THE EFFECTS OF MERCURY AND PROBIOTICS ON THE MICROBIOME AND BEHAVIOR OF THE PRAIRIE VOLE (*MICROTUS OCHROGASTER*)

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Abstract:

The digestive tract is home to trillions of microorganisms. In addition to helping with digestion, these microbes can have profound effects on host mood and behavior through a pathway known as the microbiome-gut-brain axis. By this pathway, changes in the intestinal microbial community can alter anxiety behaviors, depression, and sociability.

The aim of the current study was to characterize the impacts of mercury exposure and probiotic administration on both the gut microbiome and the anxiety and social behaviors of the prairie vole (*Microtus ochrogaster*). Following four weeks of exposure to 60 ppm mercuric chloride ($HgCl_2$), anxiety-like behaviors were significantly elevated in the voles. Subsequent administration of a potentially-probiotic *Lactobacillus* suspension (3.8×10^8 CFU / ml) for two weeks was not effective at remediating anxiety behaviors or the previously-reported decrease in sociability.

Microbiome analysis revealed significant changes in the microbial communities in response to mercury exposure and the administration of both the *Lactobacillus* suspension and its resuspension agent alone (0.15% maltodextrin). Several microorganisms were also correlated with specific anxiety and social behaviors, highlighting the strong possibility of microbiome-gut-brain axis involvement in changes in these behaviors.

Overall, the current study provides clarity into the impacts of mercury and probiotics on the microbial community and identifies several specific microorganisms that may alter prairie vole behavior via the microbiome-gut-brain axis.

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CHAPTER I

INTRODUCTION

The microbiome-gut-brain axis is a bidirectional communication pathway between the microorganisms of the digestive tract and the central nervous system. Changes in the composition of the gut microbial community have been implicated in a variety of psychosocial conditions including anxiety,[1] depression,[1] and decreased sociability.[2] The microbial changes that can alter nervous system functioning can be induced by many factors including the use of antibiotics,[3] the ingestion of toxic metals,[4] and the administration of probiotics.[5]

Probiotics are microorganisms that provide a health benefit to their host (or their host's existing microbial community) when ingested. While any bacterial species with the right beneficial characteristics could be considered a probiotic, many of the most common probiotics fall into the genera *Lactobacillus* and *Bifidobacterium.*[6] Interestingly, recent studies have highlighted the anxiolytic effects of several probiotic microorganisms on their host,[7-9] effects that are likely modulated through the pathway of the microbiome-gut-brain axis.

We sought to study the microbiome-gut-brain axis in a non-traditional lab animal – the prairie vole (*Microtus ochrogaster*). Normally a highly social rodent, the prairie vole exhibits sex-specific, selective social withdrawal following extended exposure to dilute mercuric chloride.[10]

We hypothesized that this withdrawal was due to increased anxiety, and that this anxiety could be alleviated through the administration of a potentially-probiotic *Lactobacillus* suspension.

To test our hypotheses, we employed three behavioral assessments: the Elevated Plus Maze and Open Field Test assays (to quantify anxiety) and the Social Avoidance Test (to quantify sociability). We assessed vole anxiety before and after mercury, and again after the administration of our potentially-anxiolytic probiotic suspension. We evaluated sociability at the end of the treatment course.

To determine if any behavioral changes were associated with changes in the gut microbiome, we also collected fecal pellets throughout the experimental time course and sequenced the bacterial 16S rRNA gene to determine the identity of the microorganisms present in these pellets. Using statistical analyses, we identified specific microorganisms that were significantly altered by experimental manipulations. Finally, we attempted to correlate these microbial changes with anxiety and social behavior changes.

Overall, the aim of this study was to examine the extent of the microbiome-gut-brain axis involvement in mercury-induced social withdrawal in the prairie vole. Given the similarity of this kind of withdrawal to that seen in autism spectrum disorders, this knowledge could provide invaluable insight into future treatment options for those conditions.

CHAPTER II

REVIEW OF THE LITERATURE

The Gut Microbiome

The gut microbiota is a complex microbial community composed of bacteria, fungi, and viruses. These microorganisms, coupled with their genetic information, are referred to as the gut microbiome. It is estimated that the gut microbiome contains 4×10^{13} bacterial cells,[11] representing over 1,000 different bacterial species.[12] Some of these microorganisms (referred to as the core operational taxonomic units, or core OTUs) are present in the digestive tracts of all humans at generally similar abundances; other OTUs are present at highly variable levels (or are even absent entirely) in the digestive tracts of some individuals. These variable microorganisms in particular can be useful in predicting the overall structure of the gut microbial community, known as the gut enterotype. In humans, there are three main gut enterotypes,[13] and these enterotypes are distinguished from one another by the relative abundances of microorganisms in the genera *Bacteroides*, *Prevotella*, and *Ruminococcus*.[14]

An individual's enterotype is not nearly as stable as their genotype. Indeed, several factors have been shown to dramatically alter the gut community including diet, antibiotic exposure, and probiotic administration.[15-17] Interestingly, while each of these factors are physical compounds that are ingested by the human host and ultimately come into direct contact with the bacteria of the microbiome, there are several non-physical factors (such as a host's genome,[18]

their stress level,[19] or even their age[20]) that can also shape the gut microbial community in profound ways. The goal of most modern microbiome studies is to track these microbial changes and determine what impacts those changes may have on the host.

Microbiome Analysis

Tracking changes in the gut microbial community is typically done using 16S rRNA gene sequencing. The 16S rRNA genes, found in the genomes of all bacteria, encode portions of the small subunit of the bacterial 70S ribosome. Most 16S rRNA genes are roughly 1,500 nucleotides long, and all contain both highly diverse regions and highly conserved regions.[21] The diverse regions (referred to as hypervariable regions) have DNA sequences that vary significantly between different groups of bacteria, and it is this variability that allows scientists to determine the identity of the microorganisms in their samples. The conserved regions, on the other hand, are not very different from species to species, and while they do not allow for discrimination between the microorganisms in a sample, they serve as the target sites for the nucleotide primers necessary for the process of DNA amplification and sequencing.[22]

The massively parallel approach to DNA sequencing that is necessary for the analysis of gut microbial community can be done with several different platforms, though the Illumina MiSeq platform is hailed for its accuracy and is considered an excellent choice for microbiome studies.[23] In the process of Illumina short-read sequencing, scientists first amplify a specific portion of a target gene (such as a hypervariable region of the 16S rRNA gene) in the microbiomes of each of their experimental samples. As the amplification process proceeds, the newly-synthesized DNA fragments are tagged with adaptor sequences that are complementary to short DNA fragments in the bottom of the sequencing flow cell. When the library of newly-

amplified DNA fragments is added to the flow cell, each fragment attaches to the oligonucleotide anchors in the cell, stabilizing the library fragments in place for copying by polymerase enzymes.

Following attachment of the fragments to the flow cell, rapid amplification of the original DNA strands is used to generate clusters of identical fragments in one region of the flow cell. Some of these fragments are generated from the positive ("coding") strand of the double-stranded DNA molecule while others are generated from the negative ("non-coding") strand of the DNA molecule. This bidirectional approach to synthesizing the DNA molecules decreases the error rate in subsequent sequence analysis steps.

The actual DNA sequencing process is completed using fluorescently-labeled dideoxynucleotides. The flow cell (with attached fragment clusters) is flooded with these labeled nucleotides, and as they incorporate into new DNA molecules, a color change is detected. This process, known as "sequencing by synthesis",[23] ultimately generates a FastQ file with the raw DNA sequences for each original DNA molecule as well as per base quality score information.

To make sense of the FastQ file, scientists employ processing software such as Quantitative Insights Into Microbial Ecology (QIIME).[24] QIIME sorts the raw DNA sequences and compares them to databases with sequences for the 16S rRNA gene to classify the microorganisms in the sample. The identified bacterial samples, known as operational taxonomic units (OTUs) can then be used to visualize characteristics of the gut microbiome as a whole.

Two important characteristics of a gut microbial community are alpha diversity and beta diversity. Analysis of the alpha diversity in a microbial community helps scientists to understand how many OTUs are present in an experimental sample. In general, more OTUs indicates a healthier gut community, so greater alpha diversity can be a biomarker of increased gut health. Beta diversity, on the other hand, is more of a measure of the relative abundances of the OTUs within a population. In general, decreases in alpha diversity are often an indicator of the loss of some species from the microbial community, which could be correlated with disease states.

While large-scale changes in alpha and beta diversity can indicate substantial changes on the function of the bacterial community, not all statistically-significant or biologically-significant gut community changes can be observed on these broad levels. To observe smaller-scale changes that may still be important, a more in-depth analysis of microbiome structure can be performed using linear discriminant analysis effect size (LEfSe).[25] LEfSe analysis compares the relative abundances of each OTU in the microbiomes of experimental samples and, using the Kruskal-Wallis and Wilcoxon rank-sum tests, identifies which changes reach the level of statistical significance. The specificity of LEfSe analyses better enable scientists to predict the functional impacts of the observed changes in the microbiome.

The Microbiome and The Host

The functions of the gut microbiota are numerous. Undoubtedly, one of the major functions of the bacteria of the digestive tract is host metabolism and nutrient processing. The microbiome of enteric microorganisms is enriched for genes involved in carbohydrate[20] and amino acid[26] metabolism. Additionally, gut bacteria are particularly efficient at metabolizing plant-derived polysaccharides that are too complex for direct metabolism by human enterocytes.[20] Such molecules that the host organism cannot easily metabolize (but the gut microbial community can) are often referred to as prebiotics. Prebiotics appear to be integral to the overall health and nutrition of the gut microbial community.

The diversity of molecules known as prebiotics is great. Inulin, a prebiotic originally derived from chicory roots, has been shown to selectively enhance multiple probiotic bifidobacteria.[27] Similarly, galacto-oligosaccharide, a complex polysaccharide that is made from lactose, has also been shown to increase the abundance of beneficial *Lactobacillus* strains.[28] While not as widely studied, other important prebiotics include xylan, pectin, and arabinose-containing carbohydrates.[26]

While prebiotics (which are selectively metabolized by bacterial cells) can contribute profoundly to an increase in the bacterial load of the gut, it is important to remember that even the nutrient sources that can be metabolized by the host (known as their diet) can also influence the structure of the microbiome. For individuals with a diet high in plant-based polysaccharides, the genus *Prevotella* is exceedingly abundant.[17] Individuals with a diet higher in proteins or dietary fats tend to exhibit increased levels of *Bacteroides* bacteria.[17] Interestingly, the effect of host diet on the microbial community is so powerful that within 24 hours of an extreme dietary change, measurable differences in the gut microbial community structure can be observed.[15]

While dietary changes and prebiotic exposure can lead to important changes in gut microbiome structure, even more dramatic (and typically detrimental) changes in the microbiome are noted after the administration of antibiotics. Many antibiotics are considered broad-spectrum, meaning that they indiscriminately eliminate both the beneficial and pathogenic microorganisms.[29] Commensal microorganisms, which are widely believed to maintain the proper gut community structure and function in their host, decline dramatically with antibiotic administration, and are often replaced with potentially pathogenic microbes. Such changes in microbiome structure can disrupt a variety of host functions including, unsurprisingly, host metabolism.[30]

One way that doctors are currently attempting to offset the widespread microbiome perturbances induced by antibiotics is prescribing probiotics for their patients to take concurrent with their antibiotics. For a bacterium to be considered a probiotic microorganism, it must have several beneficial characteristics such as the ability to adhere to the surface of gastrointestinal epithelial cells, a strong resistance to stomach acid and bile salts, and the ability to inhibit pathogenic microorganisms.[31] While probiotic administration has not been completely successful in protecting all of the vulnerable members of the microbiome from the ill-effects of antibiotic administration, it has been shown to temper these effects.[32] Even this tempering is a step in the

right direction, as some studies have shown that early childhood antibiotic administration can actually permanently alter the development trajectory of an individual's microbiome into adulthood.[3]

While probiotics have exhibited mixed success in addressing antibiotic-induced deficits in the microbiome, they have exhibited a great deal of potential in addressing another common human health challenge: anxiety. Several animal studies have highlighted the profound anxiolytic activities of probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium*.[7, 33, 34] Probiotics have been found to not only reduce circulating corticosterone (a stress hormone),[7] but also to reduce the behavioral manifestations of anxiety.[33] While the number of studies examining the effect of probiotics on anxiety in humans is not nearly as high as in animals, in at least one trial, individuals who received a probiotic solution reported decreased anxiety at the end of the treatment course.[33] Obviously, there is still work to be done, but the preliminary studies are promising.

The Microbiome-Gut-Brain Axis

The ability of gut microorganisms to alter the anxiety of their human host is modulated by a pathway known as the microbiome-gut-brain axis. This axis is a communication pathway between the enteric microbial community and the central nervous system, and it is likely mediated at least in part through the vagus nerve. This cranial nerve runs directly from the brain stem to the digestive tract; as bacteria activate this nerve, they may have a direct line to the brain to alter overall functionality.

Several studies have highlighted the effect of microbial community changes on host behavior,[1] but one intriguing example of this is seen in a mouse model of the autism spectrum disorders (ASD). In humans patients with ASD, levels of *Clostridium* bacteria are consistently

found to be significantly elevated.[35] Using maternal immune system activation during pregnancy, scientists were able to induce not only the behavioral characteristics typical of ASDs, but also the overabundance of *Clostridium* bacteria.[36] Interestingly, administration of a single probiotic microorganism (*Bacteroides fragilis*) not only remediated gut microbial imbalances, but also restored behavioral deficits.[36]

Given that the microbiome-gut-brain axis is bidirectional, it is important to note that changes in the functioning of the nervous system can also have a profound effect on the gut microbial community. Repeated social defeat (defined as exposure to an aggressive stimulus animal for an extended period of time) has been shown to decrease the beta diversity of the murine microbiome.[37] Similarly, mice subjected to repeated grid floor stress also developed microbiome structures significantly different both from their own baseline analyses and as compared to control animals.[38]

While the exact mechanism underlying the microbiome-gut-brain axis has not yet been fully elucidated, it is likely to be a combination of multiple pathways including stimulation of the vagus nerve, bacterial synthesis of neuroactive compounds,[39] or the activation of enteric immune system cells.[40] The vagus nerve may be the most important aspect of this axis, however, as severing this nerve has been shown to prevent the effects of neuroactive compounds or the immune system on nervous system functions.[41]

While bacteria can make a variety of neuroactive compounds, one group that is of particular interest is short chain fatty acids (SCFAs). Bacteria generate SCFAs as a byproduct of carbohydrate metabolism, and these small, lipophilic molecules can actually cross the blood-brain barrier.[42] While the gut microbiota can synthesize a variety of SCFAs (including propionate, butyrate, and acetate),[43] human cells do not possess the enzymes for this metabolic process. It is unsurprising, then, to see that animals that lack a gut microbial community (known as germ-free animals) have SCFA levels almost 100 times lower than conventional lab animals.[44]

The SCFAs made by gut bacteria contribute significantly to the behaviors of their host organism. In male rats, for example, the levels of SCFA have been directly correlated with the anxiety and aggression behaviors.[45] In mice with neonatal exposure to the SCFA propionic acid, performances in the Elevated Plus Maze and Open Field Test both indicated a significantly higher levels of anxiety than the levels seen in control animals.[46] In human patients with autism spectrum disorders, fecal SCFA levels have also been found to be significantly elevated compared to age-matched controls.[47] Taken together, these findings highlight the anxiogenic properties of SCFAs and highlight the importance of preventing an overproduction of SCFAs by the gut microbial community.

Given the role of enteric microorganisms in synthesizing SCFAs, it is no surprise that germ-free animals (which lack a gut microbial community) present with significantly lower levels of circulating SCFAs. The behavioral impacts of the absence of these compounds are profound. In the Elevated Plus Maze,[48] Open Field Test,[49] and Light-Dark box[50] tests, germ-free animals exhibit limited or no anxiety. Unfortunately, the impact of this decreased anxiety on social behavior has not been consistently identified. In some studies, germ-free mice were significantly more social when introduced to an unknown stimulus animal;[49] in other studies, however, social behavior declined dramatically.[2] These conflicting findings highlight an important aspect of behavioral assays: not all assays are equally valid for each experimental animal.[51-53]

The Prairie Vole

To more effectively study the effects of SCFAs and enteric microbes on social behavior, the prairie vole (*Microtus ochrogaster*) may serve as a better model organism than other standard laboratory rodents. Unlike mice and rats, prairie voles are highly social animals, forming stable bonds with a single partner and raising offspring together with that partner. These monogamous behaviors have been observed in prairie voles both in nature[54] and in the lab,[55] and laboratory-

based assessments have shown that the establishment of a partner preference between two voles may occur in as little as 24 hours.[55] Copulation appears to be a critical aspect of pair-bonding though, as cohabitation without successful mating often does not result in measurable partner preference.[56] Given that an important aspect of the vole monogamous relationship is the bi-parental rearing of offspring,[57] it is unsurprising that unsuccessful copulation may inhibit the formation of a pair bond.

Studies have implicated the neuropeptide vasopressin as a key regulator of monogamy in prairie voles. If a vasopressin receptor antagonist is administered to prairie voles, they will not form a pair bond even after cohabitation and successful copulation.[58] Conversely, administration of vasopressin itself dramatically expedites the pair-bond formation process, with some voles bonding with a mate after as little as 1 hour of contact.[58] It is important to note, however, that it is not just the presence or absence of vasopressin that enables pair bond formation. Indeed, non-monogamous vole species such as the montane vole (*M. montanus*) and the meadow vole (*M. pennsylvanicus*) have the same number of vasopressin-producing neurons as the monogamous prairie voles, meaning they have the ability to synthesize just as much vasopressin as their monogamous counterparts.[59] However, the distribution of the vasopressin receptors in the brain of prairie voles differs significantly from other vole species, with a significant elevation in receptors in the ventral pallidum.[60] Interestingly, when the process of viral transfection is used to increase the expression of the vasopressin receptor in the ventral pallidum, prairie voles have been shown to develop a partner preference even in the absence of a mating event.[60]

While vasopressin plays a critical role in the sexual aspect of vole monogamy, this hormone is also important in encouraging the bi-parental rearing of offspring seen in prairie voles. Though it may not be an entirely accurate measure of direct investment in offspring rearing, the amount of time spent in the nest by both male and female voles is very similar.[57] Interestingly, the administration of vasopressin further increases the involvement of male prairie

voles in pup rearing,[61] leading to increased time spent licking and grooming the offspring. This early-life parental care is critical to the establishment of normal vole social behaviors as the absence of a father in the nest during a vole's early development has been correlated with future delayed formation of pair bonds and decreased involvement in the rearing of subsequent offspring.[62]

Because the prairie vole is such a social animal, it is an ideal model to study factors that may alter social behaviors. Unsurprisingly, separation of pair-bonded voles from their respective mates has been shown to increase anxiety and depressive behaviors.[61] Isolated voles that are subjected to stressful stimuli such as the Forced Swim Test or the Resident Intruder Test have higher circulating corticosterone and exhibit more anxiety behaviors as compared to voles housed with their mate and exposed to similar stimuli.[63-65] Interestingly, the apparent anxiolytic role of another vole's companionship is not only limited to mated partners. Indeed, voles isolated from their sibling (or the age-matched, sex-matched vole they were normally housed with) also exhibit behavioral deficits such as increased aggression toward new animals.[66] Such aggression is unexpected in voles as they typically respond to the introduction to a stranger by spending extended periods of time in contact with the animal.[10]

Previous work has shown that extended exposure to dilute mercuric chloride can induce selective social withdrawal in which male prairie voles avoid unknown voles but still respond to cagemates.[10] This stranger aversion (without any aversion toward familiar animals) mimics the kind of social deficits seen in patients with autism spectrum disorders (ASDs). While the microbiome of children with ASDs has been relatively well-characterized,[35] the microbiome of the prairie vole (and especially the microbiome of prairie voles exhibiting ASD-like social deficits following mercury exposure) has not been as well clarified. By coupling 16S rRNA gene sequencing analysis with behavioral tests of anxiety such as the Elevated Plus Maze and Open

Field Test, we hope to determine the role of the microbiome-gut-brain axis in mercury-induced anxiety and social behavioral deficits in the prairie vole.

CHAPTER III

EXPERIMENTAL METHODS

Ethics Statement

The protocol used in these experiments were reviewed and approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. Procedures were found to be in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Animal Husbandry

Male prairie voles (ages 102 ± 31 days) descended from a wild southern Illinois vole population were housed with same-sex siblings or age-matched partners in plastic cages (20 x 25 x 45 cm) with pine chip bedding. The room was maintained at 21°C with a 14:10 light: dark cycle. The voles were given *ad libitum* access to Purina rabbit chow pellets (periodically supplemented with sunflower seeds) and fluids (water, 60 ppm $HgCl_2$, 0.15% maltodextrin, or probiotic solution; for details, see experimental procedures below).

Overall Experimental Design

To determine the effects of mercury and probiotics on the anxiety, sociability, and microbiome of the prairie vole, two experimental sequences were employed.

Experiment 1 focused primarily on the effects of mercury and probiotics on the anxiety and sociability of voles. Male prairie voles were randomly assigned to one of four treatment groups, and for 4 weeks they had *ad libitum* access to either water, water with low levels (0.15%) of maltodextrin, water with probiotic *Lactobacillus* bacteria (~4 x 10^8 CFU/ml), or water with mercury chloride (60 ppm). Elevated Plus Maze and Open Field Test evaluations was performed 2 times with each animal: before experimental manipulations ("Baseline") and following experimental manipulations ("Post-Treatment"). See Figure 1 for a summary of this experimental design.

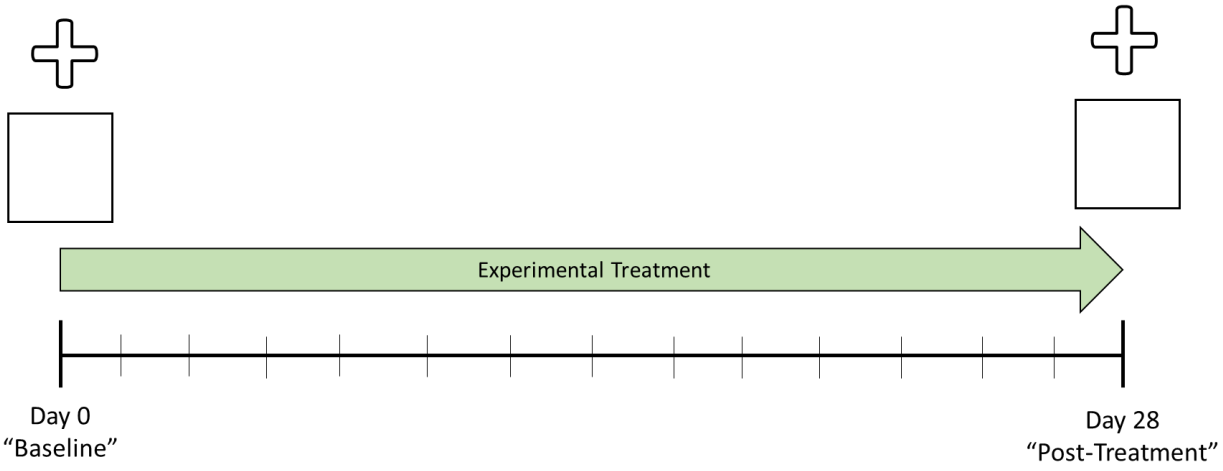

Figure 1. Experiment 1 Design. Experiment 1 spanned 4 weeks. On Day 0, animals underwent Elevated Plus Maze (+) and Open Field Test (□) testing. They were given *ad libitum* access to their experimental treatment (water, 0.15% maltodextrin, probiotic solution, or 60 ppm $HgCl_2$ for 4 weeks. Fresh bottles with the experimental solutions were administered every 2 days. At the end of the treatment, vole behaviors were again evaluated in the Elevated Plus Maze and Open Field Test.

Experiment 2 sought to extend the information obtained in Experiment 1 to assess the effects of probiotics on the gut microbiome after mercury exposure. Following 4 weeks of mercury chloride

(60 ppm) exposure, the mercury-treated voles were randomly assigned to 2 treatment groups and for 2 more weeks, they had *ad libitum* access to either water with low levels (0.15%) of maltodextrin or water with probiotic *Lactobacillus* bacteria (~4 x 10^8 CFU/ml). At the end of this additional treatment course, anxiety in the voles was re-evaluated with the Elevated Plus Maze and Open Field Test. Social behaviors were also evaluated using the Social Avoidance Test. See Figure 2 for a summary of this experimental design.

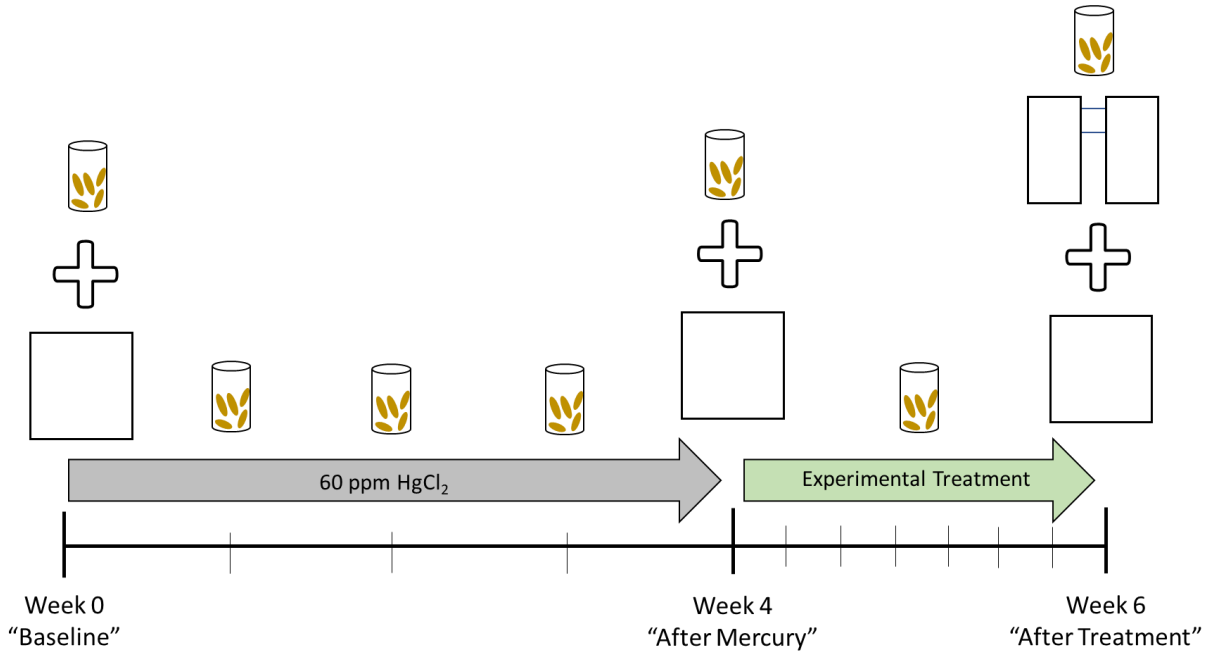

Figure 2. Experiment 2 Design. Experiment 2 spanned 6 weeks. On Day 0, animals underwent Elevated Plus Maze (+) and Open Field Test (□) testing and fecal pellets were collected (). The voles were then given *ad libitum* access to mercuric chloride for 4 weeks. Fecal pellets were collected weekly during this treatment period. After this mercury exposure, the voles underwent Elevated Plus Maze and Open Field Test testing and the experimental treatment course (either 0.15% maltodextrin or probiotic solution) began. Fresh bottles were administered every 2 days and fecal pellets were collected 7 days into the treatment course. At the end of this treatment period, voles underwent Elevated Plus Maze Testing, the Open Field Test, and the Social Avoidance test. Pellets were also collected one final time.

Behavioral Testing Protocols

To assess the impact of mercury and probiotics on anxiety levels in the prairie vole, two behavioral assays were employed: the Elevated Plus Maze and the Open Field Test.

The Elevated Plus Maze

The Elevated Plus maze is a "+" shaped apparatus that is elevated 45 cm off the ground. The maze has two open arms (35 cm long x 6.5 cm wide) and two closed arms (35 cm long x 6.5 cm wide, with 15 cm walls) that cross in the middle to form the "+" shape. Reference Figure 3 for a representation of the Elevated Plus Maze apparatus.

To perform Elevated Plus Maze analysis, a vole was placed in the center of the apparatus facing one open arm of the maze and was allowed to explore freely for 8 minutes. Behavior was recorded using a digital camera and the amount of time spent in the center and each arm of the maze was quantified using EthoVision XT software (Noldus Information Technology). The amount of experimental time spent in the closed arm of the maze compared to the open arm of the maze was used as an indicator of vole anxiety.[67]

The Open Field Test

The Open Field Test apparatus is a square field (10" long x 10" wide) with clear plexiglass walls (10" high). Reference Figure 3 for a representation of the Open Field Test apparatus.

To perform Open Field Test analysis, a vole was placed in the center of the field and allowed to explore it freely for 10 minutes. Its movements were tracked using EthoVision XT software (Noldus Information Technology). Total distance traveled, entries into the field's center, and the amount of time spent in the center of the field were recorded and used as indicators of vole anxiety and locomotor activity.[68]

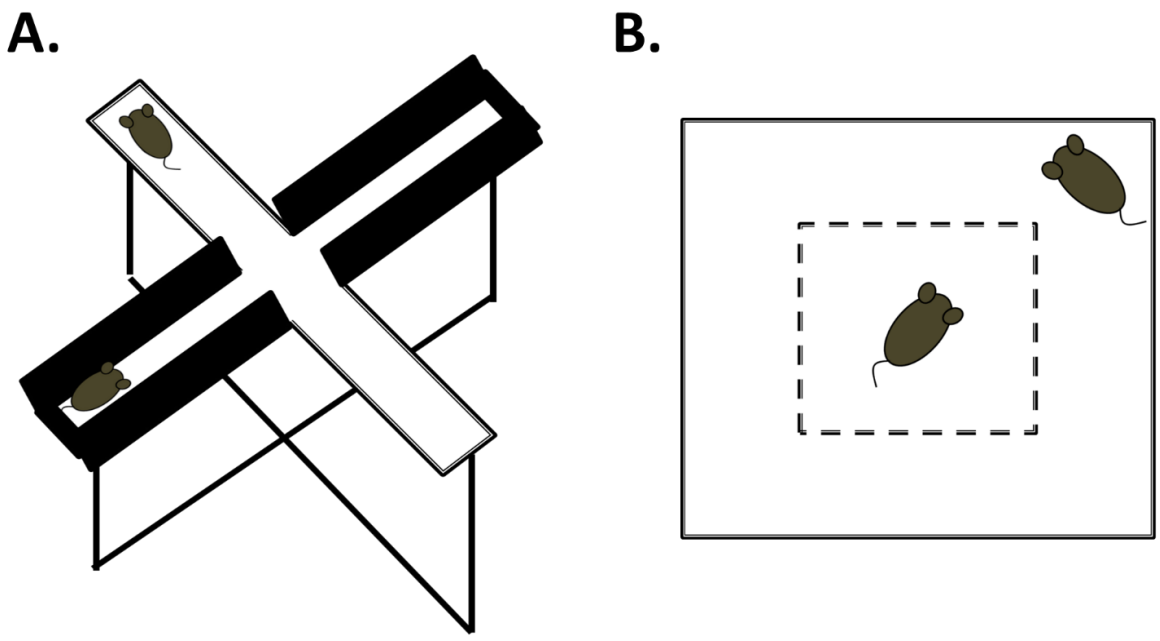

Figure 3. Anxiety Behavior Assessments. Two experimental protocols were used to evaluate anxiety in the prairie vole: the Elevated Plus Maze and the Open Field Test. **A.** The Elevated Plus Maze is an elevated "+" shaped platform with 4 arms: 2 "closed arms" with walls that enclose these arms entirely except for the open top, and 2 "open arms" with no walls. A single test animal is placed in the center of the maze and allowed to move freely for 8 minutes. The time spent in either of the open arms, either of the closed arms, and the center of the maze was quantified using the EthoVision XT software. **B.** The Open Field Test is conducted in a 10" x 10" plexiglass box with an open top. A single test animal placed in the center of the field and allowed to explore for 10 minutes. EthoVision XT software was used to quantify the total distance traveled, time spent in the center of the field, and entries into the center of the field.

The Social Avoidance Test

To assess sociability in the prairie vole, the Social Avoidance Test was used. In this test, two plastic cages were connected via a clear plexiglass tube. An unknown stimulus animal was tethered in one cage and the test animal was placed in the adjacent empty cage. The test animal's behavior was then recorded with a digital camera for three hours and the amount of time spent in contact with the stimulus animal was quantified later by review of the recording. See Figure 4 for a representation of the apparatus used in the Social Avoidance Test.

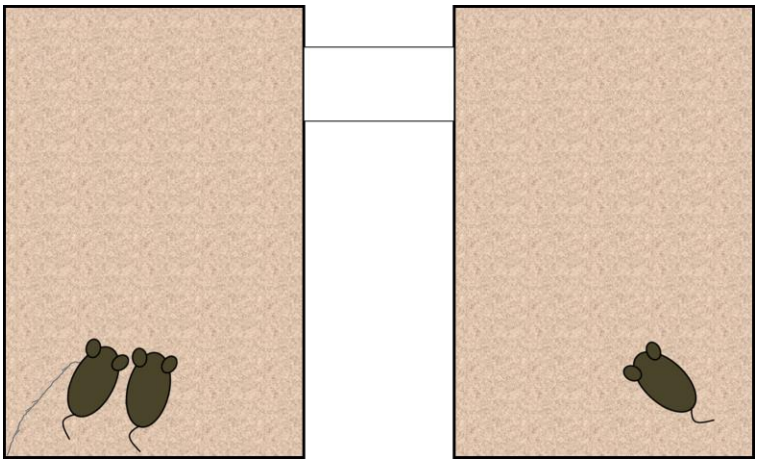

Figure 4. The Social Avoidance Test. To evaluate social behaviors in the prairie vole, the social avoidance test was used. In this test, an unknown stimulus animal is tethered in one cage and the test animal is placed in an adjacent connected cage. Time spent in stationary contact with the stimulus animal is quantified by the researcher using recordings of the test.

Statistical Analysis of Behavioral Testing Data

For the Elevated Plus Maze and Open Field Test, data were analyzed using Repeated Measures ANOVA with treatment and experimental timepoint as factors. Statistically significant ($p < 0.05$) main effects or interactions were further evaluated using Fisher's LSD tests. The Bonferroni correction was used for specific planned comparisons.

For the Social Avoidance Test, data were analyzed using the independent samples t-test.

Probiotic Synthesis & Administration

Preparation of Probiotic Solution

Three strains of *Lactobacillus* bacteria isolated from the cecum of prairie voles (PV012, PV018, and PV019) were selected for use in the experimental probiotic solution based on their beneficial properties outlined in Assefa *et. al*, 2015.[69] Cultures were grown in De Man, Rogosa, and Sharpe (MRS) broth at 37°C. To prepare the cells for administration, they were first centrifuged for 15 minutes (3,200 x g, 4°C), then washed with sterile water. These washed cells

were then re-centrifuged and washed two more times. Following the third wash, the supernatant fluid was removed from above the cell pellets and the cells were resuspended in 10% sucrose solution and incubated at room temperature for one hour. This solution was divided into 5 milliliter aliquots in 15 milliliter plastic tubes and the tubes were frozen at -80°C overnight. They were then lyophilized for 48 hours in the lyophilizer (Labconco Freezone 2.5) and stored at -80°C until time of use.

Administration of Probiotic Solution

To administer lyophilized *Lactobacillus* to the prairie voles, 5% maltodextrin solution was added to reconstitute the lyophilized samples and they were incubated at room temperature for 60 minutes. Bottles were prepared at a ratio of 3 mL probiotic suspension to 100 mL of water. This dilution correlates with a concentration of roughly 4×10^8 colony forming units per mL of water. Because the rehydrating solution contained maltodextrin, it was deemed appropriate to establish an additional control group of animals receiving diluted solution of the polysaccharide without added lactobacilli. For this group, bottles were prepared with 3 mL of 5% maltodextrin solution for every 100 mL of water. This dilution correlates with 0.15% maltodextrin per mL of water.

To ensure optimal viability of administered probiotic bacteria, bottles with fresh probiotic solution were prepared and presented every other day. Maltodextrin water bottles were prepared and provided on the same schedule.

Microbiome Evaluation Techniques

Fecal Pellet Collection

Fecal pellets were used as the source material for microbiome sequencing and analysis. For each fecal pellet collection, prairie voles were placed alone in a cage with fresh pine bedding and *ad libitum* access to Purina rabbit chow and water. They remained in this cage for one hour, at which point they were returned to a cage with their cage mate(s). All excreted pellets were collected individually in 1.5 mL snap cap tubes and frozen at -80°C until their DNA was isolated.

Fecal pellets were collected throughout the time course of Experiment 2. Collections occurred prior to experimental manipulations ("Baseline") and weekly thereafter. Ultimately, fecal pellets were collected from each vole at 7 experimental timepoints.

Fecal DNA Isolation

DNA was isolated from approximately 50 mg of each fecal sample using the ZR Fecal DNA MiniPrep Kit (Zymo Research, Irvine, CA). A Mini-Beadbeater-96 (Biospec Products, Bartlesville, OK) was used to disrupt the bacterial cell membranes, and the resulting homogenates were processed in accordance with the manufacturer's instructions (Zymo Research, Irvine, CA). Fecal DNA was quantified using a Qubit 2 Fluorometer (Life Technologies, Thermo Fisher Scientific) with the Invitrogen Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Concurrent with DNA quantification, confirmation of the quality of the isolated DNA was evaluated using 1% agarose gel electrophoresis with 1x Tris-Acetate-EDTA buffer. For each sample, a DNA band of approximately 8 kilobases was observed, correlating with the expected size of bacterial genomic DNA.

All isolated fecal DNA samples were stored at -20°C until they were prepared for use in Next Generation Sequencing (NGS).

NGS Library Preparation

To characterize the taxonomic profile of the intestinal microbiome, the V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the universal bacterial 16S bacterial primers F515 (5'- GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3'). These primers were designed to integrate the sequence of the specific Illumina multiplexing sequencing primers and dual-index-paired-end approaches following the protocols and procedures described by Kozich *et al*.[70] Briefly, each primer contained the appropriate Illumina adapter, an 8-nucleotide index sequence, a 10-nucleotide pad sequence, a 2-nucleotide linker, and the gene-specific primer. (For primer sequences, see Table 1 in Appendix A).

The 16S rRNA gene amplicons were generated using the AccuPrime *Pfx* SuperMix DNA Polymerase High Fidelity System (Invitrogen, Thermo Fisher Scientific) in 20 µl reactions. Each reaction contained 17 µl AccuPrime *Pfx* SuperMix, 2 µl of 10 µM primer mix, and 1 µl fecal genomic DNA. A PTC 200 DNA Engine thermocyler (BioRad, Hercules, CA) was used for amplification. Cycling parameters for the reaction were as follows: 95°C for 2 min; [95°C for 20 s; 55°C for 15 s; 72°C for 5 min] x 30; 72°C for 10 min]. Following amplification, the presence of PCR products was confirmed on 2% agarose E-gel® double comp gels (Invitrogen, Life Technologies). Strong amplicon bands in the expected size ranges were detected in all sample lanes, and no bands were seen in the non-template control reactions.

Library clean up and normalization was performed using a SequalPrep™ plate normalization kit (Applied Biosystems, Thermo Fisher Scientific) following manufacturer's instructions. Libraries were prepared by pooling equimolar ratios of amplicons (5 µl of the normalized libraries) from each well. These libraries were then quantified using the KAPA library

quantification kit assay (qPCR assay) with primers specific to the Illumina adapters (KAPA Biosystems, Wilmington, MA).

Fecal DNA Sequencing

The quantified library (1.4 nM) and Illumina-generated PhiX (PhiX control V3) control libraries (10 nM) were separately denatured in freshly-prepared 0.2M NaOH and diluted with chilled Hybridization Buffer to a final concentration of 4 pM. Following dilution, the samples were loaded onto a 500 cycle MiSeq reagent cartridge (Illumina, Hayward, CA) for sequencing on the Illumina MiSeq platform (Illumina, Hayward, CA,). To achieve a 15 % Phix run, 850 µl of 4 pM library was combined with 150 µl of 4 pM of Phix. 600 µl of this solution was then loaded into each of the wells on the reagent cartridge. 3 µl of each of the primers (the Read 1 sequencing primer, Index Read primer, and Read 2 sequencing primer) was added into separate wells. Ultimately, the loaded solution had a concentration of 4.0 pM, with 3.4 pM library concentration and 0.6 pM Phix concentration. (For primer sequences, see Table 1 in Appendix A.)

NGS Data Analysis and Visualization

Following MiSeq sequencing, raw sequencing data (Read 1 and Read 2) for each sample were downloaded from the Illumina MiSeq server as FastQ files. These files were processed using the open source pipeline Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1). Operational taxonomic units (OTUs) were assigned to reads using the SILVA 16S rRNA database with a 97% identity level. (See Appendix B for the QIIME analysis pathway.)

To better characterize the biologically-significant changes in the gut microbial community following experimental manipulations, linear discriminant analysis effect size (LefSE) analysis was used.[25] Comparisons were made between multiple timepoints (baseline vs.

after mercury; after mercury vs. after probiotics; after mercury vs. after maltodextrin; after probiotics vs. after maltodextrin; baseline vs. after probiotics; baseline vs. after maltodextrin). The threshold for logarithmic linear discriminant analysis (LDA) was 2.0 with comparisons made among all subclasses. The all-against-all multi-class analysis was used. LefSE graphs and cladograms were generated using the Galaxy / Hutlab website (https://huttenhower.sph.harvard.edu/galaxy).

A general outline of the DNA sequencing workflow is illustrated in Figure 5.

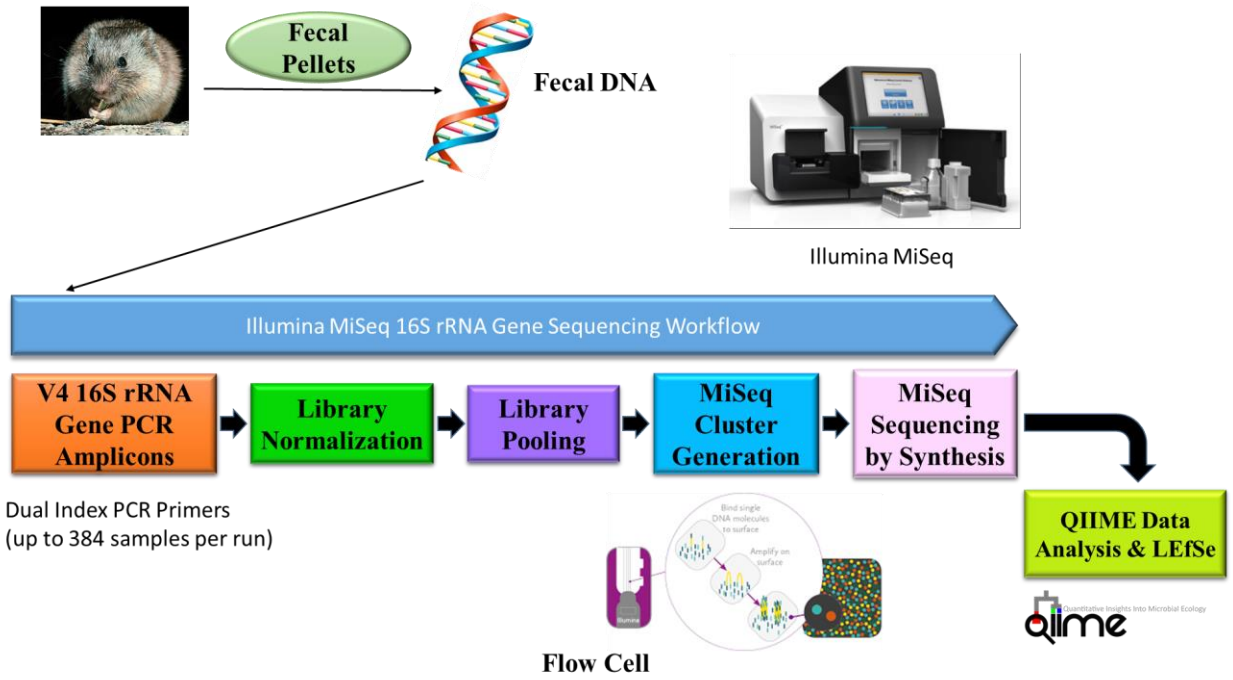

Figure 5. DNA Sequencing Workflow. To determine the gut microbiome composition of the prairie vole (*Microtus ochrogaster*), fecal pellets were collected at various experimental timepoints and their DNA was isolated. The 16S rRNA gene was selectively amplified in each sample and the resulting fragments were sequenced using the Illumina MiSeq platform. Data analysis was performed first using Quantitative Insights Into Microbial Ecology (QIIME) and then with Linear Discriminant Effect Size (LEfSe).

Correlation of Microbiome Changes with Behavior

Coding of Behavioral Data

In order to make meaningful statistical comparisons of microbiome data generated via 16S rRNA gene sequencing, it was necessary to group microbiome samples from each vole into categories that contained more than one animal sample and represented animals that performed in similar ways in each behavioral assay. To group the data, each raw data point was assigned a gradient value (typically "1" through "4"). The microbiome data from each animal was then aggregated with microbiome data from other animals with the same gradient value and statistical analyses were performed.

Gradient values for the Elevated Plus Maze were based on the equal division of total experimental time (100%) into four groups. Gradient values for Open Field exploration and entries into the field center were established by using the data collected during this experiment (mean ± 2 standard deviations). Gradient values for the social avoidance test were established using Curtis *et al.*[10] with the gradient value "3" being the approximate contact time of untreated control male voles and other gradient values 30-minute increments above or below this value.

Table 1 summarizes the transformation values for each test.

Table 1. Gradient Values for Behavioral Testing Results

Gradient Value	Elevated Plus Maze Raw Value	Exploration Raw Value	Open Field Center Time Raw Value	Open Field Entries Raw Value	Social Avoidance Test Raw Value
1	< 25% time in closed arm	< 2675 cm traveled	< 10% time in center	< 23 entries into center	0 – 30 minutes in contact
2	25% - 50% time in closed arm	2675 – 4220 cm traveled	10 – 24% time in center	24 – 40 entries into center	> 30 – 60 minutes in contact
3	> 50% - 75% time in closed arm	> 4220 – 6045 cm traveled	25 – 40% time in center	41 – 64 entries into center	> 60 – 90 minutes in contact
4	> 75% time in closed arm	6045 – 7730 cm traveled	> 40% time in center	> 65 entries into center	> 90 – 120 minutes in contact
5	N/A	> 7730 cm traveled	N/A	N/A	> 120 minutes in contact

Aggregation of Microbiome and Behavior Data

In order to perform Linear Discriminant Analysis of Effect Size (LEfSe), it was necessary to use our 16S rRNA sequencing data that had been aggregated by cage. This meant that the data used in LEfSe was the average of the raw microbiome data from both animals in the cage at a particular timepoint. To correlate this microbiome data with vole behaviors, the behavioral gradient scores from both animals in each cage at individual testing timepoints were added together. This value was used to determine the general behavioral classification of each cage.

Cages were designated as "Low" performers for a behavioral measure if the sum of the gradient values for the two animals in that cage was 2 to 5. "Low" performers exhibited low anxiety, low exploration, or low levels of sociability. Cages were designated as "High" performers for a behavioral measure if the sum of the gradient values for the two animals in that was 6 to 10. "High" performers exhibited high anxiety, high exploration, or high levels of sociability.

Analysis of the Microbiome and Behavior

Alpha and beta diversity analyses were performed in QIIME with data points from individual animals used. LEfSe analyses were performed with the Galaxy / HutLab online platform with the aggregated behavioral data for each cage.

CHAPTER IV

THE EFFECTS OF MERCURY AND PROBIOTICS ON BEHAVIOR IN THE PRAIRIE VOLE (*MICROTUS OCHROGASTER*)

Introduction

The prairie vole (*Microtus ochrogaster*) is animal model widely used in modern research to study social behaviors. Previous work has shown that exposure to dilute mercuric chloride (60 ppm) induces a state of sex-specific social avoidance toward unfamiliar animals,[10] similar to the social withdrawal seen in autism spectrum disorders. While the underlying mechanism of this withdrawal is not known, we hypothesized that it was due to an increase in anxiety. Several probiotics have been shown to reduce anxiety in humans and rodents, so we predicted that the administration of our probiotic solution may help to alleviate some of this mercury-induced anxiety.[9, 33]

The aim of Experiment 1 was to evaluate the effects of mercury and probiotics on the anxiety and social behaviors of the prairie vole. The aim of Experiment 2 was to determine whether our probiotic solution could alleviate the increased anxiety and social deficits seen in the prairie vole following mercury exposure.

Results

Experiment 1 – Characterization of the Effects of Mercury & Probiotics on Animal Behavior

Demographics

Male prairie voles (ages 83.6 ± 14.4 days) were randomly assigned to each treatment group (Control, Mercury, Maltodextrin, and Probiotics). Four animals in the Mercury group exhibited distress during the mercury treatment time course and were humanely euthanized prior to the experiment's end. Their baseline behavioral data were excluded from analyses.

T-test analysis revealed no significant differences in the body masses of animals in each treatment group before ($p = 0.22$) or after ($p = 0.08$) experimental treatments, and no significant differences ($p = 0.101$) were seen in amount of fluids consumed throughout the treatment course.

Impacts of Exogenous Substances on Elevated Plus Maze Performance

Repeated Measures ANOVA analysis revealed a main effect of experimental timepoint (baseline vs. post-treatment) on the amount of time spent in the closed arm ($F_{1, 39} = 10.26$, $p < 0.003$) and open arm ($F_{1, 39} = 13.86$, $p < 0.001$), but not the center ($p = 0.34$), of the Elevated Plus Maze. All treatment groups exhibited significantly more time in the closed arm and significantly less time in the open arm following treatment.

No main effect of experimental treatment was observed in the analysis of time spent in the closed arm ($p = 0.97$) or open arm ($p = 0.51$) of the maze, but there was a main effect of treatment ($F_{3, 39} = 3.78$, $p < 0.02$) in the amount of time spent in the center of the maze. Further analysis revealed that the time spent in the center of the maze by water-treated control animals was significantly greater than the time spent there by maltodextrin- ($p < 0.02$) and probiotic-

treated animals ($p < 0.03$), though it was not significantly different from mercury-treated animals ($p = 0.11$).

Although none of the interactions between experimental timepoint and experimental treatment were statistically significant ($p = 0.49$), specific planned comparisons were conducted with the Bonferroni correction. These comparisons revealed a significant increase in time spent in the closed arm ($p < 0.03$) and a significant decrease in time in the open arm ($p < 0.005$) by mercury-treated animals. Maltodextrin-treated animals also exhibited significantly decreased time in the open arm ($p < 0.045$).

Figure 6 shows the data collected from the Elevated Plus Maze assay.

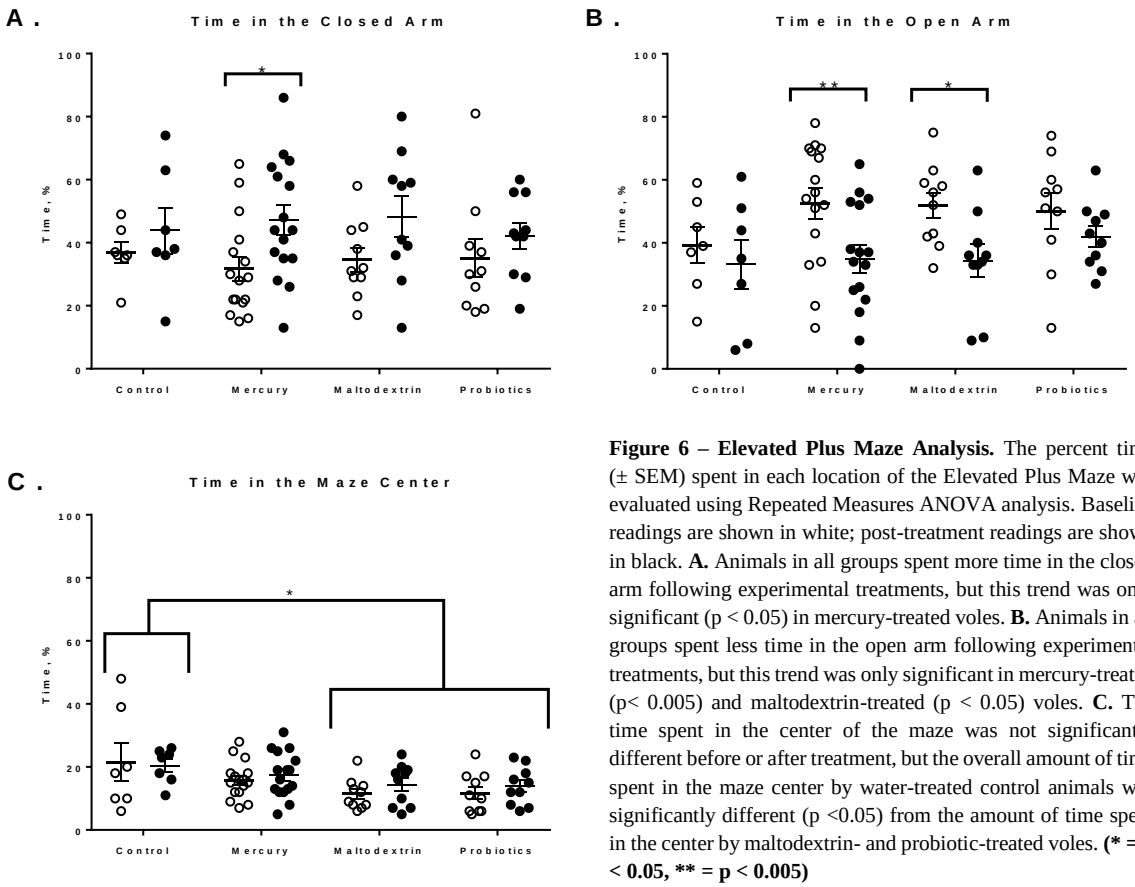

Figure 6 – Elevated Plus Maze Analysis. The percent time (± SEM) spent in each location of the Elevated Plus Maze was evaluated using Repeated Measures ANOVA analysis. Baseline readings are shown in white; post-treatment readings are shown in black. **A.** Animals in all groups spent more time in the closed arm following experimental treatments, but this trend was only significant ($p < 0.05$) in mercury-treated voles. **B.** Animals in all groups spent less time in the open arm following experimental treatments, but this trend was only significant in mercury-treated ($p< 0.005$) and maltodextrin-treated ($p < 0.05$) voles. **C.** The time spent in the center of the maze was not significantly different before or after treatment, but the overall amount of time spent in the maze center by water-treated control animals was significantly different ($p <0.05$) from the amount of time spent in the center by maltodextrin- and probiotic-treated voles. **(* = $p < 0.05$, ** = $p < 0.005$)**

Open Field Test

Repeated Measures ANOVA analysis was used to analyze the behavior of voles in the open field. A main effect of experimental timepoint was seen in the distance traveled in the field ($F_{1, 41} = 29.6$, $p < 0.0001$), the number of entries into the center of the field ($F_{1,41} = 7.97$, $p < 0.008$), and the time spent in the center of the field ($F_{1, 38} = 4.23$, $p < 0.05$). A main effect of experimental treatment was seen only in the amount of time spent in the center of the field ($F_{3, 38} = 4.38$, $p < 0.01$), with differences in distance traveled ($p = 0.929$) and entries into the center of the field ($p = 0.81$) failing to be statistically significant.

No significant interaction between experimental timepoint and experimental treatment was seen in the distance traveled in the field ($p = 0.605$), but the interaction between these factors was significant in the number of entries into the center of the field ($F_{3, 41} = 3.27$, $p < 0.04$) and the time spent in the center of the field ($F_{3, 41} = 3.46$, $p < 0.03$). Further analysis confirmed that prior to mercury treatment, voles entered the center of the field significantly more often ($p < 0.001$) and spent significantly more time in that area ($p < 0.001$) than they did after experimental treatment. Indeed, the initial amount of time spent in the center of the Open Field for mercury-treated animals was significantly different from the initial amount of time spent in the center of the field for all other treatment groups ($p < 0.04$).

Despite the lack of a significant interaction between experimental timepoint and experimental treatment on the distance traveled in the open field, specific planned comparisons were performed with the Bonferroni correction to further evaluate the experimental data. These analyses confirmed that the declines in distance traveled by animals in the control, mercury-treated, and probiotic-treated groups were statistically significant ($p < 0.05$), but declines seen in maltodextrin-treated animals were not statistically significant ($p = 0.30$).

Figure 7 illustrates the data collected in the Open Field test.

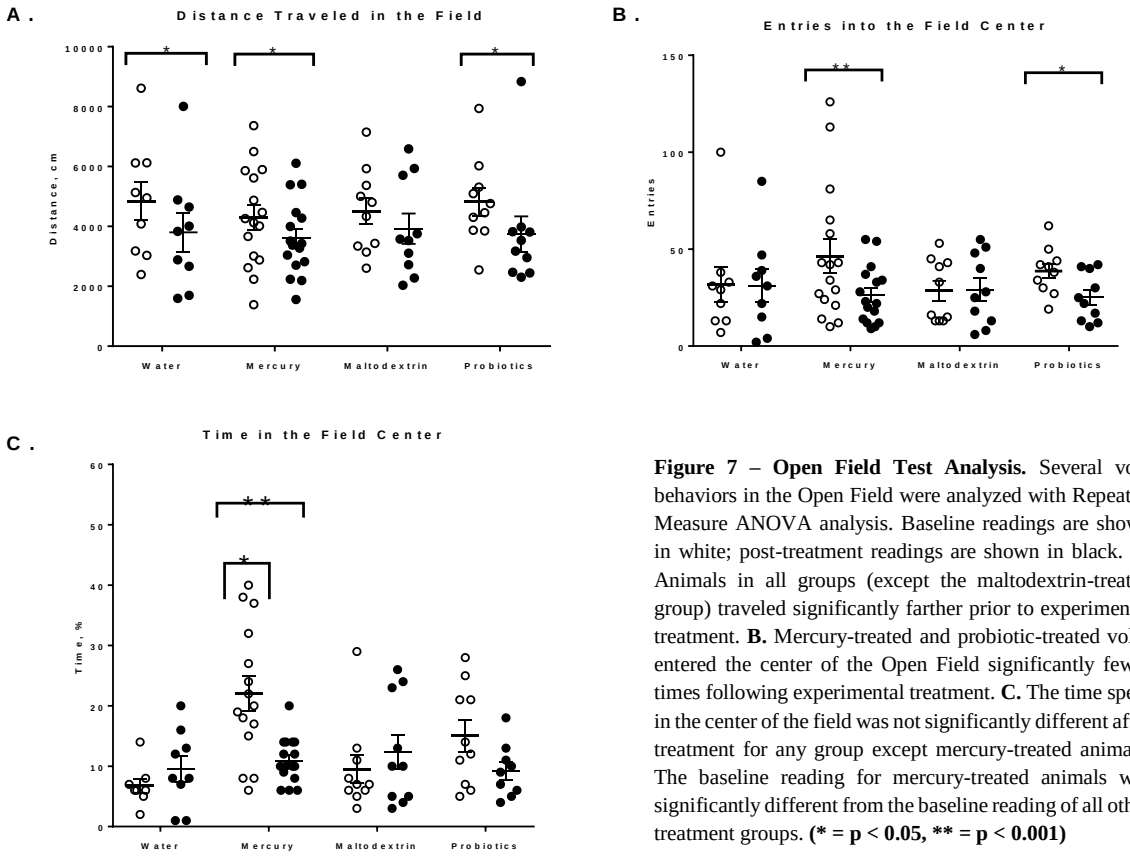

Figure 7 – Open Field Test Analysis. Several vole behaviors in the Open Field were analyzed with Repeated Measure ANOVA analysis. Baseline readings are shown in white; post-treatment readings are shown in black. **A.** Animals in all groups (except the maltodextrin-treated group) traveled significantly farther prior to experimental treatment. **B.** Mercury-treated and probiotic-treated voles entered the center of the Open Field significantly fewer times following experimental treatment. **C.** The time spent in the center of the field was not significantly different after treatment for any group except mercury-treated animals. The baseline reading for mercury-treated animals was significantly different from the baseline reading of all other treatment groups. **(* = $p < 0.05$, ** = $p < 0.001$)**

Summary of Experiment 1

The aim of Experiment 1 was to determine the effects of mercury and probiotics on the anxiety behaviors of the prairie vole. Two behavioral assays (the Elevated Plus Maze and the Open Field test) were used to evaluate anxiety before and after experimental treatment. In both the Elevated Plus Maze and the Open Field Test, main effects of experimental timepoint were observed, indicating that regardless of treatment group, all groups tended to behave differently at the second testing timepoint compared to the first testing timepoint. This difference was particularly dramatic in mercury-treated voles, with these voles spending significantly less time in the open arm (and significantly more time in the closed arm) of the Elevated Plus Maze and

significantly less time in the center of the Open Field, all behaviors indicative of increased anxiety. Interestingly, voles that received the probiotic solution also spent significantly less time in the center of the Open Field and while the trends toward anxiety behaviors in the Elevated Plus Maze failed to be statistically significant, they do support a generalized conclusion that our probiotic solution may also increase anxiety in the test animals.

Overall, the findings of Experiment 1 support the hypothesis that mercury and probiotics do alter anxiety behaviors in the prairie vole, albeit in somewhat unexpected ways.

Experiment 2 – Exploration of *Lactobacillus* effects after Mercury Exposure

Demographics

As previously described, mercury-exposed voles from Experiment 1 were further divided into maltodextrin- and probiotic-treatment groups. Six animals received dilute maltodextrin (0.15%) and ten animals received probiotics (~4 x 10^8 CFU/ml) for two weeks. All animals tolerated the treatment course, and no significant differences were seen between the average mass of animals in either treatment group before ($p = 0.15$) or after ($p = 0.14$) experimental treatment, or in consumption of the experimental solutions ($p = 0.25$) throughout the treatment course.

Elevated Plus Maze

Repeated Measures ANOVA revealed significant main effects of experimental timepoint ($F_{2,26} = 3.87$, $p < 0.04$) and experimental treatment ($F_{1,13} = 10.96$, $p < 0.01$) on the amount of time spent in the open arm of the maze. Post hoc analysis confirmed that baseline performance in the open arm was significantly different from performance after mercury exposure ($p < 0.02$) and after experimental treatment ($p < 0.03$). However, the comparison of the performance of animals after treatment with probiotics and after treatment with maltodextrin yielded no significant

differences (p = 0.86). The interaction between experimental timepoint and experimental treatment also was not significant (p = 0.96). No significant main effects or interactions were observed in analysis of time spent in the closed arms of the maze.

A main effect of experimental treatment ($F_{1, 13} = 5.70$, $p < 0.04$) was also seen in analysis of the amount of time spent in the center of the maze. Although an interaction between experimental treatment and experimental timepoint was not significant (p = 0.30), specific planned comparisons of the data were made with the Bonferroni correction. These comparisons confirmed that maltodextrin-treated animals spent significantly more time in the middle of the Elevated Plus Maze at the end of the experimental time course than did probiotic-treated animals ($p < 0.03$).

Figure 8 summarizes the data collected from the Elevated Plus Maze.

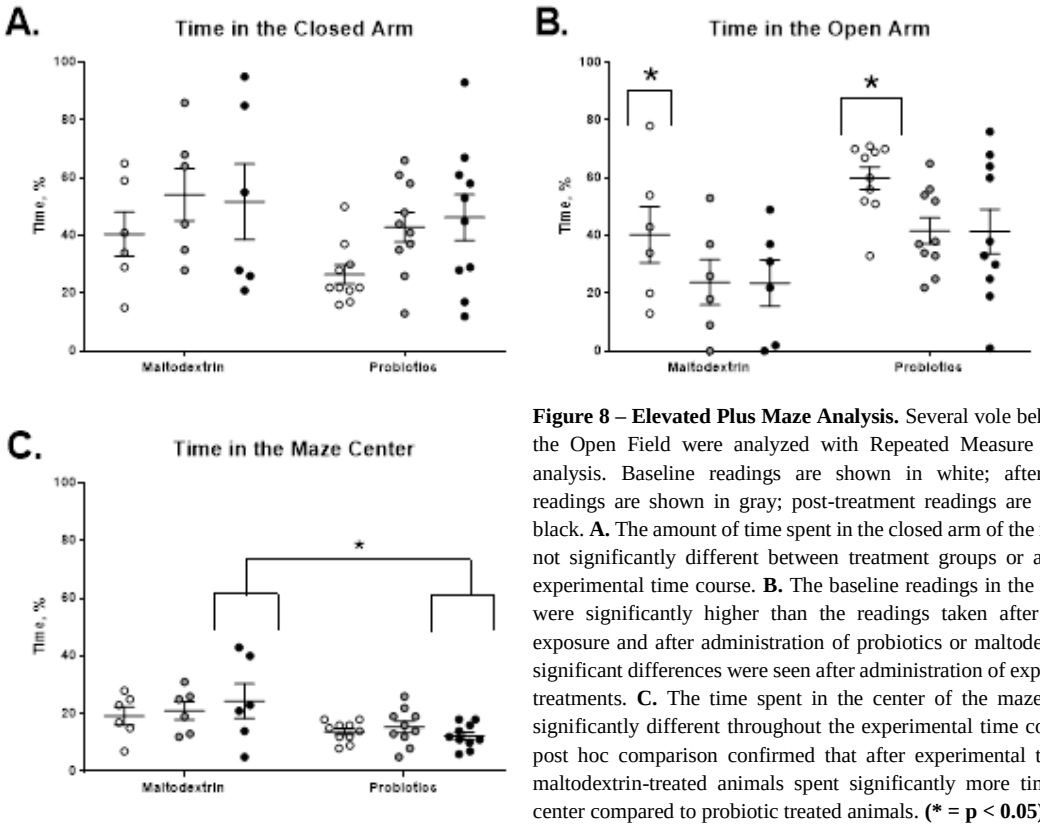

Figure 8 – Elevated Plus Maze Analysis. Several vole behaviors in the Open Field were analyzed with Repeated Measure ANOVA analysis. Baseline readings are shown in white; after-mercury readings are shown in gray; post-treatment readings are shown in black. **A.** The amount of time spent in the closed arm of the maze was not significantly different between treatment groups or across the experimental time course. **B.** The baseline readings in the open arm were significantly higher than the readings taken after mercury exposure and after administration of probiotics or maltodextrin. No significant differences were seen after administration of experimental treatments. **C.** The time spent in the center of the maze was not significantly different throughout the experimental time course, but post hoc comparison confirmed that after experimental treatment, maltodextrin-treated animals spent significantly more time in the center compared to probiotic treated animals. **(* = p < 0.05)**

Open Field Test

Repeated Measures ANOVA analysis of distance traveled revealed a main effect of time ($F_{2,26} = 8.15$, $p < 0.002$), with both treatment groups traveling significantly farther ($p < 0.03$) following treatment (compared to the distance traveled after mercury administration). No significant differences were noted between baseline and post-mercury treatment time points.

Analysis of the entries into the center of the Open Field revealed a significant main effect of experimental timepoint ($F_{2, 26} = 6.64$, $p < 0.009$), with all animals entering the center of the field significantly fewer times following mercury exposure ($p < 0.003$). No differences in entries noted when the baseline readings were compared to the post-treatment readings ($p = 0.12$). There was no significant effect of experimental treatment ($p = 0.25$) or any significant interaction between experimental timepoint and experimental treatment ($p = 0.77$) in quantification of the number of entries into the field center.

Analysis of the amount of time spent in the center of the field revealed a main effect of time ($F_{2, 26} = 5.76$, $p < 0.01$), with both treatment groups spending significantly less time in the center of the field than after mercury exposure ($p < 0.003$) and after experimental treatment ($p < 0.01$). No significant differences were noted between post-mercury and post-treatment timepoints ($p = 0.66$). No significant main effect of experimental treatment ($p = 0.87$) or interaction between experimental treatment and time ($p = 0.39$) were seen.

Figure 9 summarizes the data collected in the Open Field test.

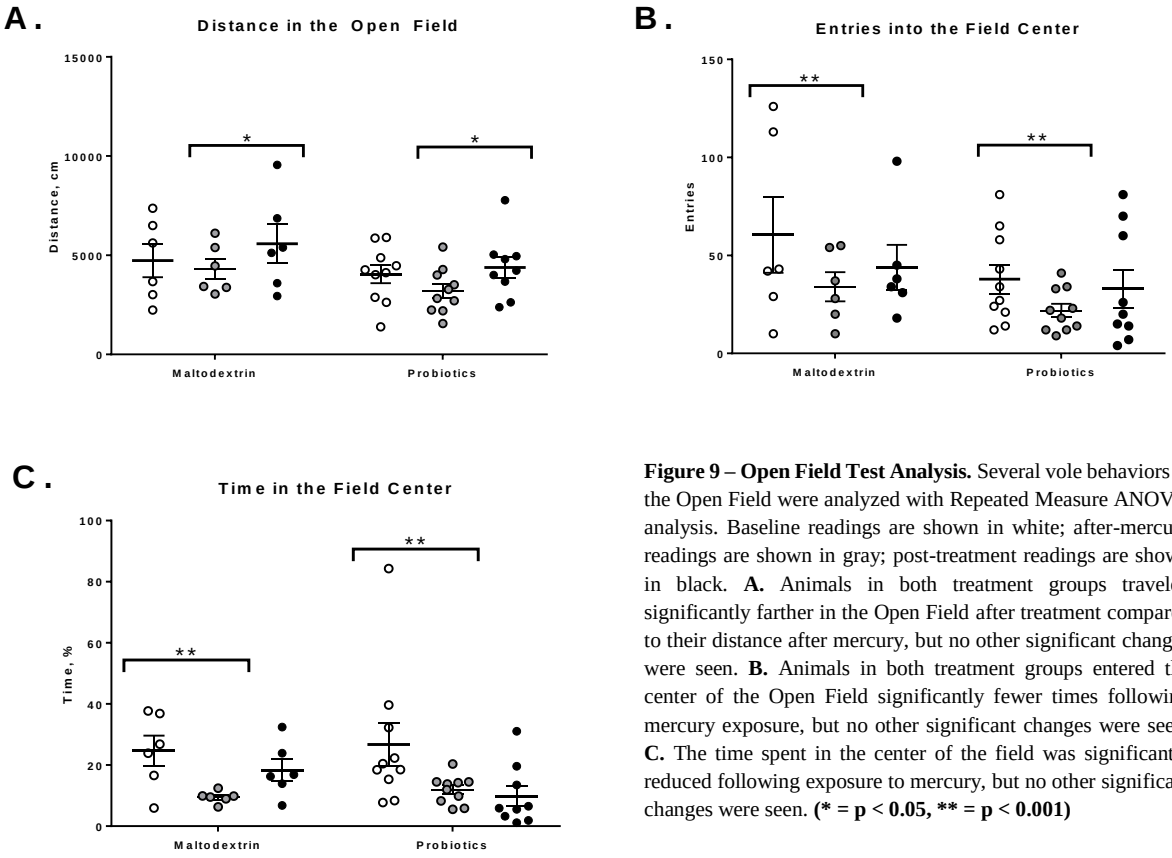

Figure 9 – Open Field Test Analysis. Several vole behaviors in the Open Field were analyzed with Repeated Measure ANOVA analysis. Baseline readings are shown in white; after-mercury readings are shown in gray; post-treatment readings are shown in black. **A.** Animals in both treatment groups traveled significantly farther in the Open Field after treatment compared to their distance after mercury, but no other significant changes were seen. **B.** Animals in both treatment groups entered the center of the Open Field significantly fewer times following mercury exposure, but no other significant changes were seen. **C.** The time spent in the center of the field was significantly reduced following exposure to mercury, but no other significant changes were seen. **(* = $p < 0.05$, ** = $p < 0.001$)**

The Social Avoidance Test

Independent t-test analysis of the time spent in contact with an unknown stimulus animal revealed a significant difference in behavior between maltodextrin-treated and probiotic-treated animals ($t = 2.796$, $p < 0.015$), with maltodextrin-treated animals spending 111.3 ± 37.1 minutes in contact with the stimulus and probiotic-treated animals spending 48.3 ± 46.8 minutes in contact with the stimulus.

Figure 10 summarizes the data collected in the Social Avoidance test.

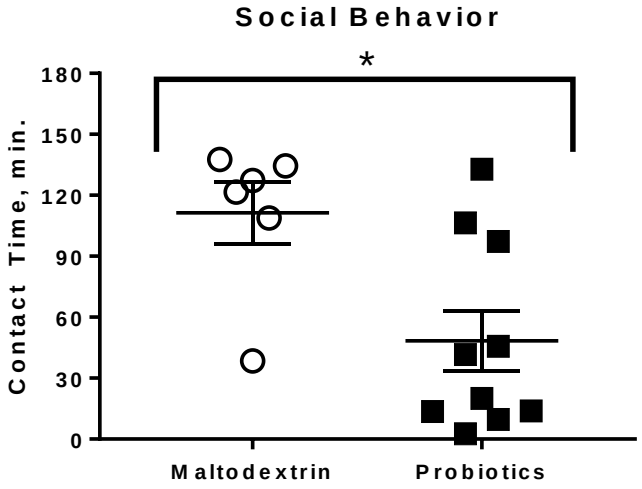

Figure 10 – Social Avoidance Test Analysis. Following mercury exposure, voles were treated with either dilute (0.15%) maltodextrin or probiotic solution. Social behavior was determined using the Social Avoidance Test. The number of minutes (± SEM) spent in contact with an unknown stimulus animal is seen at left. Student t-test analysis revealed a significant effect of experimental treatment on contact, with maltodextrin-treated voles spending significantly more time in contact with the unknown stimulus animal than probiotic-treated voles ($p < 0.015$).

Summary of Experiment 2

The aim of Experiment 2 was to explore the possible restorative effects of probiotic administration on mercury-induced anxiety and social deficits. As described in Experiment 1, the administration of mercury significantly decreased the amount time that voles spent in the open arm of the Elevated Plus Maze and the center of the Open Field. We predicted that administration of a probiotic solution after mercury exposure would increase the time in the open arm and the center of the field, returning both measurements to pre-mercury levels. However, no significant changes were seen in vole behavior in either the Elevated Plus Maze or the Open Field Test following the administration of the probiotic solution. In fact, there was a trend for probiotic-treated voles to spend even less time in the center of the Open Field following probiotic administration than they did after mercury exposure, which may indicate that these animals had become even more anxious.

In addition to anxiety-related behaviors, we also examined social behaviors following probiotic administration. As outlined in Curtis *et al.*,[10] male vole exposure to mercury decreases

the amount of time spent in contact with an unknown stimulus animal. Following mercury exposure and subsequent probiotic administration, prairie vole sociability was assessed using the Social Avoidance Test. In this test, animals treated with our probiotic solution spent an average of 48.3 minutes in contact with the stimulus while animals receiving the rehydrating agent (0.15% maltodextrin) spent an average of 111.3 minutes in contact with the stimulus. These findings are intriguing, as maltodextrin-treated voles exhibited sociability on par with untreated control animals while the probiotic-treated animals exhibited sociability on par with mercury-treated voles (reference Curtis *et al.*[10]). Given that our studies identified an increase in anxiety in mercury-treated voles (which may explain their decreased sociability), it is unsurprising that our probiotic solution, which was ineffective in alleviating anxiety, was also ineffective at restoring behavioral deficits.

General Conclusions

The aim of this study was to determine the impact of mercury and probiotics on the anxiety and social behavior of the prairie vole (*Microtus ochrogaster*). Data supported our hypothesis that exposure to dilute mercuric chloride (60 ppm) increases anxiety in the vole, offering a potential underlying mechanism of the social behavior declines seen after mercury exposure.[10] While we hypothesized that our probiotic solution may help remediate both the anxiety and social deficits of mercury-treated voles, the data did not support this hypothesis. Indeed, our selected *Lactobacillus* strains (PV012, PV018, and PV019) appear to instead increase anxiety and further suppress social behavior. Nonetheless, the results of this experiment do show a clear impact of mercury and probiotics on the behavior of prairie voles and provide a strong foundation for the correlation of behavioral changes with changes in the gut microbial community.

CHAPTER V

THE EFFECTS OF MERCURY AND PROBIOTICS ON THE MICROBIOME IN THE PRAIRIE VOLE (*MICROTUS OCHROGASTER*)

Introduction

The human digestive tract is full of bacteria, viruses, and fungi. These microorganisms and their genetic information are collectively referred to as the microbiome. The microbiome is known to change in response to a variety of factors including dietary changes, probiotic administration, antibiotic use, or host stress. In the current study, we sought to determine the nature of these changes in the prairie vole (*Microtus ochrogaster*). Voles were given *ad libitum* access to mercuric chloride (60 ppm) for 4 weeks, followed by 2 weeks of *ad libitum* access to either a probiotic suspension or 0.15% maltodextrin. Throughout the experimental course, fecal pellets were collected and processed for the isolation of bacterial DNA. This DNA was then sequenced and analyzed for differences in factors such as alpha diversity (a measurement of the total number of species present in the bacterial community) and beta diversity (a measurement of the relative abundances of the species in the community). The identification of statistically-significant abundance changes was made using linear discriminant analysis effect size (LEfSe).

We hypothesized that exposure to 60 ppm mercuric chloride would significantly alter the composition of the gut microbial community and that the administration of our probiotic suspension would return it to its baseline parameters.

Results

Microbiome Structure in Distressed and Non-Distressed Voles

Per the design of Experiment 2, twenty male prairie voles were given *ad libitum* access to 60 ppm mercuric chloride for 4 weeks. Within the first week of this treatment course, four voles exhibited distress and were humanely euthanized.

To determine if the gut microbial communities were significantly different between voles that did and did not experience distress with mercury exposure, we compared the microbiome composition of the pellets collected prior to experimental manipulations (Baseline) across the two groups. Alpha diversity, a measurement of the number of species present in the samples, was not significantly different between the two experimental groups, but weighted UniFrac analysis, a beta diversity assessment that compares the abundances of the microbes present in the microbial community, was significantly different between the two treatment groups ($t = -4.14$, $p < 0.0001$).

Bar charts illustrating the relative abundances of all microbes in the gut of distressed and non-distressed animals are shown in Figure 11.

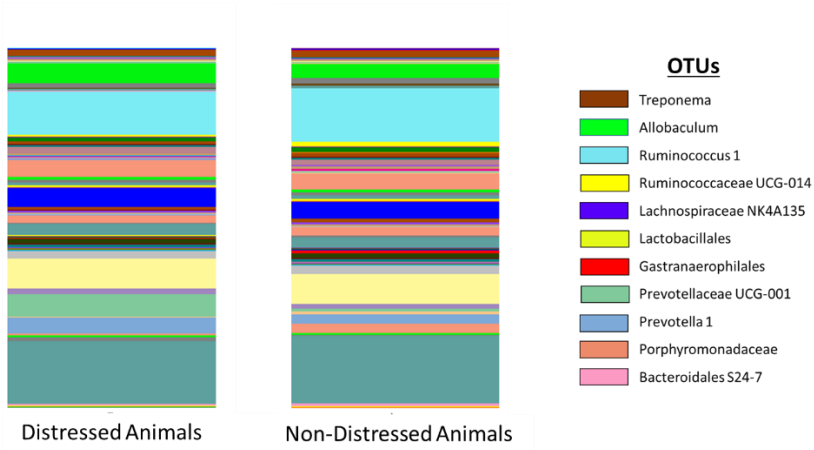

Figure 11 – General Microbiome Composition of Distressed vs. Non-Distressed Voles. During the mercury exposure period, 4 voles exhibited distress and were humanely terminated. The composition of their gut microbial community was compared to that of voles that did not exhibit distress. Bar charts were assembled to illustrate the relative abundance of all operational taxonomic units (OTUs) present in the microbiome of each group of organisms. Each color represents a different OTU; several OTUs are identified in the legend.

To determine which specific gut microbes contributed most to the differences in beta diversity between the two groups, linear discriminant analysis of effect size (LEfSe) was employed. Figure 12 summarizes the results of this analysis.

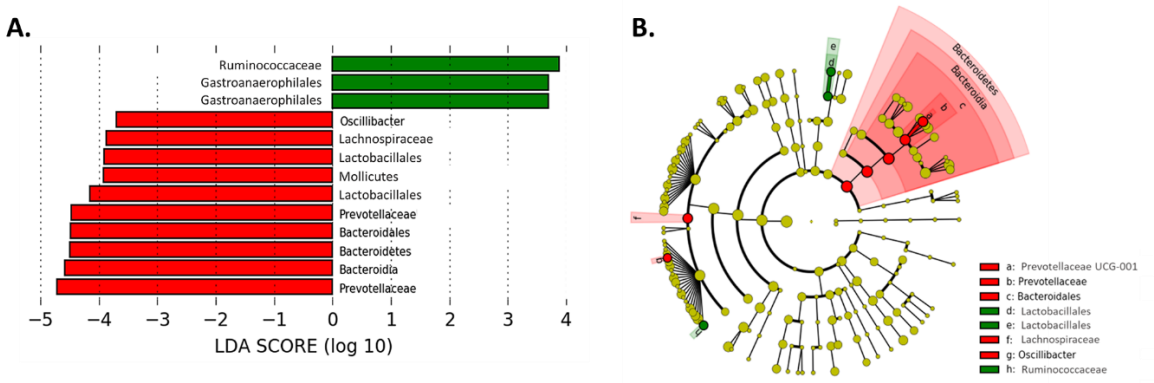

Figure 12 – LEfSe Analysis - Distressed vs. Non-Distressed Voles. The gut microbial composition of voles that did and did not tolerate mercury exposure was compared. **A.** LEfSe analysis revealed 13 operational taxonomic units (OTUs) that were significantly different between the experimental groups. OTUs elevated in animals that exhibited distress are shown in red; OTUs that were elevated in non-distressed animals are shown in green. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that several OTUs in phylum Bacteroidetes were significantly elevated in distressed animals.

Microbiome Structure Before and After Mercury Exposure

For all the voles that did not exhibit distress during the mercury exposure period, comparisons were made between the Baseline microbiome (before mercury exposure) and the After Mercury microbiome (after 4 weeks of *ad libitum* access to mercuric chloride).

Alpha diversity was significantly increased in the After Mercury samples as determined by the Chao1 index ($t = -2.02$, $p < 0.04$) and the Shannon index ($t = -2.09$, $p < 0.05$). This was due to an increased number of identified operational taxonomic units (OTUs) (391.5 ± 30.7 vs. 410.7 ± 20.1, $t = -3.00$, $p < 0.01$). Beta diversity was also significantly different as determined by both weighted ($t = -3.34$, $p < 0.001$) and unweighted ($t = -2.77$, $p < 0.006$) UniFrac analyses.

Figure 13 illustrates the differences in alpha diversity.

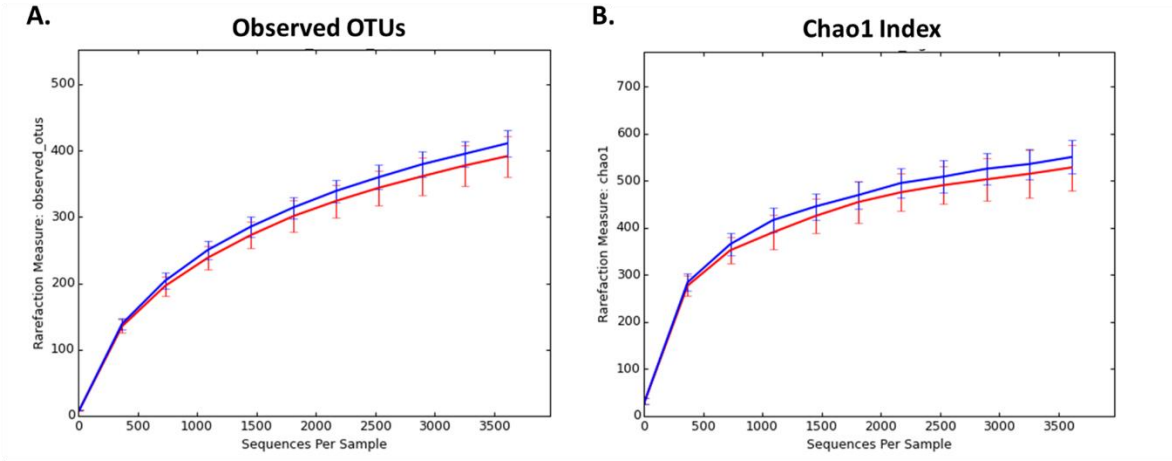

Figure 13 – Alpha Diversity Before and After Mercury Exposure. The alpha diversity of the gut microbiome before and after mercury exposure was compared. **A.** The number of observed operational taxonomic units (OTUs) was significantly elevated following mercury exposure (shown in blue) compared to those present prior to mercury exposure (shown in red). **B.** The Chao1 Index of alpha diversity was significantly higher following mercury exposure compared to the baseline timepoint.

Bar charts illustrating the relative abundances of all microbes in the gut at the Baseline and After Mercury timepoints are seen in Figure 14.

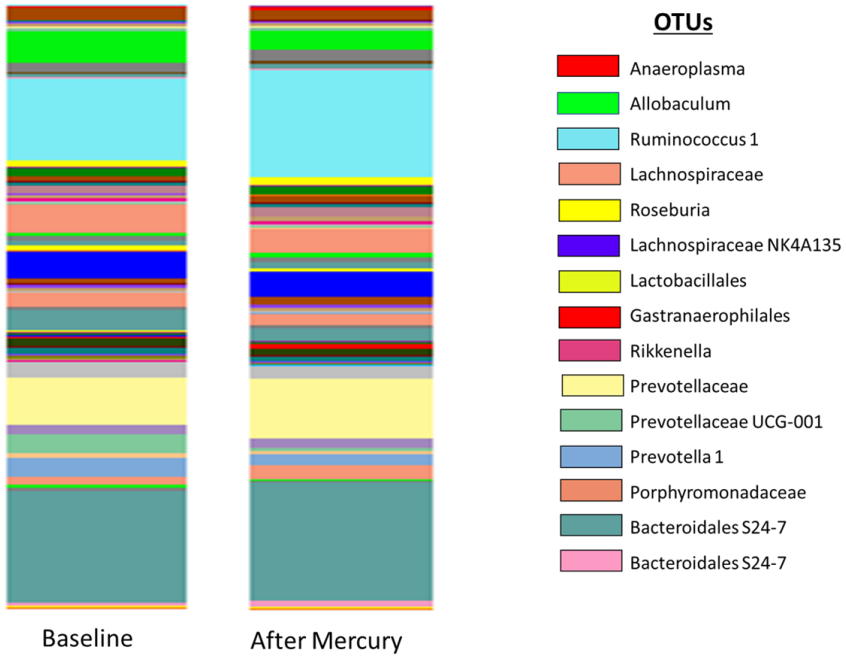

Figure 14 – General Microbiome Composition Before and After Mercury Exposure. The composition of their gut microbial communities of all voles prior to mercury exposure ("Baseline") and after mercury administration ("After Mercury") was compared. Bar charts were assembled to illustrate the relative abundance of all operational taxonomic units (OTUs) present in the microbiome, with each color representing a different OTUs. The identities of several OTUs are listed above.

To determine which OTUs were significantly different between the experimental timepoints, LEfSe analysis was employed. Figure 15 summarizes the results of this analysis.

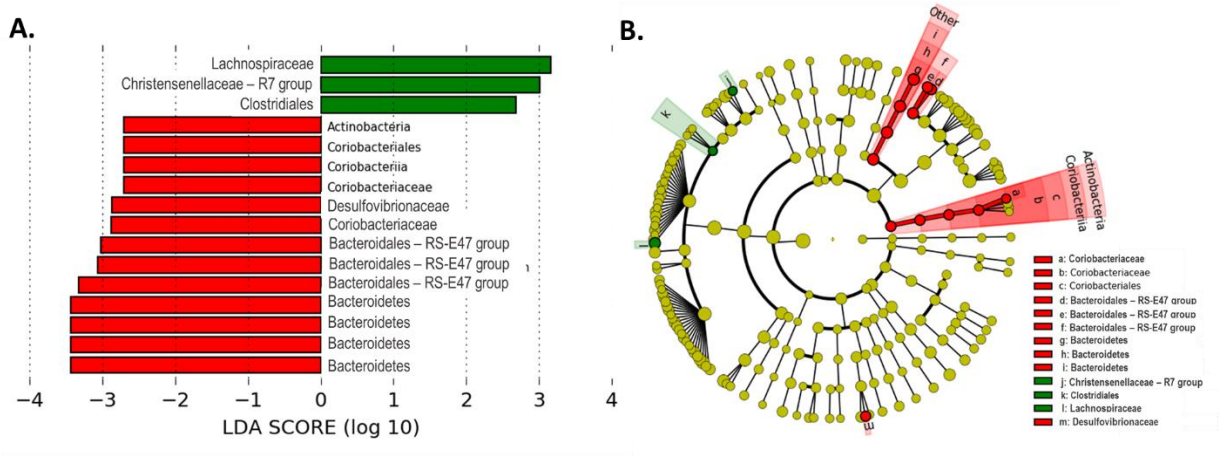

Figure 15 – LEfSe Analysis - Before and After Mercury Exposure. The gut microbial composition of voles before and after exposure to 60 ppm $HgCl_2$ was compared. **A.** LEfSe analysis revealed 16 operational taxonomic units (OTUs) that were significantly different between the two time points. OTUs elevated before mercury exposure are shown in red; OTUs that were elevated following mercury exposure are shown in green. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that several OTUs in phyla Bacteroidetes and Actinobacteria were significantly elevated prior to exposure to mercury.

Microbiome Structure After Probiotic Administration

To examine the effects of our probiotic solution on the prairie vole gut microbial community, we compared the microbiome structure of pellets collected from voles after mercury exposure and after probiotic administration.

No significant differences in alpha diversity were observed, and beta diversity differences were only significant when compared using weighted UniFrac analysis ($t = 4.22$, $p < 0.05$),

Bar charts illustrating the relative abundances of all microbes at the After Mercury and After Probiotics timepoints are seen in Figure 16.

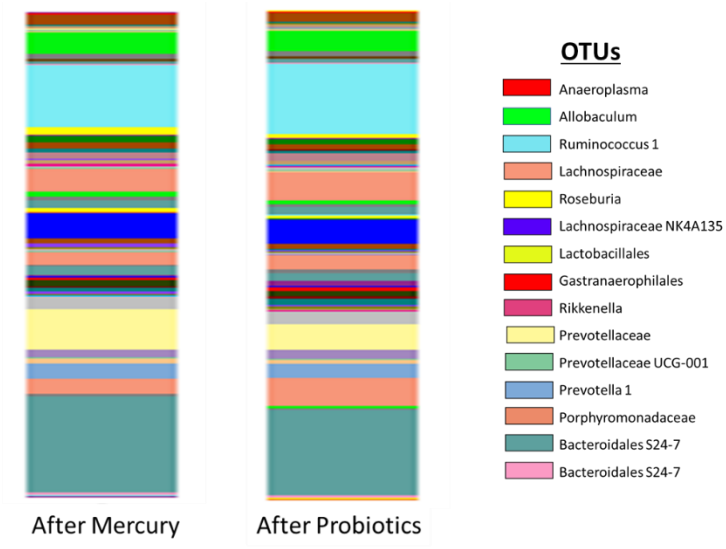

Figure 16 – General Microbiome Composition Before and After Probiotics. The compositions of the gut microbial communities of voles after mercury exposure and after probiotic administration were compared. Bar charts were assembled to illustrate the relative abundance of all operational taxonomic units (OTUs) present in the microbiome, with each color representing a different OTU. The identities of several OTUs are listed above.

To determine which OTUs were significantly different between the experimental timepoints, LEfSe analysis was employed. Figure 17 summarizes the results of this analysis.

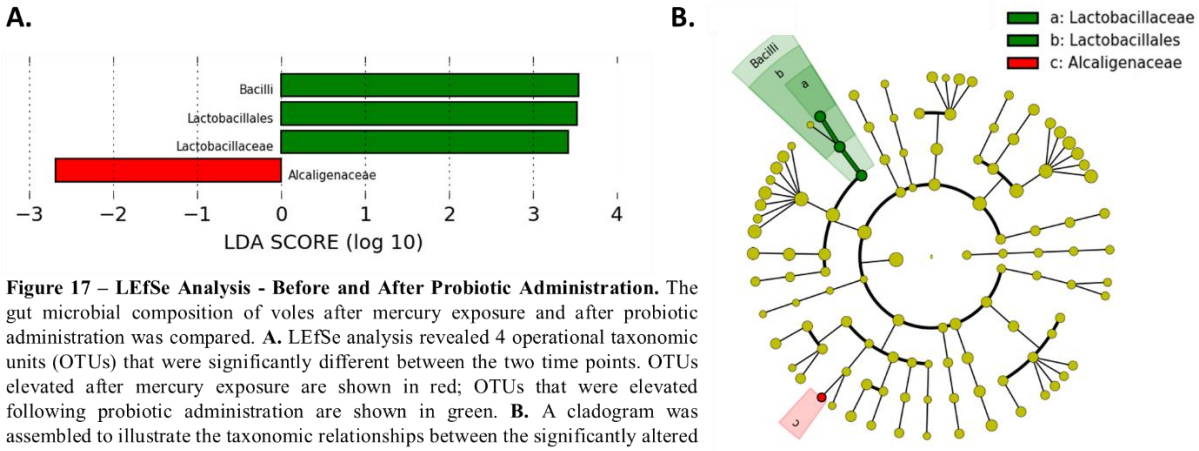

Figure 17 – LEfSe Analysis - Before and After Probiotic Administration. The gut microbial composition of voles after mercury exposure and after probiotic administration was compared. **A.** LEfSe analysis revealed 4 operational taxonomic units (OTUs) that were significantly different between the two time points. OTUs elevated after mercury exposure are shown in red; OTUs that were elevated following probiotic administration are shown in green. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that several OTUs in class Bacilli were altered by probiotic administration.

Microbiome Structure After Maltodextrin Administration

Maltodextrin was used as a rehydration agent for our probiotic suspension, but this simple sugar is also known to be a prebiotic.[71] To determine what impact our resuspension agent might have on the gut microbial community, we examined the microbiome structure of maltodextrin-treated voles at several different time points.

We first examined the effect of maltodextrin on the microbiome following mercury exposure. No significant differences in alpha diversity were observed in the comparison of the After Mercury and After Maltodextrin timepoints. However, unweighted UniFrac analysis identified a significant decrease in beta diversity following maltodextrin administration ($t = 4.45$, $p < 0.002$).

Bar charts illustrating the relative abundances of all microbes at the After Mercury and After Maltodextrin timepoints are seen in Figure 18.

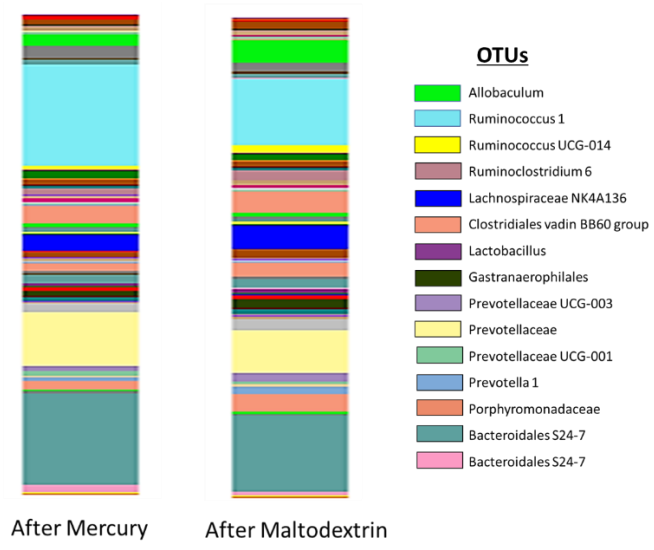

Figure 18 – General Microbiome Composition Before and After Maltodextrin. The compositions of the gut microbial communities of voles after mercury exposure and after maltodextrin administration were compared. Bar charts were assembled to illustrate the relative abundance of all operational taxonomic units (OTUs) present in the microbiome, with each color representing a different OTU. The identities of several OTUs are listed above.

To determine which OTUs were significantly different between the experimental timepoints, LEfSe analysis was employed. Figure 19 summarizes these results.

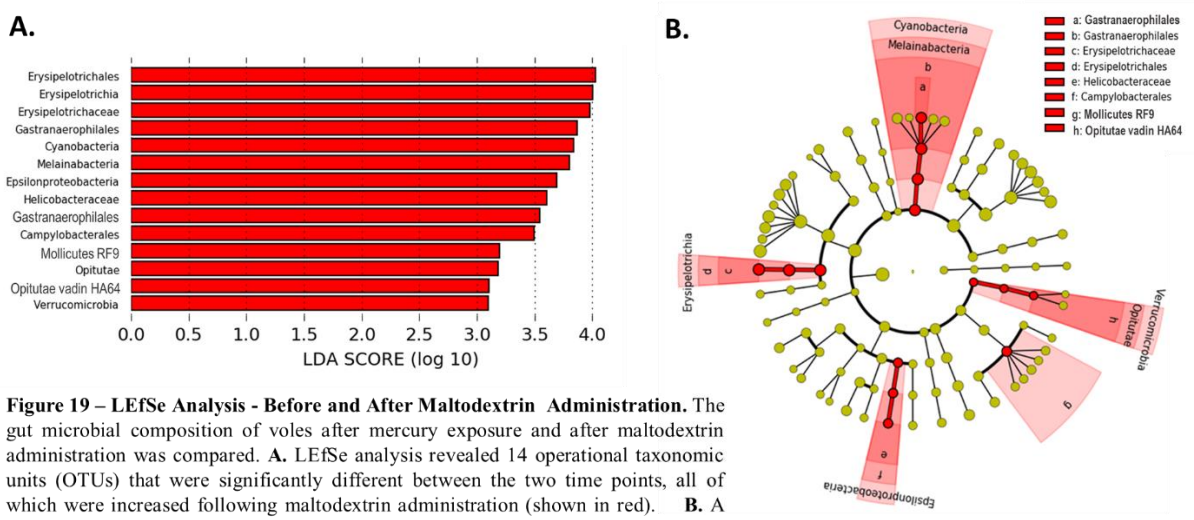

Figure 19 – LEfSe Analysis - Before and After Maltodextrin Administration. The gut microbial composition of voles after mercury exposure and after maltodextrin administration was compared. **A.** LEfSe analysis revealed 14 operational taxonomic units (OTUs) that were significantly different between the two time points, all of which were increased following maltodextrin administration (shown in red). **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that several OTUs in the phyla Cyanobacteria and Verrucomicrobia and classes Epsilonproteobacteria and Erysipelotrichia were significantly elevated

Microbiome Structure After Probiotics and Maltodextrin

Given the variety of microorganisms that were impacted by the administration of both our probiotic solution and the maltodextrin, we decided to compare the microbiomes of the voles in each treatment group to see how similar or different their microbiome structures were at the end of the treatment course.

No significant differences in alpha or beta diversity were seen between the experimental groups, though there was a trend toward significantly different beta diversity as assessed with weighted UniFrac analysis ($t = 1.96$, $p = 0.055$).

Bar charts illustrating the relative abundances of all microbes at the After Maltodextrin and After Probiotics timepoints are seen in Figure 20.

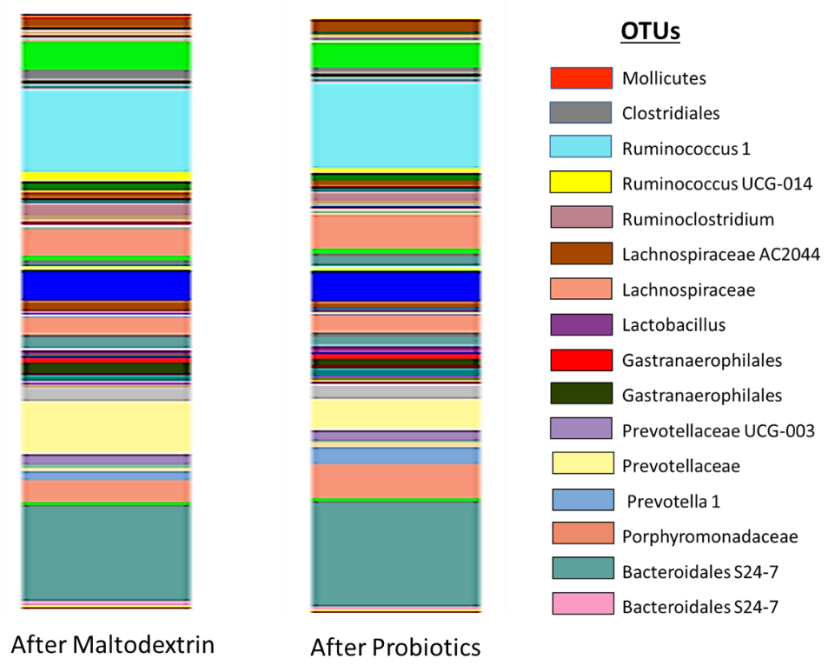

Figure 20 – General Microbiome Composition After Maltodextrin and Probiotics. The compositions of the gut microbial communities of voles after maltodextrin or probiotics administration were compared. Bar charts were assembled to illustrate the relative abundance of all operational taxonomic units (OTUs) present in the microbiome, with each color representing a different OTU. The identities of several OTUs are listed above.

To determine which OTUs were significantly different between the experimental timepoints, LEfSe analysis was employed. Figure 21 summarizes these results.

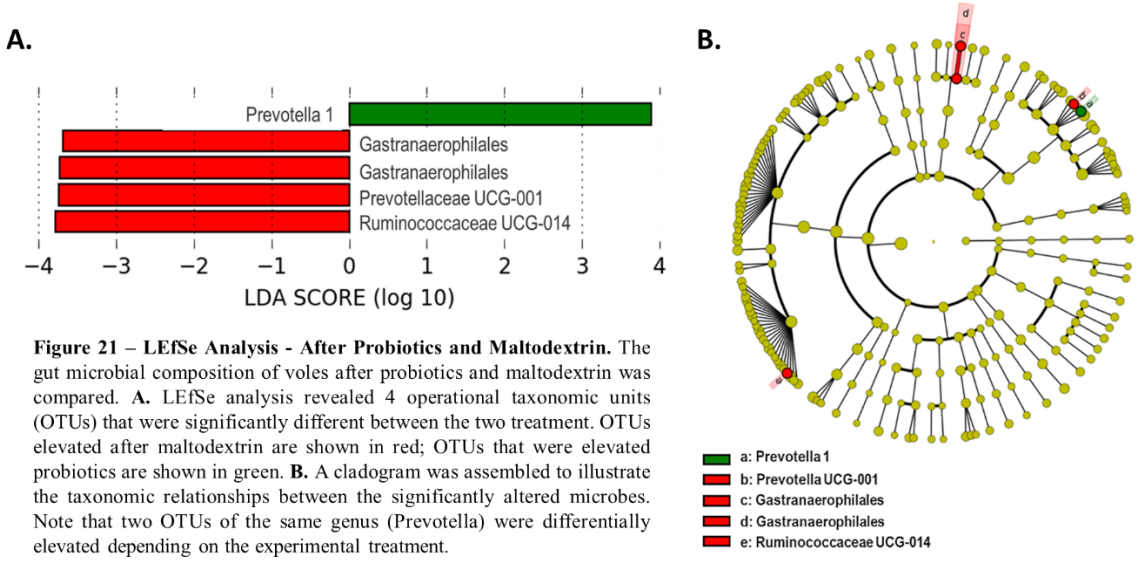

Figure 21 – LEfSe Analysis - After Probiotics and Maltodextrin. The gut microbial composition of voles after probiotics and maltodextrin was compared. **A.** LEfSe analysis revealed 4 operational taxonomic units (OTUs) that were significantly different between the two treatment. OTUs elevated after maltodextrin are shown in red; OTUs that were elevated probiotics are shown in green. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that two OTUs of the same genus (Prevotella) were differentially elevated depending on the experimental treatment.

Comparing Baseline and After Probiotic Microbiome Structures

We hypothesized that the administration of our probiotic *Lactobacillus* would restore the imbalances in the gut microbiome that were caused by mercury exposure, returning the microbiome of these probiotic-treated voles back to their original community structure. To test this hypothesis, we compared the microbiome structure of fecal pellets collected prior to experimental manipulation (Baseline) and again following the probiotic treatment course (After Probiotics).

No significant differences in alpha or beta diversity were seen between the two experimental timepoints Bar charts illustrating the relative abundances of all microbes at the Baseline and After Probiotics timepoints are seen in Figure 22.

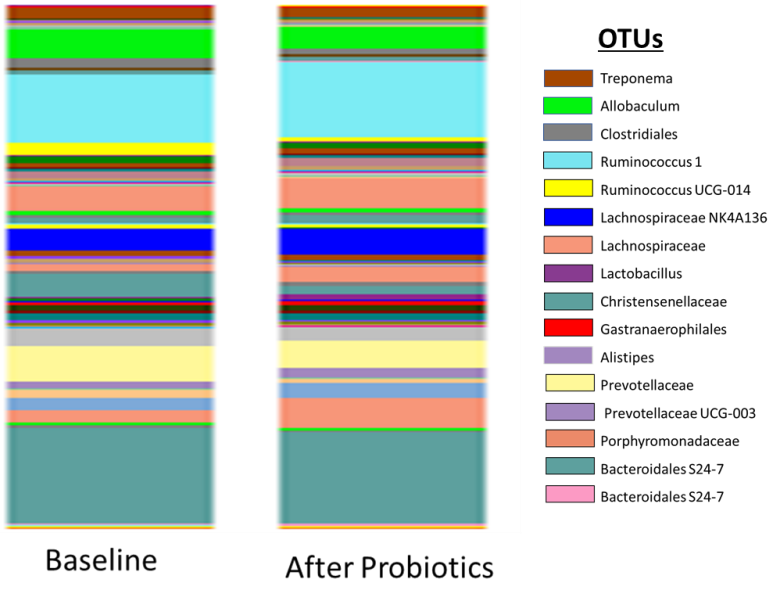

Figure 22 – General Microbiome Composition at Baseline and Following Probiotics. The compositions of the gut microbial communities of voles prior to experimentation and after administration of probiotics were compared. Bar charts were assembled to illustrate the relative abundance of all operational taxonomic units (OTUs) present in the microbiome, with each color representing a different OTU. The identities of several OTUs are listed above.

To determine which OTUs were significantly different between the experimental timepoints, LEfSe analysis was employed. Figure 23 summarizes these results.

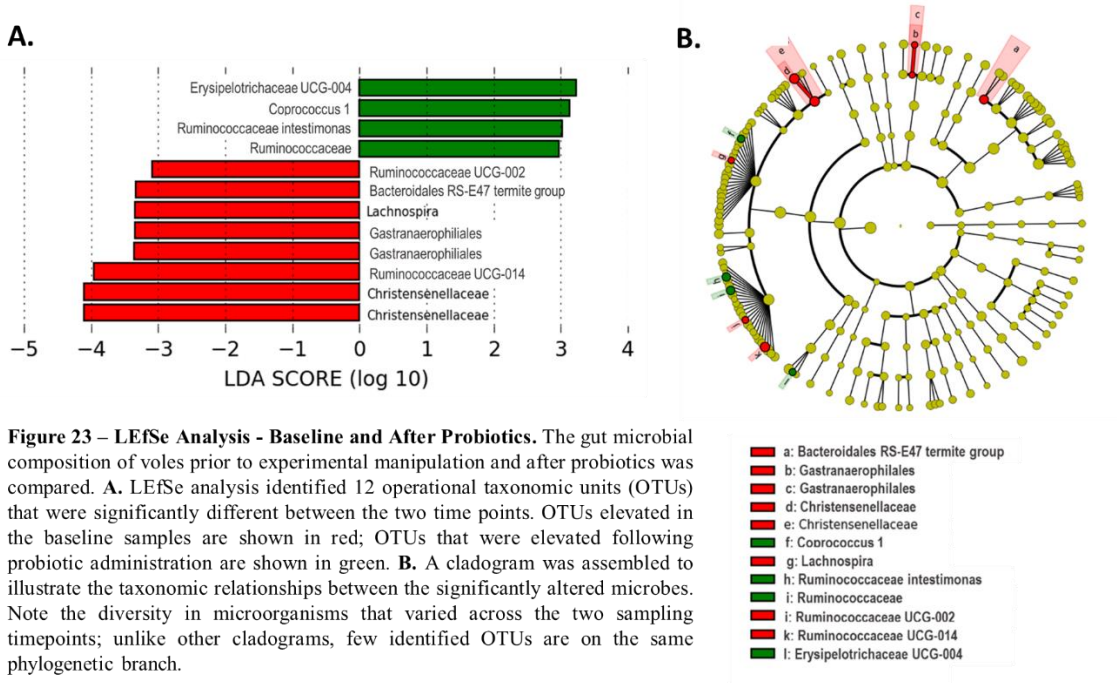

Figure 23 – LEfSe Analysis - Baseline and After Probiotics. The gut microbial composition of voles prior to experimental manipulation and after probiotics was compared. **A.** LEfSe analysis identified 12 operational taxonomic units (OTUs) that were significantly different between the two time points. OTUs elevated in the baseline samples are shown in red; OTUs that were elevated following probiotic administration are shown in green. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note the diversity in microorganisms that varied across the two sampling timepoints; unlike other cladograms, few identified OTUs are on the same phylogenetic branch.

Comparing Baseline and After Maltodextrin Microbiome Structures

We used maltodextrin primarily as a rehydrating agent for our probiotic solution, so we were not attempting to alter the gut microbial community structure by administering it to the voles. Nonetheless, given the fact that mercury so dramatically altered the microbiome from its baseline community structure and that the maltodextrin solution had a significant effect on beta diversity when administered after mercury, we decided to compare the structures of the Baseline and After Maltodextrin microbiomes.

No significant differences in alpha diversity were seen in comparing the baseline microbial communities to the communities after maltodextrin administration. While weighted UniFrac analysis did not reveal significant differences in the beta diversity at the two experimental timepoints, unweighted UniFrac analysis did ($t = 3.75$, $p < 0.02$).

Bar charts illustrating the relative abundances of all microbes at the Baseline and After Maltodextrin timepoints are seen in Figure 24.

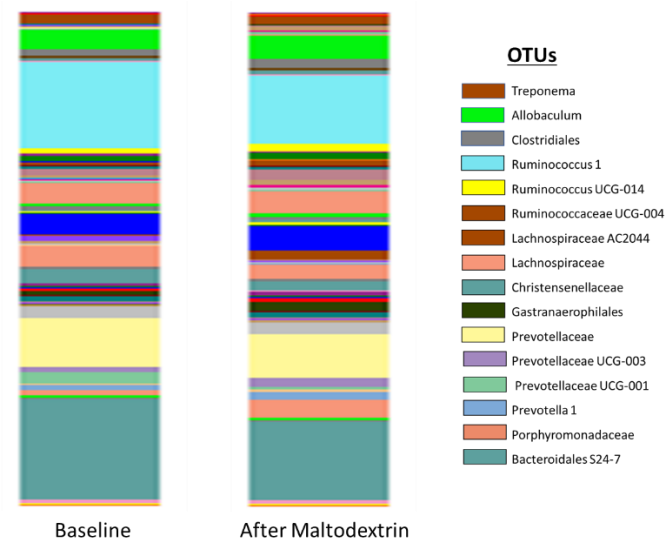

Figure 24 – General Microbiome Composition at Baseline and After Maltodextrin. The compositions of the gut microbial communities of voles prior to experimentation and after administration of maltodextrin were compared. Bar charts were assembled to illustrate the relative abundance of all operational taxonomic units (OTUs) present in the microbiome, with each color representing a different OTU. The identities of several OTUs are listed above.

To determine which OTUs were significantly different between the experimental timepoints, LEfSe analysis was employed. Figure 25 summarizes these results.

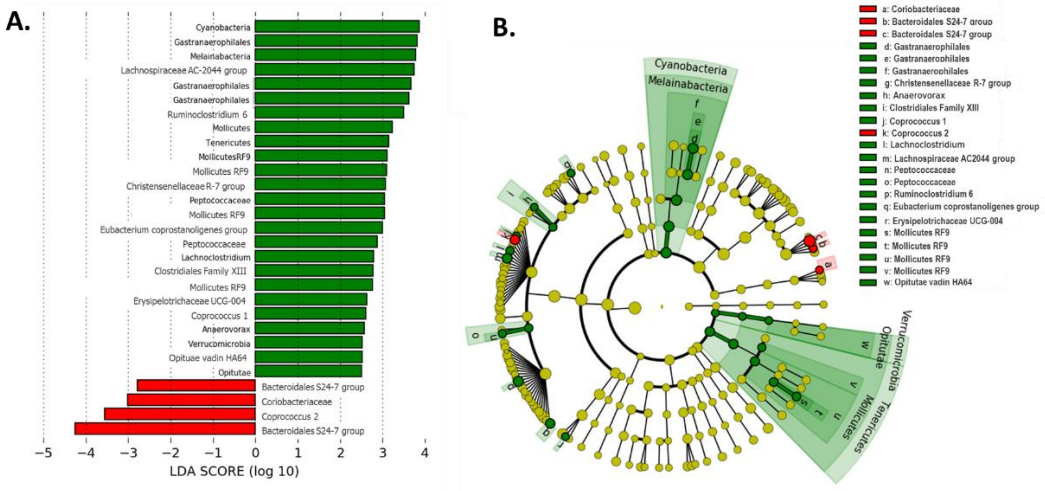

Figure 25 – LEfSe Analysis - Before and After Maltodextrin Administration. The gut microbial composition of voles prior to experimental manipulation and after maltodextrin administration was compared. **A.** LEfSe analysis revealed 29 operational taxonomic units (OTUs) that were significantly different between the two time points. OTUs elevated at baseline are shown in red; OTUs that were elevated following maltodextrin administration are shown in green. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that several OTUs in phyla Cyanobacteria, Verrucomicrobia, and Tenericutes were significantly elevated following maltodextrin administration.

General Conclusions

The aim of this experiment was to characterize the effects of mercury and probiotics on the microbiome of the prairie vole (*Microtus ochrogaster*). We examined alpha diversity (a measure of how many microorganisms are present in the gut microbial community) and beta diversity (the organization of the overall community structure).

During the initial mercury exposure period, 4 voles exhibited distress and were humanely euthanized. A comparison of their gut microbiome structure to the microbiome structure of non-distressed animals revealed a significant difference in the beta diversity of the two groups. 13 OTUs were found to contribute to this beta diversity difference, including 10 OTUs that were elevated in the microbiomes of distressed voles. The significant differences between these microbiomes highlight the role of enteric microorganisms in modulating the toxicity of mercury.

For all animals that did tolerate the mercury exposure course, profound differences in the microbial community were noted. Alpha diversity, a measure of the number of species present in the population, was significantly increased following mercury exposure. Beta diversity, a measurement of the diversity within the microbial populations was also altered by mercury exposure. 16 specific OTUs were significantly altered by mercury exposure, including several in phylum Bacteroidetes.

Compared to the effects of mercury on the microbiome, probiotic administration had a more subtle impact on gut community structure. Only weighted UniFrac analysis of beta diversity revealed significant differences, and LEfSe analysis identified only 4 OTUs that were significantly altered. Three of these OTUs were located in the same taxonomic lineage as the probiotic lactobacilli that we administered, validating the success of our experimental treatment method.

We hypothesized that the administration of our probiotic solution after mercury exposure would return the gut microbiome to its original structure. Interestingly, when the microbiomes from Baseline and After Probiotics timepoints were compared, no significant differences in alpha and beta diversity were noted, indicating that the bacterial communities were in fact quite similar to one another. However, LEfSe analysis, which is better at finding small-scale differences between microbial communities, did identify 12 OTUs that were significantly different between the two experimental timepoints, indicating that the microbial communities were still somewhat divergent.

Maltodextrin was used as a resuspension agent for our probiotic solution, but our experimental analyses highlight the profound prebiotic effects of this molecule on the vole microbiome. When administered after mercury exposure, maltodextrin increased the abundance of 14 OTUs, ultimately contributing to a change in beta diversity in the bacterial community structure. When the Baseline microbiome was compared to the After Maltodextrin microbiome, 29 OTUs were significantly different. Again, differences in beta diversity were also noted.

Taken as a whole, our experimental results support the hypothesis that mercury and our probiotic solution can alter the composition of the gut microbiome. Interestingly, the resuspension agent for our probiotic solution was also very effective at altering the composition of the microbiome, highlighting its potential importance in future discussions of microbial modulation of behavior through the microbiome-gut-brain axis.

CHAPTER VI

CORRELATION OF MICROBIOME CHANGES WITH BEHAVIORS IN THE PRAIRIE VOLE (*MICROTUS OCHROGASTER*)

Introduction

The digestive tract is home to a variety of microorganisms, including trillions of bacterial cells. Imbalances in the composition of this gut community have been implicated in a variety of neurological disorders including anxiety, depression, and autism spectrum disorders.[35, 40] Recently, the use of gut microbiota-targeted interventions have grown in popularity for treatments of these conditions.[8, 9, 33]

The ability of the enteric bacteria to influence the functioning of the nervous system is facilitated through a pathway known as the microbiome-gut-brain axis.[42, 72] By changes in the production of neuroactive compounds or stimulation of the vagus nerve, these microorganisms can profoundly alter host mood and behavior.[1]

The aim of this study was to identify potential microorganisms in the gut of the prairie vole (*Microtus ochrogaster*) that may contribute to differences in vole behaviors. We expected to find microorganisms with abundances that could be correlated with low and high anxiety levels, low and high locomotor activity levels, and low and high sociability levels.

The correlation of microbiome structure and each of these behavioral parameters are discussed individually in the sections that follow.

Anxiety and the Microbiome

Anxiety-like behaviors in the prairie vole was quantified using two behavioral assays: the Elevated Plus Maze and the Open Field Test. To correlate these anxiety behaviors with microbiome compositions, data collected from each assay were analyzed separately.

The Elevated Plus Maze Analysis

The Elevated Plus Maze is a "+" shaped apparatus with closed arms and open arms. The amount of time spent in the closed arms of this maze is considered an indicator of anxiety level. Per the table in Chapter III, the raw values for time spent in the closed arms for each experimental animal at each experimental timepoint were translated into an anxiety gradient value from 1 (lowest anxiety) to 4 (highest anxiety). The gradient value from both animals in each cage was then added together and this value was used to sort cages into two groups: "Low" or "High".

Alpha and beta diversity of the two groups were compared. No significant differences in either alpha or beta diversity were seen between any of the experimental groups.

Bar charts illustrating the relative abundances of all microbes in each of the anxiety groups are seen in Figure 26.

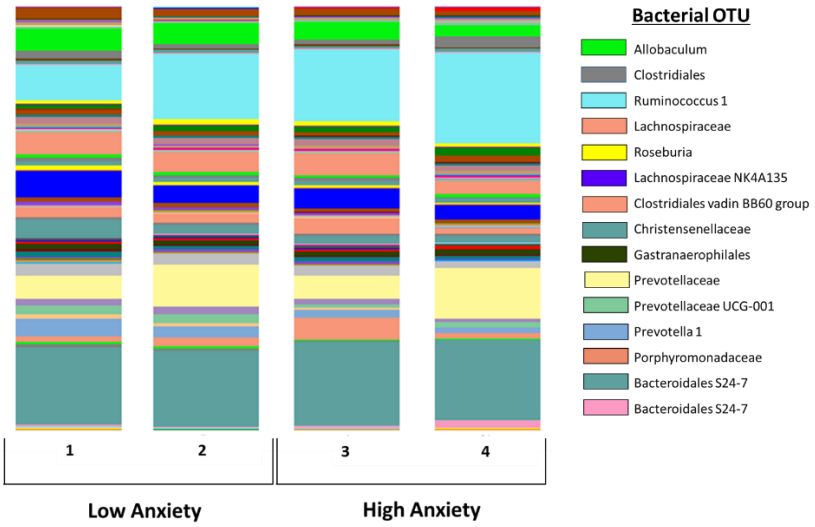

Figure 26 – General Microbiome Composition and Anxiety in the EPM. The anxiety of prairie voles was quantified using the Elevated Plus Maze (EPM) assessment. Time in the closed arm (0% - 100%) was used to sort animals into 4 groups of varying anxiety. Bar charts were assembled with the microbiome sequencing data from voles with each level of anxiety. Each color represents a different operational taxonomic unit (OTU) present in the microbiome, and several OTUs are identified above.

To determine which OTUs were significantly different across the anxiety levels, the groups were further collapsed into two levels: "Low Anxiety" (including microbiomes with behavioral scores of 1 or 2) and "High Anxiety" (including microbiomes with behavioral scores of 3 or 4). LEfSe analysis was employed to analyze the microbiomes in these two anxiety levels. Figure 27 summarizes these results.

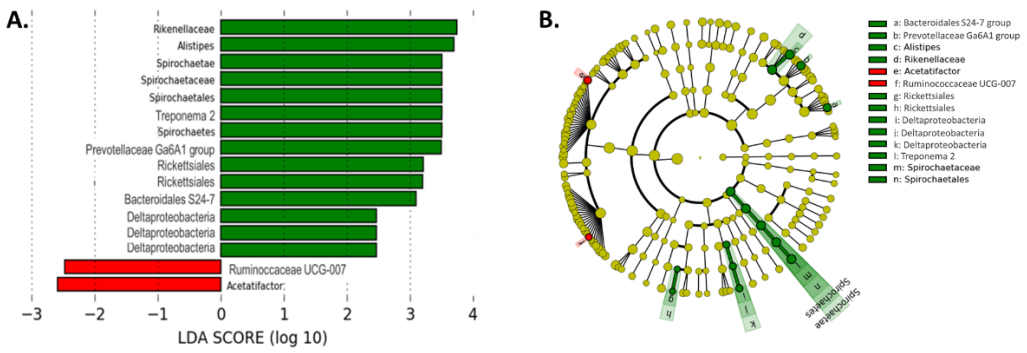

Figure 27 – LEfSe Analysis – Anxiety in the EPM. The gut microbial compositions of voles that exhibited high and low levels of anxiety in the Elevated Plus Maze (EPM) were compared. **A.** LEfSe analysis revealed 16 operational taxonomic units (OTUs) that were significantly different between the experimental groups. OTUs elevated in animals with high anxiety are shown in red; OTUs that were elevated in animals with low anxiety are shown in green. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that several OTUs in phylum Spirochaetes, class Deltaproteobacteria, and order Rickettsiales were significantly elevated in voles with low anxiety.

As an extension of the LEfSe analysis, an in-depth analysis of several significantly altered OTUs was performed (including score gradients for each group). Figure 28 summarizes these results.

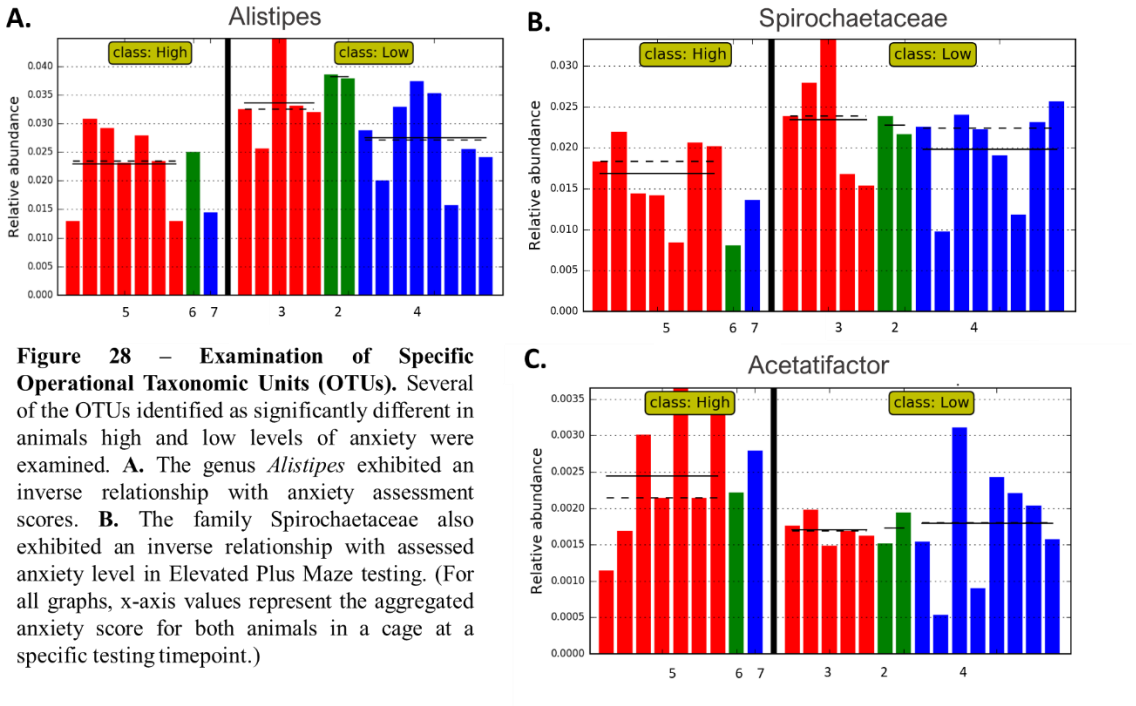

Figure 28 – Examination of Specific Operational Taxonomic Units (OTUs). Several of the OTUs identified as significantly different in animals high and low levels of anxiety were examined. **A.** The genus *Alistipes* exhibited an inverse relationship with anxiety assessment scores. **B.** The family Spirochaetaceae also exhibited an inverse relationship with assessed anxiety level in Elevated Plus Maze testing. (For all graphs, x-axis values represent the aggregated anxiety score for both animals in a cage at a specific testing timepoint.)

The Open Field Test

The Open Field Test is conducted in a square chamber with clear plexiglass walls. A test animal is placed in the center of the field and allowed to explore it for 10 minutes.

The amount of time spent in the center of the field is quantified as an indicator of anxiety level. Per the table in Chapter III, the raw values for time spent in the field's center for each experimental animal at each experimental timepoint were translated into an anxiety gradient value from 1 (lowest anxiety) to 4 (highest anxiety). The gradient value from both animals in each cage was then added together and this value was used to sort cages into two groups: "Low" or "High".

No significant differences in alpha diversity were seen between the two groups. There was a trend toward increased alpha diversity in animals with “High” anxiety (“3”) in comparison to animals with “Low” anxiety levels (“1” or “2”). Comparisons in number of observed OTUs (454 ± 8.9 vs. 420.3 ± 20.5) and the Chao1 alpha diversity index highlight differences across these groups, but these trends failed to be statistically significant.

Figure 29 Illustrates the trends observed with alpha diversity.

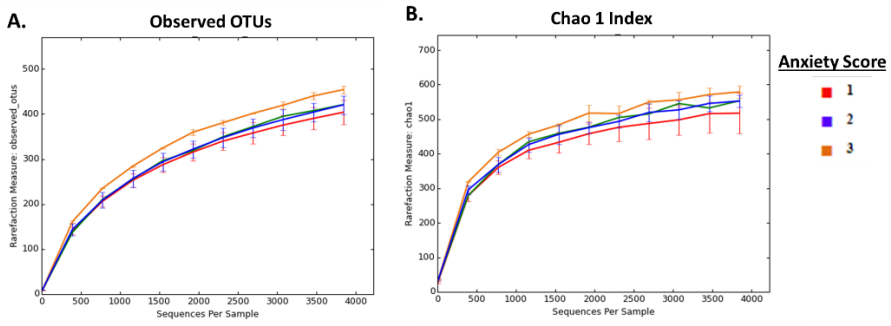

Figure 29 – Alpha Diversity and Anxiety – The Open Field Test. The alpha diversity of the gut microbial composition of voles with varying levels of anxiety was compared. **A.** The number of observed operational taxonomic units (OTUs) was the greatest in animals with higher anxiety scores (shown in orange) as compared to animals with lower anxiety score (shown in red). **B.** The Chao1 Index of alpha diversity also revealed the highest diversity in anxious animals and the lowest diversity in non-anxious animals.

Bar charts illustrating the relative abundances of all microbes in each anxiety group are seen in Figure 30.

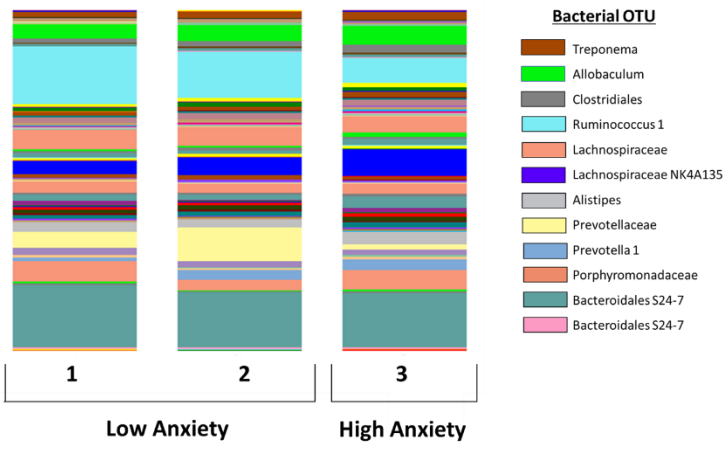

Figure 30 – General Microbiome Composition and Anxiety in the OFT. The anxiety of prairie voles was quantified using the Open Field Test (OFT). Time spent in the center of the field was used to sort animals into 3 groups of varying anxiety. Bar charts were assembled with the microbiome sequencing data from voles with each level of anxiety. Each color represents a different operational taxonomic unit (OTU) present in the microbiome, and several OTUs are identified above.

To determine which OTUs were significantly different across the anxiety levels, the behavioral groups were collapsed into two new levels: “Low Anxiety” (including microbiomes with behavioral scores of 1 or 2) and “High Anxiety” (including microbiomes with behavioral scores of 3). LEfSe analysis was employed

to analyze the microbiomes of these two anxiety levels. Figure 31 summarizes these results.

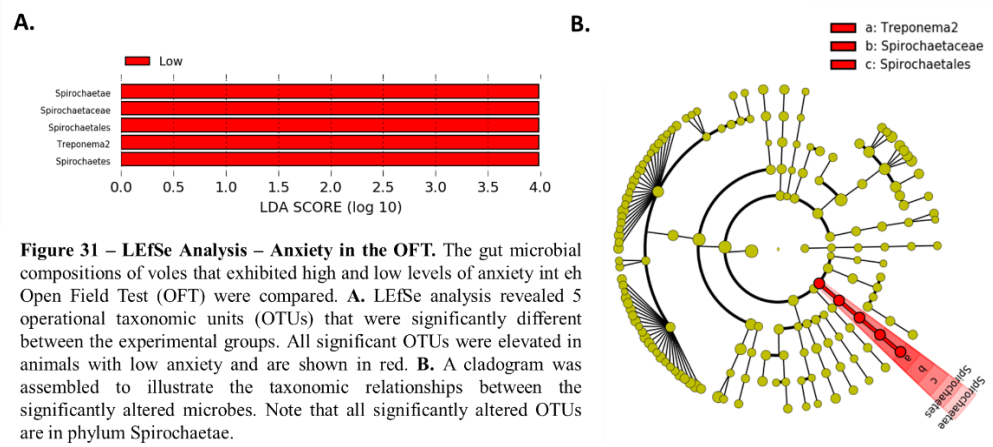

Figure 31 – LEfSe Analysis – Anxiety in the OFT. The gut microbial compositions of voles that exhibited high and low levels of anxiety int eh Open Field Test (OFT) were compared. **A.** LEfSe analysis revealed 5 operational taxonomic units (OTUs) that were significantly different between the experimental groups. All significant OTUs were elevated in animals with low anxiety and are shown in red. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that all significantly altered OTUs are in phylum Spirochaetae.

Locomotor Activity and the Microbiome

In addition to assessing anxiety in a test animal, the Open Field Test can also be used to measure the locomotor activity of a test animal. Per the table in Chapter III, the raw values for the distance traveled in the field by each experimental animal at each experimental timepoint were translated into a locomotor activity gradient value from 1 (lowest locomotion) to 5 (highest locomotion). The gradient value from both animals in each cage was then added together and this value was used to sort cages into two groups: “Low” or “High”.

No significant differences in alpha diversity were observed between the two groups. Unweighted beta diversity analysis revealed that the differences in beta diversity within activity

groups were significantly lower than the differences in beta diversity between the activity groups ($t = -3.96$, $p < 0.02$).

Bar charts illustrating the relative abundances of all microbes in each locomotor activity group are seen in Figure 32.

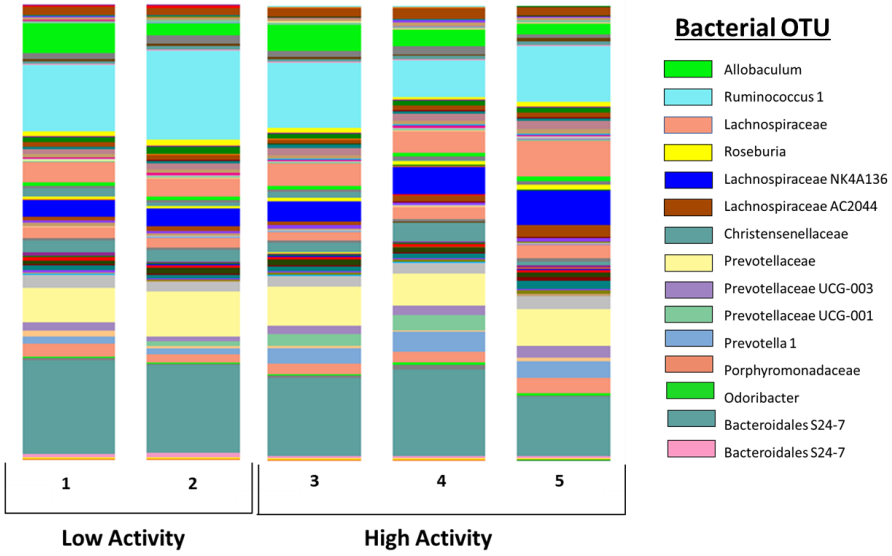

Figure 32 – General Microbiome Composition and Locomotor Activity. The locomotor activity of prairie voles was determined using the Open Field Test. The total distance traveled in the field was quantified and then ranked on a scale of 1 (smallest distance) to 5 (largest distance). Bar charts were assembled with the microbiome sequencing data from voles with each level of exploration. Each color represents a different operational taxonomic unit (OTU) present in the microbiome. Several OTUs are identified above.

To determine which OTUs were significantly different across the locomotor activity levels, the groups were collapsed into two levels: "Low Activity" (including microbiomes with behavioral scores of 1 or 2) and "High Activity" (including microbiomes with behavioral scores of 3, 4, or 5). LEfSe analysis was employed to analyze the microbiomes at each of these two activity levels. Figure 33 summarizes these results.

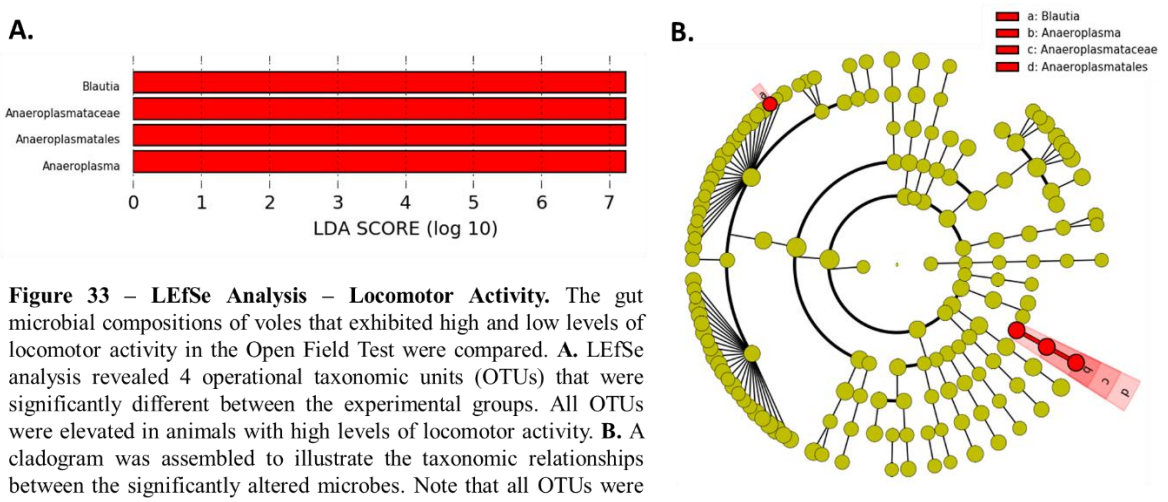

Figure 33 – LEfSe Analysis – Locomotor Activity. The gut microbial compositions of voles that exhibited high and low levels of locomotor activity in the Open Field Test were compared. **A.** LEfSe analysis revealed 4 operational taxonomic units (OTUs) that were significantly different between the experimental groups. All OTUs were elevated in animals with high levels of locomotor activity. **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that all OTUs were found in the order Anaeroplasmatales.

Social Behavior and the Microbiome

The Social Avoidance Test is used to evaluate sociability in prairie voles. In this test, a stimulus animal in tethered in one cage and the test animal is placed in an adjacent attached cage. The test animal is free to move around in either cage, but the amount of time spent in stationary contact with the stimulus animal is quantified as a measure of sociability. Per the table in Chapter III, the raw values for time spent in contact with the stimulus animal by each experimental animal were translated into a sociability gradient value from 1 (lowest sociability) to 4 (highest sociability). The gradient value from both animals in each cage was then added together and this value was used to sort cages into two groups: “Low” or “High”.

While alpha diversity analyses did not reveal any significant differences between the groups, there was a trend toward significantly lower alpha diversity for animals with the low social behavior (“2”) compared to the animals with the higher social behavior (“3” and “4”). This trend was most pronounced in the Shannon index ($t = 3.43$, $p = 0.09$) and in the number of assigned OTUs (436.9 ± 6.8 vs. 385.9 ± 9.9, $t = 7.47$, $p = 0.06$), though neither was ultimately statistically significant. No significant differences were observed in comparisons of beta diversity across the sociability groups.

Figure 34 illustrates the trends observed with alpha diversity.

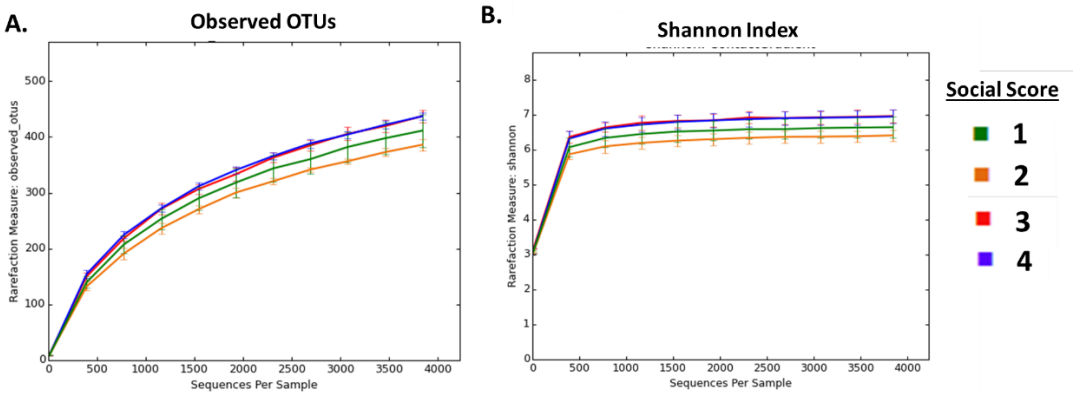

Figure 34 – Alpha Diversity and Social Behavior. The alpha diversity of the gut microbial composition of voles with varying levels of sociability was compared. **A.** The number of observed operational taxonomic units (OTUs) was the lowest in animals with lower sociability scores (shown in orange) as compared to animals with higher sociability scores (shown in red and blue). **B.** The Shannon Index of alpha diversity also revealed the highest diversity in animals with the highest levels of sociability.

Bar charts illustrating the relative abundances of all microbes in each social activity group are seen in Figure 35.

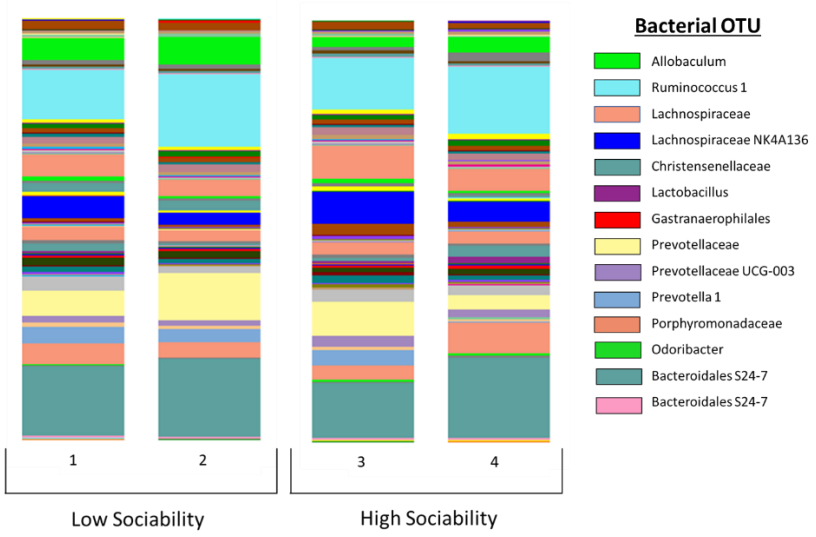

Figure 35 – General Microbiome Composition and Sociability. The sociability of prairie voles was determined using the social avoidance test. Time in contact with an unknown stimulus animal was quantified and then ranked on a scale of 1 (lowest contact) to 4 (highest contact). Bar charts were assembled with the microbiome sequencing data from voles with each level of sociability. Each color represents a different operational taxonomic unit (OTU) present in the microbiome. Several OTUs are identified above.

To determine which OTUs were significantly different across the social activity levels, the groups were further collapsed into two levels: “Low Sociability” (including microbiomes with behavioral scores of 1 or 2) and “High Sociability” (including microbiomes with behavioral scores of 3 or 4). LEfSe analysis was employed to analyze the microbiomes of these two activity levels. Figure 36 summarizes these results.

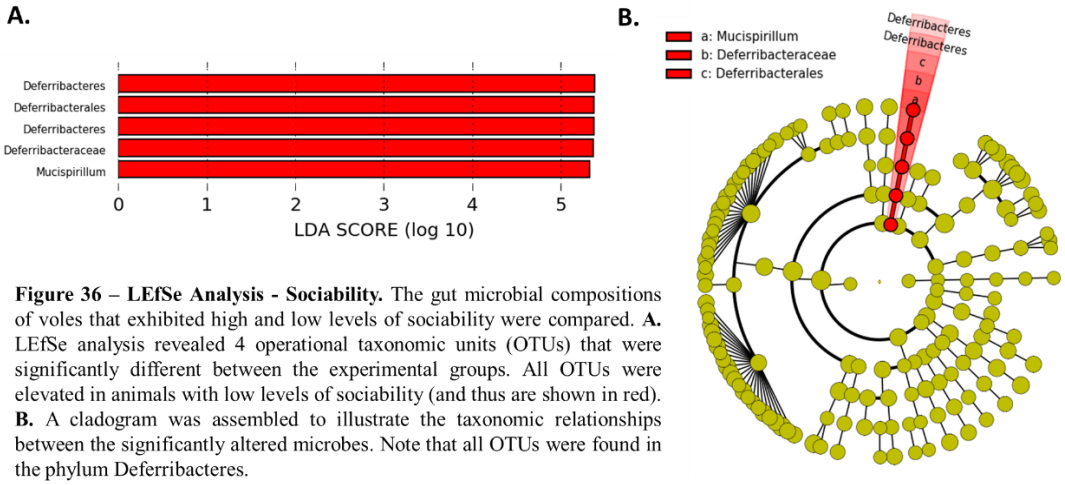

Figure 36 – LEfSe Analysis - Sociability. The gut microbial compositions of voles that exhibited high and low levels of sociability were compared. **A.** LEfSe analysis revealed 4 operational taxonomic units (OTUs) that were significantly different between the experimental groups. All OTUs were elevated in animals with low levels of sociability (and thus are shown in red). **B.** A cladogram was assembled to illustrate the taxonomic relationships between the significantly altered microbes. Note that all OTUs were found in the phylum Deferribacteres.

General Conclusions

The aim of the present study was to identify specific microorganisms that were associated with changes in anxiety, locomotor activity, or social behavior in the prairie vole (*Microtus ochrogaster*).

To evaluate vole anxiety, the Elevated Plus Maze and Open Field Test assays were employed. No significant shifts in alpha or beta diversity were correlated with anxiety behaviors in either assay, though many operational taxonomic units (OTUs) were identified as significantly different in animals with low and high anxiety. Most notably, both assays highlighted an abundance of organisms in the phylum Spirochaetae in animals with low levels of anxiety.

In addition to anxiety behavior assessments, the Open Field Test was also used to evaluate locomotor activity of the vole. Again, no significant differences in alpha diversity were

noted, but unweighted UniFrac analysis revealed significant differences in beta diversity in the microbiomes of voles exhibiting different levels of locomotor activity.

The Social Avoidance Test was used to evaluate sociability in the vole. There was a strong trend toward increased alpha diversity in the gut of animals with higher levels of sociability, but this trend failed to be statistically significant. LEfSe analysis identified changes in 5 OTUs, all in the phylum Deferrebacteres, that were significantly elevated in animals with decreased sociability.

Taken together, these results highlight a profound correlation between the bacteria of the prairie vole gut and a variety of behaviors.

CHAPTER VII

DISCUSSION

The overarching aim of this study was to better understand the involvement of the microbiome-gut-brain axis in the anxiety and social behaviors of the prairie vole (*Microtus ochrogaster*). We explored this question in three major experiments. First, we examined the effects of several ingested compounds on the behavior of the vole. Second, we examined how these ingested compounds altered the composition of the gut microbial community. Third, we correlated changes in anxiety and social behaviors with the composition of the gut microbial community.

A discussion of the results of each of these experiments is given below.

Experiment 1 – The Effects of Mercury and Probiotics on Behavior in the Prairie Vole

The prairie vole (*Microtus ochrogaster*) is a laboratory animal originally isolated from the plains of the Midwest United States. Unlike most laboratory rodents, the prairie vole is highly social; when placed in a cage with an unknown stimulus animal, prairie voles will normally huddle next to this novel animal and remain in stationary contact for extended periods of time. The sociability of voles undoubtedly contributes to their formation of life-long pair bonds, a

social construct critical to their overall well-being. Indeed, when pair-bonded voles are separated from their partner, their anxiety levels significantly increase.[73, 74]

The sociability of prairie voles appears to be vulnerable to the influence of various ingested compounds, including toxic metals such as cadmium and mercury. Previous work has shown that 4 weeks of exposure to 60 ppm mercuric chloride induces a state of social avoidance similar to that seen in patients with autism.[10] While the exact mechanism of this disruption was not previously clarified, we hypothesized that it was due to an increase in anxiety in the vole. Other toxic metals (including cadmium[75] and lead[76]) have been shown to induce anxiety, panic disorders, and depression in rats and mice, so we hypothesized that a similar effect was being seen in voles exposed to mercury.

To evaluate the potential connection between decreased sociability and increased anxiety, we employed the Elevated Plus Maze and the Open Field Test assays. In the Elevated Plus Maze (EPM), the amount of time spent in the closed arm of the maze is often used as an indicator of overall anxiety level.[67] Voles that spend more time in the shelter of the closed arm are considered more anxious; voles that spend more time in the exposure of the open arm are considered less anxious.

The Open Field (OF) Test can also be used to evaluate anxiety in a test animal.[68] The field has two zones: the outer zone (which is close to the walls of the field) and the center (which is found in the middle of the field space). Voles that spend more time in the outer zone of the field are considered more anxious; voles that spend more time in the center of the field are considered less anxious. In addition to the amount of time spent in either zone of the field, the number of entries into the center of the field can also be used to estimate the level of anxiety in the test animal. The more often an animal enters this region of the field, the less anxious they are estimated to be.

We examined vole behavior in the Elevated Plus Maze and Open Field Test before and after exposure to 60 ppm mercuric chloride. Following mercury exposure, voles spent significantly more time in the closed arm of the EPM and significantly less time in the center of the OF. Both findings suggest an increase in anxiety in these animals, supporting our experimental hypothesis.

Given the likely role of anxiety in the decline in sociability of voles with mercury exposure, we hypothesized that the administration of an anxiolytic substance could not only reduce anxiety levels, but also restore social behavioral deficits. Many studies have highlighted the anxiety-reducing properties of bacteria such as *Lactobacillus* and *Bifidobacterium*,[1, 8, 9, 41] so we generated a probiotic solution with three potentially probiotic strains of *Lactobacillus*. Several characteristics are common among probiotic microorganisms including stomach acid tolerance, bile acid resistance, the inhibition of known gut pathogens, and adhesion to intestinal epithelial cells.[31] Of the many lactobacilli our lab previously isolated from the cecum of the prairie vole, [69] we selected three (PV017, PV018, and PV019) that exhibited all of these characteristics and showed strong potential for probiotic activity.

To determine the extent of any anxiolytic properties of our probiotic solution, we administered it to mercury-treated voles for a period of two weeks, at which point we again assessed their anxiety levels with the EPM and OF test. We compared the behavior of the voles after probiotic administration to their behavior both after mercury exposure and prior to any experimental manipulations.

Unexpectedly, no significant changes in anxiety behaviors were observed in the EPM or OF test following the probiotic administration course. In the EPM, the average amount of time spent in the closed arm of the maze was roughly same after probiotic administration as it was after the mercury exposure course, and in the OF, probiotic-treated voles actually spent less time

in the center of the field after probiotic administration than they did following mercury exposure. While these trends failed to reach a level of statistical significance, they seemed to suggest that our probiotic solution was not only an ineffective anxiolytic, but also possibly an anxiogenic compound. This characteristic warrants further review.

The changes in anxiety behavior after probiotic administration did not reach a level of statistical significance, but interestingly, the changes in observed locomotor activity did. Following probiotic administration, voles traveled significantly further in the OF. In fact, the average distance traveled after probiotic administration was actually higher than the average distance traveled prior to any experimental manipulations. Literature discussing the meaning of increased locomotor activity in the OF offers two possible explanations for this phenomenon. First, the increased movement of the vole in the testing field could be an indicator of increased exploratory behaviors of the vole. Animals that are highly anxious would not so actively explore their environment, so this experimental result could be interpreted as an indicator of decreased anxiety.[77] Second, this increased movement of the vole in the testing field could be an indicator of "increased excitability", or more bluntly, hyperactivity induced by stress. Proponents of this interpretation suggest that excessive movement in the OF may be an indication of the test animal searching (unfruitfully) for an area of shelter in the field.[78] Given that the voles in our current study not only exhibited increased locomotor activity, but also decreased time in the center of the field, the latter explanation seems more logical.

Anxiety aside, one other factor that may have contributed to the increase in locomotor activity of probiotic-treated voles in the OF is the 0.15% maltodextrin solution used to resuspend the probiotics in solution. While 0.15% is an exceedingly low concentration of this simple sugar, we did observe a significant increase in distance traveled in the OF in animals that received this 0.15% maltodextrin solution after mercury exposure, implying that the changes in locomotor activity observed following probiotic administration may have less to do with the effects of our

probiotic microorganisms themselves as with the effects of the solution used to resuspend them for administration.

It is important to note that aside from locomotor activity, no significant differences in vole anxiety behaviors were identified in the EPM or OF Test following maltodextrin administration. There was a trend toward increased time spent in the closed arm of the EPM and increased time spent in the center of the OF, but neither reached a level of statistical significance. Despite this lack of significance, it is interesting to note that the amount of time maltodextrin-treated voles spent in the center of the OF was almost double the amount of time probiotic-treated voles spent in this region. Again, the comparison between these two points was not statistically significant, but this dramatic difference supports the assertion that the 0.15% maltodextrin in our probiotic solution may play an important role in addressing mercury-induced anxiety in the prairie vole.

To determine whether the behavioral impacts of 0.15% maltodextrin and our probiotic solution were universal or strictly limited to voles with previous mercury exposure, we gave voles four weeks of *ad libitum* access to either maltodextrin solution or probiotics. At the end of this treatment course, anxiety behaviors were evaluated using the EPM and the OF tests, and vole behaviors prior to and following experimental treatments were compared. In voles that had *ad libitum* access to 0.15% maltodextrin, a statistically-significant decrease in the amount of time spent in the open arm of the EPM was noted, but no significant changes in OF behavior were observed. In voles that had *ad libitum* access to our probiotic solution, no significant changes in EPM performance were noted, but these animals did enter the center of the OF significantly fewer times following probiotic administration. While this data may suggest that our maltodextrin and probiotics solutions may have some anxiogenic properties in voles without previous mercury exposure, the results overall are inconclusive and warrant further review.

Our evaluation of the impact of our probiotic solution (and 0.15% maltodextrin) on the anxiety of the prairie vole was based entirely on behavioral assays. While such assays can be invaluable for gaining a general assessment of the impact of an experimental intervention, they are susceptible to the influence of variables outside the scope of the experiment (including timing of the behavioral test, light level, ambient noise level, and odors in the testing room[51, 79]). Additionally, both the EPM and OF Test are subject to the so-called Repeated Testing Effect.[52, 80] Essentially, the repeated testing of an animal with either of these assays has been shown to correlate with an increase in observed anxiety behaviors (regardless of experimental intervention). In our own experiments, ANOVA analysis consistently identified a main effect of time on the experimental outcome (but did not consistently identify a main effect of treatment), indicating that the testing timepoint was a more consistent predictor of animal performance than the experimental treatment.

For more clarity in future analyses of anxiety, it may be beneficial to employ an alternate means of anxiety assessment such as the quantification of corticosterone levels. Known as the stress hormone, corticosterone increases with increasing levels of host stress. While corticosterone levels can be determined in both plasma and fecal samples, the repeated collection of fecal pellets (as opposed to blood samples) during a treatment course is not only logistically simpler, but it is also less likely to cause distress in the experimental animals.[81] In future analyses of the impact of mercury, maltodextrin, and probiotics on stress in the prairie vole, corticosterone analysis should be strongly considered.

Because the results of our analyses of the effects of maltodextrin and probiotics on anxiety were inconclusive, it was difficult to make an informed hypothesis of how these interventions might impact the social behavior of mercury-exposed voles. Previous work at our institution showed that voles normally spend an average of about 100 minutes in stationary

contact with an unknown stimulus animal in the Social Avoidance test; this contact time drops to about 50 minutes following extended exposure to 60 ppm mercuric chloride.[10]

We administered 0.15% maltodextrin and our probiotic solution to voles for two weeks following their four-week mercury exposure period, then assessed their sociability using the Social Avoidance Test. Voles that received our probiotic solution after mercury exposure spent an average of 48.3 minutes in contact with an unknown stimulus animal; voles that received maltodextrin solution spent an average of 111.3 minutes in contact with the unknown stimulus. The stark difference in contact times between the two groups was highly statistically significant and highlights a distinct difference in effectiveness of the maltodextrin and probiotic solutions.

Based on the results of the Social Avoidance Test, it appears that 0.15% maltodextrin solution may actually be more effective at restoring social deficits than our probiotic solution. It is important to note that the lactobacilli selected for inclusion in the probiotic were chosen based on several characteristics known to improve host gut heath, not necessarily brain health. In future experiments, it may be beneficial to explore the effects of other potentially-probiotic strains to determine if others may be more anxiolytic.

The aim of this experiment was to characterize the effect of various ingested substances on the behavior of the prairie vole (*Microtus ochrogaster*). Our experimental findings support our hypothesis that mercury exposure increases anxiety, and they provide a plausible explanation for how low-level mercury exposure can induce selective social withdrawal in the male prairie vole.[10]

While it was difficult to make an informed hypothesis as to the effect of our probiotic solution on the social behaviors of mercury exposed voles, we expected at least some tempering of the mercury-induced declines in sociability following the administration of our probiotic. While the probiotic solution was not successful in remediating these social behavioral loses, the

0.15% maltodextrin solution used to resuspend the probiotics were quite successful, increasing social behavior to levels at or above the sociability of untreated control animals.

Overall, this experiment was successful in highlighting the behavioral impacts of ingested compounds in the prairie vole. It provides a strong foundation for further exploration of the microbiome-gut-brain axis in this rodent model.

Experiment 2 – The Effects of Mercury and Probiotics on the Microbiome in the Prairie Vole

The human digestive tract is home to a complex community of bacteria, viruses, and fungi. Given that these microorganisms are in direct contact with ingested compounds, it is unsurprising that the gut microbial community is known to shift in response to factors such as changes in diet, the administration of antibiotics, and probiotic ingestion. We sought to determine how two specific compounds (60 ppm mercuric chloride and a *Lactobacillus*-based probiotic solution) could alter the gut microbial community of the prairie vole.

Mercuric chloride is a non-organic mercury salt that is not well-absorbed by the digestive tract. By some estimates, up to 90% of organic methylmercury leaves the GI tract and enters the tissues of animals; in contrast, less than 15% of mercuric chloride ever leaves the gut environment.[82] It is widely believed that the gut microbiota are responsible for keeping inorganic mercury in the gut lumen. Indeed, studies using germ-free animals[83] (that is, animals without a gut microbial community) and antibiotic administration[84, 85] (which greatly disrupts the gut microbial community) have shown dramatically increased mercury absorbance by the host organisms.

The exact mechanism of bacterial protection of the host from mercury toxicity is not fully understood, though there is mounting evidence that several microorganisms (including many strains of *Lactobacillus*) have the ability to bind to and sequester mercury that is present in the fecal material. Indeed, one study found that some *Lactobacillus* strains could bind to up to 99% of the mercury present in their growth media.[86, 87] The mercury-binding activities of *Lactobacillus* and other gut bacteria appear to be due almost entirely to the chemistry of their outer cell wall. Indeed, in some *Lactobacillus* strains, dead cells are actually more effective at sequestering mercury than living cells.[88] Living cells do have one other means of decreasing mercury toxicity, however: the demethylation of methylmercury. Several bacterial species possess the ability to remove the methyl group from this form of organic mercury,[84] a process that dramatically decreases its toxicity.

Given the variety of mercury-detoxification mechanisms present in the gut microbial community, it is unsurprising that our own experiment found a profound role of the gut microbiota in modulating mercury toxicity in the prairie vole. In our experiment, voles were given *ad libitum* access to 60 ppm mercuric chloride solution for a period of 4 weeks. During the first week of mercury exposure, four of our experimental animals exhibited distress and were humanely terminated. An examination of the overall diversity in the gut microbial communities of distressed voles compared to non-distressed voles revealed statistically significant differences in the beta diversities of the two groups.

To determine the specific microorganisms that most contributed to these beta diversity differences across the experimental group, linear discriminant analysis effect size (LEfSe) was used. This analysis compares the abundances of each microbe in the microbiome of all samples in an experimental group and then uses Kruskal-Wallis and Wilcoxon rank sum tests to identify which differences are statistically significant.[25] 13 operational taxonomic units (OTUs) were identified as significantly different between distressed and non-distressed voles.

Ten of the OTUs that were significantly altered between the two groups were elevated in voles that did not tolerate the mercury exposure course ("distressed"). Several of these microorganisms were members of the phylum Bacteroidetes. Many Bacteroidetes have been shown to exhibit low mercury resistance, either being unable to grow in the presence of mercury or even being killed when this toxic metal is added to their growth media.[89] For animals with an overrepresentation of this phyla in their microbiome, the magnitude of the microbial changes that may occur with mercury ingested is likely to be much greater. With this logic, it is unsurprising that these particular voles were strongly unable to tolerate the mercury exposure course.

We were surprised to see the elevation of one OTU in the family Lactobacillales in distressed voles. Several strains of lactic acid bacteria have been shown to be very effective at mercury sequestration both *in vitro*[86, 87] and *in vivo*,[90] so we generally expected an overabundance of such microbes to decrease mercury toxicity. Nonetheless, the presence of this OTU in distressed voles highlights an important concept in microbiology: although bacterial samples may belong to the family or genus, they may not all exhibit the same physical and biochemical characteristics. In this case, the strain of *Lactobacillus* that was elevated in distressed voles must not have possessed the mercury detoxification abilities of other strains.

As a final note about the microbiome's modulation of mercury toxicity, it is interesting to note that the four animals that exhibited distress during the mercury treatment course were all siblings born in the same litter. Many studies have highlighted the importance of the birthing process in the establishment of the early gut microbiome with bacteria being seeded into the digestive tract during passage through the birth canal.[91] Following the initial seeding event, however, many other factors are known to influence the gut community structure including, most pertinently to this discussion, the genetics of the host. A recent twin study found greater concordance of gut microbial communities in monozygotic twins as compared to dizygotic twins.[18] While the voles that exhibited distress were unlikely to be monozygotic twins, the fact

that they all share the same genetic source material and microbiome seeding event helps to explain the unanimity of mercury intolerance in this animals.

While four voles did not tolerate the mercury exposure course, sixteen other voles did, and the impact of mercury exposure on their gut microbial communities was mapped. Comparisons of baseline microbial communities to communities after mercury exposure revealed a significantly greater level of alpha diversity in the communities following mercury exposure as compared to those communities prior to mercury exposure. Similarly, the observed number of OTUs was also significantly greater following mercury treatment. Weighted and unweighted UniFrac analysis, a measure of relative abundance of microbes in the gut community, also identified significant differences between experimental timepoints.

The dramatic impact of mercuric chloride on the gut microbial community supported our hypothesis that this ingested compound would induce detectable changes in the gut community. However, the direction of these changes surprised us. We expected to see a decrease in the alpha diversity in the gut community (with several mercury-sensitive microbes being eliminated from the microbiome). Instead, the number of identified OTUs was significantly greater. This, coupled with the differences in beta diversity, highlight the profound differences before and after mercury exposure in these gut communities.

To identify the specific OTUs that contributed to the differences in alpha and beta diversity, LEfSe analysis was employed. 13 OTUs were identified as significantly decreased following mercuric chloride exposure. Interestingly, over half of these OTUs were in the phylum Bacteroidetes. As previously described, this particular phylum is known to be susceptible to the toxic effects of mercury, so it is unsurprising that these particular microbes were much less abundant at the end of the mercury exposure time course.

In addition to microorganisms in the phylum Bacteroidetes, several OTUs in phylum Actinobacteria were also significantly decreased following mercury exposure. Family *Coriobacteriaceae*, one a member of this phylum that was significantly decreased after mercury exposure, is known to produce of the compound equol.[92] Equol is a non-steroidal form of estrogen, and its level in the bloodstream has been inversely correlated with anxiety in rats.[93] (That is to say that as equol increases, anxiety decreases.) Given that the production of equol likely decreased as organisms in family *Coriobacteriaceae* declined, it would follow that anxiety behaviors should increase. In fact, our previous experiment did find an increase in anxiety following mercury exposure. This microorganism (and the equol it synthesizes) provide an intriguing potential mechanism for the microbial involvement in the mercury-induced anxiety we observed in the Elevated Plus Maze and Open Field Test.

Given the large-scale effects of mercury on the microbiome structure, we hypothesized that the administration of a probiotic suspension may help to return that community to its original structure. We generated a probiotic solution with 3 strains of *Lactobacillus* bacteria derived from the gut of the prairie vole (PV017, PV018, and PV019). These strains were selected based on several probiotic characteristics they exhibited in previous studies.[69] Voles were given *ad libitum* access to this probiotic solution for the two weeks immediately following their mercury exposure period. Comparisons of the microbiome structures before and after probiotic administration were made.

Alpha diversity, a measurement of how many microbes are present in a gut microbial community, was not changed with probiotic administration. Beta diversity, as evaluated by weighted UniFrac analysis, was significantly different between the two experimental timepoints. LEfSe analysis identified only four OTUs that contributed to this significant difference, including three that were in the class Bacilli. Our probiotic solution was made of three strains of *Lactobacillus* bacteria, a microorganism found in class Bacilli. The detection of a significant

increase in this particular class of microorganisms validates the effectiveness of our probiotic administration method. Not only do the levels of our probiotic bacterium increase to significantly detectable levels; they also reach an abundance high enough to alter the beta diversity in the gut microbial population.

In addition to the significant changes in class Bacilli, another OTU in the family *Alcaligenaceae* was found to be elevated after mercury exposure. At least one microbe in this family (genus: *Alcaligenes*) has been shown to exhibit exceptionally high mercury tolerance.[94] In an environment with extended mercury exposure (such as the gut of a prairie vole routinely ingesting 60 ppm mercuric chloride), the trait of mercury tolerance would be a distinct selective advantage. However, as the selective pressure of mercury declines (such as during the probiotic administration period), this mercury-resistance trait would no longer be necessary for survival. Given that this OTU declined significantly following probiotic administration, it seems likely that its abundance in the gut community is largely determined by the presence or absence of mercury.

While the effects of our administered probiotic solution were somewhat limited, we also sought to characterize the effects of our probiotic resuspension fluid (0.15% maltodextrin) on the microbiome. Maltodextrin is a polysaccharide made of many glucose monomers attached to one another in branched or non-branched chains. While the human body cannot use maltodextrin as a nutrient source until it has broken it down into smaller pieces, the bacteria of the gut can more easily metabolize it.

Maltodextrin was selected for use as the resuspension agent for our probiotic solution for two reasons. First, a solution supplemented with maltodextrin was most effective at reviving our lyophilized *Lactobacillus* cells (data note shown). Second, this solution prolonged the viability of the lactobacilli in our probiotic solution once they were added to the drinking water supplies of the voles (data not shown).

The ability of maltodextrin to encourage the growth of the gut microbial population has been observed in its ability to dramatically increase the bacterial mass present in fecal samples.[71] While the gut microbial community as a whole does benefit from the presence of this prebiotic, not all community members are equally impacted by it. Some (such as *Bifidobacterium*) have been shown to significantly increase following maltodextrin supplementation; others (such as *Lactobacillus*) seem relatively unaffected by its presence.[95]

To determine the extent of maltodextrin's impact on the microbiome in voles exposed to mercury, we compared the compositions of the microbial communities before and after maltodextrin administration. No significant differences in alpha or beta diversities were noted, but LEfSe analysis did identify 14 OTUs that were elevated after maltodextrin administration. Of these 14 OTUs, several were in the classes Erysipelotrichia and Verrucomicrobia. These classes were recently correlated with the levels of carbohydrates and proteins in an individual's diet.[96] Given that maltodextrin is metabolized as a carbohydrate, it is unsurprising that microbes particularly efficient at metabolizing such macromolecules would be elevated following maltodextrin administration.

After clarifying the general effects of our probiotic solution and 0.15% maltodextrin on the microbiome altered by mercury, we next compared the final microbiomes of the animals in each treatment group to one another. No significant differences in alpha or beta diversity were seen in comparing maltodextrin- and probiotic-treated voles. LEfSe analysis identified five OTUs that were significantly different between these groups.

In maltodextrin-treated voles, two OTUs in the class Gastranerophilales and family Ruminococcaceae were elevated compared to probiotic-treated voles. Genomic analysis of these microorganisms in these microbial classifications has found an overrepresentation of carbohydrate metabolism and fermentation genes,[97, 98] indicating that the metabolic processes in

these gut environments may be significantly different from the processes in the gut environments of probiotic-treated voles.

Interestingly, in the comparison of the microbiomes of maltodextrin- and probiotic-treated voles, two different members of the family Prevotellaceae were altered, one elevated following maltodextrin administration (Prevotellaceae UCG-001) and one elevated following probiotic administration (Prevotella 1). This again highlights the differences between strains within a bacterial family or genus. While Prevotellaceae UCG-001 could most likely utilize maltodextrin as a nutrient source, Prevotella 1 may have been better suited to survive in an environment with a lower pH (established by lactic acid bacteria).

Ultimately, our goal in administering probiotics and maltodextrin solutions to the prairie voles following mercury exposure was to reverse the large-scale changes seen in the gut microbial community. To evaluate the efficacy of our probiotics and the maltodextrin solution in accomplishing that goal, we compared the baseline microbial community structures of each treatment group to the final microbial community structures at the end of the treatment course.

The final microbiomes of animals that received our probiotic *Lactobacillus* solution showed no significant differences in alpha or beta diversity when compared to their baseline microbiomes. LEfSe analysis did identify 12 OTUs that were significantly different between the groups, all of which were identified at the genus level. This is intriguing, as it tends to suggest that the community structures are in fact largely similar to one another; it is only on the most specific taxonomic scale that microorganisms differ.

The final microbiomes of animals that received 0.15% maltodextrin solution showed no significant differences in alpha and beta diversity when compared to their baseline microbiomes. However, a total of 29 OTUs were significantly altered across these timepoints, including 25 OTUs with significantly elevated abundance after maltodextrin administration. Several phyla

(including Tenericutes, Verrucomicrobia, and Cyanobacteria) were increased following maltodextrin administration, indicating that these particular phyla may be best able to metabolize maltodextrin. Bacteria in the family Bacteroidales S24-7 were also significantly elevated in the baseline microbiomes of maltodextrin-treated voles. Recall that this family (a member of phylum Bacteroidetes) is one of many microorganisms that may be particularly susceptible to the toxicity of mercury. Given that this microorganism was significantly more abundant in the baseline microbiome, it is likely that mercury exposure decreased its abundance and maltodextrin administration did not restore this loss. While many microbes do increase their growth in response to maltodextrin administration, it appears that this particular mercury-sensitive class of microorganisms does not.

Taken as a whole, all of the ingested solutions that we administered to the prairie vole resulted in major changes in the gut microbial community structure. These changes were most dramatic with the ingestion of mercuric chloride, though the subsequent administration of 0.15% maltodextrin also induced several dramatic changes in the microbiome. Interestingly, while the administration of the probiotic solution did induce some changes in the microbial community, these changes were more subtle, perhaps indicating a stabilizing effect of this microorganism on the gut community structure.

The overarching aim of this experiment was to determine how exogenous compounds such as mercuric chloride and probiotics could alter the microbiome structure in the prairie vole. While there were limited differences in the large-scale measures of alpha and beta diversity following the administration of these compounds, numerous significant differences were seen on the smaller-scale level of specific operational taxonomic units (OTUs).

While maltodextrin was used simply as a rehydrating agent for our probiotic solution, it actually dramatically altered the abundance of several microorganisms in the gut microbial

community and given its effects on the social behaviors of the vole, it seems to be a prime molecule to use to include microbiome-gut-brain axis changes in future experiments.

Experiment 3 – Correlation of Microbiome Changes with Behaviors in the Prairie Vole (*Microtus ochrogaster*)

The microbiome-gut-brain axis is a bidirectional pathway of communication between the microorganisms of the digestive tract and the host's nervous system. By this pathway, changes in the gut microbial community have been correlated with changes in anxiety,[1, 7, 33, 34, 36] depression,[99] and sociability.[2, 49] Most microbiome-gut-brain axis studies have been conducted in mice and rats, and while they are excellent models for anxiety and depression, the prairie vole (*Microtus ochrogaster*) is actually better suited for sociability research.

The prairie vole is widely used in scientific research to study several atypical social behaviors including the formation of monogamous pair bonds, the biparental rearing of offspring, and an affiliative response to unknown stimulus animals. Previous work at our institution identified a significant decrease in sociability in voles when they are exposed to 60 ppm mercuric chloride for four weeks.[10] We now know that this decrease in sociability is correlated with an increase in anxiety, and given the role of the gut microbial community in modulating anxiety, it is possible that there changes in the enteric microorganisms contribute to this phenomenon.

In the current experiment, we sought to identify specific gut bacteria that were significantly altered in animals exhibiting different levels of anxiety, locomotor activity, and sociability. To perform this kind of analysis, we had to first transform our raw data into values on a behavioral scale. Animals with lower scores on this scale were considered low performers (indicating low anxiety, low motor activity, or low sociability); animals with higher scores were considered high performers (indicating elevated anxiety or increased sociability). After

categorizing all the behavioral data and correlating it with the associated microbiome data, we then used linear discriminant analysis effect size (LEfSe) to identify any changes that were statistically significant between low- and high-performing groups.

We utilized two behavioral assessments to quantify anxiety in prairie voles: the Elevated Plus Maze (EPM) and the Open Field (OF) Test. The EPM is a "+" shaped maze with two closed arms and two open arms. The amounts of time spent in the open arms and closed arms of this maze are quantified as a measure of anxiety. In general, anxious animals spend more time in the closed arms of the EPM while non-anxious animals spend more time in the open arms of the EPM. In the OF test, voles are placed in the center of a square chamber with plexiglass walls. The amount of time they spend in the center of this field (compared to the outer zone of the field) is quantified as a measure of anxiety. In general, anxious animals spend more time in the outer zone of the field while non-anxious animals spend more time in the field's center.

Interestingly, in both the EPM and OF Test, the abundance of several operational taxonomic units (OTUs) in the phylum Spirochaetes was significantly elevated in animals exhibiting low levels of anxiety. This phylum is not common in the microbiome of urban-dwelling humans, though a recent study found particularly high levels of these microbes in the gut of individuals in several small rural communities in Africa.[100] While this increased abundance was associated with the elevated levels of fiber in the diets of individuals in these communities, there is no similar dietary correlate to explain why only some voles in our experimental population exhibited increased levels of this microorganism.

The abundance of spirochetes in the gut microbial population is functionally significant as many bacteria in this phylum are efficient synthesizers of the short chain fatty acid acetate.[101] In the digestive tract, acetate increases visceral sensitivity;[102] as this sensitivity increases, anxiety behaviors are known to increase.[102] Interestingly, the neurological effects of acetate may also

occur outside of the digestive tract. The peripheral and intracerebral administration of acetate has been shown to increase anxiety behaviors in the EPM and the dark-light box test, and in the OF Test, this administration also decreases overall locomotor activity.[103] These behaviors may be indicative of increased anxiety, which is interesting given the elevation of phylum Spirochaetae in the low-anxiety populations in the prairie vole.

While many spirochetes are considered efficient producers of acetate, this experiment did not actually quantify the level of these short chain fatty acids in the cecum or fecal material of our experimental animals. The disparity between the predicted and observed anxiety behaviors (based on phylum Spirochaetae abundance) warrants further review.

In addition to changes in several spirochete bacteria, the microbiomes of animals that exhibited low anxiety behaviors in the EPM also had an elevated abundance of OTUs in classes Deltaproteobacteria and Bacteroidales S24-7. Both of these bacterial classes are depressed in a mouse model of autism,[104] a condition marked by increased anxiety. Given that they were elevated in our low-anxiety voles, it is possible that these microorganisms could possess anxiolytic characteristics.

Interestingly, the genus *Alistipes* was also significantly elevated in non-anxious voles, a finding that contradicts several studies in rats, mice, and humans. *Alistipes* is overabundant in humans with depression[105] and in a mouse model of autism.[104] When mice are housed in grid floor cages (which are known to increase rodent anxiety), the abundance of *Alistipes* also increases.[38] Taken together, these studies appear to highlight an anxiogenic (or at least anxiety-enhanced) role of this microorganisms in the gut environment.

It is also possible to interpret the abundance of *Alistipes* bacteria outside of a microbiome-gut-brain axis framework, and this interpretation may better explain the observed microbial variability in our vole population. *Alistipes* bacteria are not known to be very resistant

to the toxic effects of heavy metals. In fact, previous studies have found that both cadmium and lead can significantly decrease the *Alistipes* abundance in the animal gut microbiome.[106]

We found an increased level of *Alistipes* bacteria in voles with lower levels of anxiety, but it is important to recognize that many of the microbiome structures correlated with increased anxiety were the result of our four-week mercury exposure course. We found a distinct anxiogenic-effect of mercury administration in the prairie vole, so it is possible that the change in this particular microorganism is more an artifact of our experimental manipulations than a naturally-occurring microbial trend associated with anxiety levels.

In the OF Test, a trend toward increased alpha diversity with increased anxiety was observed. This finding was somewhat unexpected as high alpha diversity is generally considered a biomarker of a balanced and healthy gut microbial community. In a discussion of the role of the microbiome-gut-brain axis in behavioral outcomes, it would seem to follow that greater levels of diversity would correlate with improved anxiety behaviors. However, our experimental findings contradict this, with greater diversity observed in voles exhibiting greater anxiety behaviors. As with the observed changes in *Alistipes* bacteria, it is possible that this increase can be more strongly attributed to mercury and not anxiety. Both in the present study and in a recent study of expectant mothers in Africa,[107] mercury exposure has been correlated with higher levels of alpha diversity in the gut microbiome. But given that alpha diversity is also increased in the gut of patients with major depressive disorder,[108] this finding cannot be entirely ignored.

In addition to evaluating anxiety behaviors, the OF Test was also used to observe locomotor activity in the vole. Two groups of microorganisms were significantly elevated in animals with high levels of locomotor activity: organisms in the order Anaeroplasmatales and the genus *Blautia*. One microorganism in order Anaeroplasmatales (genus *Anaeroplasma*) has been shown to decline following the stress of social defeat by an aggressive stimulus animal.[37] Given

that this microbe was elevated in voles that exhibited greater locomotor activity, it is possible that their increased locomotion could be attributed to lower levels of stress. The other microorganism increased in highly active voles was found in the genus *Blautia*. Microbes in this genus are associated with decreased gut inflammation.[109] Given the role of gut inflammation in encouraging anxiety behaviors,[110] it would seem that this microorganism may lead to decreases in anxiety as well.

The final behavioral assessment we employed in this study was the Social Avoidance Test. This test measures the sociability of prairie voles in response to an unknown stimulus animal. In a comparison of voles with high sociability and low sociability, five OTUs in family Deferribacteres were significantly elevated in voles with low levels of social behavior. Interestingly, one of these OTUs (genus *Mucispirillum*) has been associated with a mouse model of colitis,[111] a disease characterized by a dramatic increase in gut inflammation. The administration of an antibiotic to eliminate these (and other inflammatory bacteria) has been shown to decrease anxiety.[112] With non-social prairie voles exhibiting high levels of this particular bacterium, it is likely that the decline in their sociability could be mediated through a gut inflammation-induced anxiety pathway.

While the number of OTUs identified as significantly different across groups exhibiting low and high levels of anxiety, locomotion, and sociability is impressive, it is important to note that this study has by no means generated an exhaustive list of the microbes that may be critical to modulation of the microbiome-gut-brain axis in the prairie vole. To identify these OTUs, we employed linear discriminant analysis effect size (LEfSe).[104] LEfSe requires that data be pooled into two main groups to enable statistically-significant comparisons to be made. For each behavioral measure, we not only had to pool the individual behaviors of both voles in a single cage to generate a cage average behavioral score, but we also had to further groups these scores into two categories: Low Performers and High Performers. Despite the collapsing of many

smaller-scale differences in behavior, we still identified a significant number of OTUs with the potential to contribute to microbiome-gut-brain axis modulation of behaviors in the prairie. In that regard, the aim of this experiment was met.

CHAPTER VIII

CONCLUSIONS

The gut microbiota is a complex community of bacteria, fungi, and viruses that are found in the digestive tract of humans. This community is known to change in response to a variety of stimuli including dietary changes, antibiotic administration, probiotic administration, and toxic metal exposure. Changes in the gut bacteria can have profound effects on the host, not only in metabolism, but also in anxiety levels and social behaviors. The ability of the enteric microbiota to alter the functionality of the nervous system is known as the microbiome-gut-brain axis, and this bidirectional communication pathway between the Central Nervous System and the bacteria of the gut is critically important to the normal functioning of the host.

The aim of this study was to modulate the microbiome-gut-brain axis in the prairie vole (*Microtus ochrogaster*). Through the administration of mercuric chloride, a probiotic solution, and 0.15% maltodextrin, we induced changes not only in the gut microbiome, but also in vole anxiety and social behaviors. Given the many behavioral similarities between prairie voles and humans, this work enhances our understanding of the role of the microbiome-gut-brain axis in our own behavior.

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APPENDIX A.
PRIMERS FOR MICROBIOME SEQUENCING

To characterize the taxonomic profile of the intestinal microbiome, the V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the universal 16S bacterial primers F515 and R806. Many gene-specific primers were also employed. Table 2 includes primer names, region of coverage, sequence, and reference. All primers used in this study were purchased from Integrated DNA Technologies, Inc. (Redwood City, CA).

In order to distinguish between each fecal DNA sample in the experiment, unique index sequences were added to the amplicons generated from each sample. Tables 3 and 4 provide the nucleotide sequences for these indices (known as i5 and i7).

Table 2 – List of Oligonucleotides (5' to 3') Used in this Study

Primer Name	Region	Oligonucleotide sequence (5'-3')	Reference
F515	**16S rRNA gene – V4 region**	GTGCCAGCMGCCGCCGCGGTAA	Kozich et al (2013)[70]
R806	**16S rRNA gene – V4 region**	GGACTACHVGGGTWTCTAAT	Kozich et al (2013)[70]
F adapter	**Illumina adapter**	AATGATACGGCGACCACCGAGATCTACAC	www.Illumina.com
R adapter	**Illumina adapter**	CAAGCAGAAGACGGCATACGAGAT	www.Illumina.com
VX.N5	**Generic PCR Primer design**	AATGATACGGCGACCACCGAGATCTACAC<i5><pad><link>< GTGCCAGCMGCCGCGGTAA >	Kozich et al (2013)[70]
VX.N7	**Generic PCR Primer design**	CAAGCAGAAGACGGCATACGAGAT<i7><pad><link>< GGACTACHVGGGTWTCTAAT >	Kozich et al (2013)[70]
Generic read 1 primer design	**Sequence primer**	<pad><link>< GTGCCAGCMGCCGCGGTAA >VX.read1	Kozich et al (2013)[70]
Generic read 2 primer design	**Sequence primer**	<pad><link>< GGACTACHVGGGTWTCTAAT >VX.read2	Kozich et al (2013)[70]
Generic index read primer design	**Index primer**	Reverse complement of (<pad><link><16S4806>)VX.p7_index	Kozich et al (2013)[70]
V4f	**Link**	GT	Kozich et al (2013)[70]
V4r	**Link**	CC	Kozich et al (2013)[70]
forward	**Pad**	TATGGTAATT	Kozich et al (2013)[70]
reverse	**Pad**	AGTCAGTCAG	Kozich et al (2013)[70]
i5	**Index 5**	i5 sequence (see Table 3)	Kozich et al (2013)[70]
i7	Index 7	i7 sequence (see Table 4)	Kozich et al (2013)[70]
P1	KAPA qPCR primers	AAT GAT ACG GCG ACC GA	www.kapabiosystems.com
P2	KAPA qPCR primers	CAA BCA GAA GAC GGC ATA CGA	www.kapabiosystems.com

Table 3 – List of i5 Sequences (5' to 3') Used in This Study

Primer Name	Oligonucleotide sequence (5'-3')	Reference
SA501	ATCGTACG	Kozich et al (2013)[70]
SA502	ACTATCTG	Kozich et al (2013)[70]
SA503	TAGCGAGT	Kozich et al (2013)[70]
SA504	CTGCGTGT	Kozich et al (2013)[70]
SA505	TCATCGAG	Kozich et al (2013)[70]
SA506	CGTGAGTG	Kozich et al (2013)[70]
SA507	GGATATCT	Kozich et al (2013)[70]
SA508	GACACCGT	Kozich et al (2013)[70]
SB501	CTACTATA	Kozich et al (2013)[70]
SB502	CGTTACTA	Kozich et al (2013)[70]
SB503	AGAGTCAC	Kozich et al (2013)[70]
SB504	TACGAGAC	Kozich et al (2013)[70]
SB505	ACGTCTCG	Kozich et al (2013)[70]
SB506	TCGACGAG	Kozich et al (2013)[70]
SB507	GATCGTGT	Kozich et al (2013)[70]
SB508	GTCAGATA	Kozich et al (2013)[70]

Table 4 – List of i7 Sequences (5' to 3') Used in This Study

Primer Name	Oligonucleotide sequence (5'-3')	Reference
SA702	ACTATGTC	Kozich et al (2013)[70]
SA703	AGTAGCGT	Kozich et al (2013)[70]
SA704	CAGTGAGT	Kozich et al (2013)[70]
SA705	CGTACTCA	Kozich et al (2013)[70]
SA706	CTACGCAG	Kozich et al (2013)[70]
SA707	GGAGACTA	Kozich et al (2013)[70]
SA708	GTCGCTCG	Kozich et al (2013)[70]
SA709	GTCGTAGT	Kozich et al (2013)[70]
SA710	TAGCAGAC	Kozich et al (2013)[70]
SA711	TCATAGAC	Kozich et al (2013)[70]
SA712	TCGCTATA	Kozich et al (2013)[70]
SB701	AAGTCGAG	Kozich et al (2013)[70]
SB702	ATACTTCG	Kozich et al (2013)[70]
SB703	AGCTGCTA	Kozich et al (2013)[70]
SB704	CATAGAGA	Kozich et al (2013)[70]
SB705	CGTAGATC	Kozich et al (2013)[70]
SB706	CTCGTTAC	Kozich et al (2013)[70]
SB707	GCGCACGT	Kozich et al (2013)[70]
SB708	GGTACTAT	Kozich et al (2013)[70]
SB709	GTATACGC	Kozich et al (2013)[70]
SB710	TACGAGCA	Kozich et al (2013)[70]
SB711	TCAGCGTT	Kozich et al (2013)[70]
SB712	TCGCTACG	Kozich et al (2013)[70]

APPENDIX B.
QIIME WORKFLOW

To analyze the raw sequencing data generated using the Illumina MiSeq platform, the software Quantitative Insights Into Microbial Ecology (QIIME) was used. Detailed below are the command line prompts used to process and interpret this data.

Table 5 – QIIME Analysis Pipeline

Step	Software	Command / Script	Options (other than default)
FASTQ file generation from MiSeq Read 1 and 2 with adapter/index trimming	Illumina MiSeq Reporter	automatically executed	
Combine individual FASTQ files from paired reads of each sample and add QIIME labels	QIIME	multiple_join_ paired_ends.py	use mapping file with SampleIDs, InputFileName, and metadata
Combine individual sample files from previous step in a single FASTA file with sample IDs retained	QIIME	multiple_split_ libraries_fastq.py	--demultiplexing_method sampleid_by_file --include_input_dir_path --remove_filepath_in_name
Identify chimeric sequences	QIIME	identify_chimeric_seqs.py	method: usearch61; reference: SILVA128/97_otus_16S.fasta
Remove chimeric sequences	QIIME	filter_fasta.py	remove chimeras.txt sequences from fasta file

Table 5, continued – Command Line Prompts

Step	Software	Command / Script	Options (other than default)
Open reference OTU picking	QIIME	pick_open_ reference_otus.py	method: usearch61 with the following parameters: pick_otus:enable_rev_strand_match True align_seqs:template_fp .../SILVA_128_QIIME_release/core_ alignment/core_alignment_SILVA12 8.fna filter_alignment:allowed_gap_frac 0.80 filter_alignment:entropy_threshold 0.10 filter_alignment:suppress_lane_mask _filter True assign_taxonomy:assignment_method rdp assign_taxonomy:reference_seqs_fp .../SILVA_128_QIIME_release/rep_s et/rep_set_16S_only/97/97_otus_16S. fasta assign_taxonomy:id_to_taxonomy_fp .../SILVA_128_QIIME_release/taxon omy/16S_only/97/majority_taxonom y_7_levels.txt assign_taxonomy:rdp_max_memory 22000
Remove spurious OTUs from the open-reference OTU table (otu_table_mc2_w_tax_no_pynast_failure s.biom)	QIIME	filter_otus_from_ot u_table.py	--min_count_fraction 0.00005
Summarize the filtered BIOM table	QIIME	biom summarize-table.py	
Perform core alpha and beta diversity analyses	QIIME	core_diversity_ analyses.py	Parameters: -e value according to lowest read number in biom summarize-table output, alpha_diversity:metrics PD_whole_tree,chao1,observed_otus, shannon; categories for group analyses were chosen from sample mapping file columns

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