THE ROLE OF THE INTESTINAL MICROBIOTA IN MODULATING BEHAVIOR IN THE ADOLESCENT PRAIRIE VOLE

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 2018

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ACKNOWLEDGEMENTS

Completing a Ph.D. is a long and challenging task. I first would like to thank my family for the support and encouragement along the way. My loving wife, Kelsi Supeck, you have sacrificed much to help me attain a dream of mine. Kelsi has given me her loving support, encouragement, held the home together, and been there through times of frustration and celebration during this amazing adventure. She is a wonderful mother to our sons, and I owe a great deal to her for her sacrifices and selflessness. My sons, you have changed my life for the better and have made me a better person. You both have provided me with many smiles and laughs just when I needed them, whether after a long day of research or after a series of failed experiments. My family, I want to thank you for the encouragement and being there when I needed someone to speak with.

I would like to extend great thanks to my major advisor, Gerwald Koehler, Ph.D. He has provided me with tremendous guidance over the past few years. He has taught me so much during my training. I want him to know that I truly cherish the knowledge and time that he has shared with me. He is an amazing person, teacher and scientist. He exhibits professionalism in everything he does, he listens to my ideals, and gives feedback that is very helpful. He has always been excited to be a part of my graduate training. Whether it's talking over coffee or just a brief two-second conversation, these are memories that I will always remember. In addition, I would like to thank Senait Assefa, Ph.D. She was so welcoming to me when I selected to work in the laboratory.

She sacrificed her time to invest into my future. During this time period she has taught me so much information and has been there to help me along the way. Thank you for your support and suggestions, I truly appreciate everything.

I would like to thank the members of my advisory committee, Drs. T. Kent Teague, Nedra Wilson, Robert Miller, and Mark Payton for their continued support and thoughtful critiques of this project as it evolved. Thank you for always making time for me whenever I had questions.

A special thank you goes to the members of Dr. T. Kent Teague's Integrative Immunology Center at the University of Oklahoma-Tulsa who were extremely helpful in my pursuit of this degree. Thank you Ashlee Rempel, MBA and Chibing Tan, Ph.D. for assisting me with the flow cytometer, helping to refine my techniques, donating supplies, and providing outstanding advice.

Additionally, I would like to thank my fellow graduate students throughout the years. There are too many to list so this is one big thank you to you all.

In closing, a heart warmed thank you to the OSU-CHS Biomedical Sciences Graduate program for providing me with the opportunity to pursue a graduate degree.

Name: DAVID JOHN SUPECK

Date of Degree: December, 2018

Title of Study: THE ROLE OF THE INTESTINAL MICROBIOTA IN MODULATING BEHAVIOR IN THE ADOLESCENT PRAIRIE VOLE

Major Field: BIOMEDICAL SCIENCES

Abstract:

Introduction: The intestinal epithelium develops during gestation and goes through many changes shortly after birth. During this time period the intestinal epithelium matures into a selective barrier to protect the host while still allowing the passage of required nutrition. Meanwhile, at birth, human and rodent intestines are colonized with microorganisms from their mother and the environment. The intestinal tract will ultimately contain trillions of resident bacteria with the colon having the greatest number of microbes. By far the largest portion of the human microbiota colonizes the intestine and plays a crucial role in maintaining the health of their host. Alterations in the bacterial communities can create an imbalance in microbial populations, which is termed dysbiosis. The intestinal microbiota is engaged in bidirectional communication with the central nervous system (CNS) and changes in the microbiota have been correlated with mood and anxiety disorders. The highly social prairie vole, *Microtus ochrogaster*, is an emerging model organism for studying the intestinal microbiota's influence on (social) behavior via the microbiota-gut-brain-behavior axis.

Methods: In order to learn more about the microbiota-host relationship in the prairie vole, intestinal barrier functioning and maturation was characterized by measuring paracellular absorption of 4 kDa FITC-dextran molecules and tight junction gene expression profiles. Subsequently, prairie voles were treated with neomycin to induce alterations in the intestinal microbiota. Fecal DNA was extracted and the compositional changes characterized using terminal restriction-fragment polymorphism analysis (T-RFLP) and next-generation sequencing of 16S rRNA genes. The effects of microbial composition shifts induced by neomycin on behavior in prairie voles were evaluated using the elevated plus maze (EPM) and forced swim test (FST).

Results: These results indicate that intestinal barrier function maturation occurs by three weeks of age and is associated with changes in the gene expression of tight junction proteins. T-FRLP and sequencing showed that neomycin treatment resulted in decreasing intestinal microbial diversity. Of interest was the decrease in lactic acid bacteria in the neomycin group. Neomycin treated prairie voles exhibited a depressive-like behavior.

Conclusion: These results indicate that microbiota play an important role in the behavior of prairie voles and that microbiota shifts induced by neomycin are correlated with depressive-like behavior.

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ABBREVIATIONS

Ab	Antibiotic
ANS	Autonomic Nervous System
BDNF	Brain-Derived Neurotrophic Factor
cDNA	Complementary Deoxyribose Nucleic Acid
Cldn3	Claudin-3
Cldn4	Claudin-4
Cldn7	Claudin-7
CNS	Central Nervous System
DNA	Deoxyribose Nucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENS	Enteric Nervous System
FCS	Fetal Calf Serum
FD4	Fluorescein Isothiocyanate–Dextran Molecular Weight 4,000
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HBSS	Hank's Balanced Salt Solution
HPA	Hypothalamic-Pituitary-Adrenal
NEC	Necrotizing Enterocolitis
Ocln	Occludin
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
RNA	Ribose Nucleic Acid
ZO-1	Zonula Occludens 1

CHAPTER I

INTRODUCTION

For many years the influence of the intestinal microbiota on behavioral health has been neglected. Many people do not think twice about taking prebiotics, probiotics, antibiotics, or drastically changing their diet. However, all of these can shift the intestinal microbiota composition. While consuming pre- and probiotic foods can elucidate behavioral health benefits, shifting the intestinal microbiota composition using broad-spectrum antibiotics, extreme diet changes, or infection with intestinal pathogens may lead to negative behavioral health outcomes. Identifying microbiota compositions that induce a specific behavioral phenotype would allow us to take a closer look at the families, species, and strains of microbes involved, and how they are interacting with each other and the host. It is important to understand how microbiota shifts can influence behavior in order to be able to develop novel, microbiota-based strategies to prevent and treat stress-related disorders, anxiety, and depression. Each of the following chapters will provide a more detailed introduction into the work covered in that section.

Review of Literature

Junctional permeability

The mammalian intestinal tract is not mature at the time of birth in humans and rodents as evidenced by intestinal epithelia that are not fully developed (Patel et al., 2012). It becomes more complex and mature weeks to months post partum depending on the animal species. Part of the intestinal maturation process involves the decreasing permeability of the intestinal epithelial

barriers (Clarke and Hardy, 1969; Lecce and Broughton, 1973; Urao et al., 1997). The intestinal epithelial barrier remains highly permeable until it finishes development (van Elburg et al., 2003). There are advantages to having this high level of permeability while *in utero*, such as enabling the exchange of molecules between fetal serum and amniotic fluid (Harada et al., 1997). However, after birth there is a vulnerability to microorganisms, microbial products, foreign antigens, and other toxins from within the lumen during the period of immaturity of the intestinal epithelial barrier (Maheshwari, 2006; Piena-Spoel et al., 2001; van Elburg et al., 2003; Weaver et al., 1987). Premature death or life-altering consequences can result if this barrier does not form correctly or if establishment of the barrier function is delayed. Therefore, intestinal maturation during the postnatal period is crucial for survival (Maheshwari, 2006; Walker, 1979). Humans and rodents both are born with immature intestines; however, rodents appear to be born with an intestinal barrier that is more immature than it is in humans (Drozdowski et al., 2010). Even though the processes may occur at different times in development, the process involved in the maturation of the intestine is similar between the two species (Drozdowski et al., 2010).

The intestinal permeability is regulated via tight junctions as the intestine matures (Bergmann et al., 2013). In the intestine, adjacent cells form a paracellular pathway barrier in the intestinal epithelium made up of several components such as tight junctions (Figure 1) (Mitic et al., 2000). This process occurs by cells forming a specialized protein complex beneath their apical surface on the lateral side of each of the cells (Balda and Matter, 2016). Tight junctions are made up of more than 40 structural and functional proteins (Bergmann et al., 2013; Mitic et al., 2000). Together these transmembrane proteins, which are composed of the claudin family and occludins, span the cell membrane and in the extracellular space between cells (Balda and Matter, 2016; Shen et al., 2011). Therefore, tight junctions ultimately restrict entry of molecules based on size, charge, and hydrophilic properties, while still allowing the paracellular absorption of necessary micromolecules and water (Balda and Matter, 2016; Bergmann et al., 2013; Groschwitz and

Hogan, 2009; Gunzel and Yu, 2013). During the maturation process, tight junctions further occlude the space between the cells preventing entry of microorganisms and macromolecules into the sub-epithelial tissue.

Figure 1.

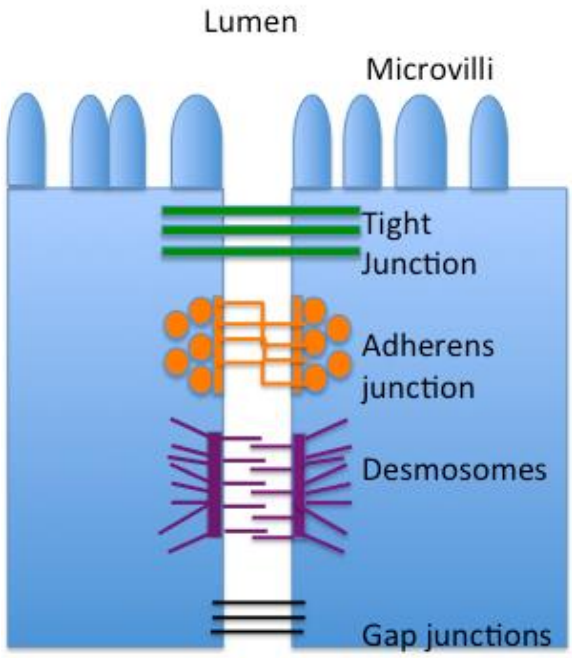


Figure 1. Cartoon of intestinal epithelial cells. This cartoon demonstrates two intestinal epithelial cells forming tight junctions, adherens junctions, desmosomes, and gap junctions. Tight junctions are involved in the regulation of intestinal permeability

The Gut Microbiota

The gut microbiota is an important component of the intestine that influences health and disease in the host. This intimate relationship begins as soon as microorganisms start to colonize the host. Some recent studies suggest that colonization occurs *in utero*, as evidenced by the prenatal presence of bacteria in amniotic fluid, placenta, and meconium of healthy neonates (Rodriguez et al., 2015). However, the vast majority of microbes that initially colonize the intestine come from the mother during vaginal birth and from the environment (Bergmann et al., 2013; Dinan et al.,

2015; Schaedler et al., 1965). Ultimately, the adult human intestinal tract will contain trillions of resident bacteria (Foster and McVey Neufeld, 2013) with the colon having the greatest number of microorganisms (Eckburg et al., 2005). Therefore, it is estimated that microorganisms outnumber the cells in our bodies by more than 2-3 times (Gill et al., 2006). Furthermore, each individual will have a specific composition of intestinal microbiota that is tailored to his or her body (Gill et al., 2006). This occurs due to many factors that influence the composition of the microbiota, such as age, antibiotic use, breastfeeding, diet, disease, genetics, geography, hospitalizations, infection, metabolism, siblings, and stress (Bennet et al., 2002; Butel et al., 2007; Cho et al., 2012; Forsythe et al., 2010; Hufeldt et al., 2010; Karlsson et al., 2012; Penders et al., 2006; Turnbaugh et al., 2009; Yatsunenko et al., 2012).

Overall, the vast numbers of microbes colonizing the intestine play a crucial role in directly influencing the host (host-microbe interactions) by inducing intestinal barrier function maturation (Patel et al., 2012), maintaining the intestinal barrier (Karczewski et al., 2010; Rakoff-Nahoum et al., 2004), and by influencing behavior, including anxiety and mood depression (microbiota-gut-brain axis) (Diaz Heijtz, 2016; Neufeld et al., 2011). Although the host and its resident microbiota share a mutualistic relationship (Backhed et al., 2005), the composition of the intestinal microbiota is not fixed and can go through shifts in bacterial species. Shifts in the microbial community structure can be beneficial or harmful to the host. Substantial alterations in the microbial communities can create an imbalance in microbial populations, which is termed dysbiosis (de Vrieze, 2013; Flak et al., 2013; Gentschew and Ferguson, 2012; Lawrence et al., 2012; Thomas, 1993). Unfortunately, dysbiosis can lead to intestinal and extra-intestinal pathologies such as colon cancer, inflammatory bowel diseases (e.g. infectious enteritis and necrotizing enterocolitis) (Bergmann et al., 2013; Clayburgh et al., 2004; Groschwitz and Hogan, 2009; Patel et al., 2012; Peterson and Artis, 2014; Rauch and Lynch, 2012; Sartor, 2008; Shiou et al., 2013), food allergies, type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis (Blaser,

2011; de Vrieze, 2013; Flak et al., 2013; Gentschew and Ferguson, 2012), and obesity in humans (Riley et al., 2013) and mouse models (Ridaura et al., 2013). Furthermore, many studies now are indicating that the intestinal microbiota are important to the function of the central nervous system (CNS) (Clarke et al., 2013; Diaz Heijtz, 2016; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004) and have been shown to influence the risk of anxiety and depressive-like behaviors (Foster and McVey Neufeld, 2013). Recent data suggest that the microbiota can influence CNS development that is related to behavior during a critical window in adolescence (Foster and McVey Neufeld, 2013). Other studies indicate that the microbiota composition along with other environmental factors likely impact DNA methylation and epigenetic alterations that are important to organogenesis, for example, the brain and the intestine (Mischke and Plosch, 2013; Weaver, 2007). Therefore, it is not surprising that intestinal microbiota composition has been directly correlated with health outcomes (Claesson et al., 2012).

In recent years it has become even more apparent that changes in the intestinal microbiota composition may play a major role in host behavior (microbiota-gut-brain axis), and current evidence has implicated that a dysfunction in this axis can be pathophysiological (Mayer, 2011). Research shows that the intestine and brain can communicate in a bidirectional manner utilizing the immune system, autonomic nervous system (ANS), enteric nervous system (ENS), and the hypothalamic-pituitary-adrenal (HPA) axis (de Jonge, 2013; Foster and McVey Neufeld, 2013).

Studies have demonstrated that the brain can be influenced by the presence or absence of specific components of the intestinal microbiota. A recent study showed that germ-free mice, which are animals that are not colonized with microorganisms, exhibited depressive-like behavior (Zheng et al., 2016), while another germ-free study in mice showed reduced anxiety-like behavior compared to conventional mice with normal microbiota (Diaz Heijtz et al., 2011). It was also reported that the behavioral phenotype could be normalized by the reconstitution of germ-free mice with mouse strain-matched microbiota early in life (Clarke et al., 2013; Diaz Heijtz et al.,

2011). Further support that microbiota can alter behavior comes from a study showing that fecal transplantation of microbiota from a strain of mice with a specific phenotypic behavior to another strain of mice induced that same phenotypic behavior in the recipient (Bercik et al., 2011; Collins et al., 2013). Furthermore, behavioral changes mediated by shifting or altering the microbiota is supported by research in germ-free mice, which exhibited no change in behavior after they were given antibiotic treatment (Foster and McVey Neufeld, 2013). This shows that the antibiotic treatment did not alter the behavior because it did not have a direct effect on the host and because no microbiota were present to be perturbed. Other studies in mice have shown that microbiota can affect central levels of Brain-Derived Neutrophic Factor (BDNF), which has been linked to depression and anxiety (Bercik, 2011; Bercik et al., 2011). In humans, subsets of patients with neurodevelopmental disorders have presented with gastrointestinal (GI) complications (Hsiao et al., 2013) supporting the conclusion that the gut-brain axis is a bi-directional pathway.

Prairie Vole

The studies of the microbiome and behavior reported in this dissertation utilize the prairie vole as a model organism. The prairie vole, *Microtus ochrogaster*, was selected because it has been the dominant model for the study of social affiliations for more than 20 years (Aragona and Wang, 2004). Prairie voles have been extensively studied because of their highly social characteristics, which contribute to monogamous relationships and bi-parental behavior (Gray and Dewsbury, 1973). Prairie vole mothers and fathers both care for the new litter of pups until they are weaned at around three weeks of age (Figure 2). This is an important distinction from other traditional rodent models, such as mice and rats. The prairie voles are small rodents that have a short small intestine and mainly utilize their hindgut for digestion (Vorontsov, 1979). They are brown in color, have a squared snout, and short tail (Carleton, 1985).

Figure 2.

Figure 2. Picture of prairie voles with a litter of pups. This picture shows a male, a female, and a new liter of pups inside the vole housing with straw and grass.

Statement of the Problem

Alterations in the composition of the intestinal microbiota may lead to various problems, including behavioral changes. The influence of the intestinal microbiota on the host gut-brain axis is only beginning to be explored. Characterization of the microbiota-gut-brain axis is in its initial stages, and questions remain to be answered about the mechanisms involved in how intestinal microbiota might affect behavior. Some investigators have reported that changes in the intestinal microbiota can lead to anxiety-like and depressive-like behaviors. The majority of these animal

studies have been in mice and rats. However, more animal models, such as the prairie voles, are required to gather critical information needed to begin to piece together a better understanding of the gut-brain axis.

Purpose of the Study

The purpose of this study was to elucidate the role of the intestinal microbiota on behavior (microbiota-gut-brain axis). Observations have been made that the intestinal microbiota can influence the health and behavior of the host. The intestinal microbiotas are suspected to be a major factor in determining host behavioral phenotypes. This study examines whether shifting the composition of the intestinal bacteria induces behavioral change. It is a necessary step to determine the potential connection between intestinal microbiota and behavioral changes in voles. Additionally, I wanted to evaluate the prairie vole as a novel animal model to study the intestinal microbiota's influences on behavior (microbiota-gut-brain axis). The prairie vole animal model has been extensively studied because these rodents' exhibit high levels of well-characterized social behaviors, which includes monogamous relationships and bi-parental care in which both mother and father tend to the young. These characteristics contribute to the fact that the prairie vole has been the dominant model for studying social affiliations for more than 20 years (Aragona and Wang, 2004). More importantly, the prairie vole has maintained this dominance because they display social behaviors similar to humans (Carter and Getz, 1993).

Significance of the Study

The benefits of this study include gaining a deeper knowledge of the importance of the intestinal microbiota in behavior. Previous studies have shown that changes in the microbiota composition

or its elimination all-together can result in changes in anxiety-like or depressive-like behavior. This study's aim was to gain a greater understanding of how shifting the microbiota composition affects the host, but it also provided analyses of the host-microbe interactions in this novel animal model. It allows for a greater understanding of how the intestinal microbiota affects the intestinal barrier as well as behavior. The conclusions from this study can form the basis for subsequent studies on specific components of the microbiota that have a role in specific behavioral phenotypes. This information may lead to novel treatments, including fecal transplanting of certain organisms, adjusting pharmaceutical regimens, or designing microbial therapies (e.g., probiotics as psychobiotics) for patients currently suffering from behavioral changes, such as anxiety and depression.

List of Hypotheses and Specific Aims

My overall hypothesis is that alteration in the prairie voles' intestinal microbiota composition induced by neomycin leads to altered behavior in adolescent prairie voles without altering the intestinal barrier. The specific aims of this study were:

Aim#1 Identify the approximate age at which the prairie vole intestinal epithelial barrier matures. I hypothesize that the intestinal epithelial barrier will mature by three weeks of age.

Aim#2 Determine the effects of neomycin on intestinal microbiota composition and intestinal barrier permeability. I hypothesize that neomycin will affect the intestinal microbiota composition without altering the intestinal epithelial barrier.

Aim#3 Determine whether manipulations of the intestinal microbial populations contribute to altered behavior. I hypothesize that shifts in intestinal microbiota composition induced by neomycin will correlate with altered behavior in adolescent prairie voles.

CHAPTER II

POSTNATAL MATURATION OF THE INTESTINAL EPITHELIAL BARRIER IN PRAIRIE VOLES

(Supeck et al., 2018)

1. Introduction

The development of the intestinal tract is a fascinating process that starts during gestation. However, it is not complete at the time of birth in humans and rodents as evidenced by intestinal epithelia that are not fully developed (Patel et al., 2012). During gestation the intestine is a basic structure, it is not until shortly after birth that it becomes more complex and mature. Part of the intestinal maturation process involves the intestinal epithelial barriers becoming less permeable (Clarke and Hardy, 1969; Lecce and Broughton, 1973; Urao et al., 1997). Until the intestinal epithelial barrier matures, it is highly permeable (van Elburg et al., 2003). While *in utero*, this high level of permeability may be advantageous, enabling the exchange of molecules between fetal serum and amniotic fluid (Harada et al., 1997). However, after birth, the period of immaturity of the intestinal epithelial barrier creates a vulnerability to microorganisms, microbial products, foreign antigens, and other toxins from within the lumen (Maheshwari, 2006; Piena-Spoel et al., 2001; van Elburg et al., 2003; Weaver et al., 1987). If this barrier does not form correctly or if establishment of the barrier function is delayed, life-altering consequences can arise and lead to premature death. Therefore, timely completion of the functional maturation

during the postnatal period is crucial for survival (Maheshwari, 2006; Walker, 1979). Rodents appear to be born with an intestinal barrier that is more immature than it is in humans (Drozdowski et al., 2010). However, the process involved in the maturation of the intestine is similar between species, even though they may occur at different time points of development (Drozdowski et al., 2010).

The maturation of the intestine involves decreasing the intestinal permeability, which is regulated by tight junctions (Bergmann et al., 2013). Tight junctions accomplish this by forming a paracellular pathway barrier in the intestinal epithelium (Mitic et al., 2000). Formation of the tight junctions occurs when neighboring epithelial cells interact and bind together leading to a specialized protein complex beneath their apical surface on the lateral side (Balda and Matter, 2016) . This apical cell-cell adherence has been described as the cells "kissing point" (Groschwitz and Hogan, 2009). During maturation, further occluding occurs and the intercellular space is sealed off to prevent entry of microorganisms and macromolecules into the sub-epithelial tissue. However, tight junctions do not completely close off and remain sensitive to a variety of stimuli (Anderson and Van Itallie, 2008; Shen and Turner, 2006). Therefore, they act like a sieve that restricts entry of molecules based on size, charge, and hydrophilic properties, while still allowing the paracellular absorption of necessary micromolecules and water (Balda and Matter, 2016; Bergmann et al., 2013; Groschwitz and Hogan, 2009; Gunzel and Yu, 2013).

Tight junctions play a fundamental role in the integrity of the intestinal barrier (Peterson and Artis, 2014). They are made up of more than 40 structural and functional proteins (Bergmann et al., 2013; Mitic et al., 2000). Together these transmembrane proteins, which are composed of the claudin family and occludins, span the cell membrane and in the extracellular space between cells (Balda and Matter, 2016; Shen et al., 2011). Claudins play an important role specifically in controlling the permeability of ions and small molecules by constituting conductive paracellular pores (Balda and Matter, 2016; Bergmann et al., 2013; Patel et al., 2012). Similarly, occludins are

an important component of tight junctions, as evidenced by alteration of occludin expression causing tight junction disruption, ultimately resulting in an increase in intestinal permeability (Noth et al., 2011). Together claudins and occludins bind to zonula occludens proteins, a family of intracytoplasmic proteins. Zonula occludens protein 1 (ZO-1) plays an important role in the formation of the tight junctions because it is a scaffold protein, which binds F-actin to the tight junction (Fanning et al., 1998; Wittchen et al., 1999). Abnormal tight junction structure can have a detrimental effect on the intestinal barrier as seen in animals carrying a inherited mutations in tight junction proteins (Bergmann et al., 2013). Therefore, this information provides evidence of the role that tight junctions play in maintaining a functional intestinal barrier.

The prairie vole, *Microtus ochrogaster*, has been extensively studied because of its social characteristics, including monogamous relationships and biparental behavior. However, knowledge of the intestinal barrier maturation in voles is lacking. Understanding the maturation of the intestinal epithelial barrier may provide information to complement the vast behavioral knowledge to inform future studies involving the gut-brain-behavior axis. In the present study, I used the prairie vole i) to determine when the intestinal permeability decreases, ii) to identify tight junction genes that undergo changes in gene expression using isolated colon epithelial cells, and iii) to determine if changes in the intestinal permeability *in vivo* are associated with changes in tight junction gene expression. See also (Supeck et al., 2018)

2. Materials and Methods

2.1. Prairie Voles Care and Handling

Voles were bred and maintained in USDA approved animal facility at the Oklahoma State University Center for Health Sciences. Male and female voles were housed in plastic shoebox style cages (17x12x22 cm) containing ~2 cm depth of pine shaving bedding at 21° C with a 14:10 light: dark cycle, with *ad libitum* access to Purina rabbit chow supplemented with black oil-sunflower seeds and water. Tissue samples were harvested at various time points from voles ranging from 2 weeks to 8 weeks of age. Voles were euthanized by decapitation after CO_2 anesthesia. The colon was immediately removed and fixed in either Carnoy's fixative or 2.5% glutaraldehyde for histological analysis or placed in ice-cold buffer solution #1 (described below) for evaluation of mRNA expression. The Institutional Animal Care and Use Committee (IACUC) at Oklahoma State University Center for Health Sciences (Tulsa, OK) approved all animal experiments. All efforts were used to minimize discomfort and the number of animals used.

2.2. Intestinal Permeability Assays

Barrier function was evaluated by measuring paracellular permeability to fluorescently labeled 4 kDa dextran *in vivo*. Randomly selected voles were fasted for a minimum of 4 hours with access to water *ad libitum* only. After the fast they were orally gavaged on a volume to weight basis with freshly prepared 40mg/ml of fluorescent isothiocyanate-ladeled 4-kDa dextran (FD4) (Sigma Aldrich, St Louis, MO) to a final administered concentration of 0.5mg/g FD4. Blood was collected in amber microcontainer SST tubes (BD, Franklin Lakes, NJ) following decapitation after CO_2 anesthesia 4.5 hours after the gavage administration. The blood samples sat at room temperature for 20 minutes and then were centrifuged at 12,000-xg for 10 minutes. The sera were diluted 1:10 in 1X PBS (Sigma Aldrich, St. Louis, MO). For FD4 fluorescence measurements,

100 microliters of each diluted sera were added to Costar black/clear bottom 96 well plate (Greiner Bio-one, Germany) in duplicate. The serum FD4 concentration was calculated by comparing the fluorescence intensities of the samples with serial dilutions of known standards using a Synergy 2 Multi-Mode plate reader (Biotek, Winooski, VT; excitation filter: 485/20 nm, emission filter: 528/20 nm). Bottom reading and a sensitivity setting of 50 was used for all experiments. The study design is shown in Figure 3. The number of animals used in this experiment was 39.

Figure 3

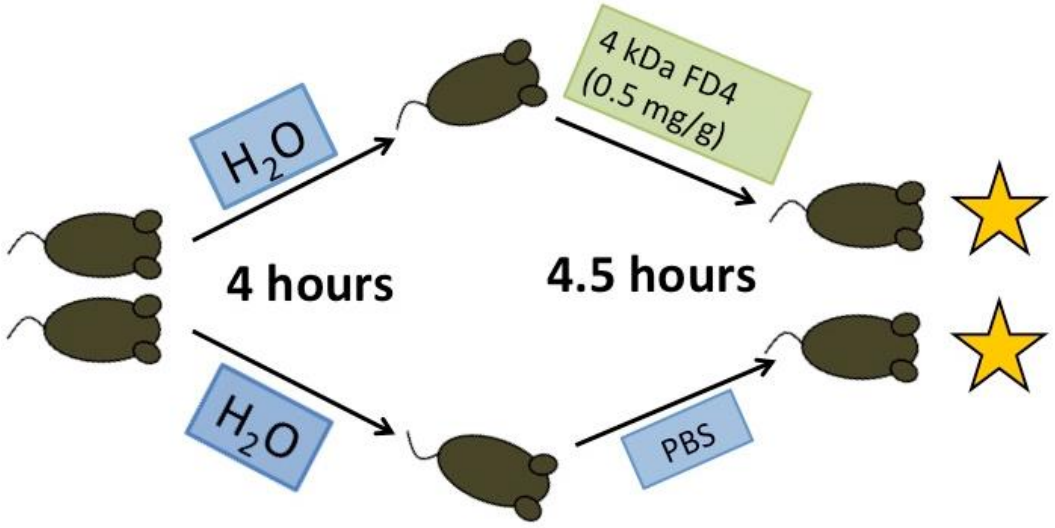


Figure 3. Prairie vole experimental design. Prairie voles were randomized into two groups: group 1 was given FITC-dextran, and group 2 was given 1X PBS and used as control. For the first 4 hours of the study, all animals were fasted and received only water *ad libitum*. After 4 hours, animals either received 1X PBS or 5mg/g (diluted in 1X PBS) of 4 kDa FITC-dextran administered via oral gavage. The animals continued on the water only restriction for 4.5 hours. Figure published in (Supeck et al., 2018). Number of animals used was 39.

2.3. Colon Epithelial Cell Isolation.

Prairie vole colon tissue samples were harvested at two and three weeks of age and immediately put into 40 mL of an ice cold buffer solution #1 consisting of Hank's Balanced Salt Solution (HBSS) (Life Technologies, Grand Island, NY), 5% FCS (Atlanta Biological, Flowery Branch, GA), and 25mM HEPES (Sigma Aldrich, St Louis, MO). The tissue was transferred into a Petri dish containing 20 mL of buffer #1 and the fat was removed from the tissue. The colon sections were then cut longitudinally to expose the lumen. The tissue samples were transferred to a 50 mL conical tube containing 15 mL of cold buffer #1 and shaken for 10 seconds to flush out the feces. The tissue was then transferred to a Petri dish and cut into 1-2 cm pieces. These pieces were transferred into a 50 mL conical tube containing 15 mL of cold buffer #1 and shaken for an additional 10 seconds to remove the mucus layer. All of the tissue pieces were collected and transferred to a 50 mL conical tube containing 25 mL of pre-warmed (37° C) buffer solution #2 consisting of HBSS, 15mM HEPES, 5mM EDTA (Fisher Scientific, Fair Lawn, NJ), 10% FCS, and 1mM DTT (Invitrogen, Carlsbad, CA). The samples were then shaken horizontally at 100 rpm for 20 minutes at 37° C. Following the incubation, the epithelial layer was removed by shaking for 10 seconds. The supernatant was collected and stored on ice. This process was repeated with 20 mL of ice-cold buffer #1. The supernatants were pooled, passed through a 40-micron nylon mesh cell filter (Fisher Scientific, USA) to remove large tissue aggregates and then centrifuged in a Thermo Scientific Sorvall Legend XTR centrifuge at 500 x g for 7 minutes. After centrifugation, the supernatants were removed and the dissociated cells were ready for use in flow cytometry and gene expression analyses. The current protocol was slightly modified from a protocol used to isolate intestinal epithelial cells from Rhesus Macaques (Pan et al., 2012).

2.4. Flow Cytometric Measurements

The prairie vole colon epithelial cells were resuspended in 1X PBS media supplemented with 2% FCS, at a concentration between 2-8×10^6 cells/ml. Cell suspensions were filtered through a 40-micron nylon mesh cell filter before sampling. Flow cytometric measurements were performed on a BD LSRII flow cytometer (BD Biosciences, USA). The intestinal epithelial cell population was identified by size using forward scatter area by side scatter area (FSC-A by SSC-A). Cell aggregates were removed from the analysis using an auto-gating population strategy. FACS data were prepared for presentation using FlowJo v. 10. GraphPad Prism was used for data analysis. Six animals were used in these experiments.

2.5. Light Microscopy

Samples were taken after the flow cytometry analysis on the remaining cells from the same tubes. The cells were stained with 0.2 μm-filtered Hyclone tryphan blue (Thermo Scientific, Logan, UT), and then a small drop of suspension was placed on a glass slide and covered with a cover slip. To visualize the cell morphology and cell viability, images were captured using a Zeiss Research Photoscope III (Carl Zeiss Microscopy, Jena, Germany). Six animals were used in these experiments.

2.6. Colon cell mRNA expression

Total RNA was extracted from dissociated epithelial cells using Quick-RNA Mini Prep Kit (Zymo Research, Irving, CA) and subsequently DNase treated with the Turbo DNA-Free Kit (Ambion, USA) following the manufacturers' protocols. RNA concentration was measured by spectroscopy (260 nm)(280 nm) using a Synergy 2 Multi-Mode plate reader (Biotek, Winooski, VT) and RNA integrity was analyzed using denaturing agarose gel-electrophoresis with a Formaldehyde Free RNA Gel Kit (Amresco, Solon, OH). All isolated RNAs had A260/A280 and A260/A230 ratios between 1.8 and 2.2. Reverse transcription was performed with 2000 ng/mL

total RNA using the High Capacity RNA to cDNA kit following manufactures protocol (Applied Biosystems, Foster City, CA). Quantitative real time PCRs were performed with the StepOne Real-Time PCR System software (version 2.2 Applied Biosystems) in duplicate for the tight junction protein gene expression survey (see Table 1 for primer sequences), with normalization to the housekeeping gene GAPDH and using the SYBR Green PCR Kit (Applied Biosystems, Foster City, CA). Relative gene expression was compared between Week 2 and 3 samples by using the comparative Ct method ($\Delta\Delta$Ct) method. Vole primer sets suitable for real-time PCR were designed using Primer3 (developed by U-Mass-Med) and prairie vole mRNA sequences available in Genbank (see Table 1). SnapGene Viewer (GSL Biotech LLC, Chicago, IL) was used for confirmation of the forward and reverse primer pair being specific for the gene of interest. All primers were purchased from Sigma Aldrich (St Louis, MO) and were used at a final concentration of 200nM (Rozen and Skaletsky, 2000).

Table 1

Oligonucleotide primers used for prairie vole tight junction protein gene expression analysis.

Published in (Supeck et al., 2018).

(Predicted) Prairie Vole Gene	Forward/Reverse Primer	Primer (5'- Sequence -3')	Amplicon Size [bp]	GenBank Accession Number
Claudin-3 (Cldn3)	Cldn3_F	CGTCTCTGCATTCATCGGTA	120	XM_005344645.1
	Cldn3_R	AGCAGCGAGTCGTACACCTT		
Claudin-4 (Cldn4)	Cldn4_F	AAGGTGTACGACTCGCTGCT	119	XM_005344646.1
	Cldn4_R	ACTTGCCTCCAACCACTGAG		
Claudin-7 (Cldn7)	Cldn7_F	CTGCAACTTCTGGGCTTTTC	117	XM_005349729.1
	Cldn7_R	CTGGGCTGTGATGATGTTGT		
Occludin (Ocln)	Ocln_F	ACGTTCGACCAATGCTCTCT	120	XM_013353536.1 , XM_005366556.1
	Ocln_R	CAATGATGAGCATGGACAG		
Tight junction protein (ZO-1) (Tjp1)	ZO1_F	GAGGAGGATCCAGCAATGAA	119	XM_005357797.1
	ZO1_R	GGCTTAAAGCTGGCAGTGTC		
GAPDH	GAPDH_F	TGACTCCCACTCCTTCCACC T	119	XM_005365241.2
	GAPDH_R	TGAGGTCCACCACTCTGTTG		

2.7. Transmission Electron Microscopy (TEM)

Three-week-old colon samples were collected and immediately fixed in a primary fixative of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30-45 minutes. The samples were then cut into 1cm pieces and secondarily fixed in 2% osmium tetroxide with 0.1M cacodylate buffer (pH 7.4) for 10 minutes. The samples were washed with 0.05 M cacodylate buffer and en bloc stained with 20% ethanol (EtOH)/uranyl acetate (UA) for 20 min at room temp. Samples were then dehydrated sequentially in an ethanol series 2 X 5-10 min washes at room temp with 50, 70, 90, and 100% EtOH. If necessary the tissues were cut into smaller pieces in 100% EtOH and then cleared with propylene oxide and embedded in PolyBed resin. Gold-silver sections were double stained with lead citrate and uranyl acetate and examined with a Hitachi H-7000 electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) operated at 75kV.

2.8. Histology and Colon Measurements

One-week-old and three-week-old vole colon tissue samples were collected and immediately fixed in Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) for 3 days and subsequently transferred to 70% ethanol. Fixed colon tissues were then sent to Histoserv, Inc. (Germantown, MD) to be processed, embedded in paraffin, sectioned, stained, and mounted. Samples were stained with hematoxylin and eosin (H&E). Tissue sections were assessed for morphology with regards to goblet cells, smooth muscle width, colonic crypt length, and colonic crypt width. Approximately 40 colonic crypts spanning the length of the distal colon were measured per animal.

2.9. Statistical analysis

Student's t-test (two groups) or one-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). p-values < 0.05 were considered to indicate

statistical significance. All quantitative data are the average of three independent experiments ± SEM.

3. Results

3.1. Time of Prairie Vole Epithelial Barrier Maturation.

To determine when the intestine matures in the vole, the serum FITC-dextran concentration was measured 4.5 hours after oral gavage administration of 0.5mg/g of 4 kDa FITC-dextran (FD4) in prairie voles between the ages of 2 and 8 weeks-old adults (Figure 4). In agreement with research showing that murine intestine maturation occurs by 3 weeks of age (Patel et al., 2012), we also found a significant decrease in intestinal permeability between the second and third week post partum. Intestinal permeability was significantly greater in the two week-old voles ($p < 0.0001$) as indicated by greater FITC-dextran serum concentration (Figure 4). These results indicate that the maturation of the intestinal epithelial barrier occurs by three weeks of postnatal life and that prairie vole's older than three weeks are suitable for studying the gut-brain-behavior axis in health and disease because they have lower permeability of macromolecules due to tight junctions (see also Figure 1).

Figure 4

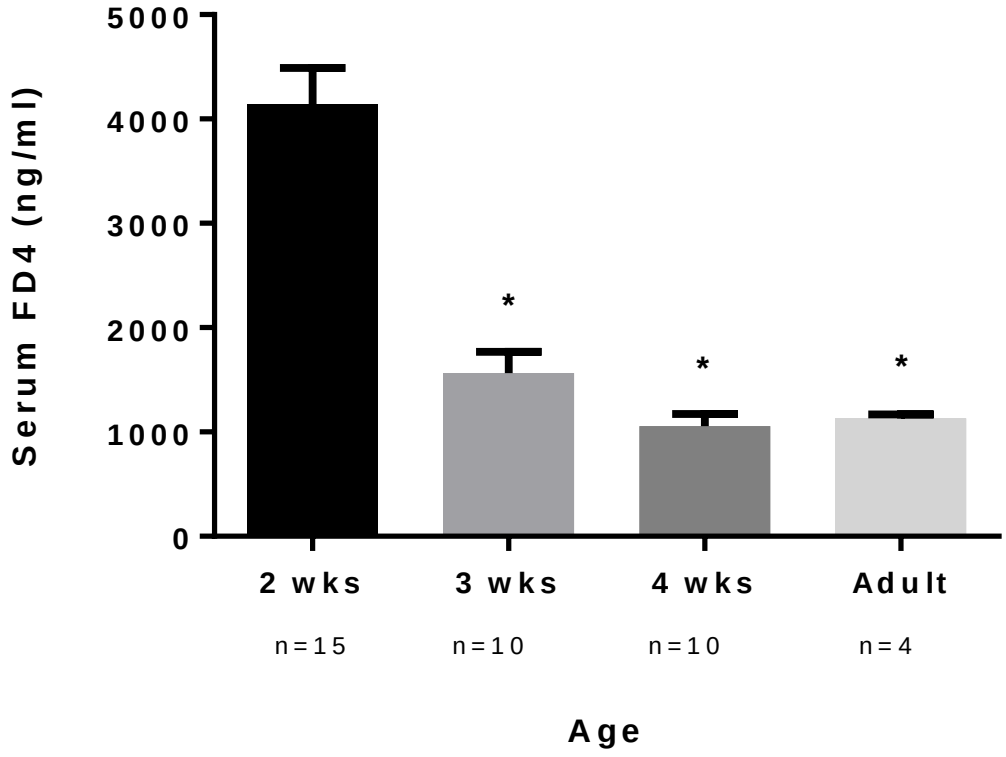


Figure 4. Prairie vole intestinal maturation of tight junctions via permeability assays. A. At the time of euthanasia, whole blood was collected, and serum 4 kDa FITC-dextran concentration was determined using a Synergy 2 Multi-mode plate reader. Data are expressed as mean ± SEM from at least three experimental repeats per condition. *$p < 0.0001$ statistically different from values from 2 week-old praririe voles. Figure modified from published (Supeck et al., 2018).

3.2. Isolation of Colon Epithelial Cells.

Prairie vole epithelial colonocytes were isolated using a multi-step process (Figure 5). Light microscopy was used to visualize the cells and confirm the isolation of epithelial cells. Epithelial cells in the suspension were stained with trypan blue and identified by their morphology using light microscopy (Figure 6A). To verify reproducibility of the procedure I performed flow cytometry. The experiments were repeated on separate occasions and the cells were run unstained

and separated by size using FSC-A by SSC-A. Epithelial cell populations consistently were observed (auto-gated cells) and the isolation was consistently replicated (Figure 6B).

Figure 5

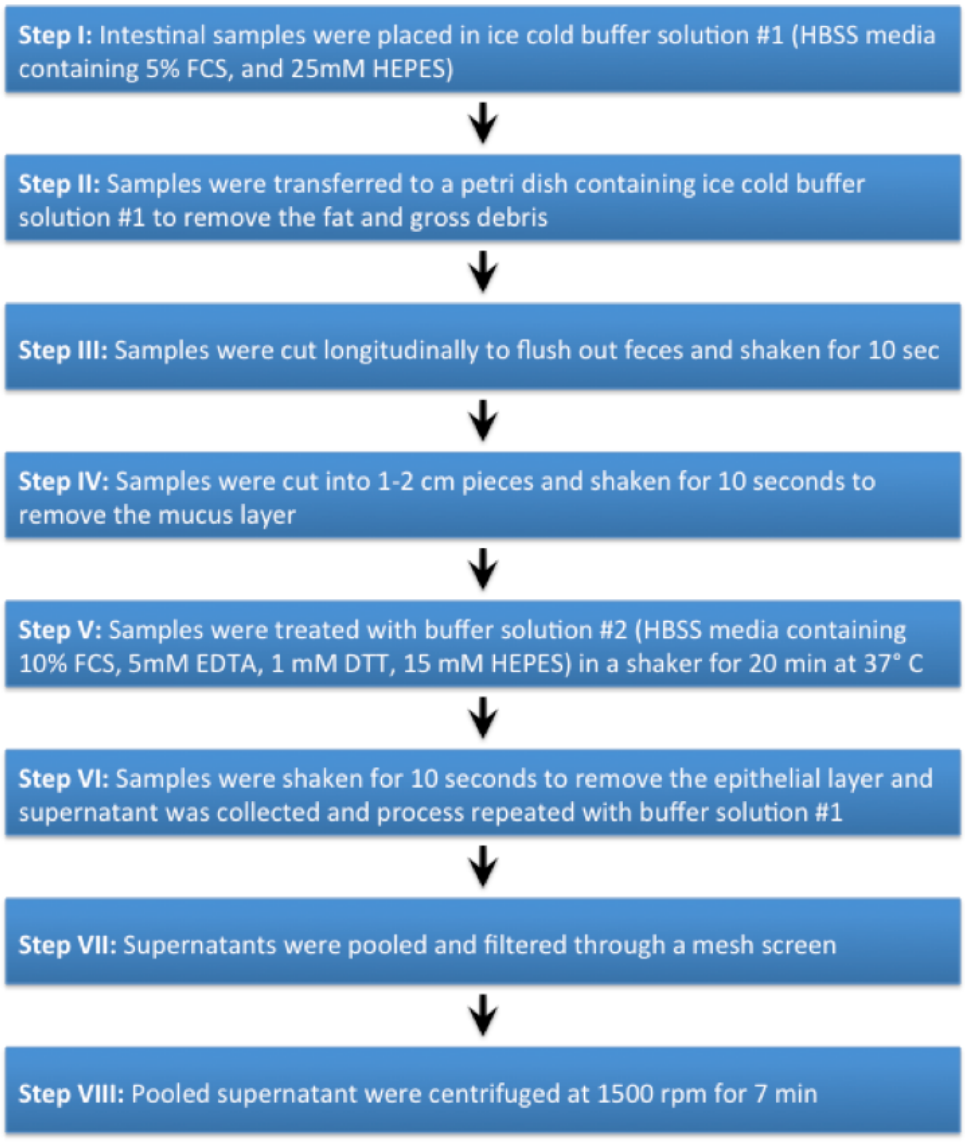
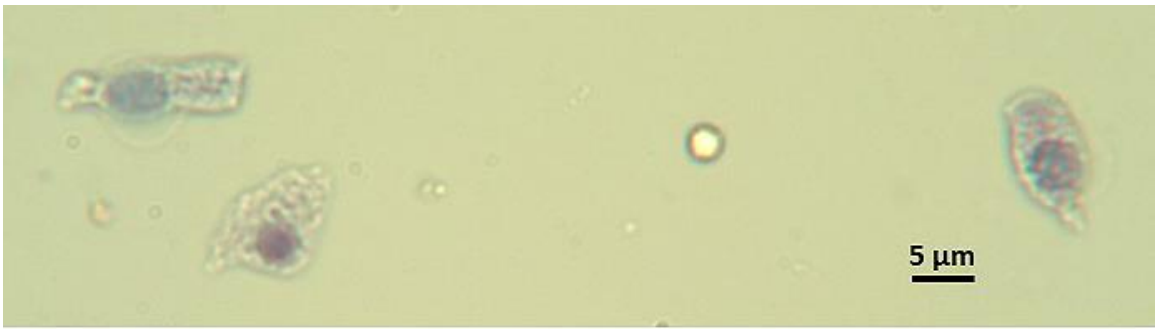


Figure 5. Schematic workflow of intestinal epithelial cell isolation. Prairie vole epithelial colonocytes were isolated using a multiple step process as presented in the flowchart diagram. The cells collected were used for gene expression studies and flow cytometry.

Figure 6

A.



B.

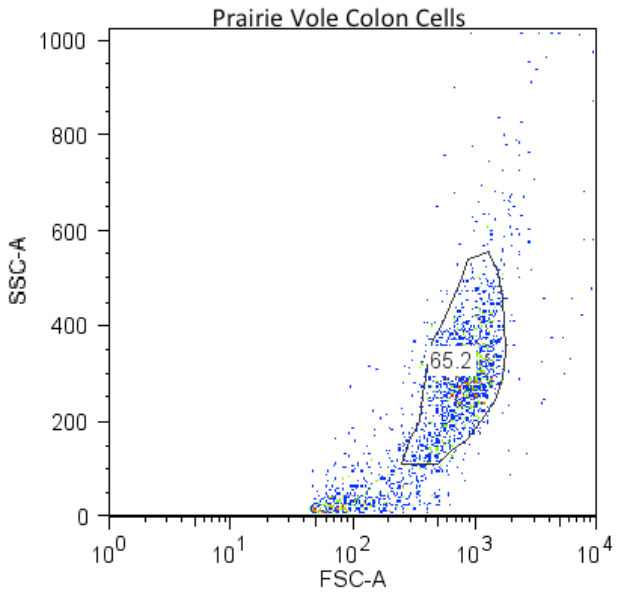


Figure 6. Microscopy and cytometry of epithelial cells isolated from intestinal tissues with the multiple step protocol. **A:** Representative photograph (10x) of the epithelial cells collected from the colon. The colon epithelial cells are stained with tryphan blue. **B:** Representative dot plot of total epithelial cells isolated (gated population) using FSC-A by SSC-A (Figure 5, Step VIII). The percentage of the autogated population is indicated. Figure 6A is shown as published and Figure 6B was adapted from the published figure (Supeck et al., 2018).

3.3. Gene Expression Is Upregulated In 3 Week Old Voles.

To further evaluate the intestinal barrier, I examined tight-junction-associated genes expression of claudins 3, 4, and 7, zonula occludens protein 1, and occludin, which had high enough expression to be measured by qPCR and normalized to GAPDH. A second housekeeping gene, KRT8 was tested and not selected due to inconsistency of expression. Most of these genes were significantly upregulated in three week-old prairie voles when compared to two week-old prairie voles (Figure 7). However, claudin-7 gene regulation did not significantly differ between the groups.

Figure 7

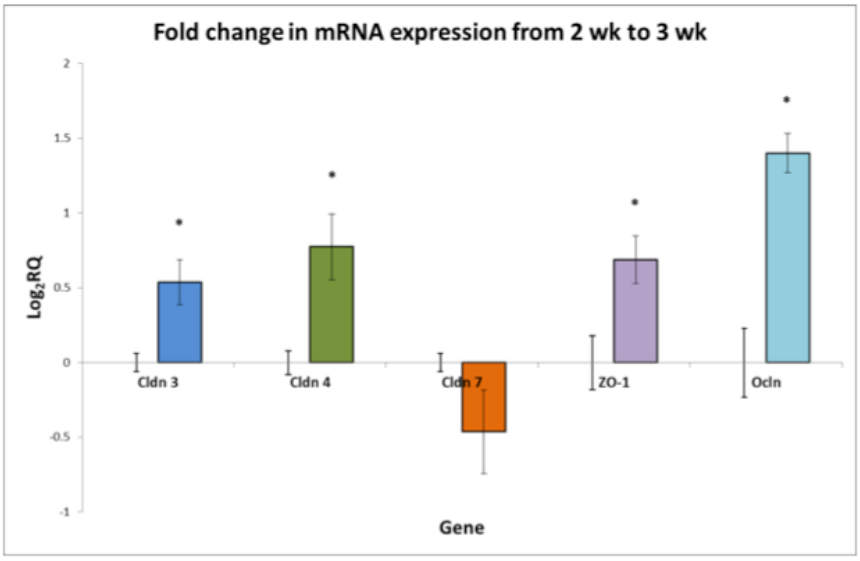


Figure 7. Quantitative RT PCR analysis of prairie vole colon epithelial cells. Tight-junction-associated mRNA expression in colonocytes isolated from 2- and 3-week-old voles was measured by RT qPCR for: claudin-3 (Cldn3), claudin-4 (Cldn4), claudin-7 (Cldn7), zonula occludens-1 (ZO-1), and occludin (Ocln). Values were normalized to the housekeeping gene GAPDH. Cldn3, Cldn4, ZO-1, and Ocln gene expression is up regulated in the 3 week-old prairie voles. Cldn 7 gene expression was not significantly different. Data are expressed as log 2 of the relative quantities (mean ± SEM from three independent experiments) in dissociated colonocytes from three-week versus two-week-old voles. Error bars on the left are control samples. n=3 samples per group, measured in duplicates. T-test was performed for each gene. *P<0.05. See also (Supeck et al., 2018).

3.4. Three-Week-Old Voles Exhibit Intact Tight Junction Structures.

Decreased intestinal permeability and an increased mRNA expression of tight junction proteins suggest that the tight junction structures are intact. I therefore utilized TEM to examine colon ultrastructure in three-week-old prairie voles. Three-week-old prairie voles exhibited electron dense structures where the adjacent apical membranes contact between the epithelium, which indicate that the tight junction structure is intact (Figure 8).

Figure 8

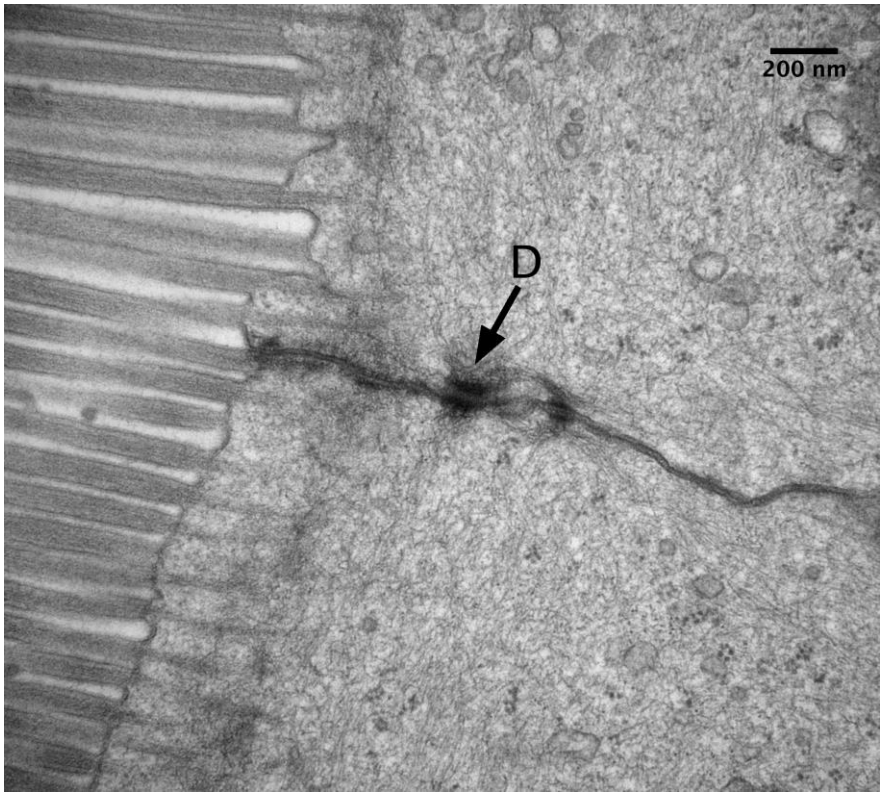


Figure 8. Colon tight junction ultrastructure micrograph of three week-old prairie voles. Images from the apical cell area reveal an intact tight junctional complex joining two intestinal epithelial cells in 3 week old prairie voles. Arrow indicates the location of desmosomes (D) with intermediate filaments extending into the dense plaque region. Magnification 40,000X. Published in (Supeck et al., 2018).

3.5. Histological Characteristics of Prairie Vole Colon Epithelium.

Histological characteristics of immature and mature colon epithelium in the prairie vole were examined in one-week and three-week-old voles. The colon samples were stained with hematoxylin and eosin, and a comparison of the colon morphology was made between seven-day-old and 21-day-old prairie voles. Light microscopy at 21 days revealed an increase in the number of goblet cells present (Figure 9) and an increase in smooth muscle width, colonic crypt length, and width (Figure 10). These data show that as the colon matures the gross morphology of the colon changes in dimension and in cell type proportions.

Figure 9

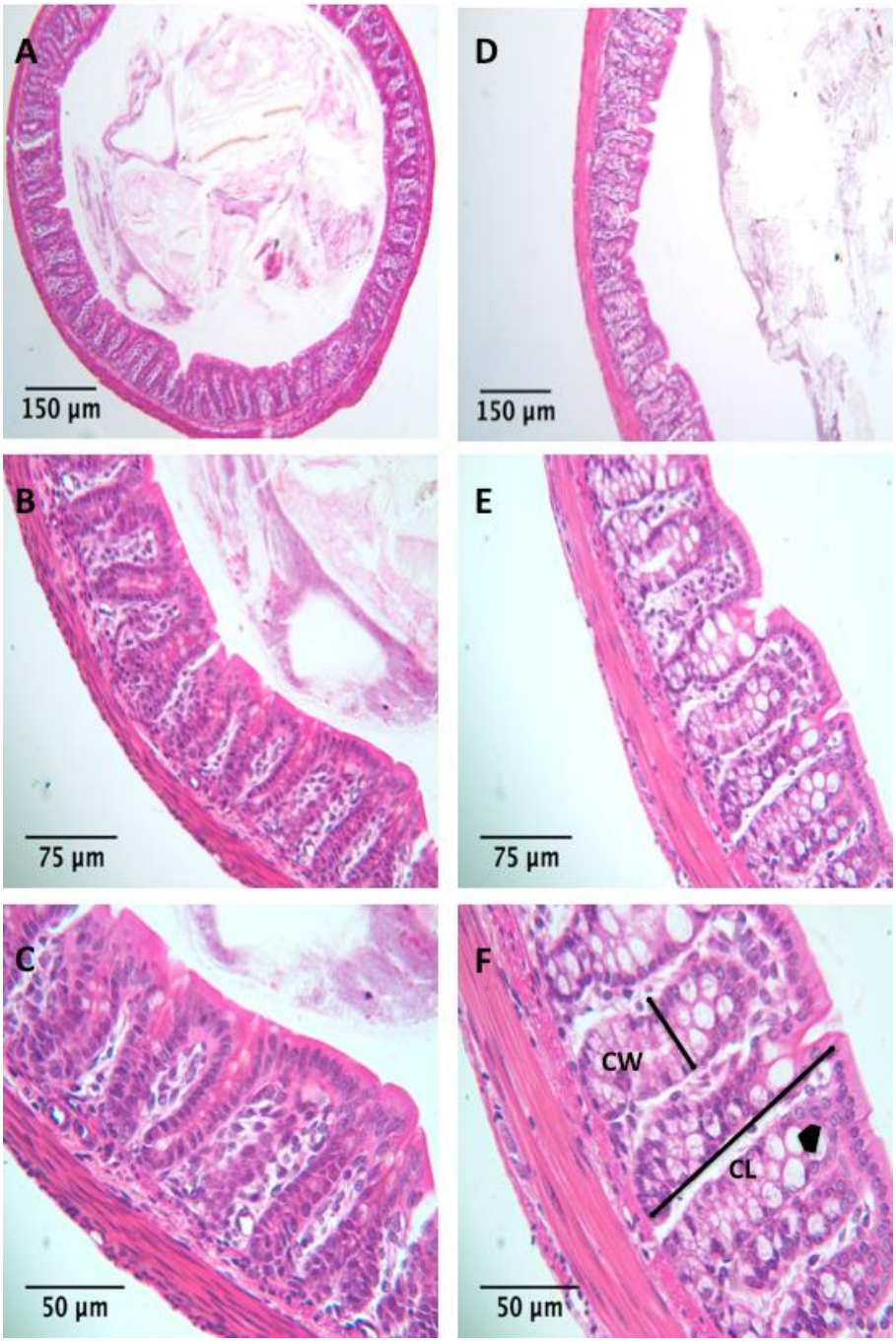


Figure 9. Colon morphology in prairie voles. Hematoxylin and eosin (H&E) histology images of 7 day old and 21 day old prairie vole intestines are represented with increasing magnifications (**A, D:** 10X; **B, E:** 25X; **C, F:** 40X). Black lines indicate dimensions measured for intestinal crypt length (CL) and crypt width (CW). Goblet cells are plentiful in the 21-day-old sample (see broad arrow in F) . Figure published with adjustments (Supeck et al., 2018).

Figure 10

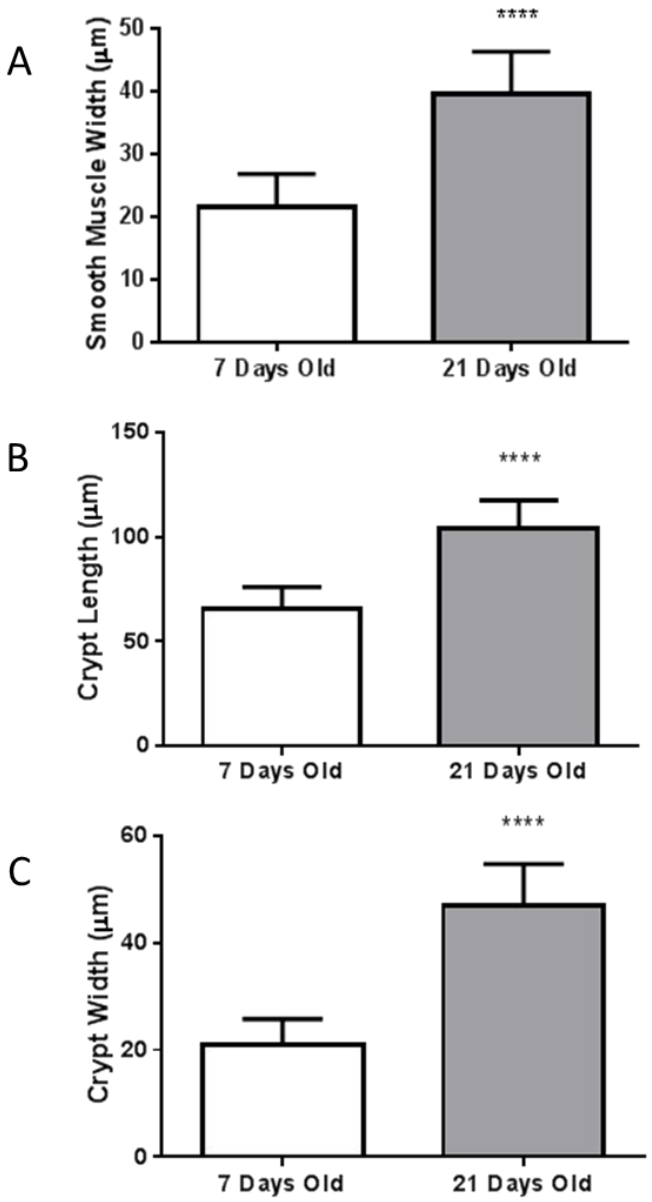


Figure 10. Prairie vole colon morphometry. A comparison of colon morphology was made between 7 and 21 day old prairie voles with regards to smooth muscle width (A), colonic crypt length (B), and width (C). Approximately 40 colonic crypts spanning the length of the colon were measured per animal (arrows in Figure 8, C and F). n=3 to 4 samples per group. All data are represented as mean ± SEM. ****P<0.0001 (T-test).

4. Discussion

Previous studies of postnatal maturation in neonatal animals (Clarke and Hardy, 1969; Lecce and Broughton, 1973; Urao et al., 1997) and premature neonates (van Elburg et al., 2003; Weaver et al., 1984) found that the intestinal barrier tightens and becomes less permeable as the individual matures. More specifically in rodents, this maturation process occurred between two and three weeks of age (Patel et al., 2012). Because prairie voles are rodents of similar size as mice, I hypothesized that maturation of the prairie vole intestinal barrier would occur within a similar time frame. We utilized the FITC-dextran assay to evaluate intestinal permeability and tight junction function (Anderson and Van Itallie, 2009; Catassi et al., 1995; Laukoetter et al., 2007). The results showed high levels of accumulation of FITC-dextran in the serum of two-week-old prairie voles. Therefore, the two-week-old prairie vole has significantly higher paracellular absorption of 4 kDa FITC-dextran molecules compared to that of three, four, and eight-week-old prairie voles. The intestinal architecture of the vole at three-weeks of age shows a mature epithelium with a predominant number of goblet cells and apparent tight junction structure (Supeck et al., 2018). Interestingly, barrier maturation coincides with weaning of vole pups and full adaptation to solid food (Cole and Batzli, 1979). These results support my hypothesis that the timing of intestinal maturation in prairie voles is similar to that seen in other rodent models

Since tight junctions form the major paracellular barrier in the intestinal epithelium (Mitic et al., 2000), I analyzed gene expression of major tight-junction-associated genes to understand which components contribute to controlling the paracellular permeability. To accomplish this, intestinal epithelial cells were isolated from the prairie vole colon. The multi-step isolation protocol was validated using light microscopy and flow cytometry. The light microscopy was helpful for visualization of the colon epithelial cells. These results demonstrated successful isolation of intestinal epithelial cells. Studies using the flow cytometry showed that my protocol consistently

isolated the desired colon cells. Ultimately, these results indicate the capability of isolating colon cells for gene expression studies.

Tight-junction gene expression was measured to identify genes that contribute to controlling paracellular permeability to FITC-dextran. To accomplish this, I evaluated the mRNA expression of tight-junction-associated genes that included claudins, occludins, and zonula occludens proteins. These results indicate that claudin-3, claudin-4, zonula occludens-1, and occludin are upregulated during this important period of development. Furthermore, the upregulation occurred simultaneously with the decrease in intestinal permeability at an age of three weeks. These results are consistent with a previous study by Holmes and coworkers (Holmes et al., 2006) showing that claudin gene expression is developmentally regulated in the intestine.

Studies examining tight junctions have shown the importance of claudins, occludins, and zonula occludens in maintaining an intact intestinal barrier; for example, the loss of claudin-3 in both humans and rodents is associated with intestinal injury (Thuijls et al., 2010). Furthermore, studies in the rat necrotizing enterocolitis (NEC) model expanded on the importance of claudin-3 by showing that stress can increase claudin-3 expression (Clark et al., 2006; Shiou et al., 2011). Other studies looked closely at the removal of claudin-4 in Madin-Darby canine kidney cells and noted an increase in epithelial permeability due to a disruption of the tight junction organization (Sonoda et al., 1999). These results have further been supported by studies showing that the loss of claudin-4 coincides with dramatic changes in the tight junction morphology (Mitic et al., 2000) and resulting in colonic disease (Prasad et al., 2005). This study supports the importance of claudin-3 and claudin-4 in maintaining an intact functional intestinal epithelial barrier.

5. Conclusion

Age is an important factor when examining the function of the intestinal barrier. In agreement with previous studies in other rodents, prairie voles absorbed much more FITC-dextran at two weeks than at three weeks, demonstrating greater intestinal permeability. These permeability changes can best be explained by the further occluding of the tight junctions in the prairie vole intestine as they age. Through the isolation of colon cells from the prairie voles, the reported gene expression profiles of tight-junction-associated-genes revealed a number of candidates that may play a role in the maturation of the prairie vole intestinal epithelial barrier. This permeability change is associated with increased gene expression of claudin-3 and 4, zonula occludens-1, and occludin in three-week-old voles compared to two-week-old voles. More importantly, this data indicate that the intestinal maturation occurs by three weeks of age. This is supported by an ultrastructural study demonstrating, that at three weeks old the tight junction structure is present. Future studies aimed at investigating the tight junction expression and barrier function following alterations in the intestinal microbiome can contribute to the development of the prairie vole as a model to study the gut-brain-behavior axis. Most importantly, future studies will investigate the intestinal barrier function during changes in the intestinal microbiome. In this context, the study is crucial to identifying when the barrier matures so that prairie voles are used at appropriate ages to study the gut-brain-behavior axis.

CHAPTER III

THE IMPACT OF NEOMYCIN ON THE PRAIRIE VOLE INTESTINAL MICROBIOTA AND INTESTINAL EPITHELIAL BARRIER

1. Introduction to Chapter III

At birth, human and rodent intestines are colonized with microorganisms from their mother and the environment (Bergmann et al., 2013; Dinan et al., 2015; Schaedler et al., 1965). The adult human intestinal tract ultimately contain trillions of bacteria (Foster and McVey Neufeld, 2013) with the colon having the greatest number and diversity (Eckburg et al., 2005). This large amount of bacteria is why microorganisms outnumber the cells in our bodies (Gill et al., 2006). The intestinal microbiota plays a crucial role in maintaining the health of the host and is important for the homeostasis of the host's epithelial barrier (Gacias et al., 2016; Willing et al., 2011). Recent studies show that microbial colonization can directly induce the intestinal barrier function maturation by promoting tight junction protein expression (Patel et al., 2012), and help to maintain the intestinal barrier function by signaling through intestinal epithelial cell toll-like receptors (Karczewski et al., 2010; Rakoff-Nahoum et al., 2004). The intestinal microbiota even influence host behavior (Diaz Heijtz, 2016; Neufeld et al., 2011). The microbiota has been shown to help with digestive metabolism by breaking down complex polysaccharides (Foster and McVey Neufeld, 2013) and aid the host by producing short-chain fatty acids (SCFAs) as end metabolites that profoundly influence intestinal barrier function, host immunity, and epithelial

proliferation (Keeney and Finlay, 2011). Since the host and its microbiota both benefit from this partnership, their intimate relationship is viewed as mutualistic (Backhed et al., 2005).

Although the host and microbiota share a mutualistic relationship during intestinal homeostasis, the composition of the microbiota is not fixed and can go through shifts in bacterial communities. These alterations can be beneficial or harmful to the host. Ultimately, many factors can influence the composition of the microbiota such as age, antibiotics, diet, disease, genetics, geography, infection, metabolism, and stress (Bennet et al., 2002; Cho et al., 2012; Forsythe et al., 2010; Hufeldt et al., 2010; Karlsson et al., 2012; Turnbaugh et al., 2009; Yatsunenko et al., 2012). Therefore, alterations in the bacterial community can result in abnormal microorganisms or increased populations of specific microbial species present in the intestinal tract (Bergmann et al., 2013; Lin et al., 2005; Sartor, 2008). This creates an imbalance in microbial populations, which is termed dysbiosis (de Vrieze, 2013; Flak et al., 2013; Gentschew and Ferguson, 2012; Lawrence et al., 2012; Thomas, 1993). Dysbiosis has been shown to cause alterations in bacteria-host signaling (Hughes and Sperandio, 2008; Sperandio, 2007; Sperandio et al., 2003), depletion of co-dependent bacterial populations (Lawrence et al., 2012), altered host immune responses (Pennisi, 2013; Sharland, 2007), disruption of epithelial barrier homeostasis via defects in tight junction protein assembly (Wlodarska et al., 2011), and alterations in mucus secretion, antimicrobial peptide production, and cytokine production (Wlodarska and Finlay, 2010).

It is not surprising that health outcomes have been correlated with bacterial composition (Claesson et al., 2012). Intestinal and extra-intestinal pathologies such as colon cancer, inflammatory bowel diseases (e.g. infectious enteritis and necrotizing enterocolitis) (Bergmann et al., 2013; Clayburgh et al., 2004; Groschwitz and Hogan, 2009; Patel et al., 2012; Peterson and Artis, 2014; Rauch and Lynch, 2012; Sartor, 2008; Shiou et al., 2013), food allergies, type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis (Blaser, 2011; de Vrieze, 2013; Flak et

al., 2013; Gentschew and Ferguson, 2012), and obesity in humans (Riley et al., 2013) and in mice models (Ridaura et al., 2013) have all been shown to stem from microorganism dysbiosis.

Recent research has shown that antibiotic therapy can cause striking shifts in the intestinal microbial composition in humans (Dethlefsen et al., 2008) and in mice (Romick-Rosendale, 2013). More specifically, the use of broad-spectrum antibiotics in water has been reported to reduce significantly the microbial number and diversity in the healthy adult mouse (Bech-Nielsen et al., 2012). The administration of antibiotics is a powerful tool to study the effects of microbial composition shifts. However, the composition of the intestinal microbiota will shift differently based on the antibiotic spectrum activity (Willing et al., 2011). Neomycin has been utilized either alone or in combination with other antibiotics to effectively deplete large amounts of bacteria from the digestive tract (Cannon et al., 2012; Emmelot and van der Waaij, 1980; Rakoff-Nahoum et al., 2004). Neomycin is an aminoglycoside that is active against a variety of gram-positive, gram-negative, and acid-fast bacteria (Waksman and Lechevalier, 1949). It also has desired characteristics, such as a lower chance of developing drug resistant strains and being both bacteriostatic and bactericidal (Waksman and Lechevalier, 1949). Neomycin is often described as being poorly absorbed systemically; however, it should be noted that it has been shown to be non-absorbable from the intestinal tract at concentrations lower than 1440 mg/kg body weight per day in mice (van der Waaij et al., 1974). This is important to my study, because most other studies do not consider whether the antibiotic would be absorbed systemically or use antibiotics that are absorbed systemically. Therefore, neomycin is an ideal antibiotic to utilize in order to study the effects of altering the intestinal microbial composition in the prairie vole.

The intestinal permeability via cellular structural components, metabolites, and indirectly via host immune cells has been shown to be directly modulated by intestinal microbiota (Camilleri et al., 2012). Maintaining the integrity of the intestinal barrier and regulating the intestinal permeability of luminal contents into the underlying subepithelium is a fundamental role that tight junctions

play (Bergmann et al., 2013; Peterson and Artis, 2014). These junctions are made up of structural and functional proteins such as claudin and occludins (Bergmann et al., 2013; Mitic et al., 2000), that together span the cell membrane and interact in the extracellular space between cells (Balda and Matter, 2016; Shen et al., 2011). Neighboring cells interact and bind together forming the tight junction which is a specialized protein complex beneath the apical surface on the lateral side of the cells (Balda and Matter, 2016). This creates a paracellular pathway in the intestinal epithelium that can regulate intestinal permeability (Mitic et al., 2000). Tight junctions restrict entry of molecules based on size, charge, and hydrophilic properties, while still allowing the paracellular absorption of necessary micromolecules and water (Balda and Matter, 2016; Bergmann et al., 2013; Groschwitz and Hogan, 2009; Gunzel and Yu, 2013). My characterization of intestinal barrier maturation in adolescent prairie voles (see Chapter II) was published with adjustments in (Supeck et al., 2018).

Knowledge about the microbiota-gut axis is lacking in the prairie vole. The information presented in this chapter contributes to our growing knowledge of behavior in the prairie vole. The prairie vole has been extensively studied because of its monogamous relationships and biparental behavior, meaning that both the father and mother care for the young. Understanding the effect of microbial composition shifts induced by neomycin and how these shifts affect the intestine epithelial barrier can provide important information needed for future studies involving the gut-brain axis. In the present study, I used the prairie vole i) to evaluate the effect of neomycin on the bacterial composition of the microbiota, ii) to investigate the effects of microbial composition shifts induced by neomycin on intestinal permeability, and iii) to assess if tight junction-associated genes undergo changes in gene expression during neomycin administration.

2. Materials and Methods

2.1. Prairie Voles

Female prairie voles (3 weeks old) were bred in an animal facility at Oklahoma State University Center for Health Sciences. The voles were housed at 21° Celsius with a 14:10 light: dark cycle, and given access to food (Purina rabbit chow) and water *ad libitum*. Antibiotic-treated prairie voles were given neomycin trisulfate (Sigma Aldrich, St Louis, MO) at 1.0 mg/ml in sterile deionized drinking water for seven days. On the fourth day, the neomycin drinking water was replaced with freshly prepared neomycin water. Untreated, control prairie voles received sterile deionized water. The prairie voles were weighed at the start of the study, and daily weights and water intake were measured and analyzed. At day seven, the antibiotic was withdrawn and the prairie voles were weighed again, then the intestinal barrier function was measured or they were euthanized by decapitation after CO_2 anesthesia, and tissue samples were harvested. The colon was immediately removed and placed in HBSS complete medium (Life Technologies, Grand Island, NY) and colon epithelial cells were isolated for evaluation of mRNA expression (Supeck Chapter II and published in (Supeck et al., 2018)). Additionally, the cecum was removed and weighed. The Institutional Animal Care and Use Committee at Oklahoma State University Center for Health Sciences (Tulsa, OK) approved all animal experiments. All efforts were undertaken to minimize the number of animals used and their suffering.

2.2. Fecal Collection and DNA Isolation

Fecal pellets were collected 30 minutes before animals were treated with water (control) or neomycin-containing water for seven days. On day seven, the treatments were stopped and pellets were collected. Samples were collected in sterile microtubes and placed on ice for 10 minutes. The pellet samples were stored at -80° Celsius until processing for DNA extraction and Terminal Fragment Length Polymorphism (T-RFLP) Analysis.

Fecal DNA extraction

DNA was extracted from the collected pellet samples using the ZR Fecal DNA MiniPrep Kit (Zymo Research, Irvine, CA). A Mini-Beadbeater-96 (Biospec Products, Bartlesville, OK) was employed for cell disruption. After initial bead beating two times for 2 min duration at 2,400 oscillations/min, the resulting fecal homogenates were processed for fecal genomic DNA isolation according to the kit's instructions (Zymo Research, Irvine, CA). The extracted DNA concentrations were determined using a Biotech Synergy 2 Multimode Microplate Reader (Biotech Instruments, Inc. Winooski, Vermont). The qualities of the fecal genomic extracts were evaluated by agarose (1%) gel electrophoresis in 1× Tris-Acetate-EDTA buffer, and a size estimate of the bulk fecal DNA of at least 8 kb when compared with the TriDye 2-log DNA marker (New England Biolabs Inc.) was indicative for good quality fecal DNA. DNA was stored at -20° Celsius until processing for Terminal Restriction Fragment Length Polymorphism (T-RFLP) study and/or other downstream analysis.

2.3. Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis.

T-RFLP analysis was comprised of the following major steps: PCR amplification and restriction enzyme digest, separation and fragment size determination of the digested products via capillary electrophoresis, analysis of data to generate the fragment profile for each sample, and clustering analysis based on the sample profiles (Supeck Chapter II). PCR amplification of the region of 16S ribosomal DNA of the bacterial genome was carried out using the two universal primers for bacteria 8F (5'-FAM-AGAGTTTGATCMTGGCTCAG -3') and 1391R (5'-PET-TGTACACACCGCCCGTC -3') labeled with 6-FAM and PET dye, respectively (Applied Biosystems). PCR amplifications were performed in a PTC-200 DNA Engine thermocycler (Bio Rad, Hercules, CA) at 50-µl total reaction volume with AmpliTaq Gold 360 master mix (25µl, Life Technologies, Carlsbad, CA), 0.2µM of each primer, 100 ng of fecal DNA, and sterile

distilled water. Thermocycling was comprised of an initial denaturing step at 95°C for 10 min, followed by 30 cycles of 15 s at 95°C, 30 s at 55°C, and 90s at 72°C and a final primer extension step at 72°C for 10 min. Aliquots of the PCRs were evaluated by agarose gel electrophoresis in 1× Tris-Acetate-EDTA buffer, and the DNA bands of the expected size (1.5kb) were detected with the Quick Load 1-Kb DNA ladder (New England Biolab) as size marker (Figure 8). The remaining amplified product was purified and concentrated using the ZR DNA Clean and Concentrator 25 kit (Zymo Research, Irvine, CA).

Two 20 μL aliquots of the cleaned PCR product were used in restriction digests with 10 U of Alu I (New England Biolabs, Inc.) and 20 U MspI (New England Biolabs, Inc.), respectively. The reaction buffers recommended by the manufacturer were used in a total reaction mix volume of 30 μL. Reactions were incubated overnight at 37 °C. The sample digests were purified using the ZR DNA Sequencing Clean-up Kit (Zymo Research, Irvine, CA). Finally, the eluted samples were run on an ABI 3130 automated gene analyzer capillary electrophoresis instrument (Applied Biosystems, Foster City, CA) with an internal lane size standard (GeneScan™ LIZ 600; Applied Biosystems). Terminal restriction fragments (TRFs) were separated and detected by capillary electrophoresis. The computer program GeneMapper 4.0 (Applied Biosystems) was used for the analysis of fragment data. Only fragments with intensity below a threshold (500 fluorescent units) were recorded. Fragments > 500 units were excluded from further analysis. The TRFs were analyzed with T-Rex software (Culman et al., 2009). Duplicate T-RFLP analyses were run for each DNA extraction from each DNA fecal sample.

2.4. Intestinal Permeability Assays

Intestinal barrier function was evaluated by measuring paracellular permeability to fluorescent-labeled dextran *in vivo*. Previously described in Supeck Chapter II and Figure 4 (Supeck et al., 2018).

2.5. Colon Epithelial Cell Isolation

At the day seven endpoint, prairie vole colon tissue samples were harvested and colon cells were isolated. Previously described in Supeck Chapter II (Supeck et al., 2018).

2.6. Colon cells mRNA expression

The mRNA expression was evaluated using the previously described method in Supeck Chapter II (Supeck et al., 2018).

2.7. Primer Design

Vole primer sets suitable for real-time PCR were designed using Primer 3 (developed by U-Mass-Med)(Rozen, 1998) and prairie vole mRNA sequences available in Genbank. SnapGene Viewer was used for confirmation of the forward and reverse primer pair sequences and target sites. All forward and reverse primers were purchased from Sigma Aldrich (St Louis, MO) and were used at a final concentration of 200nM (Supeck Chapter II, (Supeck et al., 2018)) (Rozen and Skaletsky, 2000).

2.8. Statistical analysis

Student's t-test (two groups), one-way or two-way ANOVA followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). $P < 0.05$ value was considered significant. Data are presented as mean ± SEM.

3. Results

3.1. Consumption of Neomycin-Treated Water and Measurements of Body and Cecal Weights.

The prairie voles consumed 11.72 ± 0.38 mL water daily when no neomycin was added and 10.62 ± 0.32 mL daily with the addition of neomycin (Figure 11A). The relative cecal weight was 7.718 ± 0.82 g in the water control group and 9.787 ± 0.74 g in the neomycin-treated group (Figure 11B). Initial body mass did not differ between the groups, and all prairie voles gained weight. These results show that the water consumption (Figure 11A), relative wet cecal mass (Figure 11B), and body mass (Figure 11C) did not differ between the groups.

3.2. Neomycin Treatment Alters Gut Microbiota

The principal component analysis (PCA) of fecal pellet samples showed that neomycin treatment (Neo) resulted in a left clustering of samples from the Day 0 to Day 7 in both enzyme preparations (Figure 12 A and C). Additionally, the fecal samples clustered together in the neomycin (Neo) group in the PCA (Figure 12 C and D). The result suggests a change in the prairie voles' bacterial community composition due to neomycin and a difference between the neomycin-treated and water control bacterial community composition of the distal colon as assessed by terminal restriction fragment length polymorphism (T-RFLP).

Figure 11

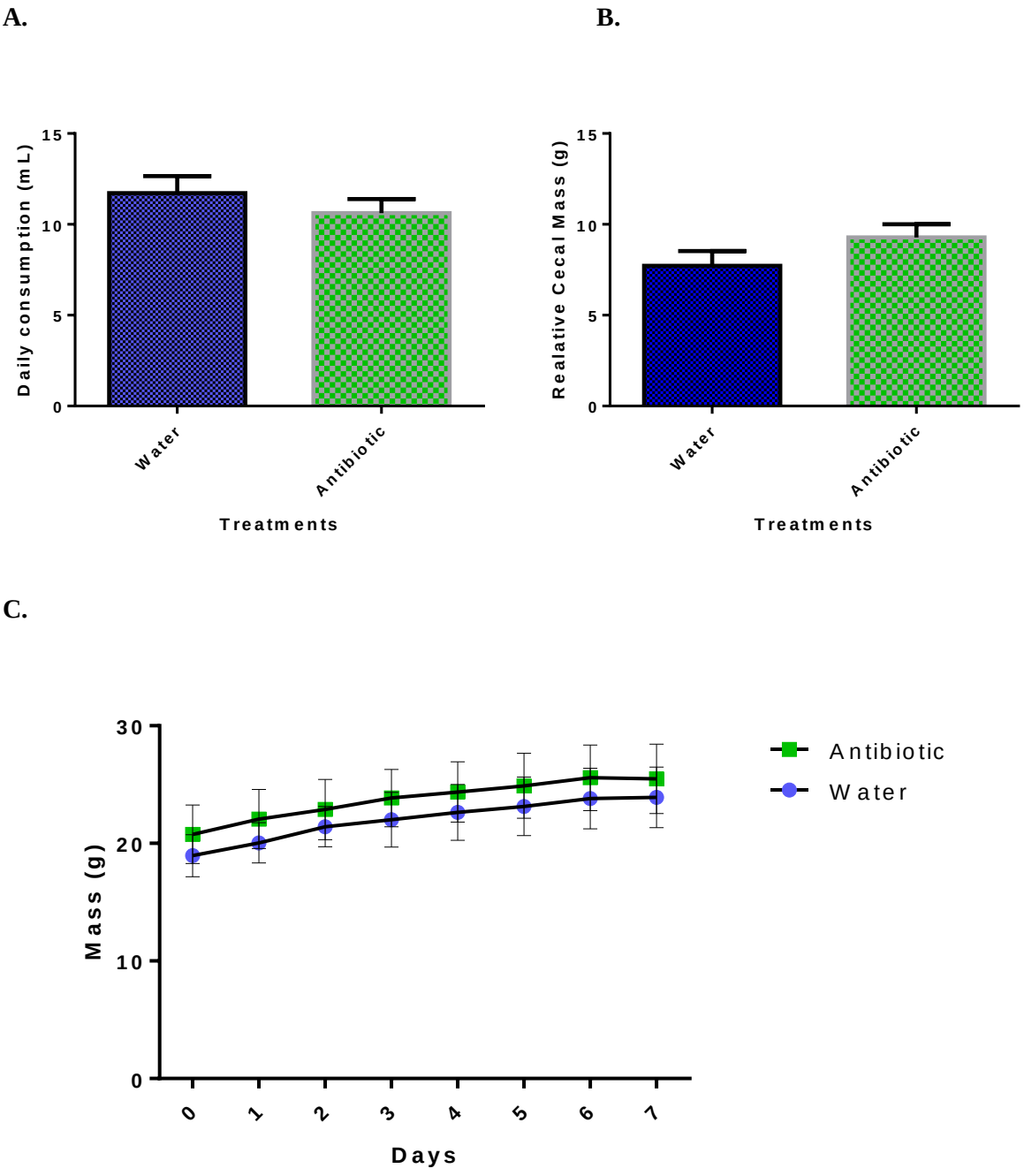


Figure 11. Effects of neomycin on water consumption, cecal and body weight.
A: Average water intake per day during the treatment period. **B:** Relative cecal weight after 7 days of neomycin treatment. **C:** Weight gain during antibiotic treatment (Day 0 to Day 7).

Figure 12

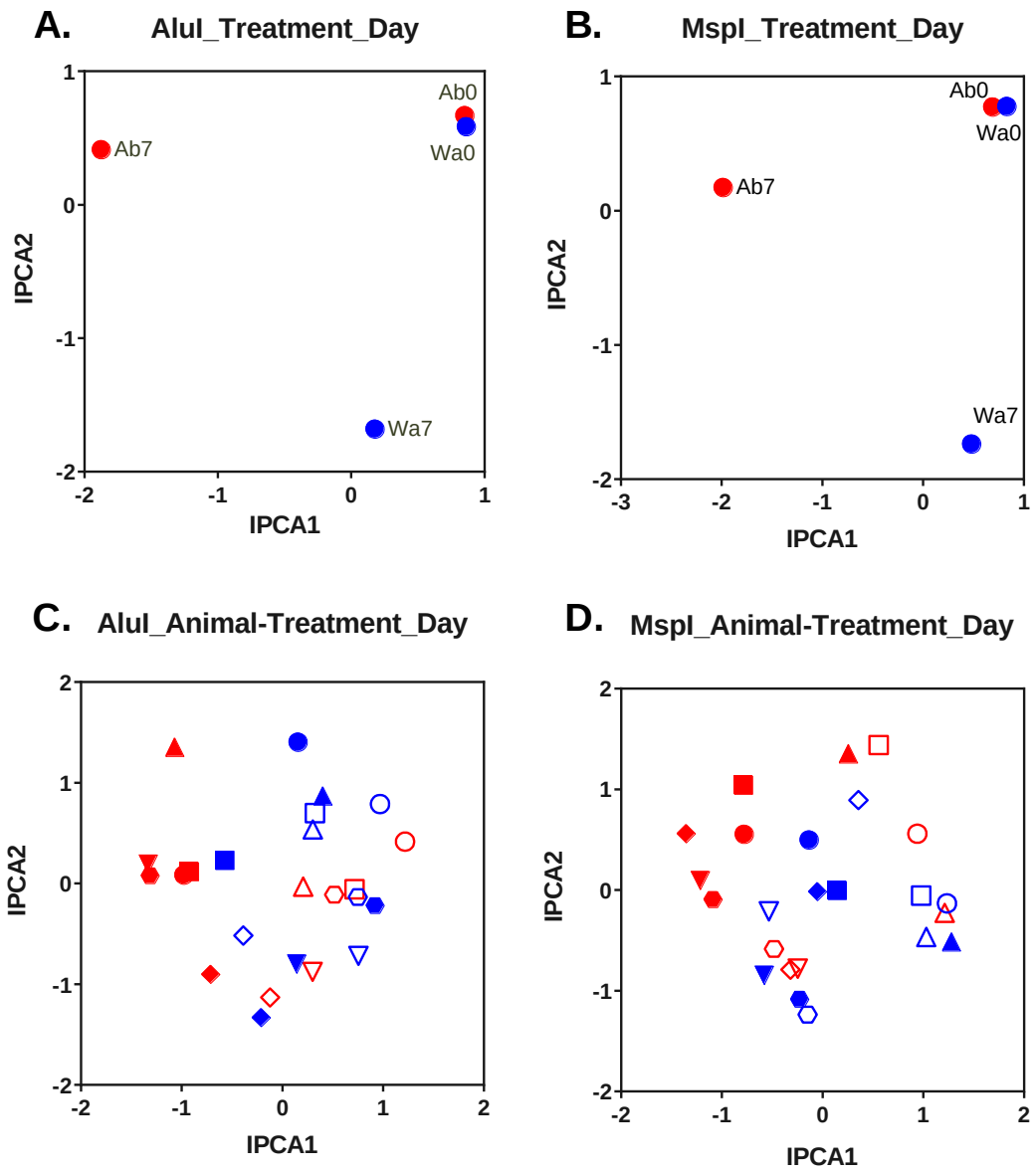


Figure 12. Principle Component Analyses (PCAs) of T-RFLP results. Interaction principal component analyses (IPCAs) of T-RFLP datasets based on 16S rRNA gene amplicon restriction digests with *Alu*I (plots A and C) and *Msp*I (plots B and D) were performed in T-REX software (Culman et al., 2009). Score plots A and B show samples combined according to treatment groups: neomycin-treated animals (red) at day 0 (Ab0) and day 7 (Ab7) compared to water control animals (blue) at day 0 (Wa0) and day 7 (Wa7). Score plots C and D depict the data points for each prairie vole in individual symbol color and shape combinations. Data points for neomycin-treated animals (red symbols) and water control animals (blue symbols) at day 0 are indicated with solid symbols. The open symbols indicate the respective data points for day 7. Six animals were used in the treatment and control groups, respectively.

3.3. Intestinal Permeability Assays.

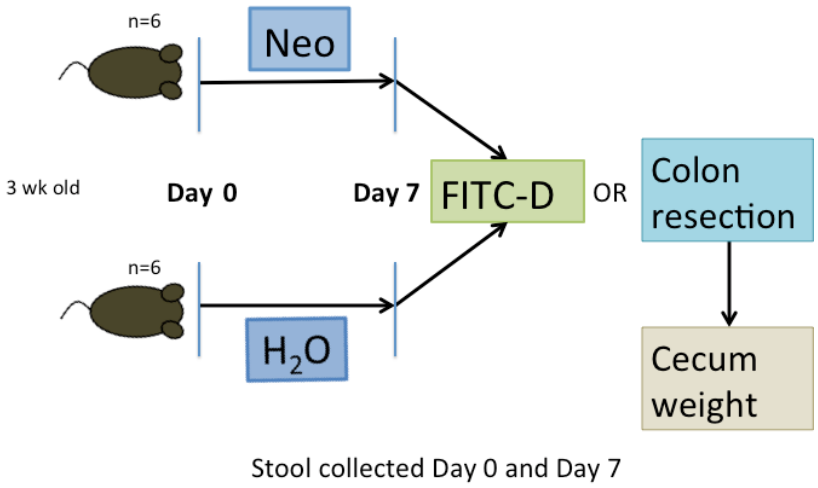
To determine if microbial composition shifts induced by neomycin treatment disrupts the intestinal epithelial barrier, the serum FITC-dextran concentration was measured 4.5 hours after oral gavage administration of 0.5mg/g of 4 kDa FITC-dextran (FD4) in two randomized groups of prairie voles (Figure 13A). I found no significant difference in intestinal permeability between neomycin-treated and the water control groups as indicated by measurements of FITC-dextran serum concentration (Figure 13B). These results indicate that neomycin does not alter the tight junctions selective paracellular pathway in the intestinal epithelial barrier when given orally for seven days.

3.4. Gene Expression in Prairie Vole Colonic Epithelium Following Neomycin Treatment.

To further evaluate if shifts in microbial composition after neomycin administration are associated with changes in tight junction gene expression, mRNA expression of tight-junction-associated genes was examined. The expression of claudin-3, claudin-4, zonula occludens-1, and occludin mRNAs were measured by qPCR and normalized to GAPDH mRNA expression. Claudin-3 gene expression was significantly upregulated in RT-PCR of neomycin-treated prairie voles when compared to water control prairie voles (Figure 14). However, claudin-4, claudin-7, zonula occludens-1, and occludin did not significantly differ between the groups (Figure 14). These results indicate that the shift in the microbiota may influence gene expression of tight-junction-associated proteins. Therefore, the neomycin-induced alteration in microbiota may modulate transcription of these proteins, but may not change the functional protein levels.

Figure 13

A.



B.

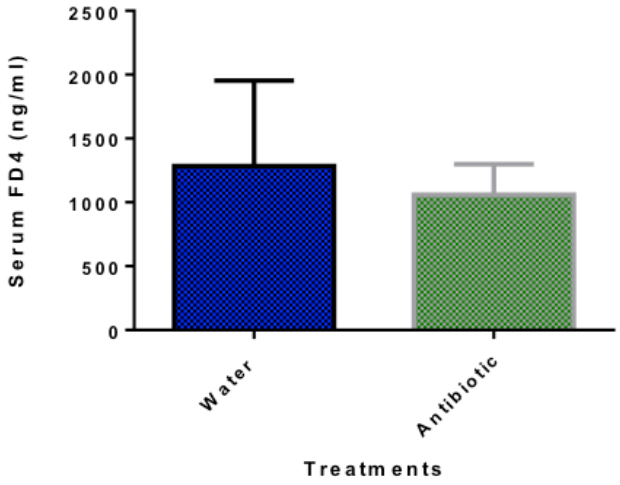


Figure 13. Prairie vole intestinal permeability following neomycin. A: Prairie voles were randomized into two groups: group 1 was given FITC-dextran, and group 2 were given 1X PBS and used as controls. For the first 4 hours of the study, all animals were fasted from food and received only water *ad libitum*. After 4 hours, animals either received 1X PBS or 5mg/g (diluted in 1X PBS) of 4 kDa FITC-dextran administered via oral gavage. The animals continued on the water only restriction for 4.5 hours. **B:** At the time of euthanasia, whole blood was collected, and serum 4 kDa FITC-dextran concentration was determined. Data in **B** are expressed as mean ± SEM from at least three experimental replicates per condition.

Figure 14

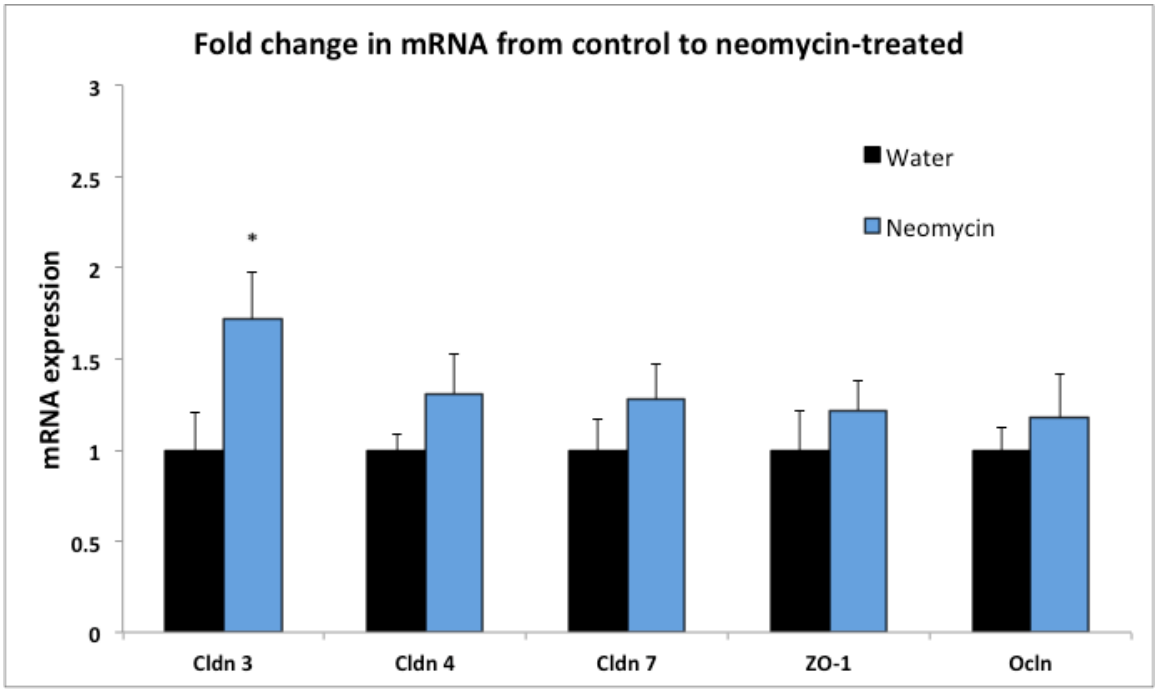


Figure 14. Quantitative Real Time PCR Analysis of Prairie Vole Colon Epithelial Cells. Messenger RNA expression was measured for: claudin-3 (Cldn3), claudin-4 (Cldn4), claudin-7 (Cldn7), zonula occludens-1 (ZO-1), and occludin (Ocln). Values were normalized to the housekeeping gene GAPDH. Cldn3 gene expression is up regulated in the neomycin-treated water prairie voles. Cldn4, Cldn7, ZO-1, and Ocln gene expression levels were not significantly different to controls. Data are expressed as fold change (mean ± SEM from at least three independent experiments) versus water control. n=3 to 6 samples per group, run in duplicate. T-test was performed for each gene. *P<0.05 was considered significant.

4. Discussion

Antibiotic treatment is generally anticipated to change the bacterial composition and diversity of the intestinal microbiota. However, the effect of neomycin on the intestinal bacterial communities and intestinal epithelial barrier has not been studied in prairie voles. Obtaining this information is needed before future gut-brain axis studies in this animal can be undertaken.

One hurdle in studying the intestinal microbiota composition comes with culturing the bacteria. Limitations exist with growing bacteria *in vitro,* and, because of this, culturing does not yield a reliable representation of the microbial composition of the gut because only a small fraction of the diversity is represented (Stewart, 2012). Therefore, I employed terminal restriction fragment length polymorphism (T-RFLP) analysis, a method that is culture-independent, rapid, and sensitive to assess the microbiota. T-RFLP is a technique based on bacterial 16S rRNA genes that is commonly used as a tool to evaluate intestinal bacterial compositions after antibiotic induced alterations in mice (Wlodarska et al., 2011) and humans (Jernberg et al., 2005). It has also been used in diverse environments to study complex microbial communities in human saliva (Sakamoto et al., 2003), soil (Derakshani et al., 2001), and marine environments (Eschenhagen et al., 2003).

In previous studies, it has been shown that antibiotic(s) can alter the daily water consumption (Marx et al., 2014). In this study, I found that the oral administration of neomycin (Neo) in drinking water for seven days did not alter the daily water consumption in prairie voles. I also examined the relative wet cecal mass, which did not differ between groups. The wet cecal mass is an important physiological measurement anytime you give a substance orally. Especially when using antibiotics, because studies as early as 1968 have shown enlarged ceca in mice which were given penicillin in drinking water at a dose of 0.3 g per liter (Savage and Dubos, 1968). Additionally, I measured the prairie vole body weights before, during, and after neomycin

treatment. These results showed no significant difference in body weight between the groups and that all prairie voles gained weight during the experiment.

Fecal pellets were used for evaluating the microbial composition depletion/shifts induced by neomycin. While this does not allow for a thorough analysis of bacteria that adhere to the mucosa (Reikvam et al., 2011), it is non-invasive and allows for repeated observations during treatment. The protocol of administrating neomycin orally had a major effect. Prairie voles that received oral administration of neomycin (Neo) in drinking water for seven days exhibited microbial composition shifts as shown by PCA analysis. Furthermore, the neomycin shifted the bacterial communities toward a similar composition, indicating a loss of diversity between individual prairie voles. Antibiotic treatment is generally anticipated to change the bacterial composition and diversity. These results align with what I report as neomycin effectively changing the bacterial community.

Next, I assessed the impact of shifts in the intestinal microbiota on intestinal permeability. The paracellular permeability of molecules occurs between epithelial cells and is regulated by tight junctions (Anderson and Van Itallie, 2009). A common way to measure intestinal barrier permeability is by orally administering a fluorescently labeled sugar molecule and then detecting its presence in serum after 4.5 hours (Supeck et al., 2018). Previously, I identified that the intestinal epithelial barrier matured by three weeks old in prairie voles, indicating that the intestinal barrier tightens and becomes less permeable (see Chapter II; (Supeck et al., 2018)). Because I previously identified that the prairie vole intestinal barriers mature by three weeks of age, I was able to answer the question: “Does microbial composition shifts induced by neomycin alter the intestinal permeability in adolescent prairie voles?” In order to answer this question, I selected adolescent prairie voles that were three weeks old since they had a mature intestinal epithelium and I utilized a selective marker (FITC-dextran) that evaluates both intestinal permeability and tight junction function (Anderson and Van Itallie, 2009; Catassi et al., 1995;

Laukoetter et al., 2007). These results showed that neomycin (Neo) neither increased nor decreased the intestinal permeability as measured by serum 4 kDa FITC-dextran molecules. These data indicate that neomycin does not alter the intestinal barrier integrity when given orally at 1mg/mL per animal. Therefore, shifts in the microbial composition induced by neomycin do not change the intestinal barrier permeability. These data support my hypothesis that prairie vole barrier integrity is not affected by the neomycin-induced shift in the microbiota.

In the intestinal epithelium, tight junctions form the major paracellular barrier, which regulates intestinal permeability (Mitic et al., 2000). Therefore, I measured tight-junction-associated gene expression in an attempt to understand which components of tight junctions contribute to controlling homeostasis of the intestinal barrier when shifts in the microbiota occur. To accomplish this, I isolated intestinal cells from the prairie vole colon as previously described (Supeck et al., 2018). Then, I evaluated the mRNA expression of tight-junction-associated genes that included claudins, occludins, and zonula occludens. These results indicate that claudin-3 is upregulated during shifts in the microbial composition induced by neomycin. Indicating that it may play a role in keeping the tight junction structure intact. Additionally, other studies reported that loss of claudin-3 in both humans and rodents is associated with intestinal injury (Thuijls et al., 2010), and studies in the rat necrotizing enterocolitis (NEC) model expands on the importance of claudin-3, by showing that stress can increase claudin-3 (Clark et al., 2006; Shiou et al., 2011). This study supports the importance of claudins-3 as a contributor to maintaining an intact functional epithelial barrier. Such a change in the microbial composition may very well contribute to the onset of intestinal stress and promote the upregulation of claudin-3 gene expression.

Future studies should take a closer look at microbial composition using sequencing to investigate the microbial composition after neomycin induced intestinal microbial shifts. This current study provides information needed for the development of the prairie vole as a model to study the gut-

brain axis. Furthermore, protein expression studies are needed to determine if the upregulation in gene expression leads to an increase in protein levels.

5. Conclusion

The oral administration of neomycin induces changes in the intestinal microbiota in prairie voles. These changes can best be explained by the activity of neomycin and the fact that it does not absorb well systemically. Neomycin-induced major shifts in the intestinal microbial composition without an effect on the intestinal permeability of the epithelial barrier. Gene expression profile revealed claudin-3 to be a candidate that may be important to the maintenance of the prairie vole intestinal barrier homeostasis. Finally, oral administration of neomycin proved to shift the microbial composition without increasing the intestinal permeability, suggesting that it is a good tool to use to increase in understanding the role of the microbiota in the gut-brain axis.

CHAPTER IV

NEOMYCIN INDUCED SHIFTS IN INTESTINAL MICROBIOTA COMPOSITION CORRELATES WITH DEPRESSION-LIKE BEHAVIOR IN PRAIRIE VOLES

1. Introduction

Anxiety and depression are both common disorders in the United States with the peak onset occurring during adolescence and early adulthood (Altemus et al., 2014). Anxiety is the most common mental illness in the United States, which affects 18.1% of the United States population and has a lifetime prevalence estimate of 28.8% (Kessler et al., 2005a; Kessler et al., 2005b). Estimates on the incidence of depression assign to this condition 6.6 % 12-month prevalence with a 16.2% lifetime prevalence (Kessler et al., 2003). Women are two times more likely to develop depression and most other anxiety disorders (Gater et al., 1998; Kessler et al., 2005b; Kessler et al., 1994).

The intestinal microbiome and the brain communicate in a bidirectional manner utilizing the immune system, autonomic nervous system, enteric nervous system, and the hypothalamic-pituitary-adrenal HPA axis (de Jonge, 2013; Foster and McVey Neufeld, 2013). Commensal gut microbes are important beyond the intestine as they contribute to brain development in mice (Collins et al., 2012; Cryan and Dinan, 2012) and in humans (Tillisch et al., 2013).

Recent studies indicate that the intestinal microbiota is crucial to the homeostatic function of the central nervous system (CNS) (Clarke et al., 2013; Diaz Heijtz, 2016; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004), while alterations in the microbiota have been shown to influence the risk of anxiety and depressive-like behaviors (Foster and McVey Neufeld, 2013). For instance, in mice, it has been shown that the intestinal microbiota can affect central levels of Brain-Derived Neurotrophic Factor (BDNF), which has been linked to depression and anxiety (Bercik, 2011; Bercik et al., 2011). While in humans, subsets of patients with neurodevelopmental disorders have presented with gastrointestinal (GI) complications (Hsiao et al., 2013). Therefore, these results support the gut-brain axis as a bi-directional pathway where microbiota can influence the brain, while the brain can influence the microbiota (microbiota-gut-brain axis).

Further support of behavioral changes mediated by shifting or altering the microbiota comes from research in germ-free mice, which exhibited no change in behavior after they were given antibiotic treatment (Foster and McVey Neufeld, 2013). This shows that the antibiotic treatment did not alter the behavior because it did not have a direct effect on the host and because no microorganisms were present to be perturbed. Furthermore, bacterial composition has been directly correlated with health outcomes (Claesson et al., 2012). Therefore, alteration in the intestinal microbiota composition can be problematic and lead to behavioral changes. It is important to understand how microbiota shifts can influence behavior to be able to develop a targeted strategy to prevent and treat stress-related disorders, anxiety, and depression via the gut-brain axis.

A common strategy to study the role that the microbiota play in the gut-brain axis is the use of antibiotics (Cryan and Dinan, 2012). Administration of antibiotics can cause striking shifts in the intestinal composition in humans (Dethlefsen et al., 2008) and in mice (Romick-Rosendale, 2013). Therefore, it is a tool that can be utilized to disrupt the microbiota composition to assess

the role of the intestinal microbiota on host behavior (Cryan and Dinan, 2012). However, the composition of the intestinal microbiota will shift differently based on the antibiotic spectrum activity (Willing et al., 2011). For instance, neomycin is an aminoglycoside that has a broad spectrum of activity against a variety of gram-positive, gram-negative, and acid-fast bacteria (Waksman and Lechevalier, 1949). In Chapter III, I showed that neomycin significantly alters the microbiota composition without altering the intestinal epithelium permeability in the prairie vole. Therefore, neomycin is an ideal antibiotic to use and assess the intestinal microbiota's role on behavior in the prairie vole.

Prairie voles display social behaviors similar to humans (Carter and Getz, 1993), thus making these rodents a great model organism to use to study behavior after microbiota composition shifts induced by neomycin. Understanding the effect of microbiota composition shifts and how these shifts alter behavior will provide a new model to gather important information needed to understand the microbiome-gut-brain axis. In the present study, I conducted behavioral tests and analyzed the fecal microbiota in female prairie voles to evaluate i) the effects of microbial composition shifts induced by neomycin on anxiety-like behavior, and ii) the effects of microbial composition shifts induced by neomycin on depression-like behavior.

2. Materials and Methods

2.1. Prairie Voles and Antibiotic Treatment

Prairie voles were bred in an animal facility at Oklahoma State University Center for Health Sciences. Three-week-old female voles were housed at 21° C with a 14:10 light: dark cycle, and given access to food (Purina rabbit chow supplemented with black oil-sunflower seeds) and water *ad libitum*. Antibiotic-treated prairie voles were given neomycin trisulfate (Sigma Aldrich, St Louis, MO) at 1.0 mg/ml in sterile deionized drinking water for seven days. On the fourth day, the drinking water was replaced with freshly prepared neomycin-supplemented drinking water. Untreated, control prairie voles received sterile deionized water. At the day seven endpoint, the antibiotics were withdrawn and behavioral testing of neomycin-treated or water control prairie voles was conducted at 4 weeks of age, from 0900-1200 hours. Subsequently, the animals were euthanized by decapitation after CO_2 anesthesia. The colon was immediately removed and flash frozen in liquid nitrogen and then stored at -80° C for future studies. The Institutional Animal Care and Use Committee at (IACUC) Oklahoma State University Center for Health Sciences (Tulsa, OK) approved all animal experiments. All efforts were used to minimize the number of animals used and their suffering. Collection and analysis of fecal samples is described below.

2.2. Study Design

A timeline of the experiment is shown in Figure 15. At three weeks of age, prairie voles were placed into two randomized groups, either neomycin-treated water or water control. The prairie voles were separated by treatments and co-housed with another prairie vole of the same gender and treatment group. At four weeks of age, which is the end of the 7-day study, the prairie voles underwent behavioral testing.

Figure 15

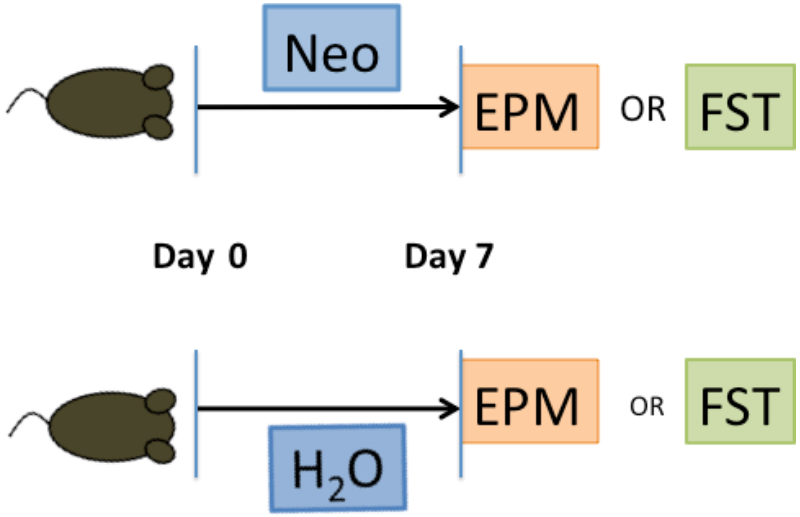


Figure 15. Study Design Timeline. Experimental timeline showing the duration of neomycin treatment, and the time point of the Behavioral Testing. Prairie voles were randomized into two groups: group 1 was given neomycin-treated water, and group 2 was given sterilized water and used as controls. After seven days, the treatments were stopped and the animal behavior was studied using the elevated plus maze (EPM) or the forced swim test (FST).

2.3. Behavioral Studies

Testing took place between 0900 and 1200 hours under low illumination to reduce stress. On the day of testing, prairie voles were transferred to clean cages and brought to the testing room where they were allowed to rest for 10 min before the elevated plus maze and 60 min before forced swim testing. The elevated plus maze was cleaned with 70% ethanol and water after each animal. The forced swim cylinder was rinsed and refilled after each animal.

2.3.1. Elevated Plus Maze

The elevated plus maze is used to assess the "emotional" state of the animal. It is a white apparatus consisted of four-arms that cross and a central area elevated approximately 40 cm

above the floor (Figure 16). Two arms lack walls and therefore are open, and two arms have high walls and therefore are closed. The elevated plus maze is placed inside a small swimming pool containing several inches of pine bedding as shown by pictures in Figure 16. This serves both to confine an animal that opts to jump from the apparatus and to provide a soft landing to preclude injury from the fall. Prairie voles were individually placed in the center facing an open arm and allowed to explore for 5 min. The behavior of the prairie vole was digitally recorded with a camera and later scored by a well-trained observer. The behaviors scored were, (1) time spent in open arms, (2) the percent of time in open arms, (3) the percent of entries into the open versus all entries, and (4) the distance traveled. The relative amount of time spent in the open or walled arms, the percentage of time in open arms, and the percentage number of entries into open arms with respect to the total number of entries are all used as an index of anxiety: more time in the open arms is considered an index of low anxiety.

Figure 16

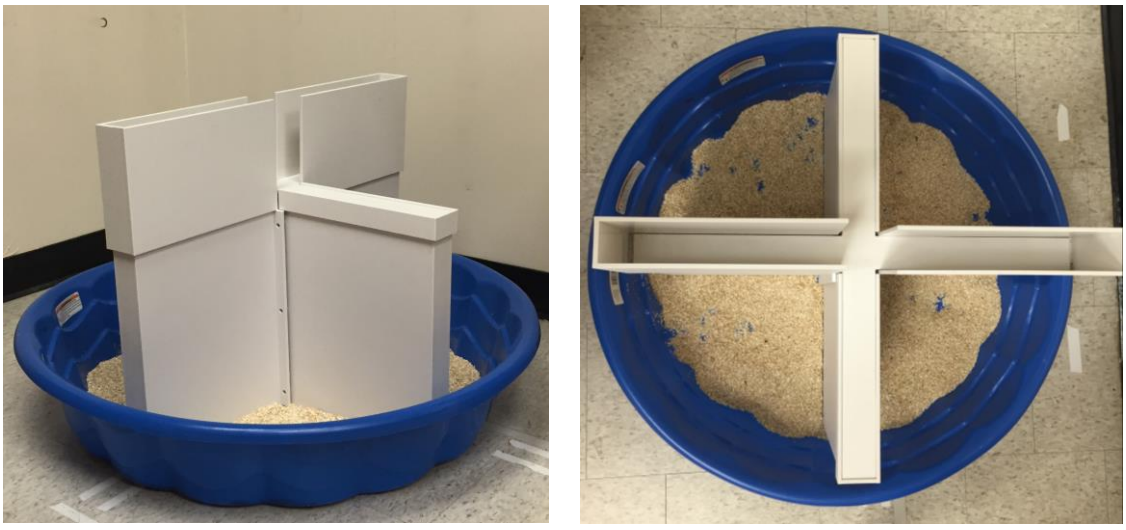


Figure 16. Pictures of the Elevated Plus Maze used for testing prairie voles. The elevated plus maze is placed on the floor with a camera system above to record the results. The elevated plus maze that I use is similar to that used with mice, but as seen in the pictures it has smaller dimensions and designed for mice and prairie voles.

2.3.2. Forced Swim Test

The forced swim test is used to assess evidence of behavioral depression in small animals. Each prairie vole was placed alone for 6 min into a glass cylinder (25 cm tall, 16 cm inside diameter) filled to a depth of 40 cm with tap water at 32° C to reduce possible complications of brain hypothermia. The behavior of the prairie vole was digitally recorded with a camera and later scored by a well-trained observer. The behaviors scored were (1) duration of mobility, defined as actively swimming around the perimeter of the cylinder, (2) duration of immobility, defined as floating with no movement of the trunk, and (3) latency to immobility, defined as the time from the beginning of the experiment to the first bout of immobility. A decrease in the mobility or a faster latency to immobility will indicate higher levels of depression. After the test, the animals were removed from the water, dried with paper towels to start the drying process. Then returned back to their home cage. All prairie voles were first-time swimmers.

2.4. Fecal Collection and DNA Isolation

Fecal pellets were collected from 12, three-week-old female voles prior to treatment with neomycin-water or pure water (control) and one week after they had received either the antibiotic or water. Previously described in Supeck Chapter III.

2.5. 16S rRNA gene fragment amplification and NGS library preparation

To characterize the taxonomic profile of the intestinal microbiome, the V4 hypervariable region of the bacterial 16S rRNA gene was targeted using the universal 16S rDNA primers v4.16Sf (5'-GTGCCAGCMGCCGCGGTAA-3') and v4.16Sr (5'-GACTACHVGGGTWTCTAAT-3'). The primers were integrated with specific Illumina multiplex sequencing primers and dual-index-paired-end approaches following the protocols and procedures described by (Kozich et al., 2013). In brief, each forward (v4.SA5xx) and reverse (v4.SA7xx) primer targeting the V4 region contained the appropriate Illumina adapter, an 8-nt index (barcode) sequence, a 10-nt pad

sequence, a 2-nt linker, and the conserved targeting sequence represented by v4.16Sf and v4.16Sr (Table 2 and Table 3). The overall dual index, paired-end read sequencing strategy targeting the bacterial V4 16S rRNA gene region is shown in Figure 17.

Table 2. List of oligonuclotides (5'-sequence-3') used in this study

Region	Primer Name	Oligonucleotide sequence (5'-3')	Reference
Illumina Adapters	Illumina VX.N5	AATGATACGGCGACCACCGAGATCTACAC	www.Illumina.com
	Illumina VX.N7	CAAGCAGAAGACGGCATACGAGAT	www.Illumina.com
Generic V4 region PCR Primer design	v4.SA5xx	AATGATACGGCGACCACCGAGATCTACAC<i5><pad><link> < GTGCCAGCMGCCGCGGTAA >	Kozich et al (2013).
	v4.SA7xx	CAAGCAGAAGACGGCATACGAGAT<i7><pad><link>< GGACTACHVGGGTWTCTAAT >	Kozich et al (2013).
V4 Sequencing primer	v4.READ1	TATGGTAATTGTGTGCCAGCMGCCGCGGTAA	Kozich et al (2013).
	v4.READ2	AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT	Kozich et al (2013).
Index read 1 primer	Illumina Index	AATGATACGGCGACCACCGAGATCTACAC	Kozich et al (2013).
Index read 2 primer	v4.INDEX	ATTAGAWACCCBDGTAGTCCGGCTGACTGACT	Kozich et al (2013).
link	V4f	GT	Kozich et al (2013).
	V4r	CC	Kozich et al (2013).
Pad	forward	TATGGTAATT	Kozich et al (2013).
	reverse	AGTCAGTCAG	Kozich et al (2013).
Index	i5	i5 sequence (see Table 2)	Kozich et al (2013).
	i7	i7 sequence (see Table 2)	Kozich et al (2013).
KAPA qPCR primers	P1	AAT GAT ACG GCG ACC GA	www.kapabiosystems.com
	P2	CAA BCA GAA GAC GGC ATA CGA	www.kapabiosystems.com

Note: All primers used in this study were purchased from integrated DNA Technologies, Inc (Redwood City, CA). Sequences listed in the generic design are the adapter sequences; i5 and i7 sequences are 8 nucleotide (index) barcodes unique to each sample.

Table 3. List of i5 and i7 index sequences (5'-sequence-3') used in this study

	Primer Name	Oligonucleotide sequence (5'-3')	Reference
Index (i5)	SA501	ATCGTACG	Kozich *et al.* (2013)
	SA502	ACTATCTG	Kozich *et al.* (2013)
	SA503	TAGCGAGT	Kozich *et al.* (2013)
	SA504	CTGCGTGT	Kozich *et al.* (2013)
	SA505	TCATCGAG	Kozich *et al.* (2013)
	SA506	CGTGAGTG	Kozich *et al.* (2013)
	SA507	GGATATCT	Kozich *et al.* (2013)
	SA508	GACACCGT	Kozich *et al.* (2013)
	SB501	CTACTATA	Kozich *et al.* (2013)
	SB502	CGTTACTA	Kozich *et al.* (2013))
	SB503	AGAGTCAC	Kozich *et al.* (2013)
	SB504	TACGAGAC	Kozich *et al.* (2013)
	SB505	ACGTCTCG	Kozich *et al.* (2013)
	SB506	TCGACGAG	Kozich *et al.* (2013)
	SB507	GATCGTGT	Kozich *et al.* (2013)
	SB508	GTCAGATA	Kozich *et al.* (2013)
Index (i7)	SA701	AACTCTCG	Kozich *et al.* (2013)
	SA702	ACTATGTC	Kozich *et al.* (2013)
	SA703	AGTAGCGT	Kozich *et al.* (2013)
	SA704	CAGTGAGT	Kozich *et al.* (2013)
	SA705	CGTACTCA	Kozich *et al.* (2013)
	SA706	CTACGCAG	Kozich *et al.* (2013)
	SA707	GGAGACTA	Kozich *et al.* (2013)
	SA708	GTCGCTCG	Kozich *et al.* (2013)
	SA709	GTCGTAGT	Kozich *et al.* (2013)
	SA710	TAGCAGAC	Kozich *et al.* (2013)
	SA711	TCATAGAC	Kozich *et al.* (2013)
	SA712	TCGCTATA	Kozich *et al.* (2013)
	SB701	AAGTCGAG	Kozich *et al.* (2013)
	SB702	ATACTTCG	Kozich *et al.* (2013)
	SB703	AGCTGCTA	Kozich *et al.* (2013)
	SB704	CATAGAGA	Kozich *et al.* (2013)
	SB705	CGTAGATC	Kozich *et al.* (2013)
	SB706	CTCGTTAC	Kozich *et al.* (2013)
	SB707	GCGCACGT	Kozich *et al.* (2013)
	SB708	GGTACTAT	Kozich *et al.* (2013)
	SB709	GTATACGC	Kozich *et al.* (2013)
	SB710	TACGAGCA	Kozich *et al.* (2013)
	SB711	TCAGCGTT	Kozich *et al.* (2013)
	SB712	TCGCTACG	Kozich *et al.* (2013)

Figure 17

Figure 17. PCR amplification and Illumina MiSeq sequencing strategy for variable region 4 of the bacterial 16S rRNA gene. The dual-index, paired read sequencing strategy was adapted from Kozich and coworkers (Kozich et al., 2013). As an example, the V4 region of an *E. coli* rRNA gene is depicted flanked by the targeting and Illumina adapter sequences. Additionally, the schematic shows the variable region sequencing primers (v4.READ_1, v4.READ_2), which sequence into the V4 region from both directions as well as the index read primers IlluminaINDEX and v4.INDEX which detect the SA5xx and SA7xx indexes, respectively. The I5 and I7 indexes shown here are SA501 and SA701, respectively. Primers v4.16Sf and v4.16Sr indicate the conserved targeting regions flanking V4. Oligonucleotides v4.SA501 and v4.SA701 are examples of the indexed primers used to prepare the sequencing libraries.

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 (containing 17 µl AccuPrime *Pfx* SuperMix, 2 µl of 10 µM forward/reverse primer mix and 1 µl fecal genomic DNA) on a PTC 200 DNA Engine thermocyler (BioRad, Hercules, CA). PCR conditions for construction of the sequencing libraries consisted of one initial cycle at 95°C for 2 min; 30 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 5 min; and a final incubation at 72°C for 10 min (Kozich et al., 2013). Following amplification, the presence of PCR products were examined (from random samples) by running aliquots (2 µl) of the reactions on 2% agarose E-gel® double comp gels (Invitrogen, Life Technologies). Strong amplicon bands at the expected size ranges were detected in all samples except in the no template control reactions (negative control).

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 the SequalPrep wells, and then quantified using a KAPA library Quantification Kit assay (qPCR assay) with primers specific to the Illumina adapters (Kappa Biosystems, Boston, MA). Subsequently, the quantified library (1.4 nM) and Illumina-generated PhiX control (10 nM) libraries were separately denatured in 0.2N NaOH (fresh) and diluted with ice-cold Illumina Hybridization Buffer to a final concentration of 4 pM before being loaded onto a Illumina reagent cartridge for sequencing on the Illumina MiSeq Platform (Illumina, Hayward, CA, USA). For a 15 % PhiX run, 850 µl of 4 pM library and 150 µl of 4 pM of PhiX were combined in a tube and 600 µl of the combined library/PhiX solution were then loaded into well number 17 on the reagent cartridge and sequenced on the MiSeq platform. (Note that the solution loaded was 4.0 pM overall with 3.4 pM library concentration, 0.6 pM PhiX concentration, and 0.000515N NaOH). As described by Kozich and coworkers (Kozich *et al.* (2013)), 3 µl (at 100 µM) of the

read 1 sequencing primer v4.READ1, the v4.INDEX read primer, and the v4.Read 2 sequencing primer were added into the reagent cartridge well number 12, 13 and 14, respectively.

2.8. Sequencing Data Analysis

Following sequencing, raw sequence data for each sample were downloaded from the MiSeq instrument as unaligned/unmapped reads (fastq, barcodes and adapters removed) and imported into the Quantitative Insights Into Microbial Ecology software package, QIIME version 1.9.1 (Caporaso et al., 2010). Forward and reverse read sequences were combined in QIIME using the multiple_join_paired_ends.py script and subsequently quality controlled using the multiple_split_libraries_fastq.py script. Sequence chimera were removed using the usearch61 method, *de novo* and in combination with the SILVA version 128 16S rRNA reference database (Quast et al., 2013). The resulting quality controlled sequence reads were classified individually in QIIME version 1.9.1 (Caporaso et al., 2010; Navas-Molina et al., 2013) by open reference operational taxonomic unit (OTU) picking using the usearch61 method in combination with the SILVA 16S rRNA database (Release 128; 97% identity level) and RDP Classifier for taxonomic assignments with at 90% cutoff (Edgar, 2010; Liu et al., 2007; Quast et al., 2013; Yarza et al., 2014). The resulting OTU table was filtered to remove spurious OTUs (number of sequences < 0.005%). A core set of diversity analyses was run in QIIME (core_diversity_analyses.py) to compute the phylotype diversities (alpha, beta diversities) of the fecal samples' bacterial communities (Caporaso et al., 2010; Lozupone and Knight, 2005; Navas-Molina et al., 2013). The even sampling (rarefaction) depth was adjusted to the lowest number of reads in the samples. Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al., 2014) software was used to analyze and compare taxonomic profiles of the fecal microbiotas of control and neomycin treated animals.

2.9. Statistical analysis

Two-sided Student's t-tests were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) when the means of two groups were compared. P values < 0.05 were considered to indicate significance. In the default QIIME core diversity analysis workflow, Non-parametric t-tests with 999 Monte Carlo permutations followed by Bonferroni post hoc tests were employed in comparative analyses of alpha diversity and bacterial abundances.

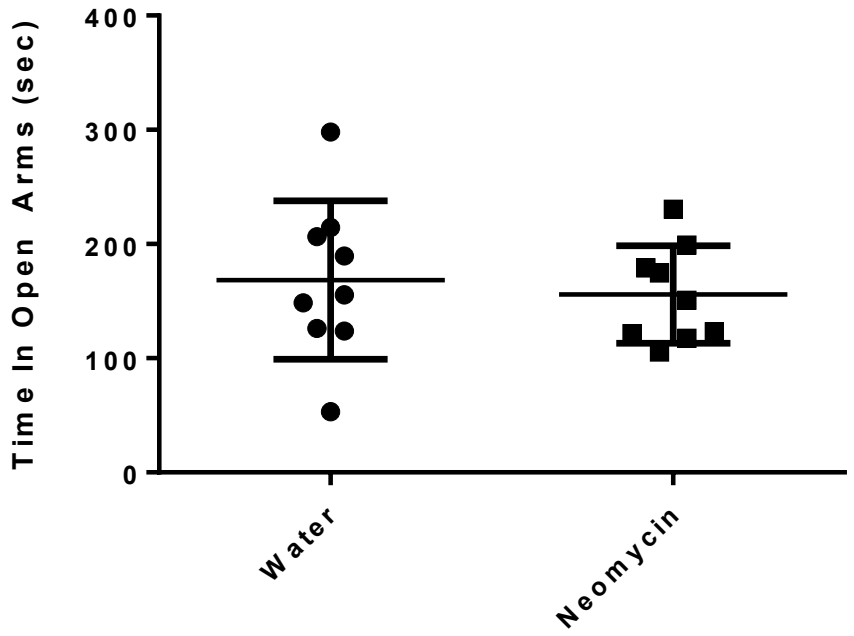
3. Results

3.1. Elevated Plus Maze Behavioral Test.

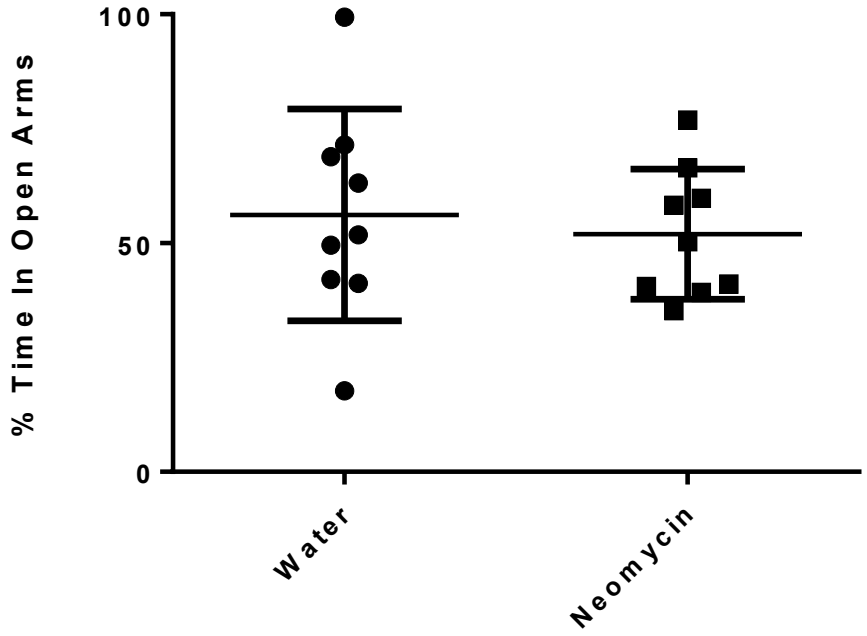
Neomycin-treated prairie voles did not spend significantly more time in the open arms of the elevated plus maze compared to water control prairie voles (P=0.5313; see Figure 18A). Additionally, neomycin treatment did not significantly change the percentage of time spent on the open arms compared to the control group (P=0.5313; see Figure 18B). Furthermore, the neomycin treatment did not significantly change the percentage of open/total arm time (P=0.6936; see Figure 18C). These results show that neomycin-treated water does not significantly change the anxiety-like behavior in prairie voles and therefore no behavioral differences between groups were observed during the elevated plus maze test. However, data points from neomycin treated voles are more clustered together than, controls, which might indicate a loss of microbial diversity.

Figure 18

A.


Time In Open Arms (sec)
400
300
200
100
0
Water
Neomycin

B.


% Time In Open Arms
100
50
0
Water
Neomycin

C.

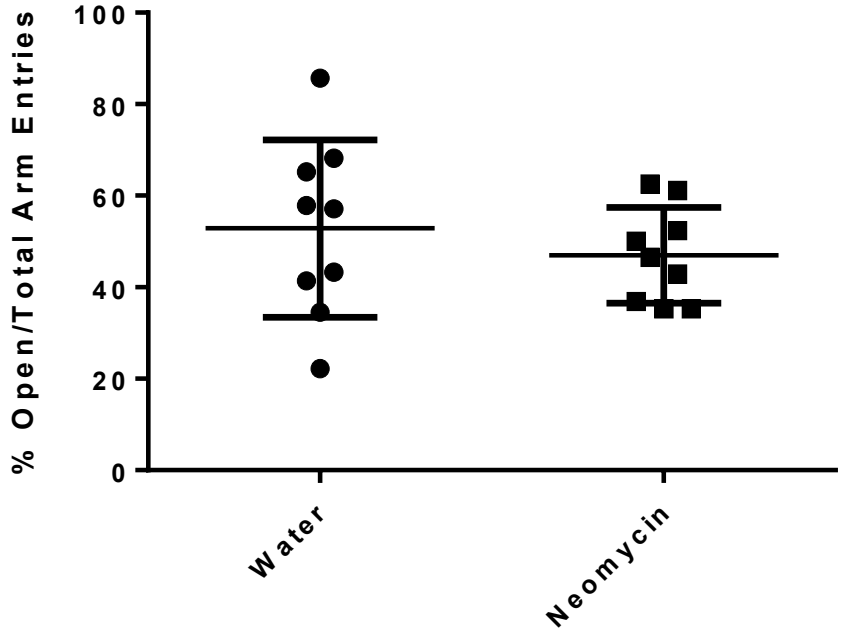


Figure 18. Elevated Plus Maze testing anxiety index. Testing was for 5 min. **A:** Time spent on the open arms by prairie voles receiving neomycin-treatment or water control. **B:** Percentage of permanence time on the open arms with respect to the total duration of the experiment by prairie voles receiving neomycin-treatment or water control. **C:** The percent number of entries into the open arms with respect to the total number of entries by prairie voles receiving neomycin-treatment or water control. Data in are expressed as mean ± SEM from at least three experimental repeats per condition. Nine animals were used per group.

3.2. Locomotor Activity

The total distance traveled by the neomycin-treated prairie voles was 1321 ± 159.2 cm and 1528 ± 242.9 cm for water control prairie voles. Figure 19 shows the distance traveled in the elevated plus maze after a 5 min testing period. No difference was observed in these values between the neomycin and the water control prairie voles, indicating no significant difference in generalized locomotor activity (P=0.4879).

Figure 19

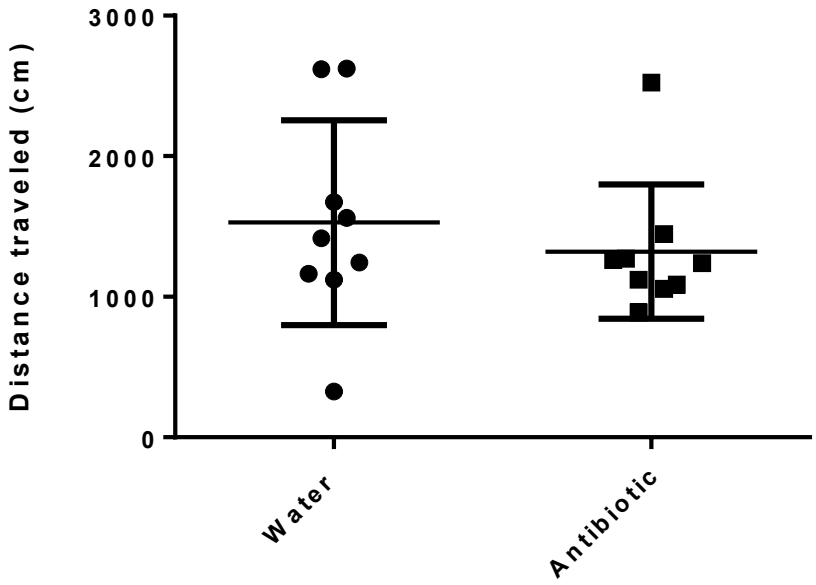


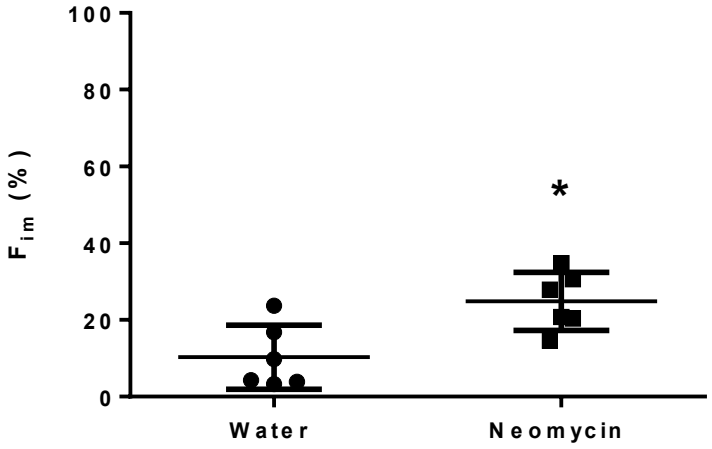
Figure 19. Quantitative evaluation of locomotor activity. Testing was for 5 min using the elevated plus maze. **A:** Total distance traveled by prairie voles is shown. Data are expressed as mean ± SEM from at least three experimental repeats per condition. Nine animals were used per group.

3.3. Forced Swim Test

There was a significant difference between the fractions of total immobile time (F_{im}) of neomycin and water prairie voles (neomycin 24.85 ± 3.069 %, water 10.30 ± 3.406 %, *P*=0.0099; Figure 20A). Additionally, the fractions of total mobility time (F_{mob}) showed a significant difference between the two groups (neomycin 75.15 ± 3.069 %, water 89.70 ± 3.406 %, *P*=0.0099; Figure 20B). I also noticed neomycin-treated prairie voles became immobile faster than water control prairie voles during the forced swim test, indicating that there was a significant difference in the latency to immobility (t_{lat}) between the two groups (neomycin: 58.95 ± 4.648 sec; water: 130.7 ± 19.65 sec; *P*=0.0137; Figure 21). The results indicate that neomycin treated voles significantly exhibit a more depression-like behavior.

Figure 20

A. Total Immobile Time



B. Total Mobile Time

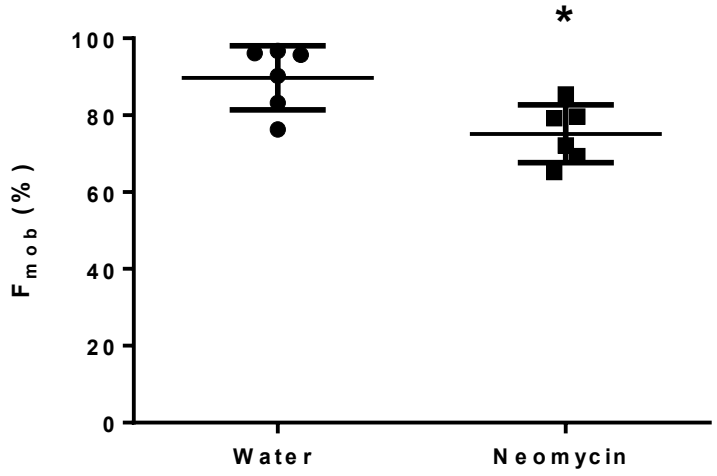


Figure 20. Forced Swim Testing. A: The fractions of total immobile time (F_{im}) of neomycin-treated and water control prairie voles showing a significant difference **B:** The fractions of total mobile time (F_{mob}) of neomycin-treated and water control prairie voles showing a significant difference. *P<0.05 significant value was used to denote a significant difference compared to the water control group. Data are expressed as mean ± SEM. Six animals were used per group.

Figure 21

Latency to Immobility

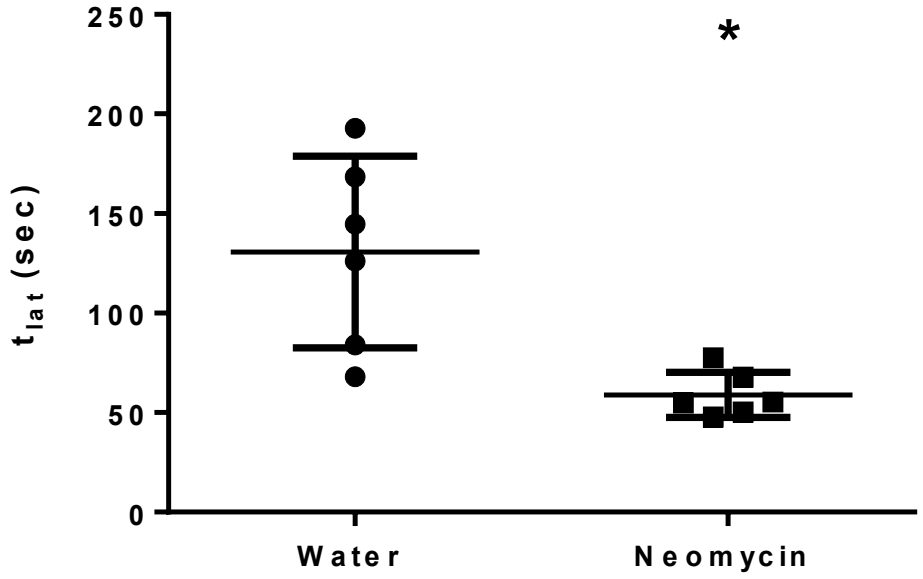
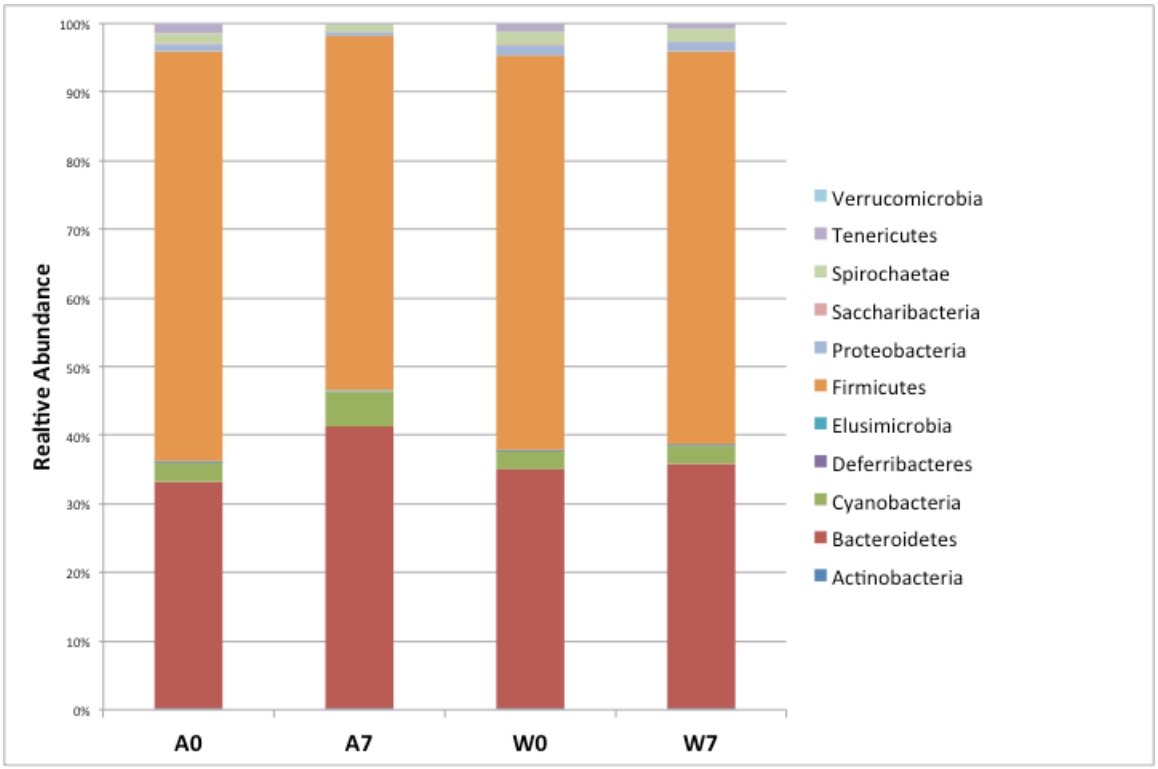


Figure 21. Screening for the first critical immobility. Latency to immobility (t_{lat}) in seconds comparing neomycin-treated and water control prairie voles showed a significant difference. *p<0.05 significant value was used to denote a significant difference compared to the water control group. Data are expressed as mean ± SEM. Six animals were used per group.

3.4. Sequencing Data Analysis of Fecal Microbiome in Prairie Voles Given Neomycin.

Sequencing the fecal pellets revealed that exposure to the neomycin affected various bacterial groups in the intestinal microbiome. Examining the relative abundance of bacteria in the treatment vs. control groups at the phylum level revealed that Bacteriodetes and Cyanobacteria were increased while Firmicutes and Proteobacteria decreased after seven days of treatment with Neomycin (Figure 22).

Figure 22



Proteobacteria

Firmicutes

Cyanobacteria

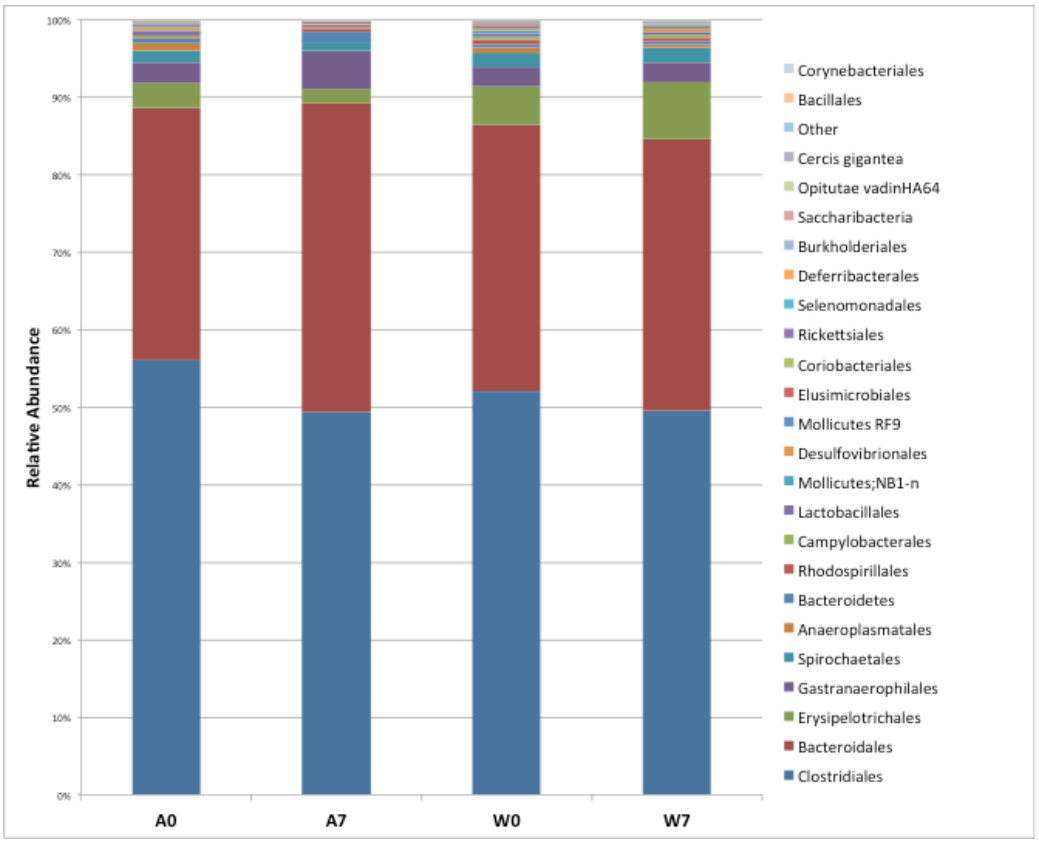
Bacteroidetes

Figure 22. Relative abundance of bacterial phyla in the fecal microbiota from neomycin treated and control animals. Bacteriodetes and Cyanobacteria appear to be increased while Firmicutes, Proteobacteria and Tenericutes decreased after seven days of antibiotic treatment. A0= Neomycin day 0; A7= Neomycin day 7; W0= Water day 0; W7=Water day 7.

Evaluation at the bacterial order level revealed that Bacteroidales, Gastroanaerophilales, and Bacteroidales increased, while Erysipelotrichales (Figure 23A), Anaeroplasmatales, Rhodospirallales, Campylobacterales, and Lactobacillales decreased after seven days of neomycin antibiotic treatment (Figure 23B).

Figure 23

A.



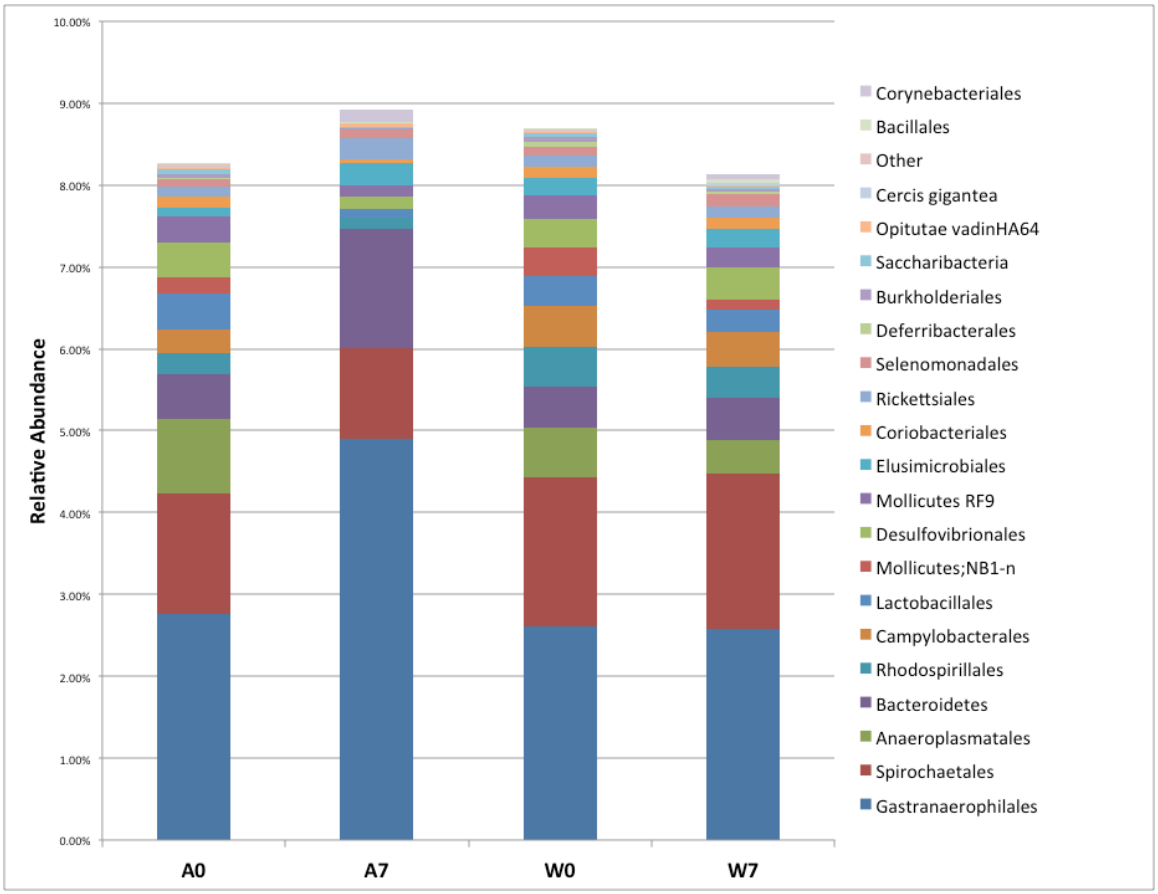
Erysipelotrichales

Bacteroidales

Gastroanaerophales

Bacteriodetes

B.



Anaeroplasmatales

Lactobacillales

Rhodospirallales

Campylobacterales

Figure 23. Relative abundance of bacterial order in the fecal microbiota from neomycin treated and control animals. A. The orders Bacteroidales, Gastroanaerophales, and Bacteroidetes increased, while Erysipelotrichales decreased after seven days of antibiotic treatment. **B.** Zooming in on the smaller population of microbe's show that orders Anaeroplasmatales, Rhodospirallales, Campylobacterales, and Lactobacillales decreased after seven days of antibiotic treatment. A0= Neomycin day 0; A7= Neomycin day 7; W0= Water day 0; W7=Water day 7.

Finally, evaluating the relative abundance of bacterial phylotypes at the genus level showed that the operational taxonomic units (OTU) Lachnospiraceae;other, Ruminiclostridium 6, Prevotellaceae;other, and Gastranaerophilales increased, while, the OTUs Allobaculum, Clostridiales vadin BB60 group, Coprococcus 2, and Ruminococcaceae UCG-014 decreased after seven days of antibiotic treatment (Figures 24, 25, and 26).

Figure 24

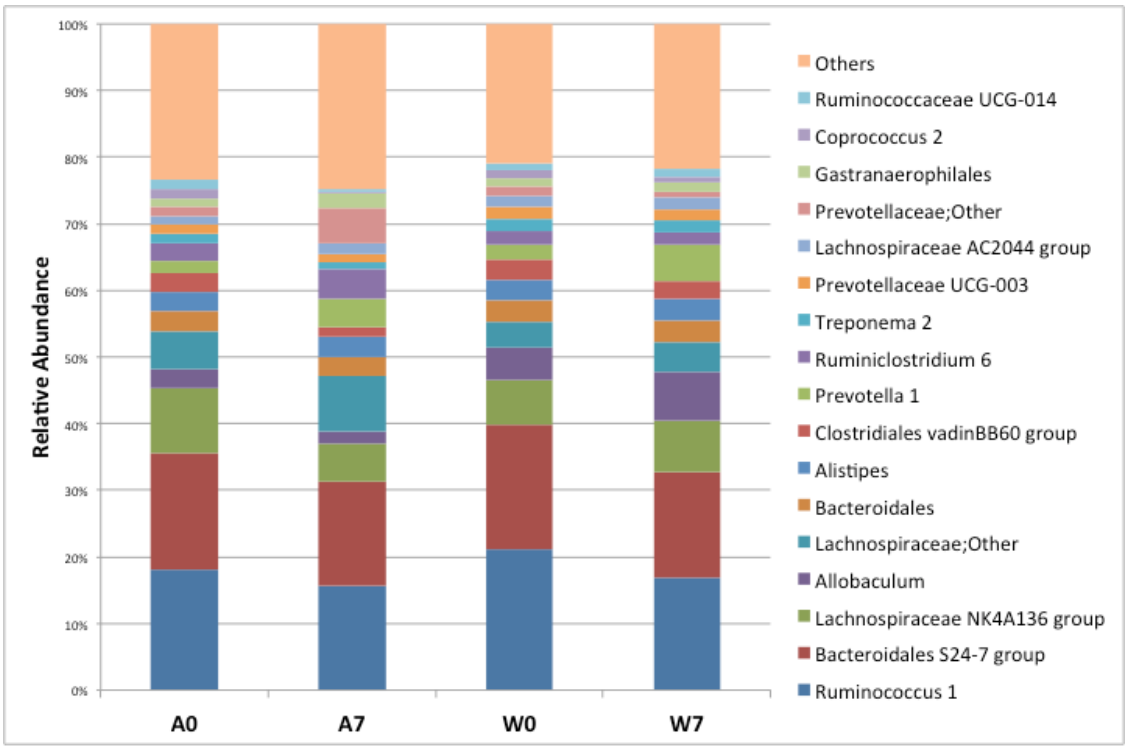


Figure 24 Relative abundance of bacterial genera in the fecal microbiota from neomycin treated and control animals. The genera Lachnospiraceae;other, Ruminiclostridium 6, Prevotellaceae;other, and Gastranaerophilales increased. The genera Allobacterium, Clostridiales vadin BB60 group, Coprococcus 2, and Ruminococcaceae UCG-014 decreased after seven days of antibiotic treatment. A0= Neomycin day 0; A7= Neomycin day 7; W0= Water day 0; W7=Water day 7.

Figure 25

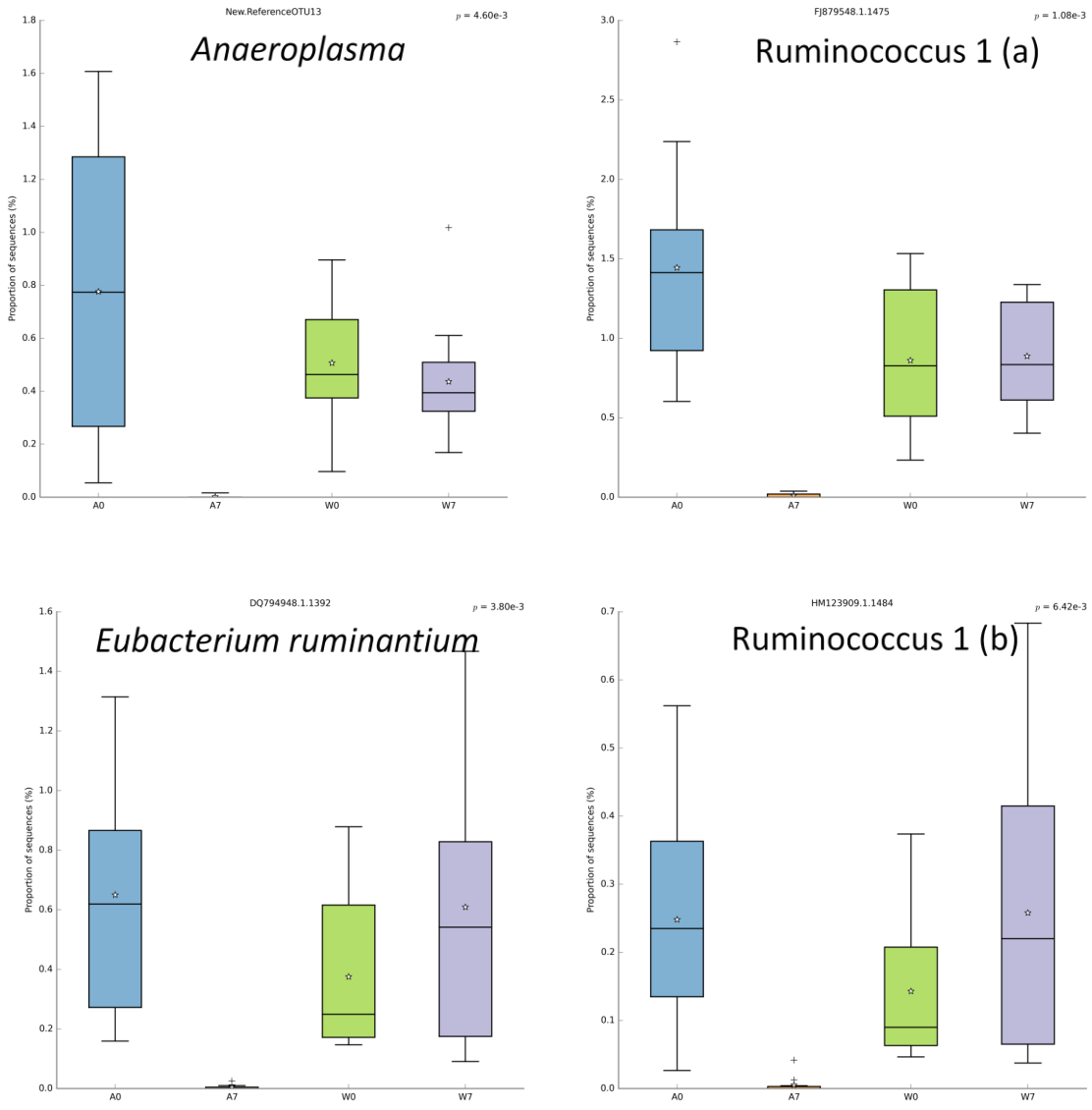


Figure 25 Boxplots depicting OTUs with significant decreases in relative abundance in the fecal microbiota from neomycin treated animals. Examples of taxa with reduced abundances are shown. The OTU sequence identifiers following OTU picking in QIIME are indicated (New.ReferenceOTU13, DQ794948.1.1392, FJ879548.1.1475, HM123909.1.1484) with their respective taxonomic assignments. The letters a and b were randomly allocated to the two Ruminococcus 1 assignments. The indicated p values were generated by Kruskal-Wallis-H tests followed by Games-Howell post-hoc tests with Bonferroni multiple comparison correction. Plus signs (+) indicate outliers. A0= Neomycin day 0; A7= Neomycin day 7; W0= Water day 0; W7=Water day 7.

Figure 26

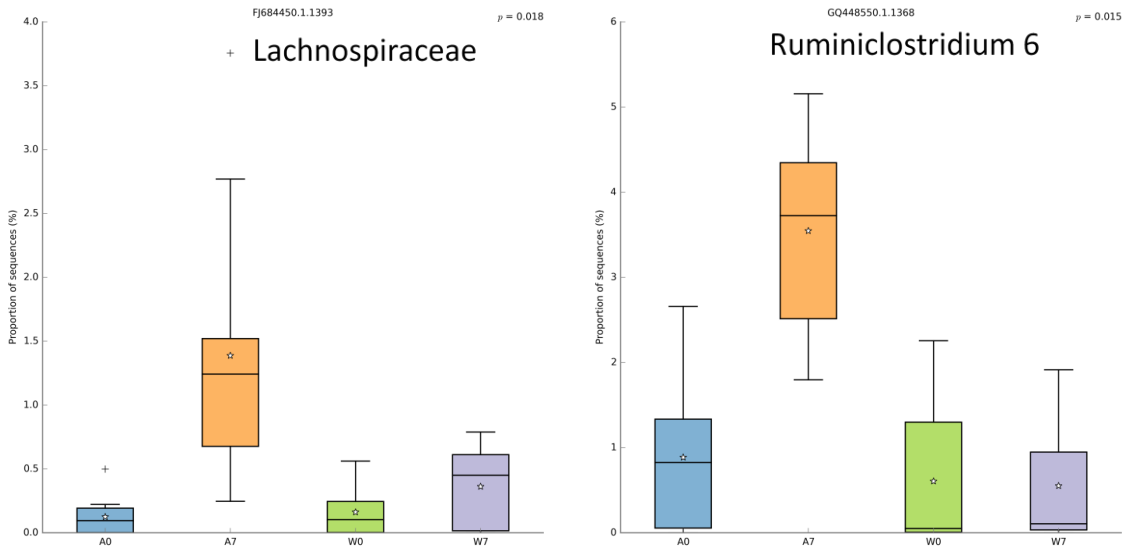
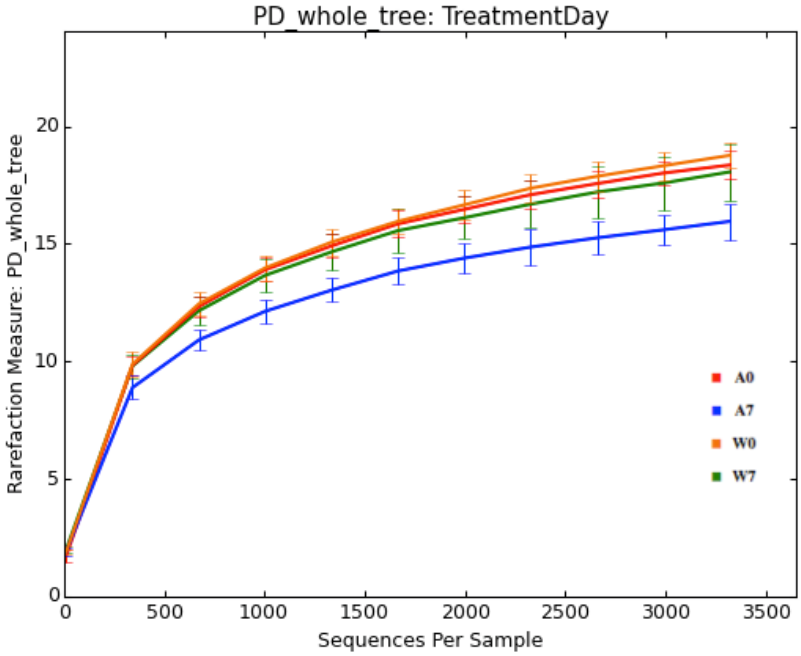


Figure 26 Boxplots depicting OTUs with significant increases in relative abundance in the fecal microbiota from neomycin treated animals. Examples of taxa with increased abundances are shown. The OTU sequence identifiers following OTU picking in QIIME are indicated (FJ684450.1.1393, GQ448550.1.1368) with their respective taxonomic assignments. The indicated p values were generated by Kruskal-Wallis-H tests followed by Games-Howell post-hoc tests with Bonferroni multiple comparison correction. Plus signs (+) indicate outliers. A0= Neomycin day 0; A7= Neomycin day 7; W0= Water day 0; W7=Water day 7.

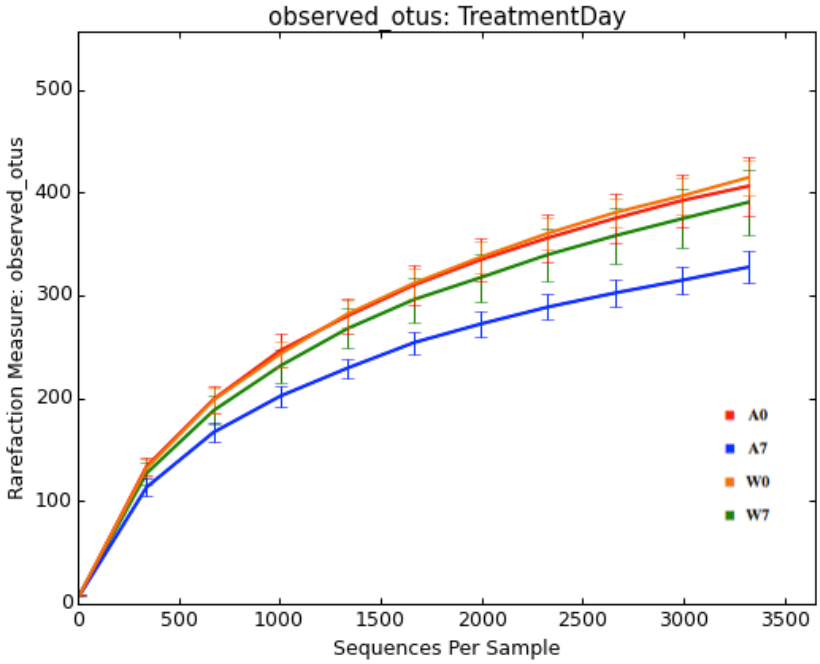
Analysis of alpha diversity rarefaction curves following even sampling (to 3326 reads/sample) of the individual sample OTU tables indicated reduced microbial diversity in the neomycin-treated group, both in the phylogenetic diversity (PD_whole_tree) and number of OTUs (observed-OTUs) metrics. The rarefaction curves also indicated that the overall sequencing depth was sufficient for analysis (Figure 27 A,B). Boxplots for phylogenetic diversity and the non-phylogenetic observed OTUs metrics confirmed that the differences in the neomycin-treated group mean from the means of all the other groups were significant after non-parametric t-tests with 999 Monte Carlo permutations and Bonferroni correction (Figure 27 C,D).

Figure 27 (A-D, 2 pages)

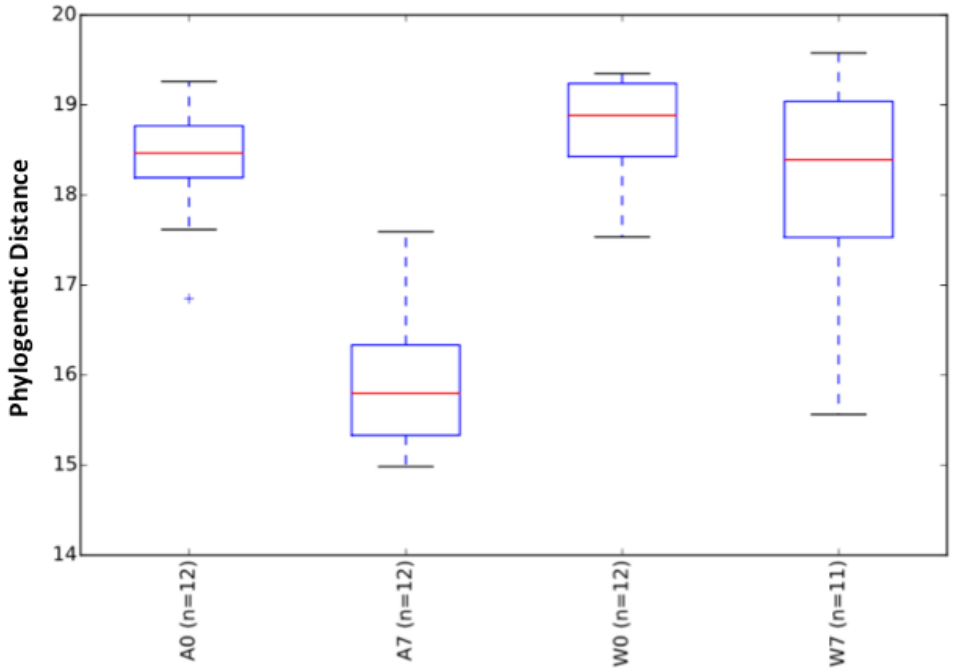
A.



B.



C.



D.

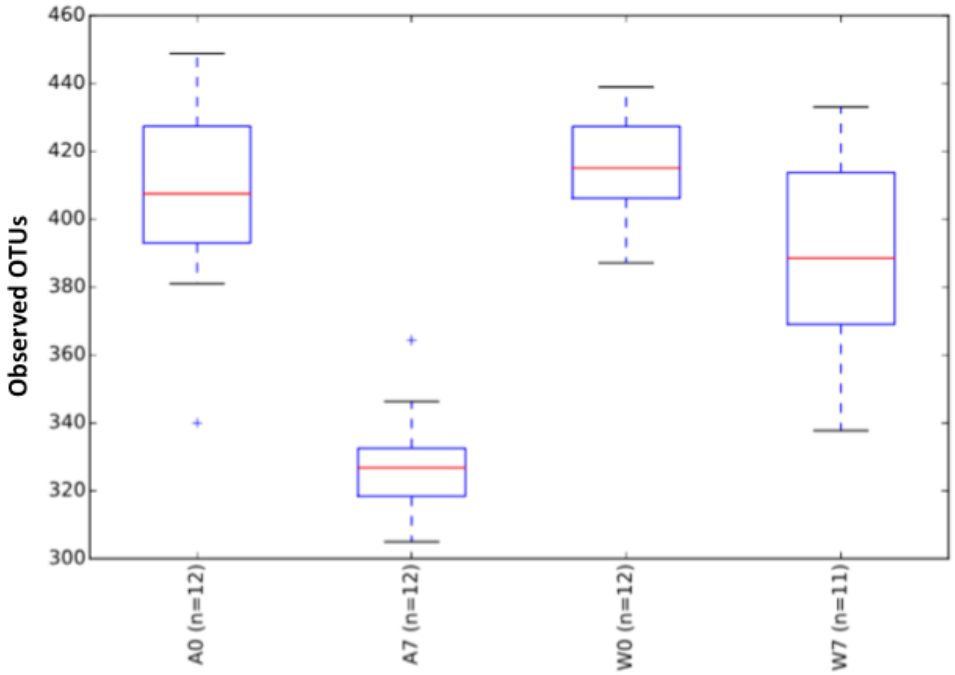


Figure 27. Alpha diversity analyses. Refraction curves of the phylogenetic diversity (**A**) and OTUs (**B**) of experimental and control groups revealed reduced microbial diversity in the neomycin treated group (A7). Boxplots for phylogenetic diversity (**C)** and OTUs numbers **(D)** show significant differences in the neomycin treated group (A7) from all the other groups. A0= Neomycin day 0; A7= Neomycin day 7; W0= Water day 0; W7=Water day 7. n=11-12 per group.

Analysis of the beta diversity using principal coordinate analyses of unweighted and weighted phylogenetic UniFrac distances (after random subsampling to even numbers of sequences, i.e. 3326 reads/sample) reveals separation of the neomycin treatment group at day 7 from the other three groups (neomycin day 0; water day 0; water day7) – see Figure 28.

Figure 28

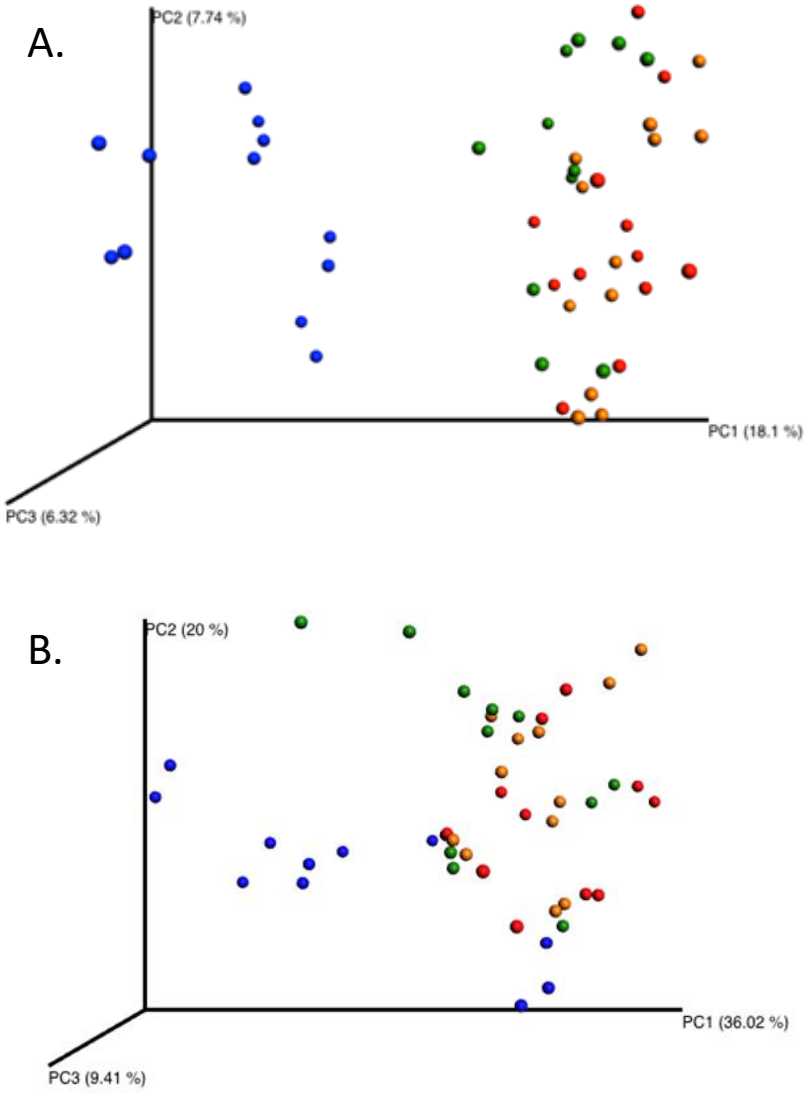


Figure 28. Beta diversity analyses. The principal coordinate analyses of unweighted (A) and weighted (B) phylogenetic UniFrac distances (after random subsampling to even numbers of sequences) reveals separation of the neomycin treatment group at day 7 (blue spheres=neomycin day 7) from the other three groups (red spheres = neomycin day 0; yellow spheres = water day 0; green spheres = water day7). Sample number n=11-12 per group.

4. Discussion

In previous studies of behavior in animal models, it has been found that perturbing the intestinal microbiota could alter behavioral phenotype (Cryan and Dinan, 2012). More specifically in mice models, this critical window appeared to occur between three and ten weeks of age (Foster and McVey Neufeld, 2013). Prairie voles have been extensively studied because of their highly social characteristics that are similar to humans; thus, the following question can be asked: "Does the microbiota composition shift induced by neomycin alter prairie vole behavior?" In order to answer this question, I sequenced the fecal microbiome before and after treatment and utilized the elevated plus maze and forced swim tests to evaluate changes in behavior.

The elevated plus maze was used to test if changes in the intestinal microbiota influence the prairie vole's anxiety-related behavior (Walf and Frye, 2007). For this analysis, I used the time in the open arms of the elevated plus maze, the percentage of time spent in the open arms, and the percentage of open/total arm time as an anxiety-index. The prairie voles that received neomycin treatment for seven days displayed similar behavior as the water control group. Additionally, the total distance traveled was measured to examine if there was a difference in locomotor activity. These data indicated no significant differences between the groups. Therefore, these results suggest that the intestinal microbiota shift induced by neomycin does not alter the microbiota to a composition that is correlated with increased anxiety-like behavior.

The forced swim test was used to further evaluate the prairie vole behavior. The forced swim test is used to evaluate the depressive-like state in mice (Can et al., 2012) and prairie voles (Grippo et al., 2008). I measured the time spent mobile and the latency to immobilization in an attempt to understand if shifting the intestinal microbiota would influence prairie vole behavior. Voles that received neomycin treatment for seven days had an increased time spent immobile as well as a faster latency to immobilization. Both of these features are characterized as depression-like

behaviors in rodents (Chen et al., 2015). These results demonstrate that microbiota may play an important role in behavior and that microbiota composition shifts induced by neomycin can induce a depressive-like behavioral phenotype in prairie voles. These findings not only support the hypothesis that shifts in intestinal microbiota can alter prairie vole behavior, but also support another recently published paper reporting that the absence of gut microbiota induces depression-like behavior in mice (Zheng et al., 2016). The current study supports the importance of the intestinal microbiota in behavioral health.

To further evaluate the shifts in microbial composition after neomycin administration, the bacterial 16S rRNA gene region V4 was sequenced. These next-generation sequencing results showed that some microbial communities increase while others decrease. Of interest is the fact that the Lactobacillales relative abundance decreased only in the neomycin group. This is an interesting finding due to the fact that lactic acid bacteria have been shown to be important in reducing anxiety (Rao et al., 2009) and depression (Steenbergen et al., 2015) in humans. Overall, neomycin treatment reduced the diversity of the intestinal microbial communities. This loss of diversity may have played a role in the behavioral changes observed.

It is important to recognize that antibiotics are one of the most powerful interventions to effectively inhibit the growth or kill bacteria that cause infections and disease (Prevention, 2008). However, with recent studies showing that antibiotics are prescribed in nearly 101-million adult ambulatory visits annually, with broad-spectrum antibiotics being the most common (Shapiro et al., 2014), caution should be exercised when prescribing broad-spectrum antibiotics. Especially broad-spectrum antibiotics have been shown to create an imbalance in microbial populations, which could lead to dysbiosis (de Vrieze, 2013; Flak et al., 2013; Gentschew and Ferguson, 2012; Lawrence et al., 2012; Thomas, 1993). My results indicate the intestinal dysbiosis is correlated with depression-like behavior. Therefore, a need exists for future, more detailed studies on the

effects of antibiotics on the microbiota-gut-brain axis, as well as investigation of alternative medications and therapeutic options for patients.

Most importantly, future studies should be conducted to investigate the prairie vole behavior after shifts in the microbiota induced by other antibiotics. The current study introduces a novel model that could be used to study how the gut microbiota is integrated with behavior via the microbiota-gut-brain axis. Future studies of neomycin absorption could be beneficial to address any concerns for systemic side effects.

5. Conclusion

No previous studies have been conducted in prairie voles to assess the role of intestinal microbiota on behavior. Taking advantage of antibiotics as a tool to disrupt and induce compositional shifts in the intestinal microbiota, I have shown that neomycin reduces the diversity of the intestinal microbiota, which appear to play a role in the behavioral health of prairie voles. More importantly, the data indicate that shifting the intestinal microbiota using neomycin induces a depressive-like behavioral phenotype in the prairie voles. The results indicating that lactic acid bacilli are depleted produces a starting point to identifying microbes that maybe be very important in behavioral alterations. These results help to validate the prairie vole as a model for the study of microbiota-gut-brain axis. Future studies examining brain tissue, and utilizing other broad-spectrum antibiotics would help to identify the role of specific microbes in determining behavior.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Discussion

The goals of this study were to gain deeper understanding of how shifting the intestinal microbiota composition affects the host and to evaluate the prairie vole as a novel model to study the influences of the intestinal microbiota on host behavior (microbiota-gut-brain axis). Chapter II, III, and IV addressed necessary components to establish the prairie vole as a model for the study of the microbiota-gut-brain-behavior axis.

Various studies have shown that the intestinal microbiota is important to the function of the central nervous system (CNS) (Clarke et al., 2013; Diaz Heijtz, 2016; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004), for example, by influencing the risk of anxiety and mood depressive-like behaviors (Foster and McVey Neufeld, 2013). Data suggest that the microbiota can modulate CNS connections that are related to behavior during a critical window in adolescence (Foster and McVey Neufeld, 2013). In order to study the effects of neomycin-induced dysbiosis, I needed to know at what age the prairie vole intestinal barrier matured. This information was crucial because I did not want to administer the non-absorbable antibiotic orally to adolescent voles with an immature intestinal barrier due to the associated risks neomycin entering the systemic circulation.

Previous studies in mice have shown that this maturation process occurs between two and three weeks of age (Patel et al., 2012). To determine the age at which the intestinal epithelial barrier matures in prairie voles, I studied the intestinal permeability of 4 kDa FITC-dextran *in vivo* of four age groups; two weeks, three weeks, four weeks, and adult (Chapter II; (Supeck et al., 2018)). It was found that two-week-old prairie voles exhibited significantly higher intestinal permeability for 4 kDa FITC-dextran molecules as determined by the concentration of the fluorescent marker in the sera. The results indicate that the two-week-old voles had a more immature epithelium with higher permeability than their older conspecifics. The levels of 4 kDa FITC-dextran molecules in the sera significantly decreased at three weeks of age, followed by a slight but not significant decrease at four weeks and adulthood. These results provided evidence that the intestinal barrier was tightening by three weeks of age. Thus, postnatal maturation of the intestinal epithelium barrier in this rodent species likely contributes to the decrease in intestinal permeability.

Intestinal barrier maturation can be further studied by examining the tight junctions between adjacent epithelial cells since they form the major paracellular barrier in the intestinal epithelium (Mitic et al., 2000). To better understand the tightening of the tight junctions, I extended the study by evaluating the gene expression of tight junction-associated proteins (Chapter II; (Supeck et al., 2018)). I found that claudin-3, claudin-4, zonula occludens-1, and occludin were the most upregulated among the investigated genes during this important period of development. Furthermore, the upregulation occurred simultaneously with the decrease in permeability of the intestinal barrier in three-week-old voles. These results provided further evidence that the intestinal barrier matured by three weeks of age. They indicate that the tight junctions-associated gene expression in the intestinal epithelium is affected by the age of the animal and contributes to the selectivity of the paracellular pathway.

The intestine can be further evaluated via morphology and ultrastructure. It is accepted that the intestinal morphology changes drastically after birth, with an increase in size, length, and depth of the colon crypts, and the increase in goblet cells present in the colon. I conducted light microscopy and transmission electron microscopy to further evaluate the architecture of the colon (see Chapter II; (Supeck et al., 2018)). The architecture of the vole colon at three weeks of age shows a mature epithelium with a significantly longer and wider crypts, predominant number of goblet cells, and intact tight junction structure. Therefore, the data supported my previous findings, which indicate that the intestinal barrier maturation occurs by three weeks postpartum in the prairie vole.

In order to study the microbiota's influence on behavior, a strategy is needed to perturb the microbiota without damaging the intestinal permeability. There are five strategies that have been used: germ-free animals, introducing pathogenic bacteria, fecal transplantation, using probiotic microbes, or administering antibiotics (Cryan and Dinan, 2012). The first strategy, use of germ-free animals, is not available in the prairie vole species, and the OSU-CHS Institutional Animal Care and Use Committee did not approve creating such a model. The second strategy uses infection studies with pathogenic bacteria to assess their effects on behavior. This method requires knowledge of bacteria that are pathogenic to the prairie vole and establishment of appropriate infection models, which currently do not exist. The third strategy uses transplantation of human fecal samples in the prairie vole to "humanize the animal" or vole to vole fecal transplants. However, currently there are no studies involving the microbiota-gut-brain axis in the vole and this would be more advantageous for follow-up studies once proof-of-concept has been established. The fourth strategy is uses probiotics. While this could be a powerful approach, it requires knowledge of what microbes are probiotic in prairie voles. The fifth strategy uses antibiotics. This method shifts the microbiota composition in a controlled manner to assess the role of the microbiota. Different antibiotics can be used to induce different changes in the

composition depending on the antibiotic's spectrum activity. I utilized oral administration of the antibiotic neomycin, as described in detail in Supeck Chapter III.

Antibiotic treatment is generally anticipated to change the bacterial composition and diversity of the microbiome. However, the effects of antibiotics on intestinal bacterial communities and intestinal epithelial barrier have not been studied in prairie voles. Obtaining this crucial information was the next step needed for gut-brain axis studies in prairie voles. One hurdle in studying the intestinal microbiota composition comes with culturing the bacteria. Limitations exist with growing bacteria on various media. This method does not give a good representation of the true microbial composition, only a small fraction of the diversity is identified (Stewart, 2012). Therefore, I used methods that are culture-independent, rapid, and sensitive, called Next Generation Sequencing (NGS) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis. T-RFLP based on the bacterial 16S rRNA gene is commonly used as a tool to evaluate intestinal bacterial community after antibiotic induced alterations in mice (Wlodarska et al., 2011) and humans (Jernberg et al., 2005).

Chapter III describes the use of fecal pellets evaluating the depletion/shifts in microbial population in the vole gut induced by neomycin. This is a non-invasive approach that allows for repeated sampling during treatment with the drawback being that it does not allow for a thorough analysis of bacteria that adhere to the mucosa (Reikvam et al., 2011). I observed that administrating neomycin orally for seven days had a major effect on microbiota composition as shown by principle component analysis (PCA). Furthermore, neomycin shifted the bacterial communities toward a similar composition, indicating a loss of microbiota diversity. These results align with what I anticipated, since neomycin has broad-spectrum activity that is effective against gram-negative and gram-positive bacteria. Furthermore, the shifts in the intestinal microbiota did not alter the intestinal permeability as measured by serum 4 kDa FITC-dextran molecules, thus indicating an intact intestinal barrier. Results reported in Chapter III provide the information

needed to support the use of neomycin as a tool to perturb the intestinal microbiota allowing assessment of the microbiota influence on animal behavior (microbiota-gut-brain axis).

Thus far, studies reported in Chapter II and III built a foundation that was needed to be able to study the gut-brain axis in prairie voles. In Chapter IV, I wanted to evaluate the role of the microbiota on prairie vole behavior. I used female prairie voles that were three weeks old, since human females are twice as likely to have anxiety and/or depression (Gater et al., 1998; Kessler et al., 2005b; Kessler et al., 1994) and since I previously showed that the intestinal barrier was mature at that age. I utilized the non-absorbable antibiotic neomycin as a tool to disrupt the intestinal microbiota composition.

When using the Elevated Plus Maze, it was observed that microbiota composition shifts induced by neomycin did not alter prairie vole anxiety-like behavior. However, the Forced Swim test results indicate that microbiota play an important role in behavior and that microbiota composition shifts induced by neomycin can induce a depressive-like behavioral phenotype in prairie voles. Additionally, the total distance traveled was measured to examine if there was a difference in locomotor activity, which indicated no significant differences between the groups. These findings not only support my hypothesis that shifts in intestinal microbiota can alter prairie vole behavior, but also confirm the recently published report by (Zheng et al., 2016) that the absence of gut microbiota induces depression-like behavior in mice. My studies also indicate that the prairie vole is an excellent model to study the microbiota-gut-brain axis. My approach is the only study in prairie voles and one of the few studies that demonstrates the role of microbiota in depressive-like behavior. Compared to the studies in mice, the prairie vole provides a behavioral model that is more similar to humans.

Limitations of the Study

The research limitations are more prevalent in the prairie vole than in other rodent models such as mice and rats. The prairie vole is unique in that it requires United States Department of Agriculture (USDA) approval and inspection of the animals. This creates additional cost and paperwork for someone interested in starting a colony. The available antibodies designed for the prairie vole are very limited. I conducted experiments to find human, mice and rat antibodies that would cross-react with the prairie vole intestinal cells. I was unable to find any antibodies that would cross-react or that were available to purchase. The possibility of designing antibodies exist, however this can be very time consuming and expensive to create eight different antibodies. During the intestinal permeability experiments, timing was very important with multiple steps in the process. Therefore, there is a limit to the number of prairie voles that can be tested each day. Utilizing the T-RFLP analyses was a cost effective method. However, it did not provide any information about microbial taxa. I chose to utilize the next generation sequencing to determine this information, which is a more expensive test and requires very time consuming analysis of the data. The Elevated Plus Maze and Forced Swim Test both were constricted to a single experiment on each animal. This is because after the animal is tested one time on the apparatus then they can learn from that and change how they would behave on follow up experiments. Finally, the experiments were conducted in prairie voles and therefore assumptions about the gut microbiota and behavior would have to be further characterized in humans.

Summary of the Study

Age is an important factor when examining the function of the intestinal barrier. In Chapter II, results are reported that show decreased absorption of FITC-dextran at three weeks old, demonstrating decrease in intestinal permeability (Supeck et al., 2018). These permeability

changes can best be explained by testing for the tightening of the tight junctions in the prairie vole intestinal epithelium. Through the isolation of colon epithelial cells from the prairie voles, gene expression study of tight-junction-associated genes revealed increased gene expression of claudin-3, claudin-4, zonula occludens-1, and occludin in three-week-old voles compared to two-week-old voles. These data support my study demonstrating that at three weeks old the tight junction structure is present on ultrastructural micrographs. More importantly, my data indicates that intestinal maturation occurs by three weeks of life. Therefore, it is important to use adolescent prairie voles that are at least three weeks of age in the studies reported here.

Chapter III reports on studies that further explored the changes in the intestinal microbiota composition and intestinal barrier function induced by oral administration of neomycin. These changes can best be explained by the activity of neomycin and the fact that it does not absorb systemically through the intestinal barrier. Neomycin induced major shifts in the intestinal microbial composition without an effect on the intestinal permeability of the epithelial barrier. Through my gene expression study of tight-junction-associated genes in colon epithelial cells, I determined that claudin-3 could be important to the maintenance of the intestinal barrier. Additionally, oral administration of neomycin proved to shift the microbial composition without increasing the intestinal permeability, supporting the hypothesis that this antibiotic it is a good choice for use in studies designed to increase the understanding of the role that microbiota may play in the gut-brain axis.

Chapter IV reports on the studies that utilized the knowledge from Chapters II and III to evaluate the role of the microbiota on behavior. This study showed that the intestinal microbiota is critical to the behavioral health of prairie voles. More importantly, these data indicate that shifting the composition of the intestinal microbiota using neomycin induces a depressive-like behavioral phenotype in the prairie voles. It also helps to validate the establishment of the prairie vole as a

model for the study of the microbiota-gut-brain axis. Additionally, more studies are needed to elucidate the specific bacterial families and species involved.

Future directions

The main future research direction should continue the investigation in the prairie vole to elucidate the connections between the microbiota and behavior. No other studies have used the prairie vole to explore how microbiota can influence behavior (microbiota-gut-brain axis). It would be beneficial to take a closer look at the microbial composition in neomycin treated voles versus the water control voles, as well as compare the microbiota composition before neomycin treatment and after the treatment within the same animal. Furthermore, it should be considered to examine brain tissue to see if it affects physiological functions or gene expression.

Detailed analysis of the microbiota composition using other antibiotics is another area that can be explored. For such a study, it would be ideal to test antibiotics with different spectrum activities to affect different microbial populations. It would be important to choose one that does not absorb well and analyze if it alters the barrier permeability, because some are systemically toxic if absorbed or may have an unwanted or unknown side effect.

Administration of probiotics is an additional approach that can be used to assess the bacterial effects on behavior. It could be utilized as a tool to perturb the microbiota or used after the administration of the antibiotic to see if it can decrease the depressive-like phenotype. This approach will ultimately help to identify bacteria that are beneficial to prairie vole health.

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VITA

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