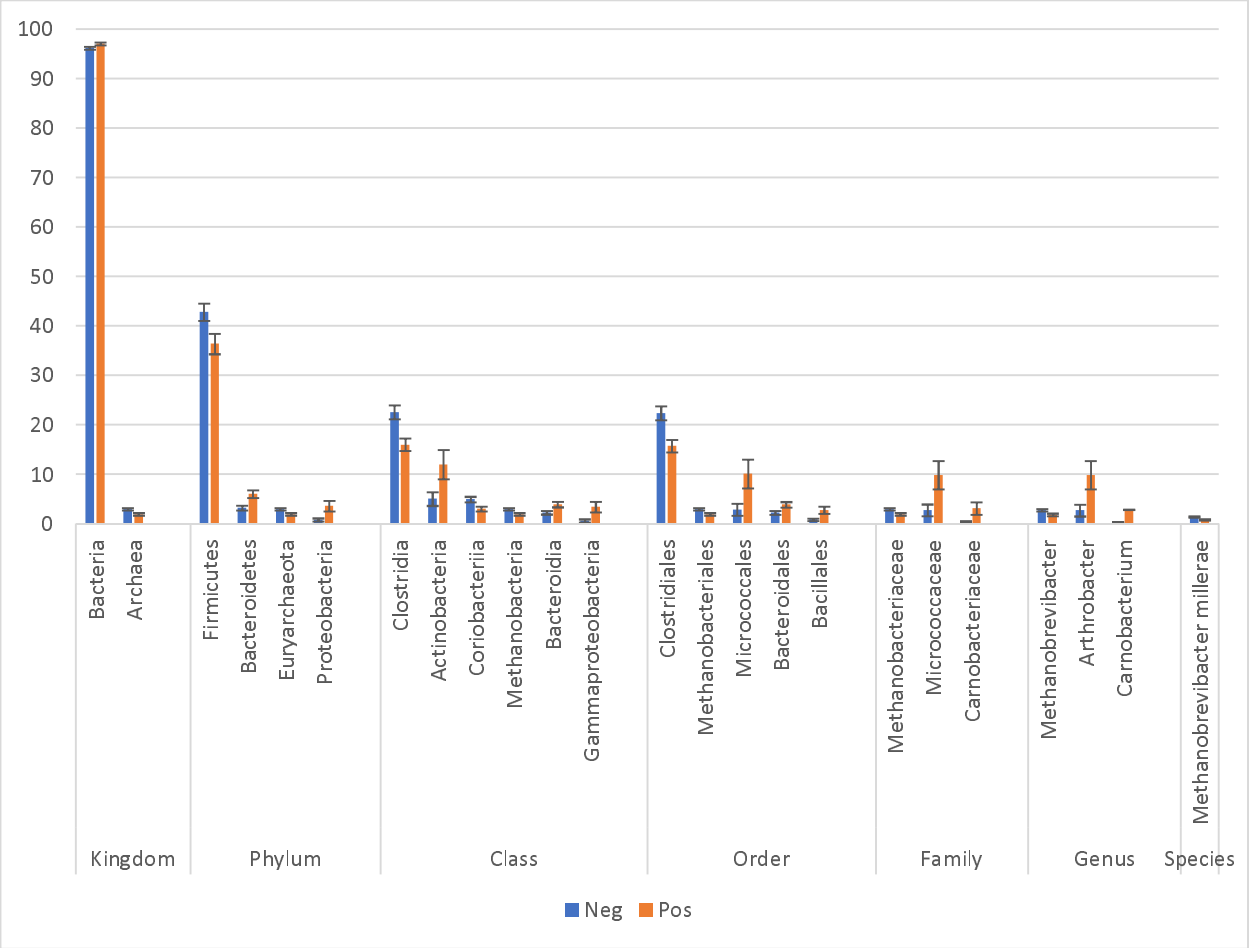

Figure 2. Differences in microbiota between feces from CWD positive and negative deer at different taxonomic levels. Taxa with >1% abundance in at least one type of the fecal samples are shown. Bar charts showing average % abundance of each taxa in the control (Neg), versus CWD-positive (Pos) samples are shown. Error bars represent SEM, n=50 for each set. See table S3 for the full list of hits and values for the abundance and p-value change in each pair.

Fig.3

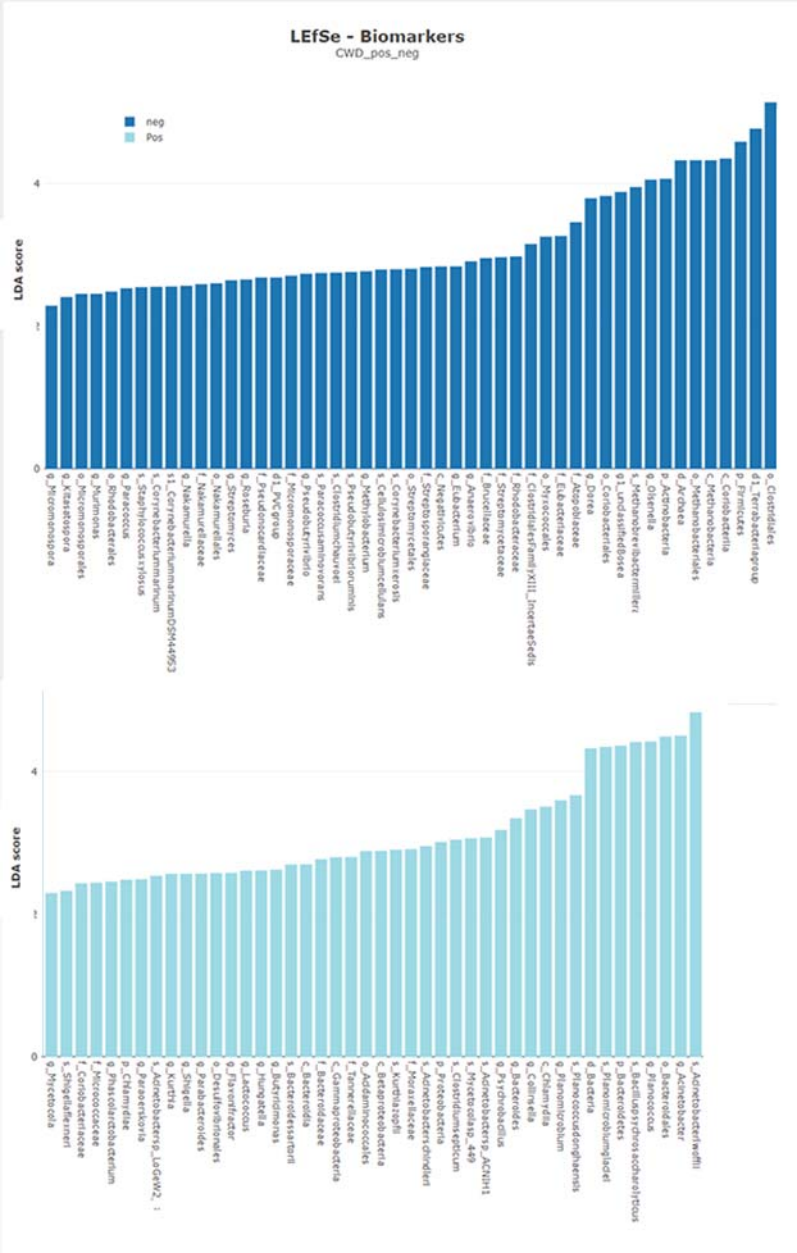

Figure 3. LefSe analysis bar plots. for the control (Neg), versus CWD-positive (Pos) samples. See Tables S4 for the values.

Fig. 4.

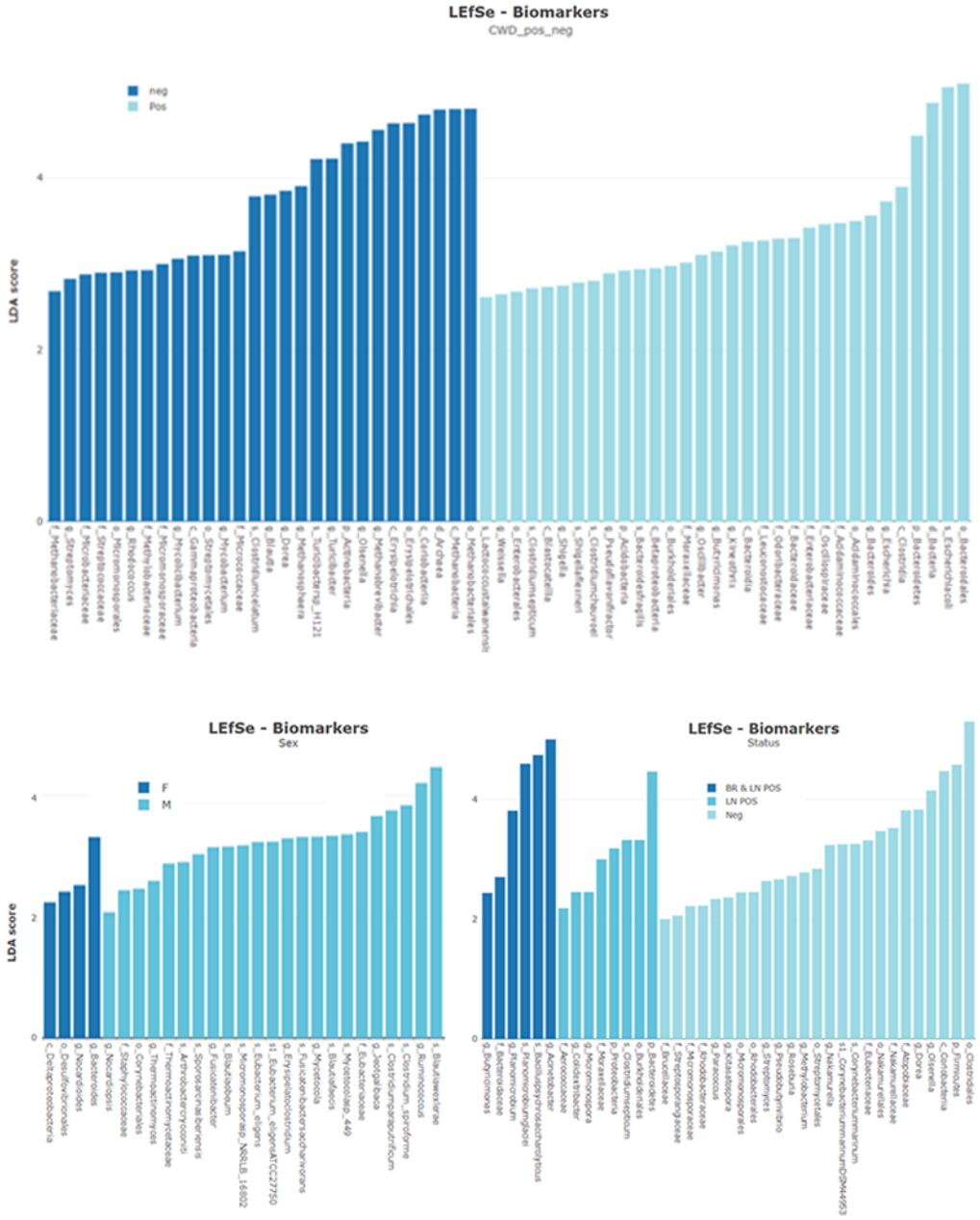

Figure 4. LefSe analysis bar plots for sex and CWD disease status. Neg: control, BR&LN Pos: advanced CWD, characterized by positive prion signal in the brain and lymph nodes; LN POS: Early CWD status, characterized by positive prion signal only in the lymph node. See table S4 for the values.

Fig. 5

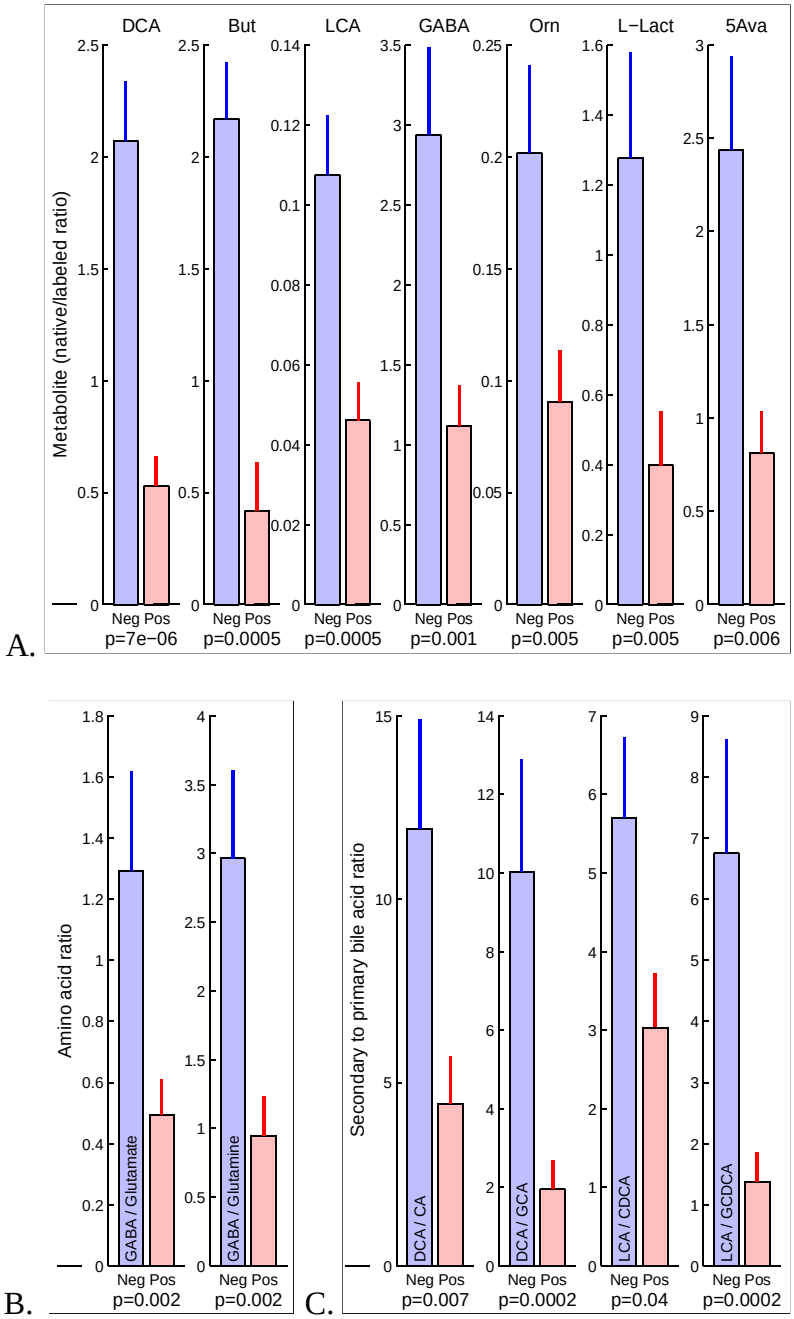

Figure 5. Difference between metabolites in feces of CWD positive and negative deer. The bar charts are for average metabilites and their ratios in the control (Neg, n=38), versus CWD-positive (Pos, n=31) samples. Error bars represent SEM for each set. A. These metabolites have significant CWD-dependent changes (FDR<0.05). B. GABA to glutamate and glutamine ratios. C. Secondary to primary bile acid ratios. 5Ava - 5-Aminopentanoic acid, But - butyrate, CA - cholic acid, CDCA - glycodeoxycholic acid, DCA - deoxycholic acid, GABA - gamma-aminobutyric acid, GCA - clycocholic acid, GCDCA - glycochenodeoxycholic acid, LCA - lithocholic acid, L-Lact - l-lactic acid, Orn - ornithine.

Fig 6

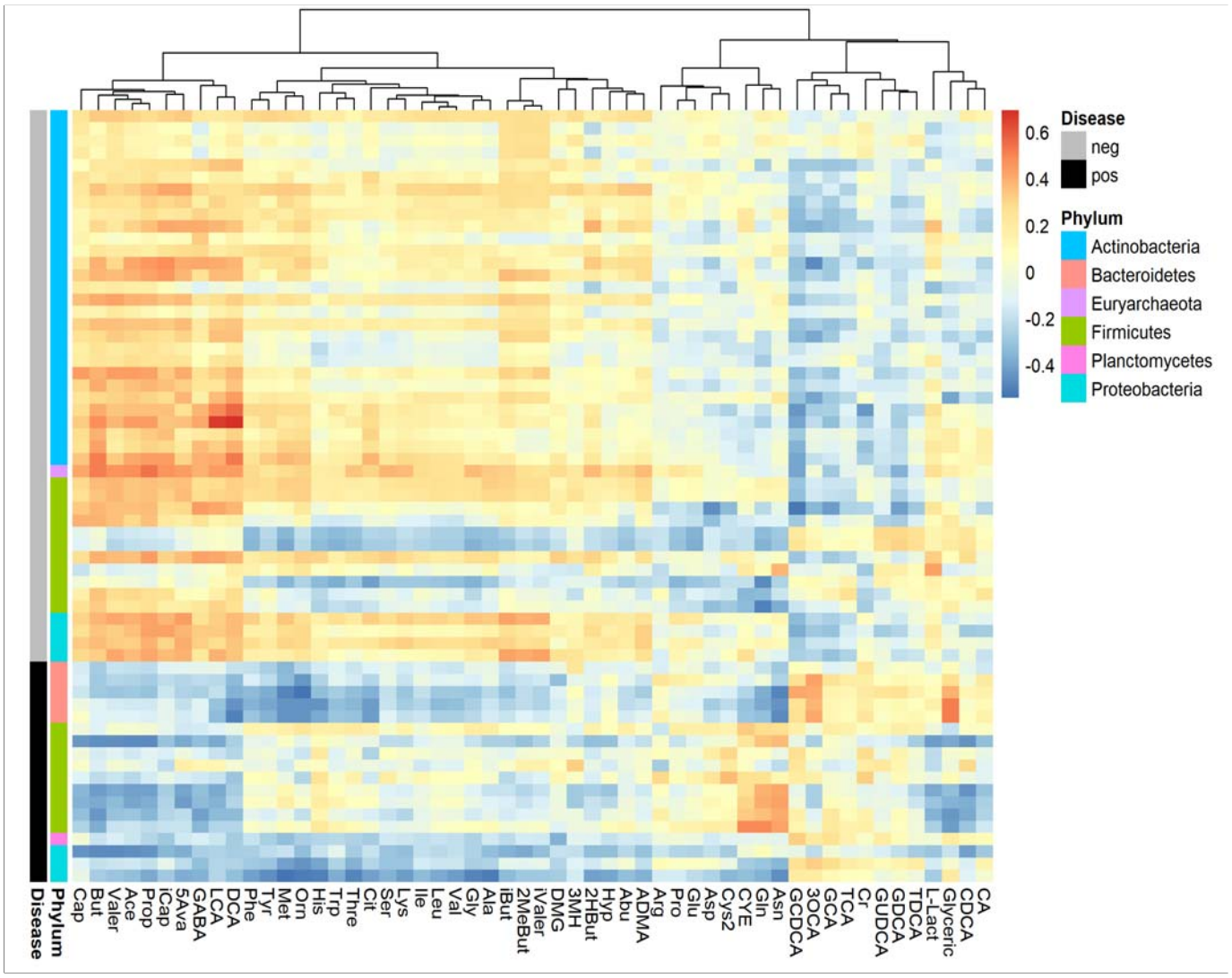

Figure 6. Heatmap of the correlation coefficient between metabolites and microbial abundance. The correlation coefficient of 63 significantly enriched microbial taxa (as rows), to 54 metabolites (columns). The color-scaled heatmap corresponds to Spearman's rank correlation coefficients. The microbial taxa (rows) are sorted for enriched in negative control or CWD disease positive fecal samples. Microbial taxa annotation represents the taxonomic profile at the phylum level.

Fig. 7

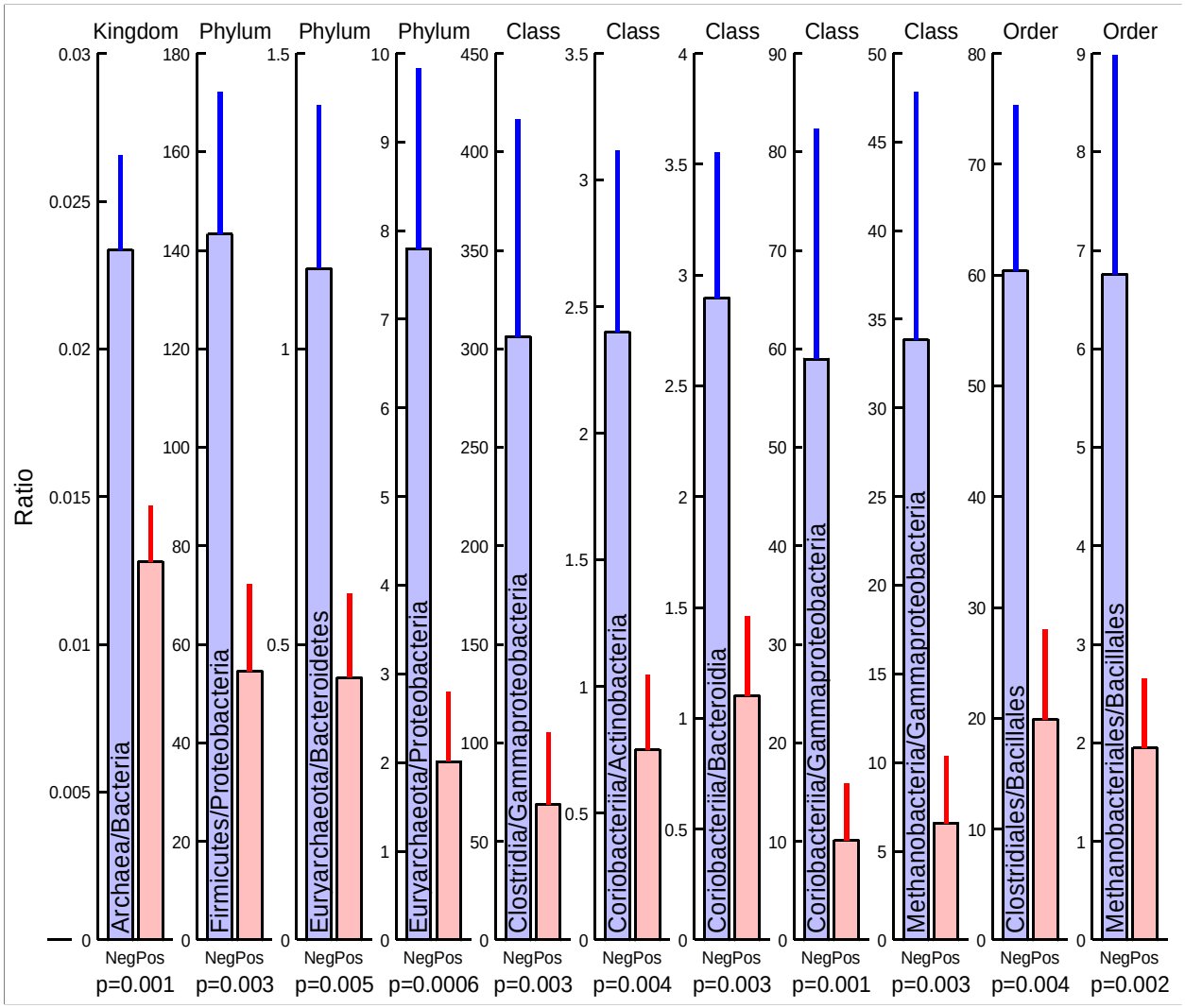

Figure 7. Taxa ratios as potential diagnostic markers of CWD. The bar charts are for average ratios of each pairs of taxa in the control (Neg), versus CWD-positive (Pos) samples. Error bars represent SEM, n=50 for each set. These taxa ratios represent the most significant CWD-dependent changes (p-value<0.005 and fold change>1.5) in fecal microbiome and can potentially serve as a diagnostic marker for CWD. See Table S3 for the value change in each pair and for p-value<0.05.