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Alterations in gut microbiota linked to provenance, sex, and chronic wasting disease in white-tailed deer (*Odocoileus virginianus*)

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Chronic wasting disease (CWD) is a fatal, contagious, neurodegenerative prion disease affecting both free-ranging and captive cervid species. CWD is spread via direct or indirect contact or oral ingestion of prions. In the gastrointestinal tract, prions enter the body through microfold cells (M-cells), and the abundance of these cells can be influenced by the gut microbiota. To explore potential links between the gut microbiota and CWD, we collected fecal samples from farmed and free-ranging white-tailed deer (*Odocoileus virginianus*) around the Midwest, USA. Farmed deer originated from farms that were depopulated due to CWD. Free-ranging deer were sampled during annual deer harvests. All farmed deer were tested for CWD via ELISA and IHC, and we used 16S rRNA gene sequencing to characterize the gut microbiota. We report significant differences in gut microbiota by provenance (Farm 1, Farm 2, Free-ranging), sex, and CWD status. CWD-positive deer from Farm 1 and 2 had increased abundances of *Akkermansia*, *Lachnospiraceae* UCG-010, and RF39 taxa. Overall, differences by provenance and sex appear to be driven by diet, while differences by CWD status may be linked to CWD pathogenesis.

Chronic wasting disease (CWD) is a fatal, contagious, neurodegenerative prion disease affecting both free-ranging and captive cervid species, including white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), elk (*Cervus elaphus elaphus*), and moose (*Alces alces*). First identified in Colorado, USA in the 1960s, CWD was given the designation as a transmissible spongiform encephalopathy (TSE) in 1978^{1,2}. Other TSEs include bovine spongiform encephalopathy, transmissible mink encephalopathy, kuru, and variant and sporadic Creutzfeldt-Jakob Disease (CJD)¹. Since the 1960s, CWD has spread across North America and has been identified in cervids in 26 states³. Outside of the United States, CWD has been documented in Korea, Canada¹, Sweden, Finland³, and Norway⁴. Clinical signs of CWD include progressive weight loss, altered posture, head tremors, ataxia, and polydipsia and polyphagia¹. Pathologically, CWD causes spongiform lesions within the central nervous system caused by an abnormal, diseased isoform (PrP^{CWD}) of the normal cellular prion protein (PrP^C). PrP^C is typically composed of multiple alpha-helices, but the abnormal isoform undergoes a transformation into a beta-sheet conformation, making it resistant to proteases, high temperatures, and standard disinfection protocols¹. The extreme hardiness of the diseased prion, as well as an incubation period ranging from 18 months to 5 years¹, makes CWD extremely challenging to control and manage.

CWD is commonly shed in the saliva, urine, and feces and is spread via direct or indirect contact with infectious prions and environmental fomites⁵. There is evidence that after oral ingestion and passage into the intestinal tract, prions enter the body through microfold cells (M-cells)^{6,7}. M-cells are specialized cells found in lymphoid follicles, the appendix, mucosal associated lymphoid tissue (MALT), and in the follicle-associated epithelium (FAE) of Peyer's patches in the gut⁸. M-cells are considered the gatekeeper of the intestine, as they continuously sample and internalize material from the lumen of the intestine via transcytosis to the underlying lymphoid tissue in the Peyer's patch for initiation of mucosal and systemic immune responses⁷⁻⁹. Studies in mice have shown that after oral entry of a TSE agent, prions initially accumulate in Peyer's patches and mesenteric lymph nodes in the gut^{7,10}. Increased M-cell abundance has been linked to an increased susceptibility to orally acquired prion

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diseases, and the absence of M-cells at the time of oral exposure to infectious prions blocks neuroinvasion and disease development⁶. Importantly, M-cell abundance can be influenced by microbes in the gut as well as by enteric inflammation, and M-cell induction and development has been linked to inflammatory cytokine stimulation and pathogen infection^{11–13}. Further, a 2009 study¹⁴ found that mice with intestinal inflammation as a result of increased levels of *Salmonella* had a significantly higher risk of prion disease. Therefore, increased abundance of M-cells in the gut due to a concurrent inflammation or due to increased levels of specific microbes, such as *Salmonella*^{14,15}, could potentially enhance uptake of prions from the gut lumen¹².

The gut microbiota serves as a defense system against pathogens and other disease-causing agents¹⁶. Furthermore, the gut microbiome plays an important role in host immune development¹⁷, neurogenesis¹⁸, brain development¹⁹, and microglia function in the central nervous system (CNS)^{20,21}. The gut microbiome has also been linked to human neurologic conditions via the “gut-brain axis”²¹. Both Parkinson’s Disease (PD) and Alzheimer’s Disease (AD) have similarities to prion diseases and involve abnormal protein aggregates and protein misfolding occurring in the brain, including a conversion of alpha-helical structures to beta-sheet structures in PD^{22–26}. As a result of these similarities, the “prion hypothesis” suggests that PD is a prion-like disease²⁷. Studies indicate a critical relationship between the gut microbiota and neurologic diseases, including PD, AD, amyotrophic lateral sclerosis (ALS), and autism^{21,28–32}. In a 2016 study, alpha synuclein-overexpressing mice (a mouse model for PD) treated with antibiotics had an altered gut microbiota and exhibited reduced brain pathology and motor deficits, identifying direct links between alterations in the gut microbiota and brain pathology associated with PD. Further, microbial colonization of germ-free mice with stool samples from patients with PD resulted in the disease-typical protein-misfolding-mediated motor deficits³¹.

Although there is growing evidence for the role of gut microbes in neurologic diseases, there has been relatively little work examining gut microbes in prion disease, and none to our knowledge, on chronic wasting disease. Earlier work on scrapie, a prion disease of sheep and goats, explored the role of the microbiota on scrapie pathogenesis by inoculating germfree or conventional mice with prions³³. Germfree mice demonstrated increased survival as compared to conventional mice, suggesting a role for microbes in enhancing prion disease³³. Subsequent work, using different approaches, found no difference in survival between germfree mice or mice colonized with a defined microbiota; although, disease progression was more rapid in mice colonized with microbiota³⁴. More recent work reported no difference in survival or disease duration in germfree or conventional mice inoculated with scrapie but suggested that the microbiota could exacerbate prion disease³⁵.

In this study, we used 16S rRNA gene sequencing to examine the gut microbiota of white-tailed deer (*Odocoileus virginianus*) from two deer farms (breeding facilities) that were depopulated due to the presence of CWD. Additionally, we characterized the gut microbiota of free-ranging white-tailed deer harvested from Cleveland Metroparks in northeast Ohio as part of its deer population management program. Based on previous studies that have reported differences in the gut microbiota of wild and captive ruminants, including deer³⁶, we hypothesized that microbial communities would differ between deer by provenance (Farm 1, Farm 2, and Free-ranging) with the greatest differences being observed between farmed and free-ranging deer. Based on studies that have reported alterations in gut microbiota associated with neurologic disease, we hypothesized that we would observe differences in farmed-deer gut microbial communities by CWD status (CWD-positive, CWD non-detect).

Methods

Farmed deer fecal sample collection. All deer on Farm 1 (n=101) and Farm 2 (n=30) (Midwest, Wisconsin, USA) were humanely euthanized under United States Department of Agriculture (USDA) purview and in accordance with USDA CWD Program Standards and the American Veterinary Medical Association (AVMA) guidelines for euthanasia after a deer from each farm tested positive for CWD at harvest. Fecal samples were opportunistically collected digitally from the rectum of each deer in cooperation with USDA Veterinary Services (Farm 1, n=101, May 2018; Farm 2, n=30, May 2019; Wisconsin, USA; Table 1). Samples were stored on dry ice until they were transferred into a –80 °C freezer. Fresh gloves were donned for sampling each deer. Samples remained at –80 °C until DNA extraction was performed. We sampled all deer without exclusion in order to maximize our likelihood of obtaining samples from deer that tested positive for CWD, which is diagnosed postmortem. Fecal samples were collected under USDA Animal and Plant Health Inspection Service (APHIS) permit #136,689 (for the transport of controlled material).

Free-ranging deer fecal sample collection. As part of the Cleveland Metroparks deer population management program (Midwest, Ohio, USA), free-ranging white-tailed deer were humanely euthanized under Cleveland Metroparks purview and in accordance with AVMA guidelines for euthanasia. This population management program was approved by the Ohio Department of Natural Resources, Division of Wildlife under Deer Damage Control Permit #3377. Fecal samples were opportunistically collected from a total of 100 deer over six months old including 50 males and 50 females (January–March 2018; Table 1). Fecal samples were digitally collected from the rectum of each deer, placed in a sterile plastic bag, and frozen at –20 °C. Samples were transferred into a –80 °C freezer within 24 h of collection where they remained until DNA extraction.

The Metroparks population management program also includes regular CWD testing, and Cleveland Metroparks deer herds were tested for CWD in 2008 (125 deer), 2011 (53 deer), 2012 (50 deer), 2016 (277 deer), and 2020 (135 deer). No deer was found to have detectable PrP^{CWD}. The free-ranging deer sampled in this study (2018) were not tested for CWD but were presumed CWD non-detect based on extensive CWD testing on Cleveland Metroparks deer herds in years antecedent and subsequent to 2018. Further, until December 2020, CWD had never been detected in any free-ranging deer in the state of Ohio.

All samples from farmed and free-ranging deer were collected opportunistically in cooperation with regulatory agencies and as part of routine surveillance programs; no deer were euthanized specifically for the purposes

	CWD positive	CWD non-detect	p-value
Farm 1			
Total number of deer	20	81	
Age at depopulation (mean ± SD)	2.59 ± 1.02	4.37 ± 3.43	0.14
Sex (n, %)			0.001
Female	1 (1)	35 (35)	
Male	19 (19)	46 (45)	
Farm 2			
Total number of deer	6	24	
Age at depopulation (mean ± SD)	2.76 ± 0.39	3.22 ± 2.71	0.32
Sex (n, %)			0.03
Female	0 (0)	12 (40)	
Male	6 (20)	12 (40)	
Free-ranging deer			
Total number of deer	100		NA
Age at depopulation (mean ± SD)	1.70 ± 1.01		
Sex (n, %)			
Female	50 (50)		
Male	50 (50)		

Table 1. Farm 1, Farm 2, and free-ranging deer demographics.

of this study. Post-mortem collection of feces from farmed and free-ranging deer was deemed exempt by the Ohio State University Institutional Biosafety Committee and the Institutional Animal Care and Use Committee. This study was carried out in accordance with ARRIVE guidelines.

CWD sample collection and testing. CWD testing on farmed deer was conducted per USDA CWD Program Standards and under the purview of USDA Animal and Plant Health Inspection Service (APHIS). Testing was performed using standard operating protocols approved and audited by the USDA and documented in the quality system of the Wisconsin Veterinary Diagnostic Laboratory (WVDL). The WVDL is a National Animal Health Laboratory Network (NAHLN) Level 1 laboratory and is accredited by the American Association of Veterinary Laboratory Diagnosticians. The heads were removed from all farmed deer greater than one year of age and the obex region of the brainstem and medial retropharyngeal lymph nodes (MRLN) were collected following USDA APHIS guidelines³⁷. Regulatory surveillance samples were shipped to the National Veterinary Services Laboratory (NVSL) for CWD Immunohistochemistry (IHC). Enzyme-linked immunosorbent assay (ELISA) testing was performed at WVDL. IHC and ELISA-based testing for the abnormal prion protein were performed on the dorsal motor nucleus of the vagus nerve in the obex and MRLNs. For IHC testing, tissues were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm, mounted on slides, and examined using IHC with monoclonal antibody (Mab) F99/97.6.1³⁸. All testing was performed using USDA-APHIS-NAHLN approved protocols (IHC: SOP-PS-0002.02; ELISA: SOP-PS-0007.01). Animals were considered CWD-positive if any one of the tissues examined contained detectable PrP^{CWD}. Animals in which tissues did not contain detectable PrP^{CWD} were considered CWD non-detect animals. CWD testing was performed under USDA regulatory guidelines and not for the purposes of this study. CWD test results generated under USDA purview were shared with investigators on this study for the purpose of assessing deer gut microbiota in relation to CWD status.

DNA extraction, amplification, and sequencing. DNA extraction on all fecal samples was performed as follows: Samples were randomized for extraction to minimize batch effects by kit. Approximately 0.25 g of stool was used for each extraction with QIAamp PowerFecal DNA Kits (Qiagen, Venlo, Netherlands). Following DNA isolation, DNA concentration and purity was measured using a Qubit Fluorometer 4 (Invitrogen, Carlsbad, CA, USA) and a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), respectively. Farm 1 DNA samples had below optimal A260/A280 (average: 1.53; optimal range 1.8–2.0) and A260/230 ratios (average: 1.70; optimal range 2.0–2.2) while Farm 2 and free-ranging deer DNA ratios were all within the expected range. An ethanol precipitation was performed on DNA samples from Farm 1 to improve DNA purity using a protocol³⁹ from MRC Holland (Amsterdam, Netherlands). Briefly, 4 µl of sodium acetate and 132 µl of 200 proof ethanol was added to 40 µl of the DNA. This was incubated for 30 min at 4°C then centrifuged for 30 min at 4°C. After removing the supernatant, 250 µl of 70% ethanol was added to the DNA and centrifuged for 15 min. The supernatant was again removed and the DNA pellet was resuspended in 40 µl of the C6 elution buffer from the PowerFecal (Qiagen) DNA isolation kits. All DNA samples were submitted for library preparation and 16S rRNA gene sequencing on an Illumina MiSeq (Farm 1 and Free-ranging: The Ohio State University Molecular and Cellular Imaging Center; Farm 2 samples: Argonne National Laboratory). Earth Microbiome Project primers (515F and 806R) were used to amplify the V4 hypervariable region of the bacterial 16S rRNA gene⁴⁰. Negative (no template) controls were extracted with each kit and underwent amplification,

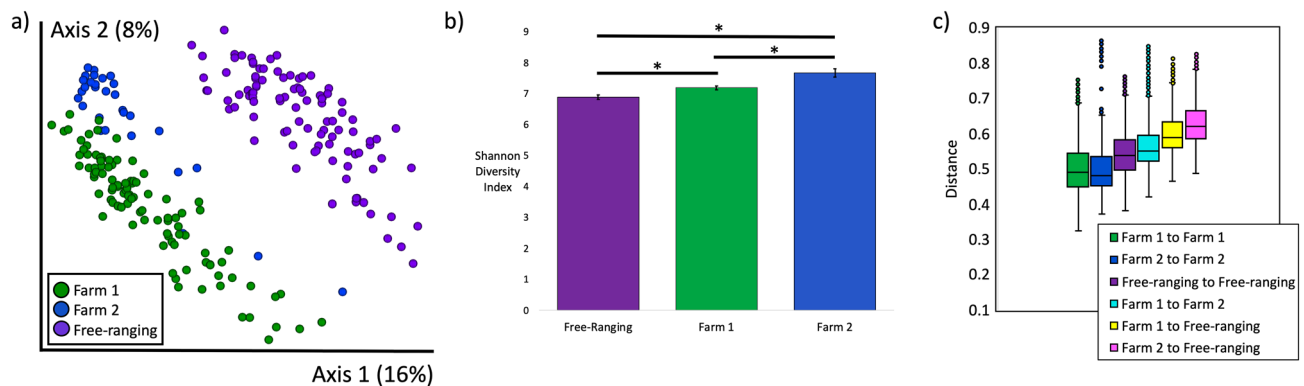


Figure 1. Microbial composition and diversity by provenance. (a) Microbial composition (Unweighted UniFrac) differed significantly by provenance (PERMANOVA $p=0.001$). (b) Microbial diversity as measured by the Shannon Diversity Index differed significantly by provenance ($p=6.5 \times 10^{-11}$). All pairwise comparisons $*p < 0.001$. (c) Farmed deer have more similar microbial communities to each other than to free-ranging deer (Unweighted UniFrac pairwise PERMANOVA: Farm 1 to Farm 2 pseudo-F = 9, $q=0.001$; Farm 1 to Free-ranging pseudo-F = 38, $q=0.001$; Farm 2 to Free-ranging pseudo-F = 18, $q=0.001$).

library preparation, and sequencing along with the rest of the samples. A positive control sample (DNA from a mixed microbial community) underwent amplification, library preparation, and sequencing along with the rest of the samples in each sequencing run, allowing to compare results between sequencing runs.

Sequence processing and analysis. A total of 231 samples were submitted for sequencing. Raw, paired-end reads were processed and denoised in QIIME2 v. 2020.2⁴¹. Taxonomy was assigned using the SILVA 132 99% amplicon sequence variants (ASVs) database from the 515F/806R classifier^{42,43}, and samples were filtered at a sequencing depth of 10,000 features. This resulted in the retention of 229 samples with the loss of 2 samples—one CWD non-detect male deer from Farm 1 and one CWD non-detect female deer from the free-ranging population. After filtering, 5,803,410 reads from 229 samples were used for analysis (average of 25,342 reads per sample). Reads per sample ranged from 10,049 to 92,179 reads. Alpha (Shannon Diversity Index) and beta diversity were analyzed using QIIME 2⁴¹. Beta diversity indices were compared using permutational multivariate analysis of variances (PERMANOVA) between weighted and unweighted UniFrac distance matrices. P -values were corrected for multiple comparisons using the Benjamini–Hochberg FDR correction, and values less than 0.05 were considered significant.

An analysis of composition of microbes (ANCOM) was used to determine differentially abundant taxa between groups after filtering out taxa that had fewer than 10 reads and taxa that occurred in fewer than two deer. ANCOMs generate W values, which represent the number of times the null hypothesis is rejected in pairwise comparisons of microbial species ratios between groups. For example, a W value of 80 would indicate the null hypothesis was rejected 80 times when comparing microbial species ratios between different groups. We performed ANCOMs at both the L7 and amplicon sequence variant (ASV) levels. The L7 level is roughly equivalent to a species level while an ASV is roughly equivalent to a bacterial strain and may differ from another ASV by as few as one nucleotide⁴⁴. Multiple ASVs may be classified as a single L7 level taxa. However, deeper genome sequencing is necessary for true species and strain differentiation. The single CWD-positive female was not included in statistical analyses comparing CWD-positive and CWD non-detect animals to reduce any confounding introduced by sex. Age was compared using a Kruskal–Wallis test between CWD-positive and CWD non-detect deer by farm after testing the data for normality using a Shapiro Wilk Test (R Studio, version 1.3.1093. Sequencing data are available at NCBI Bioproject PRJNA688284.

Results

Microbial composition and diversity by provenance and sex. When we examined the gut microbiota of all deer ($n=229$), we found significant differences in gut microbial composition and diversity by provenance (Farm 1, Farm 2, Free-ranging), with farmed deer having greater microbial diversity than free-ranging deer (Unweighted UniFrac PERMANOVA $p=0.001$, Shannon Diversity Index $q=6.5 \times 10^{-11}$, Weighted UniFrac PERMANOVA $p=0.001$; Fig. 1a, b, Supp. Fig. 1). Moreover, farmed deer from both farms had more similar gut microbiota to each other than to free-ranging deer (Farm 1 to Farm 2 pseudo-F = 9, $q=0.001$; Farm 1 to Free-ranging pseudo-F = 38, $q=0.001$; Farm 2 to Free-ranging pseudo-F = 18, $q=0.001$; Fig. 1c).

To identify microbial taxa that were differentially abundant between farmed and free-ranging deer, we combined all deer from Farm 1 and 2—excluding CWD-positive deer—and compared these against the free-ranging deer. Through an ANCOM at the L7 (roughly species) level, we identified 82 taxa that were differentially abundant (Supp. Table 1). Twenty-six of these taxa were in the order Bacteroidales (phylum Bacteroidetes) and seven of these were in the family Prevotellaceae. The vast majority of the Bacteroidales taxa (22 of 26) were significantly increased in the farmed deer. On the other hand, free-ranging deer had significantly greater abundances of taxa (25 of 38) in the Clostridiales order (phylum Firmicutes), all of which were in the Ruminococcaceae and

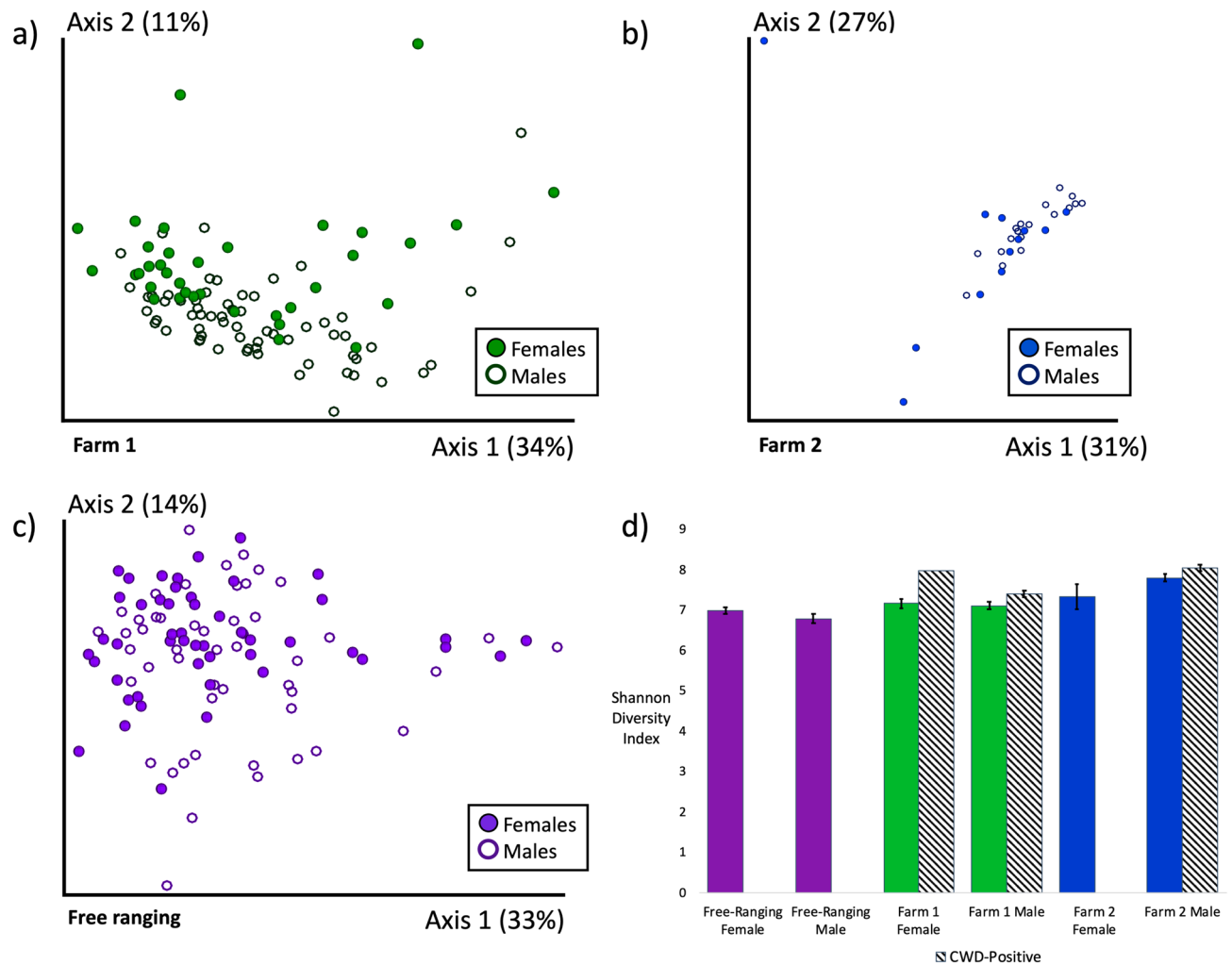


Figure 2. Microbial composition and diversity by sex. Gut microbial composition (weighted UniFrac) by sex on (a) Farm 1, (b) Farm 2, and (c) free-ranging deer. Gut microbiota differed significantly by sex on Farm 1 (PERMANOVA $p=0.008$) and in Free-ranging deer (PERMANOVA $p=0.018$), but not on Farm 2 (PERMANOVA $p=0.179$). (d) Microbial diversity (Shannon diversity index) did not differ significantly by sex (Farm 1: $p=0.34$, Farm 2: $p=0.15$, Free-ranging: $p=0.53$) or CWD status (Farm 1: $p=0.07$, Farm 2: $p=0.26$). Only CWD non-detect deer were included in statistical analyses by sex.

Lachnospiraceae families. Based on these results, we decided to compare log Firmicutes:Bacteroidetes (F:B) ratios for farmed and free-ranging deer. Log F:B ratios are associated with dietary energy harvest and higher ratios indicate greater energy extraction^{45–47}. We found significantly higher F:B ratios in the free-ranging deer as compared to the farmed deer (Log F:B ratios (mean \pm SE), Free-ranging: 0.39 ± 0.03 ; Farmed: 0.08 ± 0.02 ; Kruskal–Wallis $p < 0.0001$).

We also discovered significant differences in microbial composition but not diversity by sex on Farm 1 and in free-ranging deer (CWD non-detect deer only; Farm 1: Unweighted UniFrac PERMANOVA $p=0.008$, Shannon Diversity Index $p=0.34$, Weighted UniFrac PERMANOVA $p=0.003$; Free-ranging: Unweighted UniFrac PERMANOVA $p=0.018$, Shannon Diversity Index $p=0.53$, Weighted UniFrac PERMANOVA $p=0.066$; Fig. 2a, c, d Supp. Fig. 2a, c). No significant differences in microbial composition or diversity were detected by sex on Farm 2 (CWD non-detect deer only; Farm 2: Unweighted UniFrac PERMANOVA $p=0.179$, Shannon Diversity Index $p=0.15$, Weighted UniFrac PERMANOVA $p=0.115$; Fig. 2b, d Supp. Fig. 2b). There were also no differentially abundant microbial taxa detected by sex on Farm 2. However, on Farm 1, we identified a single taxa that was significantly increased in males. This was an uncultured bacterium from the order Bacteroidales, family RF16 (ANCOM, L7—roughly species level, $W=626$). In the free-ranging deer population, there were multiple differentially abundant taxa by sex, with the two most differentially abundant including a microbe in the genera *Oscillibacter* and a microbe in the family *Lachnospiraceae*, genera GCA-900066575. Both of these taxa were significantly increased in males (Supp. Table 2).

Microbial composition and diversity by CWD status. Based on the microbial composition differences observed by sex and the fact that there was only one CWD-positive female in the entire data set, we opted to ana-

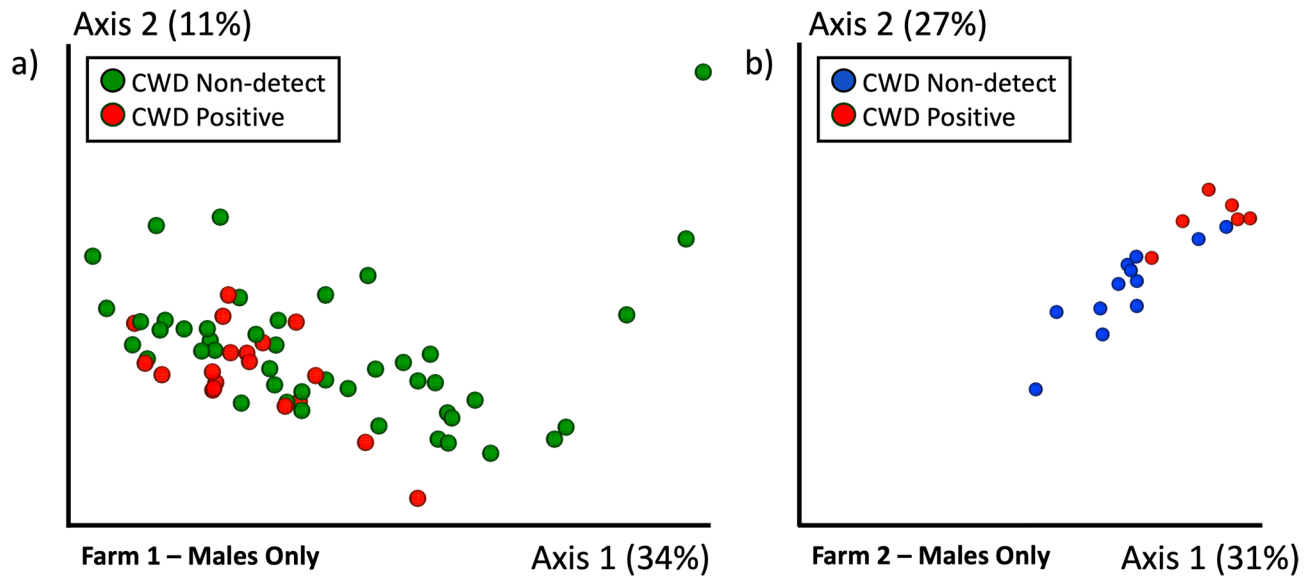


Figure 3. Microbial composition by CWD status. Gut microbial composition (weighted UniFrac) differed significantly by CWD Status on (a) Farm 1 (PERMANOVA $p=0.011$), (b) Farm 2 (PERMANOVA $p=0.002$).

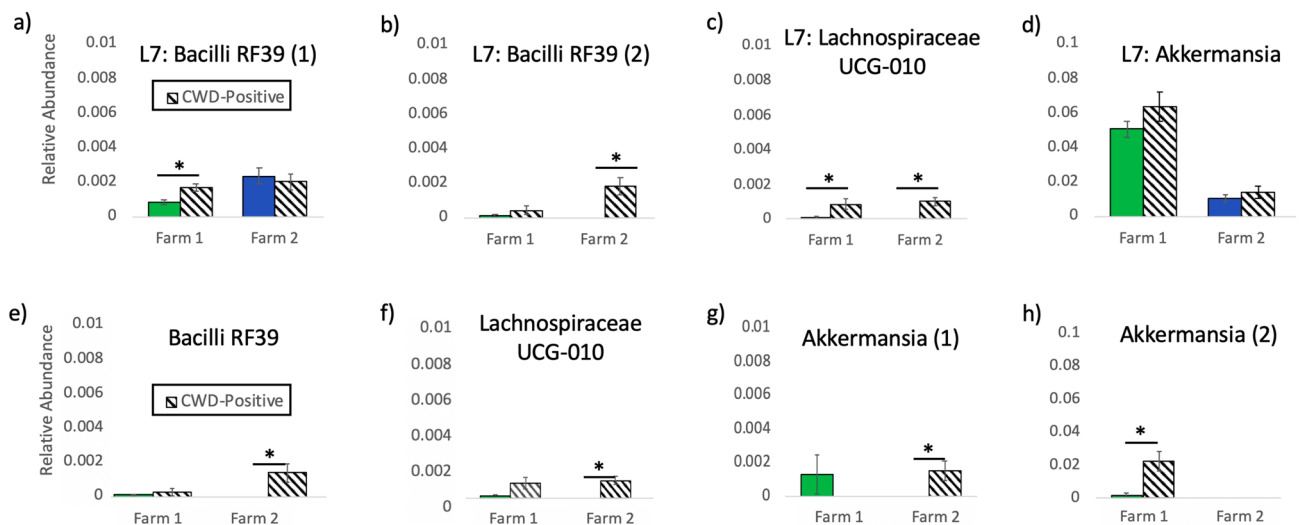


Figure 4. Differentially abundant microbial taxa by CWD status. Differentially abundant taxa by CWD Status, including (a) an L7 (roughly species) level taxa in the Bacilli class, order RF39, (b) a second L7 level taxa in the Bacilli class, order RF39, (c) an L7 level taxa in the Lachnospiraceae UCG-10 family, (d) an L7 level taxa in the *Akkermansia* genera, (e) an ASV (roughly strain level) in the Bacilli class, order RF39 (formerly Mollicutes RF39), (f) an ASV in the Lachnospiraceae UCG-10 family, (g) an ASV in the *Akkermansia* genera, (h) a second ASV also in the *Akkermansia* genera.

lyze only male deer in relation to CWD status. The single CWD-positive female deer was still included in data visualizations. Microbial composition differed significantly in CWD-positive deer on both farms (Males only; Farm 1: Unweighted UniFrac PERMANOVA $p=0.003$ Weighted UniFrac PERMANOVA $p=0.011$; Farm 2: Unweighted UniFrac PERMANOVA $p=0.003$, Weighted UniFrac PERMANOVA $p=0.002$; Fig. 3, Supp. Fig. 3). Increased microbial diversity (Shannon Index), although not significant, was also observed in CWD-positive males on both farms (Farm 1 $p=0.07$; Farm 2 $p=0.26$; Fig. 2d).

We further discovered several differentially abundant microbes at the L7 and ASV levels between CWD-positive and CWD non-detect males on both farms. (Note, multiple ASVs may be classified as a single L7, roughly species level, taxa.) On Farm 1, at the L7 level, multiple taxa were differentially abundant between CWD-positive and CWD non-detect males, the top four of which included: an uncultured bacterium from the class Bacilli (formerly Mollicutes), order RF39, increased in CWD-positive males (ANCOM $W=80$; Fig. 4a); an uncultured *Paludibacter* species increased in non-detect males (ANCOM $W=54$); an uncultured bacterium

in the order Gastranaerophilales also increased in non-detect males (ANCOM $W = 34$); and a microbe in the family Lachnospiraceae UCG-10 increased in CWD-positive males (ANCOM $W = 28$; Fig. 4c) (Supp. Table 3).

On Farm 2, at the L7 level, two microbes were found to be differentially abundant. Both were increased in CWD-positive males and included a unidentified rumen bacterium from the class Bacilli, order RF39 (ANCOM $W = 132$; Fig. 4b) and a microbe from the family Lachnospiraceae UCG-10 (ANCOM $W = 115$; Fig. 4c). On Farm 2, multiple ASVs were also differentially abundant (Supp. Table 4), the top three of which, all increased in CWD-positive males, were an ASV from the class Bacilli (formerly Mollicutes), order RF39 (ANCOM $W = 132$; Fig. 4e), an ASV from the family Lachnospiraceae UCG-10 (ANCOM $W = 176$; Fig. 4f), and an ASV from the genera *Akkermansia* (ANCOM $W = 90$; Fig. 4g). On Farm 1, only one ASV in the *Akkermansia* genera was found to be differentially abundant and increased in CWD-positive males (ANCOM $W = 1958$; Fig. 4h). This *Akkermansia* ASV differed from the *Akkermansia* ASV on Farm 2, suggesting species or strain level variance in *Akkermansia* by farm; although, deeper sequencing is required to assess this. *Akkermansia* taxa at the L7 level were not differentially abundant on either farm (Fig. 4d).

Discussion

In this study, we used 16S rRNA gene sequencing to compare the gut microbiota of farmed and free-ranging white-tailed deer (*Odocoileus virginianus*). We hypothesized that deer gut microbiota would differ by provenance (Farm 1, Farm 2, and Free-ranging) and disease status (CWD-positive, CWD non-detect). Indeed, microbial composition and diversity did vary with provenance. Moreover, composition but not diversity varied with sex (Farm 1 and Free-ranging only) and with CWD status (Farm 1 and Farm 2).

Drivers of microbial community composition by provenance. Multiple factors could contribute to the gut microbial differences we observed based on provenance, including diet, spatial proximity⁴⁸, host genetics⁴⁹, and biogeography⁵⁰. Although, diet likely has the strongest influence on gut microbial composition and diversity as compared to the other factors^{51–53}. The free-ranging deer in this study had diets that primarily consisted of browse, small plants, shrubs, grasses and occasional agricultural, landscaping, and garden plants⁵⁴. The farmed deer had access to pastures and were also fed a variety of commercial deer feeds, grains, hay, and supplemental items, including peanuts, roasted soybeans, and dandelions. As diets differed between farmed and free-ranging deer, it was not surprising that farmed and free-ranging deer had significantly different microbial communities or that there were significant differences in gut microbiota between the two farms with different feeding regimens. Multiple previous studies have also reported gut microbial differences between wild and captive animals^{55–57}, including ruminants^{36,58}.

We predicted that based on differing diets, host genetics, and biogeography, we would observe distinct microbial signatures in deer from each location (Farm 1, Farm 2, Free-ranging) (Fig. 1a). We further predicted that within locations, farmed deer would have more similar (less distant) microbiota due to more regulated diets and more limited “home ranges” as compared to free-ranging deer (Fig. 1c, Farm 1 to Farm 1, Farm 2 to Farm 2, Free-ranging to Free-ranging). Finally, we hypothesized that the greatest differences in microbial communities would be observed between farmed and free-ranging deer since farmed deer generally share more similar diets (formulated commercial feeds, grains, hay, pasture) than free-ranging deer (Fig. 1c, Farm 1 to Farm 2, Farm 1 to Free-ranging, Farm 2 to Free-ranging). Our results supported each of these predictions. Although we cannot parse the individual effects of diet, spatial proximity, host genetics, and biogeography in this data set, the differentially abundant taxa identified between farmed and free-ranging deer strongly support a role for diet as a key driver of the microbial community differences we observed. Free-ranging deer consume a plant and fiber-rich diet full of shrubs and browse, while farmed deer consume a starchier diet of grains and commercial feed in addition to pasture and hay. Microbial taxa in the Lachnospiraceae and Ruminococcaceae families were increased in abundance in the free-ranging deer, while Bacteroidales taxa, like Prevotellaceae, were increased in the farmed deer (Supp. Table 1). Lachnospiraceae and Ruminococcaceae taxa are associated with plant-rich diets, and these taxa metabolize plant materials such as cellulose and hemicellulose^{57,59,60}. Bacteroidales and Prevotellaceae are more commonly associated with starch consumption, and in ruminants, Bacteroidales, including *Prevotella*, increase in animals on concentrate/grain diets^{57,61–63}.

Increased Firmicutes:Bacteroidetes ratios in free-ranging deer further suggests increased energy extraction and fermentation efficiency. In humans, increased F:B ratios are associated with obesity; in farmed ruminants, increased ratios are positively correlated with average daily gain^{45,46}. In foregut-fermenting primates (which have ruminant-like digestion), wild primates exhibited higher F:B ratios than captive primates⁴⁷. This was attributed to the need for the wild primates to maximize energy extraction from “low-quality” food items such as fibrous plants, bark, and seeds, while captive primates, with “high quality” diets rich in soluble carbohydrates, were less dependent on efficient energy harvest⁴⁷. Similarly, free-ranging deer gut microbiota may maximize energy extraction from a fibrous browse diet, while the grain-rich diets of farmed deer reduces the need for fermentation efficiency and creates a niche for microbial taxa capable of metabolizing soluble starches and sugars.

Microbial community structure by sex. Interestingly, we also identified microbial composition differences by sex on Farm 1 and in free-ranging deer. No differences were observed on Farm 2, which also had the smallest sample size ($n = 18$ males, 12 females), limiting our power to detect these differences. Microbial community alterations associated with sex could be attributed to differential feeding / diets by sex or hormonal influences on the gut microbiome. We received anecdotal reports of differential feeding by sex on Farm 1 based on deer breeding and growth requirements. While we did not characterize the diet of free-ranging deer in this study, a previous study on white-tailed deer reported that, in winter, female deer in the Midwest consumed more grass (higher quality feed) and less browse than male deer⁶⁴. Our samples were also collected from free-

ranging deer in the Midwest during winter. Differentially abundant taxa observed between sexes (Bacteroidales, Lachnospiraceae, *Oscillibacter*—all increased in males) also supported a role for diet in microbial community differences by sex. Notably, *Oscillibacter* species increase in humans on diets high in resistant starch and low in carbohydrates⁶⁵, which is consistent with the browse-rich winter diet of free-ranging male deer⁶⁴. Breeding hormones have also been linked to gut microbial changes by sex in wild animals^{66,67}. However, a 2017 study on white-tailed deer observed no differences in microbial composition between sexes⁵²; although, sampling season differed from this study. Our samples were collected January through March, which corresponds to estrous cycling or pregnancy in females and post-rut (declining testosterone levels) in males⁶⁸.

Chronic wasting disease and the gut microbiota. On both farms, we observed significant differences in microbial composition in CWD-positive deer as compared to non-detect deer. Twenty-five of the 26 total CWD-positive deer across both farms were male (Table 1). Previous studies in wild deer have reported that CWD prevalence is two times higher in males, and that males have a threefold greater risk of CWD infection as compared to females⁶⁹. These differences in infection risk and prevalence by sex are thought to be linked to increased CWD transmission amongst male social groups outside of breeding season⁶⁹. Alternately, models of CWD outbreaks in captive deer predict that density-dependence and indirect transmission⁷⁰ play an important role in CWD spread. Although penning arrangements on Farm 2 are unknown, on Farm 1, male deer were penned with females during rut (fall) and then separated into bachelor herds for the remainder of the year. As such, transmission through male social groups and indirect, density-dependent transmission (in bachelor pens) could have played a role in the predominantly male infections observed in farmed deer. Because of this skew by sex, we opted to analyze only males in relation to CWD status. This within-farm, male-only analysis mitigated potential gut microbial confounders, including sex, diet, and biogeography.

Differentially abundant microbial taxa common across both farms and increased in CWD-positive animals included: two different microbes in the class Bacilli, order RF39 (formerly Mollicutes RF39)—one increased on Farm 1 and one increased on Farm 2; a microbe in the family Lachnospiraceae UCG-10; and two different ASVs in the *Akkermansia* genera—one increased on Farm 1, and one increased on Farm 2 (Fig. 4). The fact that these three taxa (RF39, Lachnospiraceae UCG-10, *Akkermansia*) emerged as CWD-associated on two independently run farms over 100 miles apart is intriguing and merits further attention. In a previous study, RF39 was found to be increased in a mouse model of the relapse-remitting form of multiple sclerosis (MS)⁷¹, which is a disease that shares many features with prion diseases, including CJD⁷².

Besides RF39, we also observed an increase in Lachnospiraceae UCG-010 in CWD-positive animals on both farms at the L7 level (Fig. 4b, c). Lachnospiraceae taxa have been reported in other studies on wild and captive deer gut microbiota^{73,74}. Lachnospiraceae has also been noted in association with neurologic diseases. However, it is decreased, rather than increased, in studies on Parkinson's disease (PD)^{75,76}, Alzheimer's Disease (AD), and amyotrophic lateral sclerosis (ALS)^{28,77}. Moreover, multiple studies highlight the ability of Lachnospiraceae species to produce butyrate which helps maintain the epithelial barrier^{78,79}. However, Lachnospiraceae family taxa have also been associated with type 2 diabetes⁷⁹ and intestinal inflammation⁸⁰.

Like Lachnospiraceae, *Akkermansia* is commonly associated with health^{81,82} and has been shown to reduce pathological alterations (amyloid beta-protein accumulation) and cognitive impairments in one mouse model of AD⁸³. However, more recent evidence has promoted caution in defining *Akkermansia* as a “good bug”⁸⁴. In fact, the mucin-degrading *Akkermansia* is reportedly increased in multiple neurologic diseases, including PD, multiple sclerosis, and AD^{32,85–88}. *Akkermansia* has additionally been associated with fasting and malnutrition, as it can utilize host mucin as its sole energy source while other microbes rely on dietary substrates consumed by the host^{57,89}. Importantly, recent work has positively correlated *Akkermansia muciniphilia* abundance with M-cell density and function in the gut⁹⁰, an intriguing finding as M-cell density is linked with increased susceptibility to orally acquired prion diseases⁹.

How and why these three taxa (RF39, Lachnospiraceae UCG-010, *Akkermansia*) are associated with CWD are the next important questions to answer. Do these taxa contribute to a gut environment that is more permissive to orally-ingested prions? Can *Akkermansia* directly or indirectly stimulate M cell density and function? For example, *Akkermansia* degrades host mucin, thinning the protective mucus barrier that lines the gut. In concert with a pro-inflammatory Lachnospiraceae species, these microbes could create an inflammatory environment that induces colonic M-cells^{11,14,15,91}, potentially enhancing prion uptake^{6,92}. Gut inflammation has also been linked to the progression of neurodegenerative disease including AD and PD^{30–32}. Alternately, are these taxa increased as a result of prion disease? Early clinical signs of CWD can include behavioral and locomotive changes followed by eventual wasting and weight loss¹. Subtle behavioral changes could conceivably alter diet and drive dietary differences in the gut microbiota between deer with and without CWD. *Akkermansia* relies exclusively on host mucin as a nutrient source; therefore, *Akkermansia* could feasibly increase in relative abundance in a host that is consuming less food; although, anorexia is typically only associated with terminal stage disease, and most of these deer were pre-clinical. Finally, could these taxa be providing protective effects in the presence of a prion disease? Lachnospiraceae and *Akkermansia* are associated with many health benefits, and increased relative abundances of these species are associated with protection against metabolic diseases and reduced pathological changes in AD^{28,79,83,84,93}. Bacilli (e.g. RF39—formerly in phylum Tenericutes, now in Firmicutes) have also been posited to play a protective role in the gut as they are decreased in relative abundance in the presence of colitis⁹⁴.

This study represents the first investigation, to our knowledge, of white-tailed deer gut microbiota in relation to CWD. We acknowledge several limitations to the present study. First, while our results suggest that differential diets are the major driver of microbial community differences by provenance and sex, we cannot explicitly rule out the potential effects of spatial proximity, host genetics, or biogeography. Polymorphisms in the deer PRNP gene can significantly influence susceptibility to CWD⁹⁵, but host genotypes and genotype/microbiome

interactions were not evaluated in this study. Second, as farmed and free-ranging deer had markedly different microbial communities, we cannot be certain that microbial composition differences observed in farmed deer based on CWD-status are generalizable to free-ranging deer. Third, microbial composition is not representative of microbial function⁹⁶, and future studies using shotgun metagenomics and metabolomics are warranted to capture function. Fourth, while *Akkermansia*, RF39, and *Lachnospiraceae* UCG-010 are associated with CWD, further work is needed to clarify if these differences preceded or succeeded disease. Fifth, Farm 1 DNA samples underwent ethanol precipitation to improve DNA purity, which could have altered the microbial community of these samples in comparison to Farm 2 and free-ranging deer samples. However, inter-individual differences in gut microbiota largely overwhelm differences introduced by extraction method, including ethanol precipitation^{97,98}. Finally, fecal samples from Farm 1 and free-ranging deer underwent library preparation and sequencing on an Illumina MiSeq at The Ohio State University Molecular and Cellular Imaging Center, while fecal samples from Farm 2 underwent library preparation and sequencing on an Illumina MiSeq at Argonne National Laboratory. While differences between laboratories and sequencing facilities can lead to differing results in microbiome studies⁹⁹, we limited these effects by using the same methodology and kits (Qiagen PowerFecal) for all DNA extractions, the same region and primers for sequencing (V4 -515F and 806R), and all sequencing data was combined and underwent sequence processing and taxonomy assignment together. Further, results by sex and CWD status were analyzed independently for each location (Farm 1, Farm 2, Free-ranging).

In conclusion, we report differences in gut microbiota in white-tailed deer by provenance (Farm 1, Farm 2, Free-ranging), sex, and CWD status. Differences by provenance and sex are likely driven by diet, while differences by CWD status are more challenging to interpret and include increased abundances of *Akkermansia*, *Lachnospiraceae* UCG-010, and RF39 taxa in CWD-positive deer. Priorities for future research include determining how these taxa may be associated with CWD susceptibility or pathogenesis, characterizing the gut microbiota of free-ranging cervids with CWD, examining the gut microbiota within a host before and after CWD infection, and assessing M-cell presence and abundance in CWD-positive and CWD non-detect animals to elucidate potential relationships between gut microbiota, M-cells, and chronic wasting disease.

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Author contributions

D.M. collected the samples from the farmed deer, performed DNA extraction on all samples, assisted with data analysis of sequencing results using QIIME 2, and drafted the manuscript. C.M. assisted with DNA extraction of all samples, DNA purification of Farm 1 samples, coordinated laboratory activities, and assisted with editing of the manuscript. M.E. assisted with data analysis and processing of sequencing results using QIIME 2 and provided feedback on the manuscript. G.B. provided expertise on neuropathology in cervids and feedback on the manuscript. D.B. and K.P. facilitated CWD testing and sample collection from farmed deer, provided expertise on CWD in cervids, and provided feedback on the manuscript. P.D. facilitated sample collection from free-ranging deer and provided feedback on the manuscript. V.L.H. conceived the idea and designed and directed the study. Additionally, V.L.H. assisted with analysis of sequencing results, manuscript preparation, and figure generation.

Competing interests

The authors declare no competing interests.

Additional information

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