5-2022

Gastrointestinal Alterations in Two Mouse Models Associated with Social Behavior Deficits

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By

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An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
Fine and Performing Arts Scholars Program
Honors College
and the
Department of Public Health
East Tennessee State University

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ABSTRACT

Gastrointestinal alterations in two mouse models that are associated with social behavior deficits

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The gastrointestinal (GI) tract is a diverse habitat for multiple microorganisms. Disturbances in the microbiome of the GI tract have been associated with psychiatric disorders including autism spectrum disorder (ASD). Individuals with ASD, when compared to neurotypical individuals, have demonstrated differing gut species. Also, it has been shown that microbial transplant therapies impact ASD symptoms in patients. Animal models of behaviors associated with ASD might offer insight for the actual role these microbial differences may occupy regarding symptoms. Unfortunately, ASD does not have an accepted animal model where the GI alterations have been thoroughly explored. In this study, we sought to determine if the microbiome and other GI alterations were observed in two potential mouse models of social behavior deficits, the genetic BTBR T+Itpr3tf/J (BTBR) mouse strain and an environmental mouse strain consisting of offspring of valproic acid (VA) treated pregnant controls. Both mouse models have been shown to exhibit social and repetitive behaviors that are found in human ASD. Using the Illumina MiSeq, we were able to identify taxonomy associated with 16S ribosomal DNA sequences extracted from fecal matter. We were able to compare the sequencing results from the two affected strains and a control C5BL/6J mouse strain for both female and male animals using the Qiagen CLC Genomics Workbench software. Overall, microbiome composition was found to be significantly different between the male control animals (N=6)

when compared to the VA (N=5; p-value=.00216; F-score 11.20904) or the BTBR (N=7; p-value=.00216; F-score 18.47839) males using a PERMANOVA analysis. This was replicated in female groups where composition significantly differed between the control (N=14) and VA (N=14; p-value=.00001; F-score 3.53307) or BTBR (N=14; p-value=.00001; F-score 11.23443) females. Additionally, short-chain fatty acid analysis using gas capillary-based chromatography was used to examine acetate, butyrate, propionate, and valerate levels in feces. Only valerate levels were significantly lower ($p<.05$) in BTBR males (N=18) when compared to control males (N=13). In females, both acetate ($p<.05$) and propionate ($p<.01$) were significantly decreased in BTBR females (N=13) when compared to control female mice (N=14). Additionally, we compared the thickness of the intestinal mucosal and muscular layers in the three groups for males and females. Across all three groups, neither male nor female showed notable significant differences in the thickness of either the muscular or mucosal layers. This data will be useful in continuing to identify the complex pathologies present in potential animal models used for research into social deficit disorders.

ACKNOWLEDGEMENTS

I would like to extend my sincerest gratitude to a multitude of people who made this study possible.

To begin, I would like to thank my research advisor Dr. Michelle Chandley for guiding me through this process, being exceedingly patient with me, and welcoming me with open arms into her laboratory. Funding for the work was provided by a major grant award from the RDC major grant program at ETSU as well as the National Institutes of Health R-15 award.

A special thank you to everyone who was of assistance through the process of my research---Dr. W. Andy Clark, Lindsey Stallworth, Evan Mason for their help on gas chromatography analysis and DNA purification.

A thank you to Dr. Ranjan Chakraborty for being gracious and patient by agreeing to be my second reader.

Thank you to ETSU College of Public Health, Quillen College of Medicine, and the Molecular Biology Core Facility for allowing me to use their facilities to complete this work.

And a thank you to my Honors advisor Dr. Scott Koterbay of the FPA Program who has stuck by me every step of the way for the last four years.

And, finally, a thank you to the Lord for giving me the wisdom, courage, and will to complete this monumental accomplishment.

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INTRODUCTION

Autism Spectrum Disorder Diagnosis

Autism Spectrum Disorder (ASD) is a neurological disease that is characterized by deficits in social behaviors as well as relative behaviors. These behaviors include but are not limited to lack of eye contact, inability to control and distinguish emotions, and overall activity restrictions (Park, 2016). ASD is commonly diagnosed at approximately three years of age and has a four to one ratio in the diagnosis of males to females (de Giambattista, 2021). This disorder can only be identified by behavioral deficiencies. Currently, there is no treatment exclusively for ASD symptoms. Most likely the disorder is affected by countless variables including a genetic etiology; however, it is possible that environmental factors may influence the development. Biomarkers associated with ASD have not been identified, but physiological markers such as microbiota profiles and metabolic function markers are being explored (Peralta-Marzal, 2021). The identification of specific biomarkers and the etiology of the disorder are lacking in the ASD literature (Peralta-Marzal, 2021).

ASD Brain Pathology

The brain pathology of ASD is an essential part of research efforts, but has not been completely characterized. Neuropathological abnormalities between neurotypical and ASD individuals have been observed in both cortical and non-cortical regions of the brain. Early studies like one done in 2015 indicated that ASD has an increased head circumference and total brain volume (TBV) when compared to typically developing subjects by use of MRI. The same study used twenty-seven ASD patients ranging from one to thirty-eight years of age to find increased head circumference in both males and females. Similarly, increased TBV was found using forty-four males and females ages two to forty years old with a structural MRI scan

(Sacco, 2015). In addition, increases in both gray and white matter have been shown in children with ASD in a cohort study using twenty-seven subjects of eighteen males and nine females ranging from four to sixty-nine years of age (Wegiel, 2010). Disruption in neuronal signaling during brain maturation likely plays a key role in the pathophysiology of ASD. Interestingly, clinical symptoms and traits have been linked to disruptions in unique brain areas in ASD. For instance, brain areas associated with verbal communication, such as the Broca and Wernicke's areas, demonstrated less brain activity in ASD children when compared to neurotypical children in a 2008 study of thirty-five subjects (twenty-nine males and seventeen females) ranging from two to three years old using functional magnetic resonance imaging (fMRI) (Redcay, 2008).

Gastrointestinal Disorders in ASD

Gastrointestinal (GI) alterations are associated with ASD. Studies have shown that it is not uncommon for children with ASD to have GI inflammation, gut microbiota variation, and occasional gut infections (Ristori , 2019). Dysbiosis is a notable GI disorder associated with ASD (Mangolia, 2021). Dysbiosis has been coined as a term describing the disturbance in the gut microbiota equilibrium caused by an imbalance in the microbial population, metabolic, composition or distribution change (DeGruttola, 2016) Dysbiosis can consequently lead to increased permeability of the intestinal tract (Ristori, 2019). Another disorder closely linked to dysbiosis is irritable bowel syndrome (IBS). A 2017 study using sixty adult subjects (fifty-four males and ninety-five females) found that IBS symptom severity was associated with dysbiosis and intestinal permeability (Tap, 2017). Symptoms associated with IBS such as inflammatory systemic responses as a result of increased permeability have been found in ASD individuals (Ristori, 2019). It seems likely that intestinal permeability deficits are associated with ASD. It is tempting to speculate that bacterial products are able to travel across the gut barrier into the

bloodstream and past the blood-brain-barrier (BBB). Thus, increased permeability is perhaps a noteworthy event in ASD.

Gut-Brain Axis

The association of gut disorders in the neurodevelopmental disorder ASD has prompted investigators to contemplate the relationship between the digestive and central nervous systems. The gut-brain axis can be described as the intimate connection between the GI system and the central nervous system (CNS). The brain and the GI system are able to communicate and influence one another. They can be mediated by a plethora of pathways that are neural, endocrine, immune, and metabolic in nature and are not fully understood (Mörkl, 2020). These bacteria and other metabolites are able to pass over the BBB due to its permeability and can contribute to the CNS function and disease (Rutsch, 2020).

In psychiatric disorders, the gut-brain axis can be disrupted by dysbiosis in the gut in terms of altered permeability of the BBB and neuroinflammation. These events can impact the central nervous system's (CNS) homeostasis by changes in inflammation. Inflammation in the brain can occur due to stress that is accompanied by the presence of a different bacterial profile in the gut and the of bacteria producing inflammatory metabolites (Rutsch, 2020). The alteration of bacterial species in the gut and related products can directly affect individual behaviors. It has been shown that ninety percent of serotonin required for mood, behavior, sleep, and several other functions within the CNS and gastrointestinal (GI) tract is produced in the gut (Rutsch, 2020). A disruption of CNS blood-brain barrier permeability and inflammation can be related to the gut. Additionally, it has been shown that pathogens and aberrant molecules are able to enter the CNS via the blood-brain barrier (Osadchiy, 2019). Consequently, it seems that gut microbial

alterations and increased BBB permeability could be involved in the pathophysiology of psychiatric and neurodevelopmental disorders.

Various therapeutic research has begun to identify the outcomes of modifying the gut-brain axis. For instance, probiotics have been found to diversify the microorganisms available in the gut for transportation. And in a similar case, prebiotics have been shown to lead to modulation in brain function and behavior as well (Mörkl, 2020). It is thought that diet can clinically improve the gut microbial function in adults by individualized treatments following dysbiosis specificity (Osadchiy, 2019). Personal therapies of probiotics, prebiotics, and diet are hypothesized as an effective way to modulate a disrupted gut-brain axis as a result of dysbiosis (Osadchiy, 2019). The human body houses trillions of cells that contribute to the benefit and detriment of human life. The gut holds the densest amount of these microbial cells (Milani, 2017). Over 70% of the microorganisms making up the gut microbiome come from the colon (Hold, 2002). The gut microbiome is influenced by factors including genetics, environment, and individual nutrition (Mukherjee, 2018). Per the Human Microbiome Project, a healthy gut is defined as "a core set of microbial taxa universally present in healthy individuals who lack overt disease phenotypes under the hypothesis that the absence of such microbes would indicate dysbiosis" (Redondo-Useros, 2020).

Gut Microbiota Alterations in ASD

In addition to cognitive behaviors and functions, individuals on the ASD spectrum have been observed to have an altered gut microbiome when compared to the microbiome of neurotypical individuals. Apart from overall contrasting microbiomes, the most notable difference in the quantities of SCFAs found in the gut that are able to cross the BBB into the CNS (Macfabe, 2012). There is interest in the possibility that altering GI tract factors in terms of

microbiota could improve ASD symptoms. This correlation can be seen in a study using microbiota transfer therapy (MTT) in eighteen children seven to eight years old. The results indicate that this 10-week antibiotic treatment improved GI factors and ASD symptoms on average (Kang, 2017). Further understanding and acknowledgement of the importance of the gut microbiota and its effect on the brain is an important element in understanding ASD.

Various analyses of fecal matter, serum, and urine between neurotypical controls and ASD individuals have resulted in comparable differences in terms of what molecules are present within the microbiome. (Sharon, 2019). The gut serves to be an impactful conduit in other disorders such as obesity, IBS, allergies, and other non-communicable diseases (NCD) as shown in a 2015 open study (West, 2015). The specificity of the microbes present in individuals are 1/3 similar in all individuals and 2/3 specific in every individual almost like a microbial fingerprint.(Salami M. 2021). A study done in 2015 sequenced 242 individual DNA and found distinct differences in personal microbiota (Franzosa, 2015). Thus, the differences in the gut of a neurotypical individual and an ASD individual are vital to understand, as an imbalance may lead to behaviors of the disorder (Grace Lucas, 2018).

Role of Metabolites

Venegas defines short-chain fatty acids (SCFAs) as “carboxylic acids with aliphatic tails of 1-6 carbons...produced by anaerobic fermentation of dietary fibers in the intestine” (Venegas, 2019). They are significant metabolites that can have positive effects on the intestines and can affect processes such as gene expression, cell proliferation and differentiation. SCFAs act as important energy and signaling molecules that contribute to an individual’s overall health by improving metabolism and glycemic control (Koh, 2016). Though not fully understood, SCFAs may hold a key role in regulating the gut-brain axis. In recent studies, there have been

differentiations between the gut microbiota in association with SCFAs analyzed in fecal matter of ASD individuals in comparison to neurotypical A 2019 study's results showed that between fifty 3-6 year old children, there was a significant decrease in acetate and significantly high increase in valerate in the ASD children observed (Liu, 2019).

Notable SCFAs associated with impacts on human health include acetate, propionate, butyrate, and valerate. Acetate, propionate, and butyrate are the most abundant SCFAs and are most commonly used in studies when researching certain metabolites that can cross the BBB and come in intimate contact with the brain (Wenzel, 2020). Valerate, a five-carbon chain, is included in many studies as well despite a lack of abundance. Acetate, a two-carbon chain, encourages cholesterol production in the liver and has a key role in health but, additionally, neurological diseases (Lobzhanidze, 2020). It has been shown that increasing propionate, a three-carbon chain, can impact social behaviors in animal models (Frye, 2015). It also affected gastric motility, smooth muscle contractions, and dilation of intestinal mucosa in these same animals (Frye, 2015). Butyrate, a four-carbon chain, can impact the gut barrier by way of an increase in transepithelial electrical resistance (TER) and insulin permeability decrease. These factors encourage the gut function (Peng, 2007). Butyrate has been shown to elevate occludin protein expression, a transmembrane protein that is linked to decreasing and revitalizing BBB permeability (Braniste, 2014). Valerate has been shown to be elevated in young children with ASD when compared to typically developing control subjects in a study of forty-one males and nine females (Liu, 2019). Interestingly, valproate or valproic acid is commonly used as an antiseizure medicine and does seem to have an increased incidence of risk in children whose mothers were exposed during pregnancy (Ornoy, 2009). Notably, data published concerning

SCFAs is not always consistent concerning levels of propionate, acetate, butyrate, and valerate associated with ASD (Liu, 2019).

Pathology of the Digestive System in ASD

The purpose of the intestinal lumen, notably the mucosal and muscular layers, is to filter harmful and harmless stimuli (Thoo, 2019). There are four general layers to the digestive tract: the mucosa, submucosa, muscular layer, and serous layer. The mucosa is the innermost layer of the tract that lines the lumen consisting of connective tissue and smooth muscle called the epithelium. The submucosa encloses the mucosa in loose connective tissue. It contains the blood vessels, lymphatic vessels, nerves, and occasional glands (reference general structure of the digestive). The muscular layers, otherwise known as the muscular propria, aid in the transportation of food through the gut and lie just beyond the submucosa. Finally, the serosa is the very outermost layer made up of loose connective tissue whose primary role is to reduce friction. The intestinal lumen, attributed to its anatomical characteristics, is hypothesized to contribute to the gut-brain axis.

The enteric nervous system (ENS) is the nervous system related to the intestinal tract. It is a series of neurons that contribute to sensory and motor functions in the digestive tract. The ENS plays a role in intestinal homeostasis and controls processes such as gut motility, secretion, and blood flow and are able to interact with afferent/sensory neurons that travel to the CNS to relay signals (Margolis, 2016). This network is arranged in the gut in two layers: the myenteric and submucosal plexus. The myenteric plexus of muscular layers influences gut motility. The submucosal plexus is responsible for secretion and blood flow within the intestines. Although working through independent neuronal signals, the ENS has an intimate connection with the

central nervous system through autonomic regulation. Both sympathetic and parasympathetic signaling can influence ENS function.

Information associated with the parasympathetic division of the autonomic nervous system can occur between the ENS and the vagus nerve. The vagus nerve is the tenth cranial nerve whose cell bodies are housed in the brainstem and offers the most direct route for communication between the digestive tract and the brain axis. It has extensive innervation including many organs of the digestive system. The direct communication allows for the stimulation of the vagus nerve to result in the activation of neurons in the CNS by way of gut microbiota signaling. Another key CNS regulator of the gut is the sympathetic nervous system. The sympathetic trunk relays direct efferent and afferent information between the ENS to the CNS via the spinal cord (Furness, 2014). Though clear in its role as a conduit for gut-brain signaling, more research is needed to identify specific CNS to ENS contributions to the pathophysiology of neurological, neurodevelopmental, and psychiatric disorders.

The digestive system can become compromised and lead to pathogenic intestinal symptoms including diarrhea, bloating, constipation, etc. as discovered in various neuro-related disorders such as ASD, Parkinson’s disease (PD) and Alzheimer's disease (AD) (Fu, 2021, Wang, 2011). A large study in 2011 was conducted to determine the prevalence in children with autism of GI symptoms. The study found that diarrhea and constipation were significantly correlated with ASD (Wang, 2011). In Parkinson’s Disease, probiotics were successful in alleviating gastrointestinal symptoms such as bloating (Cassani, 2011). A meta-analysis of fifty-six studies concerning PD and AD found that there was indeed a relationship between these diseases and intestinal disorders (Fu, 2020). The association of digestive system alterations with neurological and neurodevelopmental disorders highlights the need to understand the actual

microbiome makeup within the gut. The pathology and microbiota mechanisms and connections are not widely understood; however, it has been observed that interruptions in the digestive system are a direct result of gut microbiota instability and lack of diversity (Lombardi, 2018).

Mouse Models of ASD

A viable tool for exploring the relationship between brain and intestinal pathophysiology of neurodevelopmental disorders is the use of animal models. However, it is noted in the literature that animal models are only accepted for study when a defined behavior or event can be identified for study. Animal models used in the study of neurodevelopmental or psychiatric disorders have been highly criticized due to the complex nature of human behavior and neuronal signaling between executive order brain areas. In ASD, animal models associated with behaviors such as repetition, social deficits, and other impairments contributing to communication are being investigated.

While no animal model has been accepted in the field of ASD study there are a few in the literature that have emerged as potential candidates. The valproic acid (VA) model injects the five-carbon SCFA valproic acid into pregnant mouse dams during mid-gestation (Kultima, 2004). Offspring from pregnant mouse dams injected with valproic acid demonstrate social behavior deficits and repetitive behaviors (Yamaguchi, 2017). Interestingly, valproic acid or valproate is commonly used as an anti-seizure medication. As mentioned previously, it was observed that exposure to valproic acid during pregnancy resulted in an increased risk of ASD in the children of these mothers (Nicolini, 2018). It was shown in a 2020 animal study that valproic acid-treated rats demonstrated neuronal apoptosis resulting in neurotransmitter alterations (Bittigau, 2002).

The BTBR strain is another model that has been used to examine pathophysiological differences associated with social behavior deficits. It is an inbred mouse strain of animals using C57BL/6J with tan mice that exhibit low sociability and repetitive grooming (McTighe, 2013). In addition, this strain exhibits abnormal immune responses (Queen, 2021). Originally, this strain was bred for use in studies concerning abdominal obesity, insulin resistance, diabetes-induced nephropathy and phenylketonuria (Queen, 2021). This model may be useful in exploring disorders with social behavior deficits. Studies have shown altered gut permeability and colon inflammation by altered cytokine patterns in adult female and male rats of BTBR when compared to wild-type control samples (Cortetti, 2017). Though there is no accepted animal model for ASD as of late, the BTBR strain is a promising one to use as the inbred strain elicits mice with deficits in social behaviors.

Hypothesis:

The study described here compared C57BL/6 wild type mice to two different animal models that have been associated with social behavioral deficits in an attempt to identify intestinal pathology. We hypothesize that differences in the intestinal makeup present in the mouse colon may include differences in gut microbiota, SCFA expression and thickness of intestinal layers between at least one of the mouse models and wild type control mice.

METHODS AND MATERIALS

Animals and Tissue Collection

Both male and female mice of C57BL/6 wild-type control mice, BTBR strain, and valproic acid-treated used in all experiments were group-housed and fed ad libitum. All mice used in the study were obtained from The Jackson Laboratories including C57BL/6J (strain #000664) and BTBR T^{+} *Itpr3*^{tf}/J (strain # 002282). For the valproic acid model, pregnant C57BL/6J dams were injected with valproic acid (600 mg/kg; #4543; Sigma Aldrich; St. Louis, MO) at gestational day 11. Females used in the study were from similar time periods, while the males were obtained for two different batch dates. All animals used in this study were aged to postnatal day 105 (P105). At P105 fecal matter was collected for approximately 14 days and stored in -80°C ultra-low freezer immediately following collection. Intestinal tissue was obtained at sacrifice and placed in 4% paraformaldehyde for 24 hours followed by storage in 70% ethanol at 4^{o}C. All animal protocols were approved by the ETSU University Committee for Animal Care (UCAC).

DNA Isolation

DNA isolations were performed using the Power Soil Pro extraction system from Qiagen (#47014 Qiagen, Germantown, Maryland). Fecal matter used for DNA purification was between 75 to 150 mg total. DNA concentrations and OD 280/260 readings were obtained using nanodrop 2200. Library construction and next-generation DNA sequencing was performed by the Genomics Core Laboratory at the University of Tennesse Genomics Center using the Illumina Miseq. The variable V3 and V4 region specific primers for the ribosomal subunit 16 were used in library construction.

Analysis of DNA sequences

All data analysis was performed using the Qiagen CLC Genomics Workbench. The DNA was trimmed and clustered into Operational Taxonomical Units (OTU) in which reads of 97% or more similarity represent a unit. These sequences were then aligned and sorted into the different taxonomy classifications using the Green Genes Data Dank. Afterwards, OTUs were analyzed to create alpha diversity plots, phylogenetic diversity analysis, beta diversity graphs, a differential abundance analysis file and a PERMONOVA of beta diversity. There were a total of 14 wild type mice, 14 BTBR mice, 14 valproic acid female samples and 13 wild type control mice, 15 BTBR, and 14 Valproic Acid male samples used for analysis.

SCFA Methodology

Approximately 500 mg of fecal sample was used for short-chain fatty acid identification of acetate, propionate, butyrate, and valerate. Freeze drying of fecal samples was performed for 24-48 hours prior to analysis. The procedure for identifying SCFA acids using gas chromatography was adapted from a previously described protocol (Schwiertz 2010). In brief, approximately 80 mg of freeze sample remained after freeze drying and was added to 1 mL of SCFA solution that contained oxalic acid (0.1 mol/L), sodium azide (40 mmol/L) and caproic acid (0.1 mmol/L). Tubes was placed on shaker for one hour followed by centrifugation at 4,000 rpm for 20 minutes. Supernatant was removed and centrifuged again at 12,000 rpm for 15 minutes. Samples were analyzed using a Shimadzu GC2010 gas chromatograph machine that obtained measurements through increasing the temperature from 50^{o}C to 140^{o}C using a ramp rate of 15 degrees per minute. Following a five-minute hold at 140^{o}C, the temperature then rose at 10 degrees per minute until reaching 175^{o}C with a three-minute hold at 160^{o}C. Two replicates were performed for each sample.

Intestinal Sectioning and Staining

Large Intestine tissue from P105 male and female mice (P105) was stored in 70% ethanol solution at 4^{o}C until use. Each sample was cut into an approximately .5 cm diameter section and placed in an embedding tissue cassette. The embedding process was performed by ETSU Department of Pathology. Paraffin embedding was performed, and sections were obtained to provide a cross-sectional view of each large intestine. The sections were then exposed to using a hematoxylin and eosin solution for morphological identification of intestinal layers.

Intestinal Analysis

A Zeiss AxioObserver using an AxioVision 4.8 camera hookup was used to acquire all images. Images were viewed using the Brightfield setting for a 5X objective. Image J software provided by NIH was used to determine the distance of the tunica mucosa and tunica muscularis for each section. The average muscular and mucosal measurements for each of the three groups (Control, VA, and BTBR) in both male and females resulted from a total of 60 different animal intestines made up of male controls (N=10), valproic acid (N=11), BTBR N=9, female controls (N=10), valproic acid (N=10), and BTBR (N=10). Each animal has three cross-sections from the colon for analysis. Every cross-section had 2-4 images to obtain the entire diameter. Each cross-section had a total of 8 muscular and mucosal measurements each. To ensure this quantity of measurements stayed as similar as possible, cross-sections small enough to be captured in only two images had 4 measurements taken for each layer per image. See Appendix Table 1 and Table 7 to further analyze the quantity of animals, cross-sections, images, and measurements that were taken for each individual animal. The table indicates that the number of measurements for each layer relies heavily on the number of cross-sections. Averages for each layer were obtained using the three different measurements for cross-sections from each subject. Each cross-section

that made up a specific animal's intestine was given an average muscular and mucosal measurement from the 8 measurements for each individual layer calculated. Following, these cross-sectional averages were then averaged for the animal's overall muscular and mucosa average. The averages of each of the animals for each group were displayed in the figures discussed below and investigated for significance in comparison to other groups.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism Version 9. The p-value was set a-priori at $p>.05$. Data was examined for outliers using a ROUT test. Equal variance was assessed through the use of Kolmogorov-Smirnov test. A one-way Analysis of Variance (ANOVA) was used to determine statistical significance between the three groups for the short-chain fatty acid and intestinal thickness studies.

RESULTS

Microbiota expression in mice

The CLC Workbench, per Qiagen, was used to compute the alpha diversity, beta diversity, and PERMONOVA for the three groups in each gender. Alpha diversity is used to illustrate diversity within each sample. It is sometimes referred to as richness amongst the control, VA, and BTBR groups. An alpha diversity analysis was run for both female and male separately. A Shannon entropy test evaluates the alpha-diversity within sample groups **(Figure 1A**) and does indicate that there was sufficient sequencing to explore microbiota investigations for each sample in the males. Beta diversity analysis was used to illustrate an overall evaluation of the different taxonomy between the three groups. The Bray-Curtis analysis indicates that differing central tendency measures can be observed for each group in the males (**Figure 1B**). A PERMONOVA is an analysis of variance that compares all groups side-by-side to display distinct differences in the sample groups by determining an overall level of significance between microbiota expressions. Using the Bray Curtis data, the PERMANOVA does indicate that both the BTBR and VA groups demonstrate a significantly different microbiota profile ($p<.05$) when compared to the wild-type control group in the males (**Table 1**).

A B

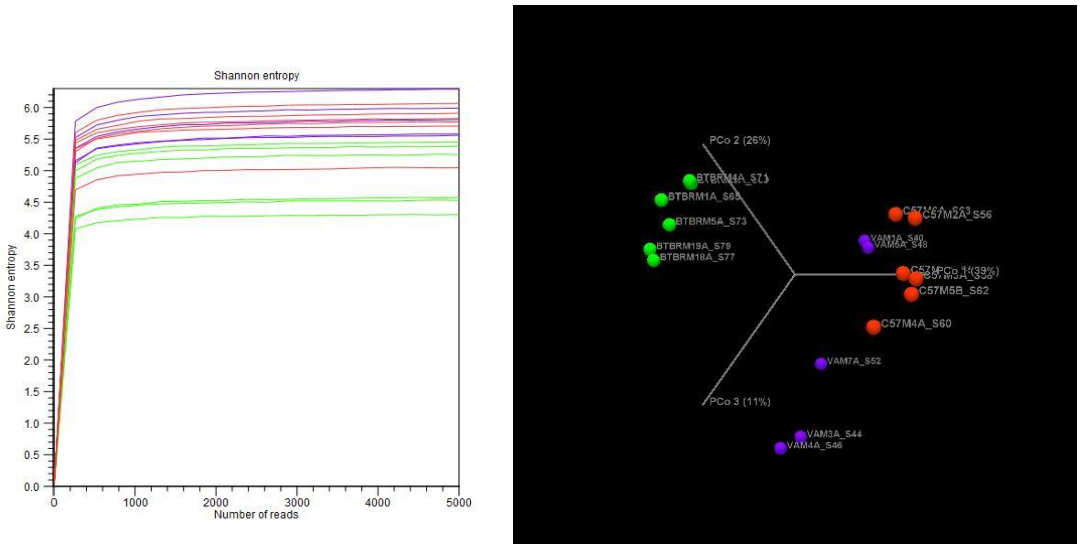


Figure 1: (Panel A) Male alpha diversity across groups (Control N=6 red, BTBR N=8 green, VA N=5 purple). Each line represents a sample that was analyzed in its respective group. The BTBR group has the lowest richness, while on average, valproic acid has the highest. (Panel B) The 3-D Bray-Curtis graphic shows the points of central tendency for each group (N=6 red), VA (N=5 purple), and BTBR (N=8 green) male groups.

Table 1. PERMONOVA comparing male Control (Type 1), BTBR (Type 2), and Valproic Acid (Type 3) groups. The p-values were all <0.05 to indicate significance.

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Type	1, 3, 2	12.36146	0.00001

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
1	3	11.20904	0.00216	0.00649
1	2	18.47839	0.00216	0.00649
3	2	8.79017	0.00216	0.00649

In females, the Shannon entropy analysis indicated that there was sufficient sequencing that demonstrated richness within each sample to warrant further evaluation in the female samples (**Figure 2A**). The Bray-Curtis measurement demonstrated unique measures of central tendency for each group (**Figure 2B**). Analysis using PERMANOVA did indicate in females that the wild-type control group did express a significantly different microbiota expression profile when compared to BTBR and VA mice (**Table 2**).

A B

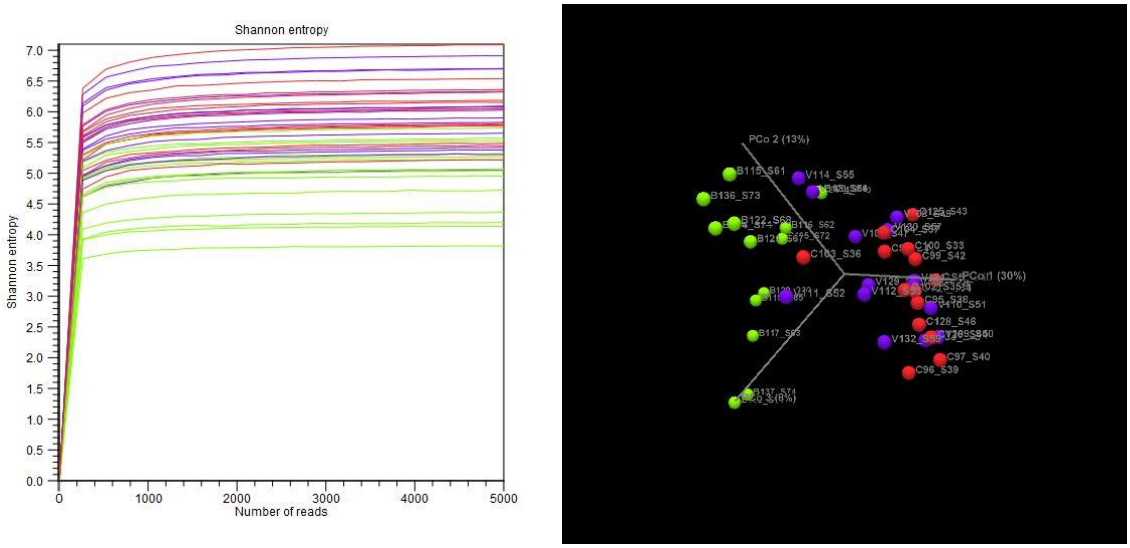


Figure 2: (Panel A) Female alpha diversity across groups (Control N=14 red, BTBR N=14 green, VA N=14 purple). Each line represents a single sample with color indicating the group. The BTBR group has the lowest richness, while on average, valproic acid has the highest richness. (Panel B) The 3-D Bray-Curtis graphic shows the differentiation between the control (N=14; red dots), VA (N=14; purple dots), and BTBR (N=14; green dots) mice.

Table 2. PERMONOVA comparing female wild type control (Type 1), BTBR (Type 2), and valproic acid (Type 3) groups. Significance was found using $p < 0.05$.

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Type	2, 3, 1	8.40268	0.00001

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
2	3	9.32504	0.00001	0.00003
2	1	11.23443	0.00001	0.00003
3	1	3.53307	0.00001	0.00003

SCFA expression in animal models

Gas chromatography was used to measure acetate, propionate, butyrate, and valerate levels in male mice. The data shown found the quantity of nanomoles for each SCFA per milligram of freeze-dried feces used for analysis (80 mgs). There were no significant differences found for acetate, propionate or butyrate in either BTBR or VA male mice when compared to control male mice (**Figure 3A-C**). Valproic acid was significantly different in the BTBR group when compared to control wild-type mice using an ANOVA for analysis (**Figure 3D**).

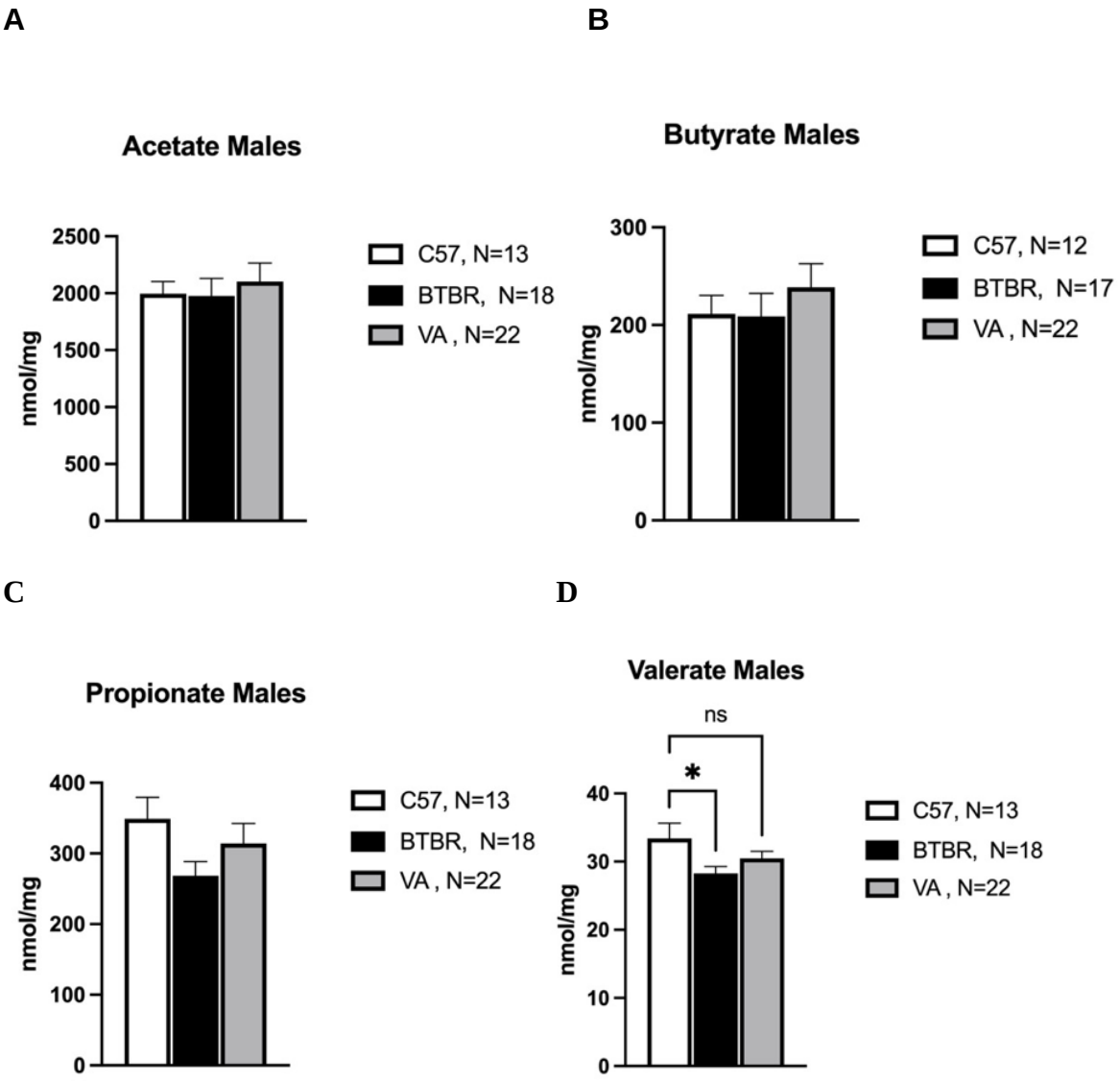
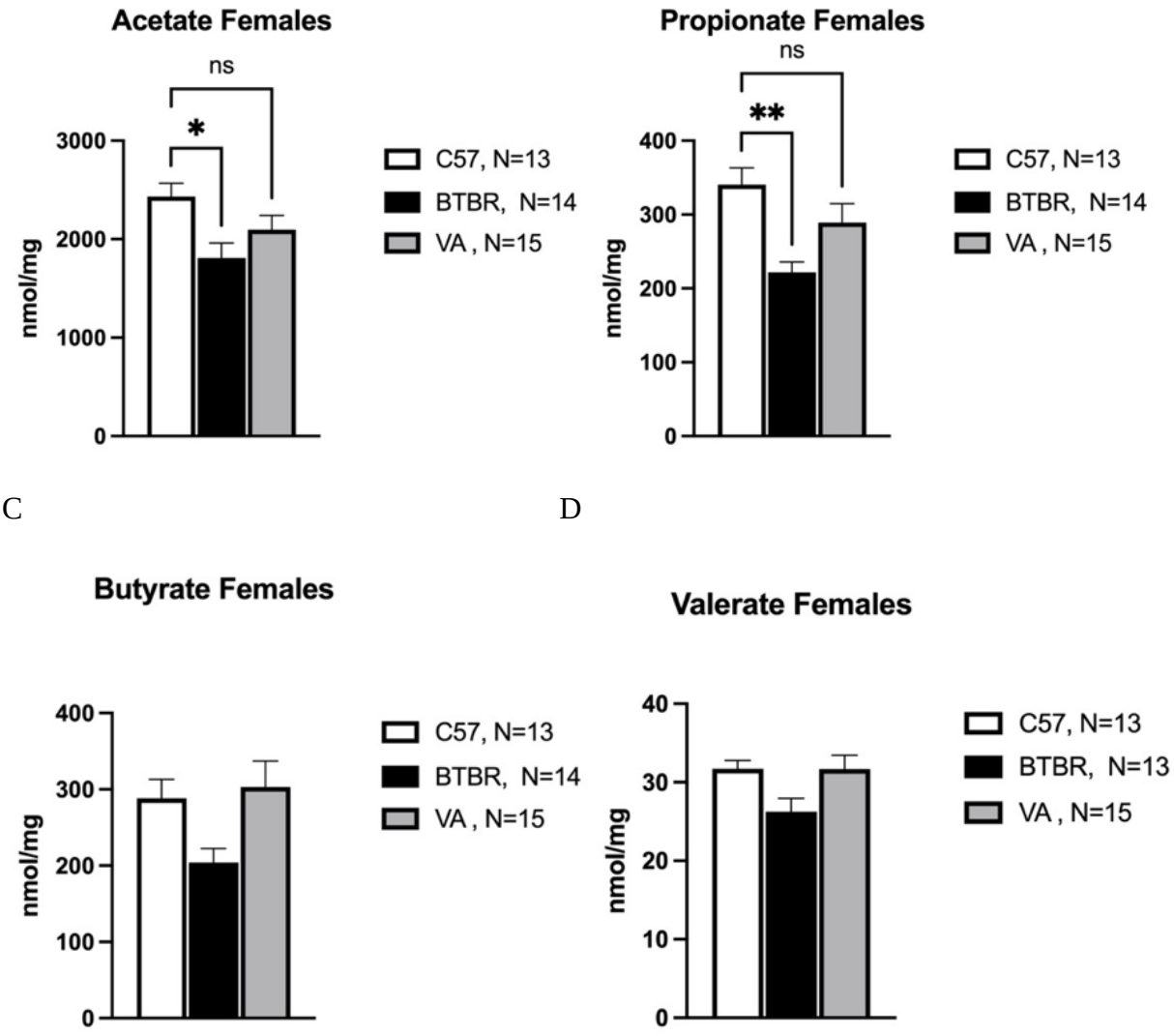


Figure 3: (Panel A) Nanomoles (nmol) of acetate per 80 milligrams (mg) of feces from male mice (N=13 unfilled bars), BTBR (N=18 black bars), and VA (N=22 gray bars). (Panel B) Nanomoles of propionate in 80 mgs of feces. (Panel C) Nanomoles of butyrate found per 80 mgs of feces. (Panel D) Nanomoles of valerate in 80 mgs of feces. Asterisks (*) indicates a p-value <.05.

Short chain fatty acid levels were also compared between female groups. Interestingly, a one-way ANOVA found that acetate ($p<.05$) and propionate ($p<.01$) demonstrated significant differences in the BTBR group when compared to C57 wild type control mice (**Figure 4A and 4B**). Butyrate nor valproic acid did not demonstrate any significant differences when compared to the wild type control mice (**Figure 4C and 4D**).

A B



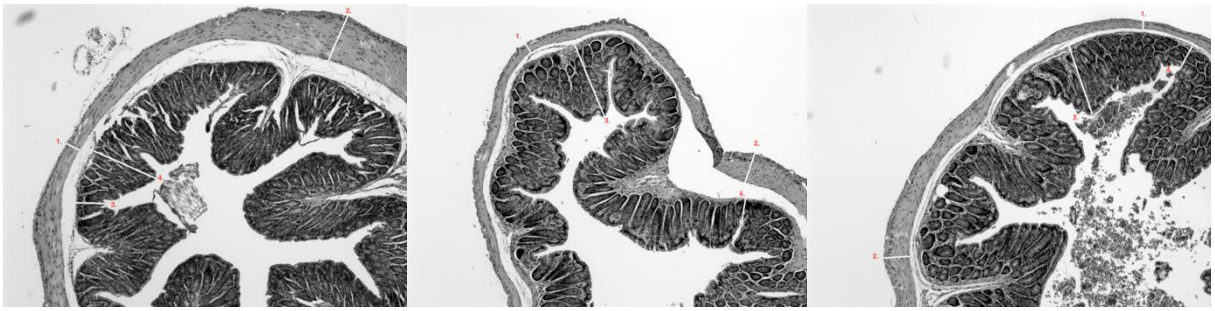
C D

Figure 4: (Panel A) Nanomoles (nmol) of acetate per 80 milligrams (mg) of feces from female mice (N=13 unfilled bars), BTBR (N=14 black bars), and VA (N=15 gray bars). (Panel B) Nanomoles of propionate in 80 mgs of feces. (Panel C) Nanomoles of butyrate found per 80 mgs of feces. (Panel D) Nanomoles of valerate in 80 mgs of feces (N=13 control, N=13 BTBR, and N=15 VA). Asterisks (*) indicates p <.05 and ** is p<.01.

Mucosal and Muscular Layer thickness in Models

Two layers of the colon were measured to determine if intestinal pathology could be found in the models. The tunica mucosa is the layer closest to the lumen, while the tunica muscularis is the smooth muscle layer. Representative images of the colon for each group including C57 wild type control (**Figure 5A**), BTBR (**Figure 5B**) and VA female mice (**Figure 5C**) are show in **Figure 5**. Neither layer demonstrated any significant differences in thickness in either the BTBR or VA group when compared to the wild type control mice for the males (**Figure 5D and 5E**).

A B C



D E

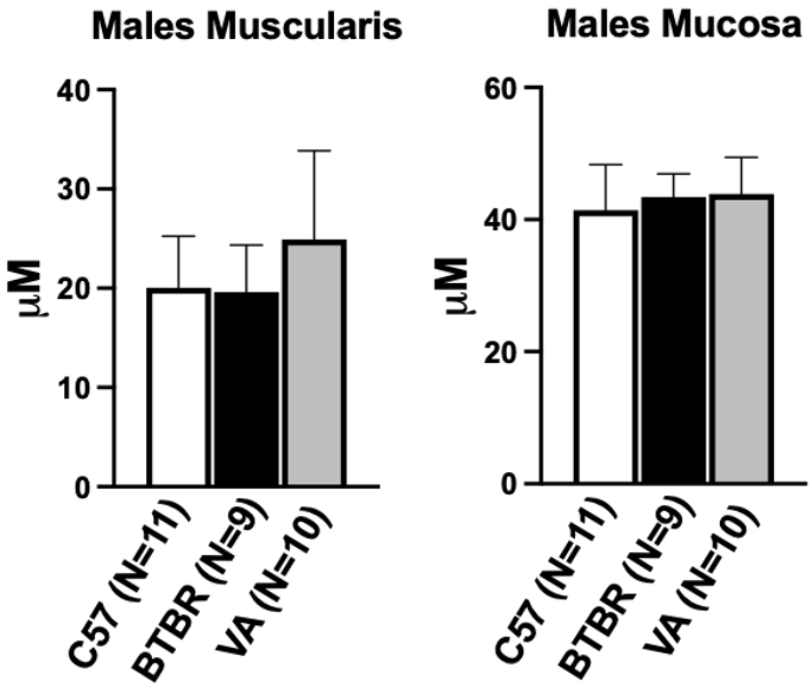


Figure 5: Representative images of the colon used for image analysis from C57 male (panel A), BTBR male (panel B) and VA male (panel C). Panel D is the thickness of the tunica muscularis of C57 control (N=11; unfilled bars), BTBR (N=9; filled bars), and VA (N=10; gray bars) showing no significance between the average muscular measurements (µm) between the groups. Panel E is the thickness of the intestinal mucosal layer of male C57 control (N=11; unfilled

bars), BTBR (N=9; filled bars), and VA (N=10; gray bars) showing no significance between the average mucosa measurements (μm) taken for each group in the males.

Tunica muscularis and tunica mucosa thickness were measured in the C57 wild type control, BTBR and VA female mice. Representative images are shown for each group in **Figure 6A-C**. The tunica mucularis nor the tunica mucosa demonstrated any significant differences in the females between any groups (**Figure 6D and 6E)**.

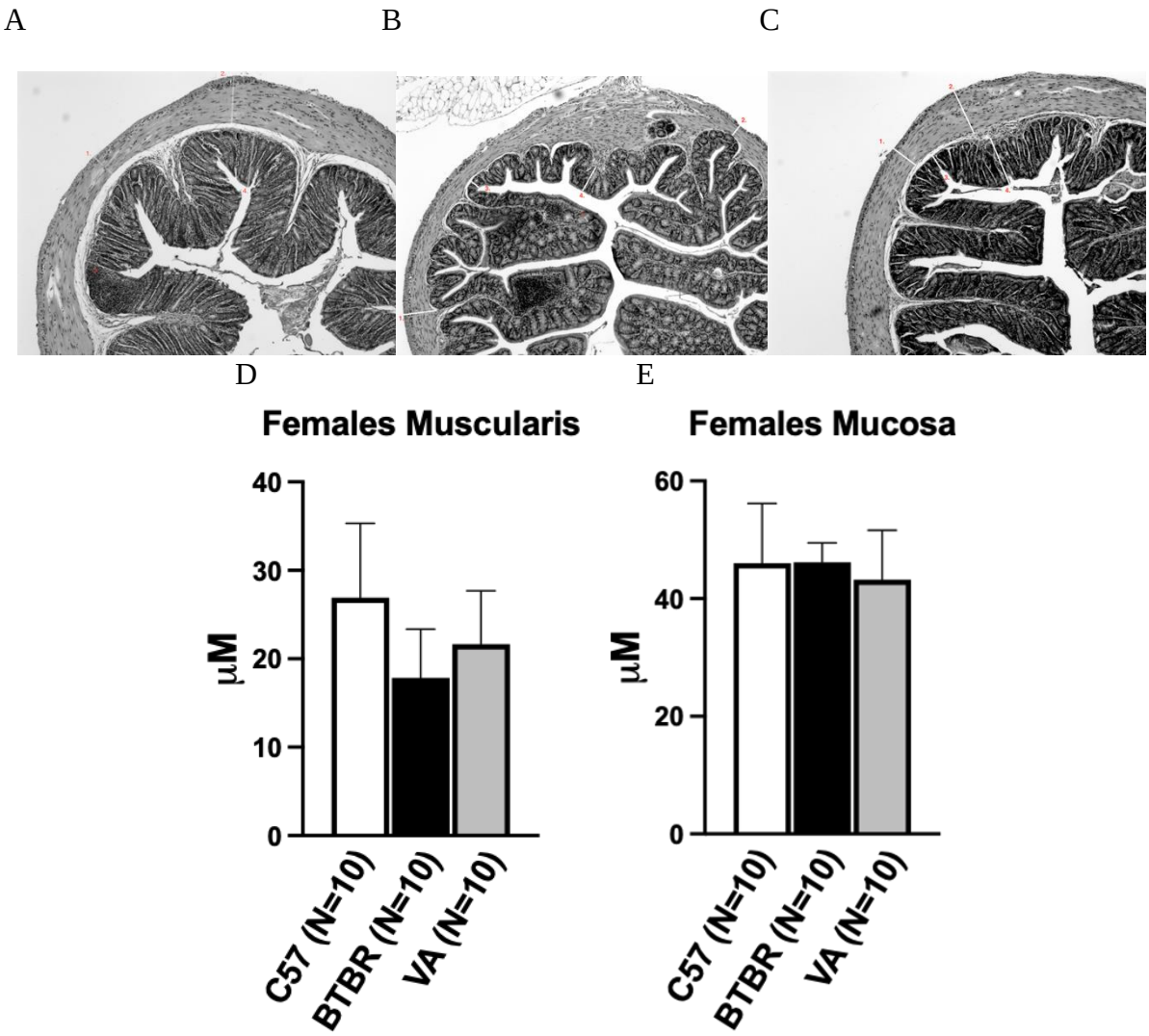


Figure 6: Representative images of the colon used for image analysis from C57 (panel A), BTBR (panel B) and VA (panel C). Panel D is the thickness of the tunica muscularis of C57 control (N=10; unfilled bars), BTBR (N=10; filled bars), and VA (N=10; gray bars) showing no significance between the average muscular measurements (μm) between the groups. Panel E is the thickness of the intestinal mucosal layer of female C57 control (N=10; unfilled bars), BTBR (N=10; filled bars), and VA (N=10; gray bars) showing no significance between the average mucosa measurements (μm) taken for each group in the females.

DISCUSSION

The study shown here was designed to evaluate whether intestinal pathology is associated with potential mouse models that could be used to investigate ASD. Microbiome expression patterns, short-chain fatty acid production, and intestinal thickness were assessed to compose an exhaustive picture of the overall gut health of these mouse models. While the concept and even some of the experimentations aren't novel, the use of all three methods to create a comprehensive examination of gut pathology in the same animals has never been done.

Using 16S ribosomal RNA sequencing to identify bacterial taxonomic expression levels from feces revealed vastly different expression profiles between the models and the control mice. The alpha diversity shows a significant differentiation in the richness across samples. Interestingly, the BTBR model, in both males and females, depicts the lowest richness while the valproic acid treated control group exhibits the highest diversity within a sample. It can be concluded that individual samples within groups have similar diversity. The BTBR group and control group are notably the most different in measures of central tendency shown by the beta diversity plots, while the VA group lies somewhere in between.

The intestinal pathology data found that there is no significant difference concerning muscular and mucosal widths across all three groups. It is possible that pathological differences of layer thickness would not be affected by the alteration of bacterial phyla found in the lumen. While we determined that microbial communities are significantly different, the short chain fatty acid profiles were not significantly different in the VA model and only marginally different in the BTBR; thus, making it acceptable that pathology of the intestinal layers may not have been present. These findings certainly question the validity of the VA animals as a model of social behavioral deficits since they are created from the control animals.

With multiple physiological and psychological functions affected by the microbiome, the direction of treatment for ASD and other disorders may lie in the restoration of a normal microbiome. The microbiome can be affected by multiple factors such as diet, probiotics, prebiotics, antibiotics, one's psychiatric health, etc. More clinical studies need to be performed in order to completely understand the complexities involved in the gut and brain interactions. It is known that the presence of unfavorable bacteria in the gut can negatively impact gastrointestinal health as well as behavior. For example, antibiotics lead to a less diverse microbiome as well as impact mood (Karakan, 2021). Prebiotics have been shown to improve gut microbiome by supplying dietary fiber that results in the production of healthy SCFAs. However, this type of treatment relies solely on the bacteria already present within the microbiome. In cancer research in particular, probiotic usage has been shown to influence microbiome makeup as well. Changes in the gut can promote expression of anti-inflammatory cytokines, while decreasing the production of pro-inflammatory ones. The characterization of the "ideal" microbiome in humans is unclear and needs more conclusive information especially regarding behavior.

There have been extensive studies done concerning diet and its effects on the microbiome in ASD children; specifically, the ketogenic diet (KD) which is also used to treat epilepsy. Factors such as anti-inflammatory activity, oxidative stress, metabolism, neurotransmitter regulation, and GI symptoms are much approved alikely due to the altered gut microbial composition restored by a KD (Li, 2021). The long-term effects of KD on ASD patients are unknown. Further studies need to be done to identify future reactions.

Limitations

Some experimental limitations could be found in this study. Ribosomal sequencing using variable regions of the 16S area can be considered a limitation. The variable region can only

identify to the genus level. A PERMONOVA analysis was run to determine initially if male and female mice could be grouped together. However, as shown in the appendix, male and female control microbiome profiles were significantly different indicated by a p-value <0.05 (Appendix Table 2). Additionally, the male groups were obtained in two different time periods and the microbiome RNA sequencing data were obtained from two different sequencing runs. Analysis was performed to determine if results would be affected if both batches were grouped together. To illustrate the microbiome differences that were found, a Bray-Curtis beta diversity analysis and PERMONOVA on the Qiagen CLC Workbench was performed to compare batch one controls to batch two controls, batch one VA to batch two, and batch one BTBR to batch two (Appendix Figures 2-4; Appendix Tables 4-6). From the results, the microbiome profiles of the batch controls, VA, and BTBR samples are distinctly different. In the same way, a p-value<0.05 indicates significant differences. Therefore, the data shown in the manuscript body only includes the most recent batch of males instead of the initial combined.

The mice models themselves used served as a limitation. This is because there is no universally accepted animal model that has been found for ASD study use. Studies observing strains that exhibit behaviors such as social behavior deficits or excessive grooming such as the BTBR and VA models are used. It does not eliminate the issue that the mechanisms of these models are not fully understood. Additionally, the BTBR model is a knock-out of an inositol receptor and while C57 mice are used as control mice throughout the literature, it is quite possible that C57 mice are not the appropriate group to use for comparisons.

Another limitation worthy to note is the use of only the colon for evaluation of intestinal pathology. Some studies show thickness of multiple areas of the digestive tract including the jejunum, ileum and even the stomach. We chose to only evaluate the part of the digestive tract

that housed the greatest diversity of bacterial species. The number of measurements per muscular and mucosal layer were based on samples that had been obtained from a relatively small study number. Additionally, the measures of the thinnest and thickest layer of the colon were obtained by observational methods. A representative image for each animal is shown in Appendix Figure 7. Approximately 1,000 images were taken, and 5,500 measurements were obtained for both the muscular and mucosal layers. However, it would enhance the study to do other intestinal areas.

Future studies

Future studies are needed for the identification of specific biomarkers contributing to ASD. The microbiome analysis of the different groups created expression differential abundance tables for the different taxonomy for both the male and female comparisons. An exhaustive list of the identified taxonomy related OTU comparisons can be found for the BTBR and VA to the wild-type control group. Each genus, phylum, order, etc. of identified bacterial taxonomy is included in the table with a p-value that indicates if there is a significant difference between each of the three groups. The next step is to identify specific taxonomy that are significantly different between the VA or BTBR group and the control group. Identification of these bacterium could lead to specific therapies that target and/or promote production. Additionally, examination of the maternal gut microbiota would be a valid future investigation. It can be inferred from this initial study that there is reason to analyze mother-child gut microbiome profiles, as the valproic acid treated offspring resulted in social deficits and excessive repetitive behaviors. Also, many other markers including glia activation and serotonin expression in the gut could be evaluated to identify a more specific pathology of the model.

Conclusions

The importance of a healthy microbiome may be incredibly important to understanding and treating ASD. Animal studies may provide a viable route to examine the relationship between the gut brain axis. It was shown that the microbiome profiles of control samples and mice models of abnormal social behaviors for both males and females were significantly different. However, there were no pathological differences in intestinal anatomy nor were consistent changes found for short chain fatty profiles between the groups. It is possible that pathological levels of aberrant bacterial expression are not reached in these models. However, there are multiple markers that could be investigated to determine the extent of gut pathology in behavioral studies. A need for further clarity remains to understand the complexities of ASD pathology.

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APPENDIX

Appendix Table 1: Breakdown of muscular and mucosal measurements.

Animal	Number of Cross-Sections	Number of Images	Number of Muscular Measurements	Number of Mucosal Measurements
C50M	6	20	49	52
C53M	3	14	30	30
C54M	7	26	56	56
C55M	3	17	34	34
C59M	3	10	24	24
C60M	2	9	18	18
C63M	2	8	16	16
C139M	4	19	38	42
C2375M	8	16	62	62
C2376M	8	20	52	52
C2377M	7	17	54	54
V72M	4	23	46	46
V75M	4	13	32	32
V76M	5	16	44	44
V77M	5	12	40	40
V83M	4	17	38	38
V85M	3	10	24	24
V86M	3	14	30	32
V622M	6	21	40	44
V624M	5	20	44	44
V625M	6	15	46	46
B85M	8	21	58	58
B86M	9	19	68	68
B89M	8	16	64	64
B92M	8	19	62	62
B93M	8	23	58	60
B513M	8	21	66	66
B5128M	6	14	42	42
B5129M	8	21	66	66
B5130M	8	17	56	56
C95F	5	21	48	50

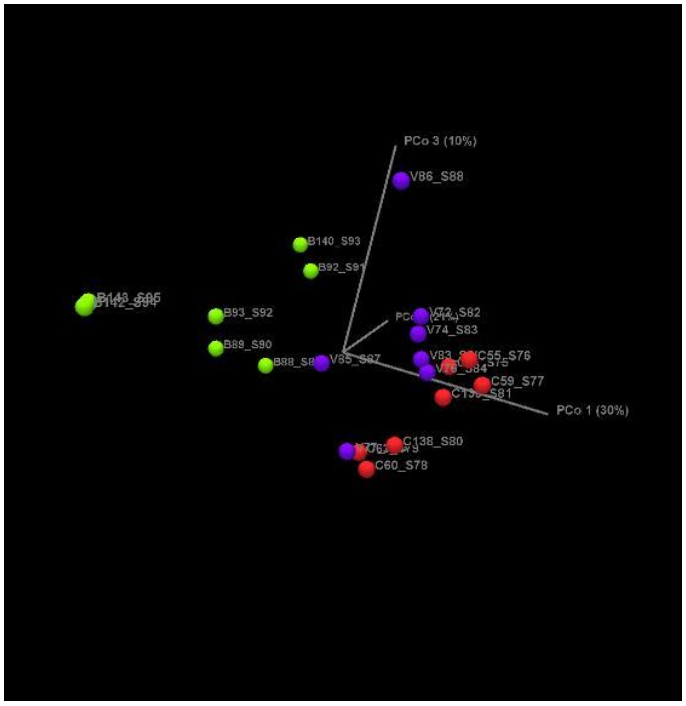
C97F	3	8	24	24
C98F	4	16	33	38
C100F	2	10	18	20
C125F	3	14	30	32
C126F	8	25	64	64
C127F	3	11	19	22
C128F	3	12	24	24
C2378M	7	25	56	56
C2379M	6	13	44	44
V105F	4	26	44	52
V106F	4	19	42	42
V107F	5	25	50	52
V108F	7	37	71	78
V110F	4	13	28	30
V111F	3	10	18	22
V113F	6	18	40	48
V129F	6	35	56	76
V133F	7	25	54	54
V637F	7	16	56	56
B115F	8	29	61	61
B116F	8	22	60	60
B118F	6	15	42	42
B119F	8	18	56	56
B121F	8	16	62	62
B122F	7	15	52	52
B135F	9	17	66	66
B136F	8	19	60	60
B137F	9	19	66	66
B5122F	7	19	52	52

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Type	1, 2	10.84591	0.00003

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
1	2	10.84591	0.00003	0.00003

Appendix Table 2: PERMONOVA comparing male controls (Type 1) to female controls (Type 2). P-value <0.05 indicates significant difference in microbiome profiles.



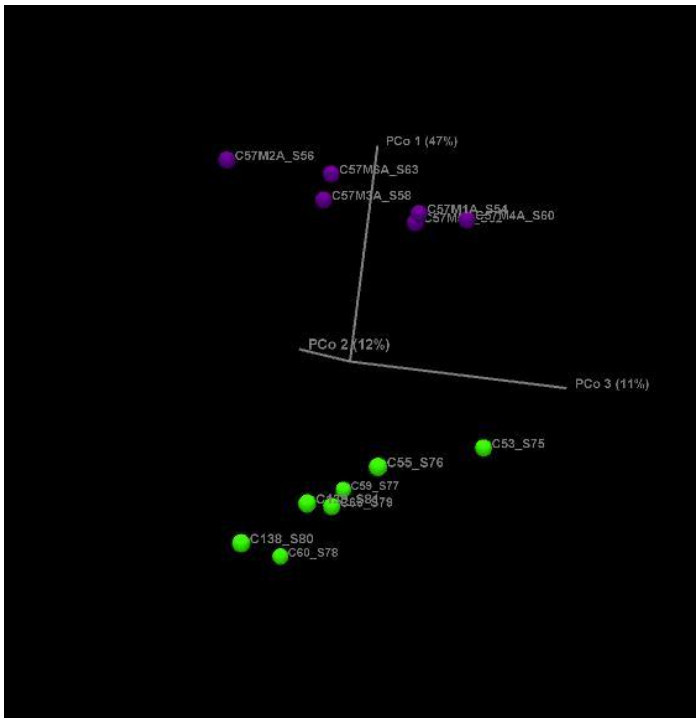
Appendix Figure 1: The 3-D Bray-Curtis graphic shows the differentiation between the batch 1 control (N=7 red), and BTBR (N=7 green), and VA (N=7 purple) male groups.

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Type	2, 3, 1	8.39295	0.00001

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
2	3	9.31605	0.00001	0.00003
2	1	11.22082	0.00001	0.00003
3	1	3.52983	0.00001	0.00003

Appendix Table 3: PERMONOVA comparing batch 1 control (Type 1) to BTBR (Type 2), to VA (Type 3) males. All p-values <0.05 indicate significant difference.



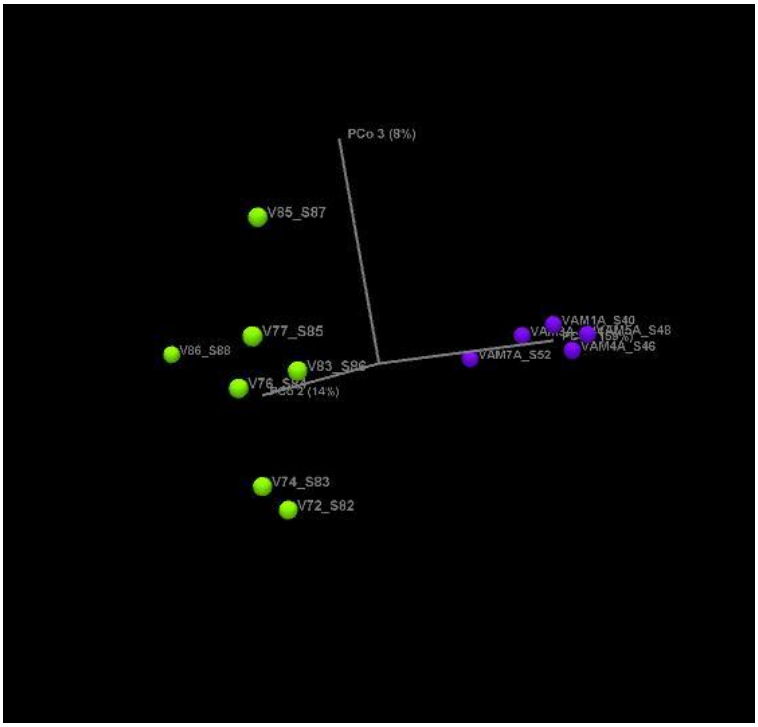
Appendix Figure 2: The 3-D Bray-Curtis graphic shows the differentiation between the batch 1 control (N=7 green) and batch 2 control (N=6 purple) male groups.

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Type	2, 1	9.15070	0.00058

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
2	1	9.15070	0.00058	0.00058

Appendix Table 4: PERMONOVA comparing batch 1 control (Type 1) to batch 2 control (Type 2) males.



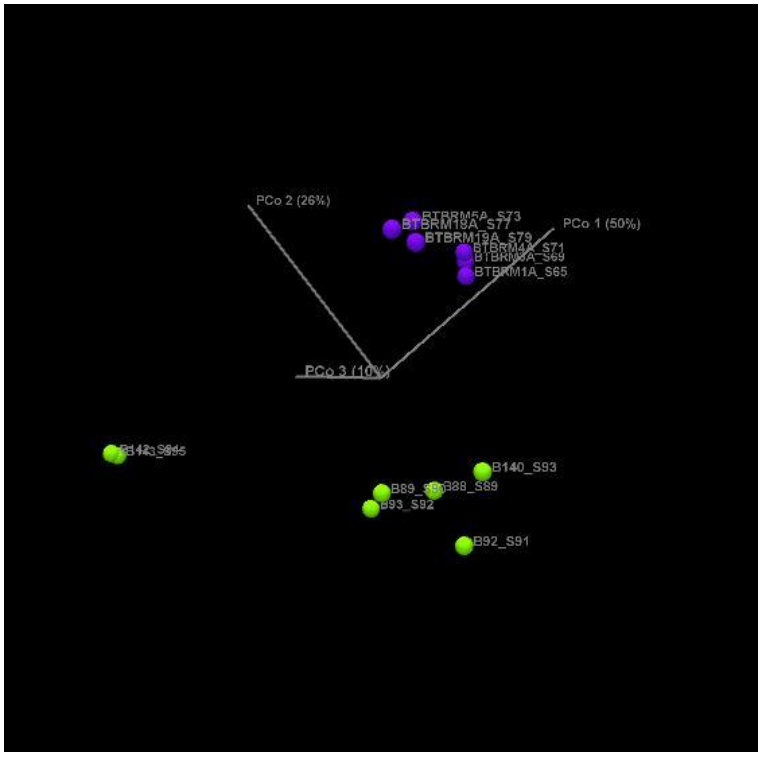
Appendix Figure 3: The 3-D Bray-Curtis graphic shows the differentiation between the batch 1 VA (N=7 green) and batch 2 VA (N=7 purple) male groups.

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Type	1, 2	8.00899	0.00126

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
1	2	8.00899	0.00126	0.00126

Appendix Table 5: PERMONOVA comparing batch 1 VA (Type 1) to batch 2 VA (Type 2) males.



Appendix Figure 4: The 3-D Bray-Curtis graphic shows the differentiation between the batch 1 BTBR (N=7 green) and batch 2 BTBR (N=8 purple) male groups.

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Type	1, 2	7.86252	0.00058

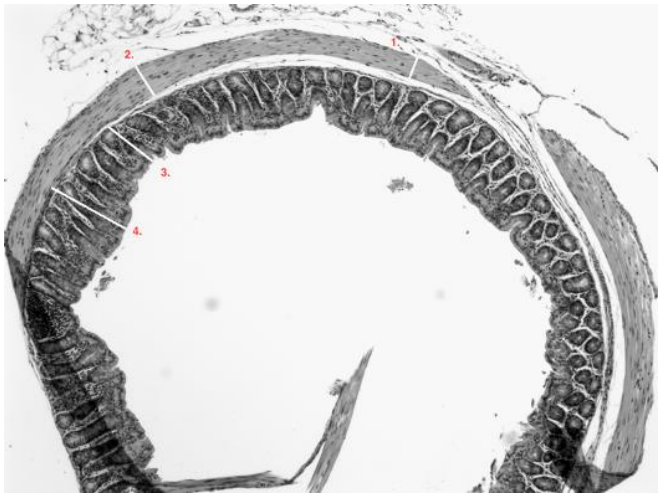
Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
1	2	7.86252	0.00058	0.00058

Appendix Table 6: PERMONOVA comparing batch 1 BTBR (Type 1) to batch 2 BTBR (Type 2) males.

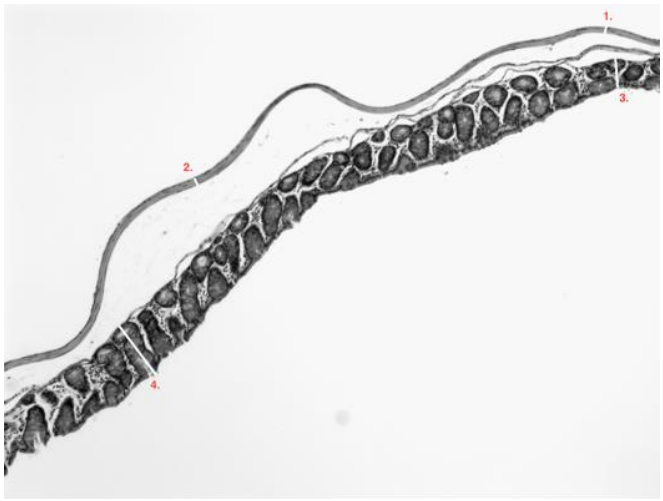
Appendix Figure 7: Representative image for all samples used for intestinal lumen measurements and accompanying image.

Animal	Gender	Cross-Section	Image

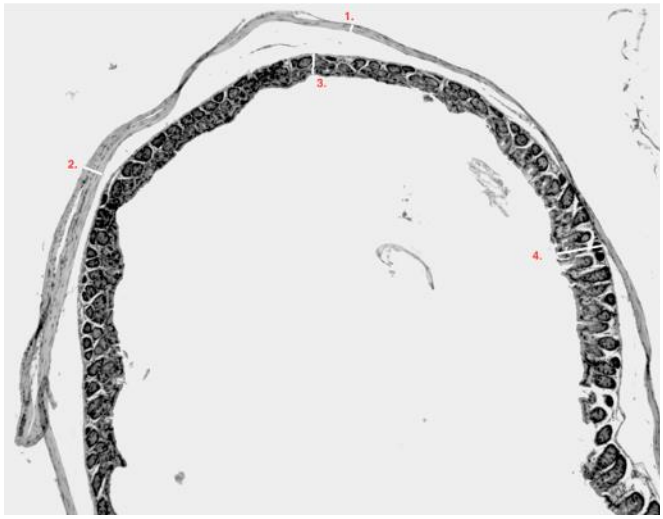
C50M Male 1



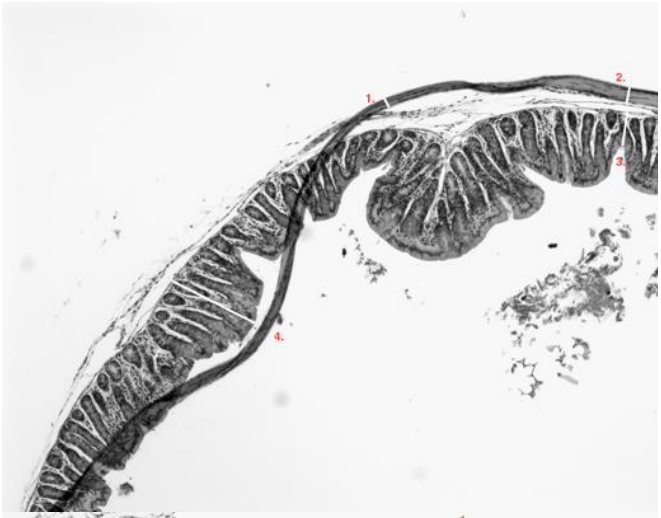
C53M Male 2



C54M Male 1

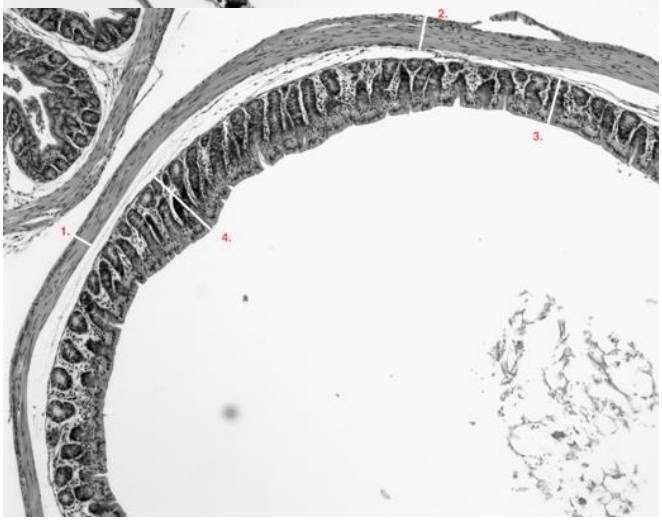


C55M Male 1

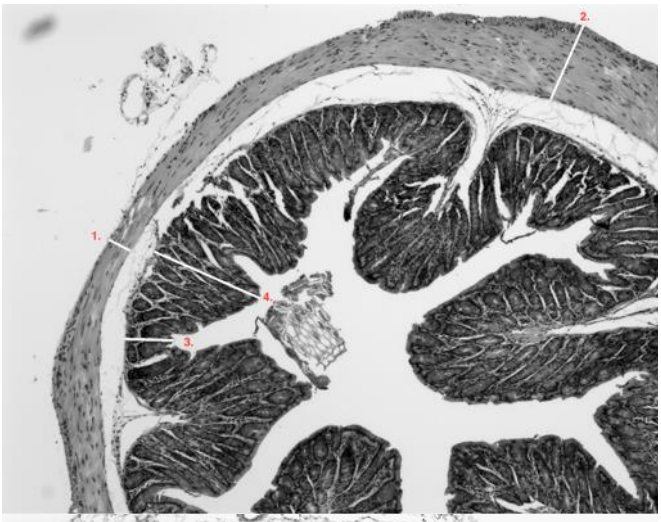


C59M Male 1

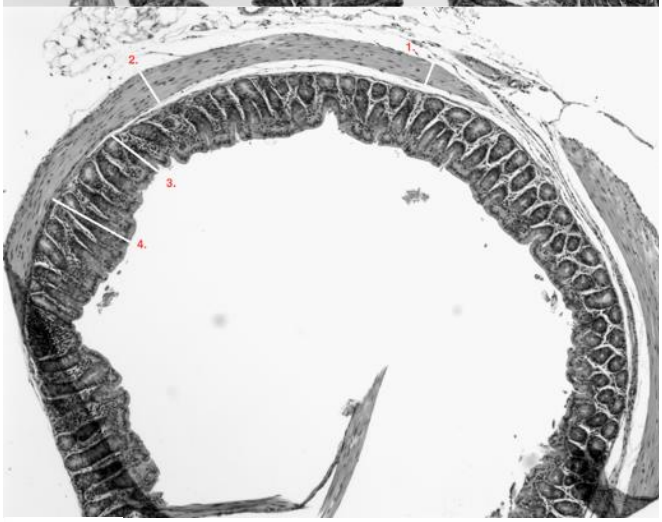
C60M Male 1



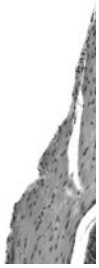
C63M Male 1

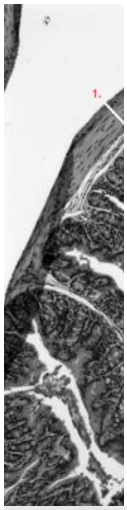
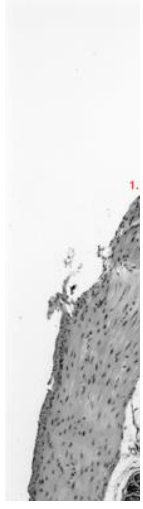


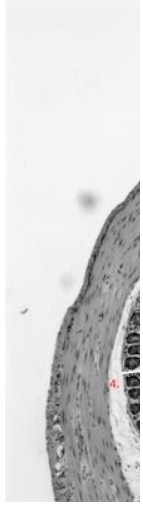
C139M Male 4

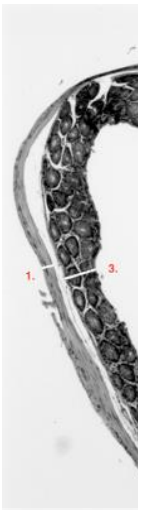
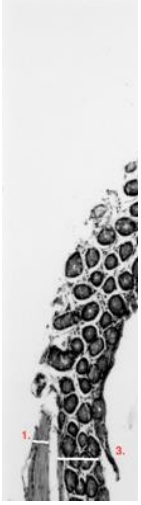


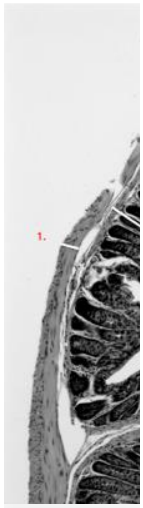
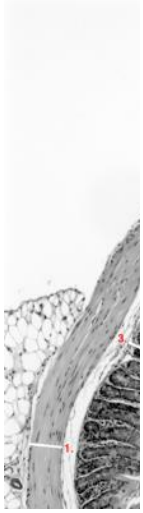
C2375M Male 1

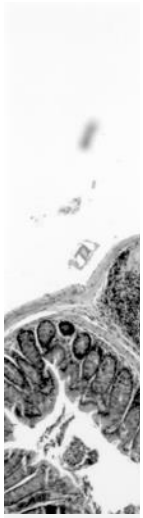
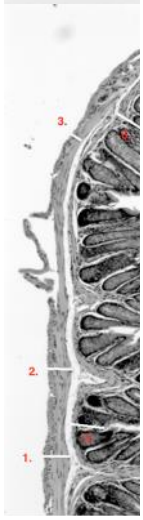


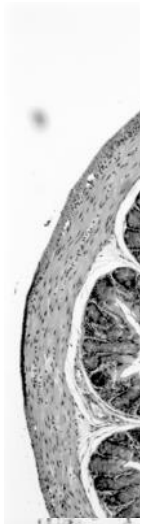
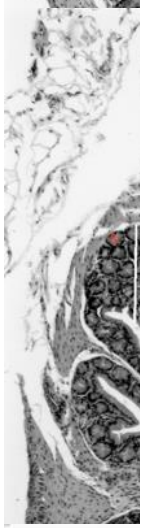
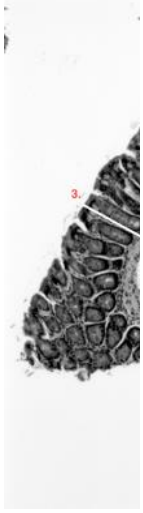
C2376M	Male	2	
C2377M	Male	6	
V72M	Male	1	

V75M	Male	1	
V76M	Male	1	
V77M	Male	1	

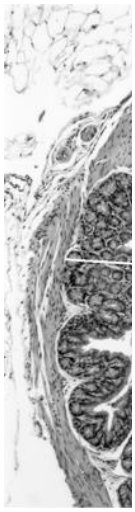
V83M	Male	1	
V85M	Male	1	
V86M	Male	2	

V622M	Male	2	
V624M	Male	5	
V625M	Male	3	

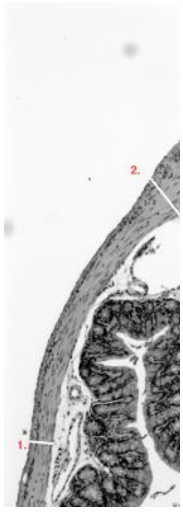
B85M	Male	2	
B86M	Male	7	
B89M	Male	2	

B92M	Male	3	
B93M	Male	7	
B513M	Male	6	

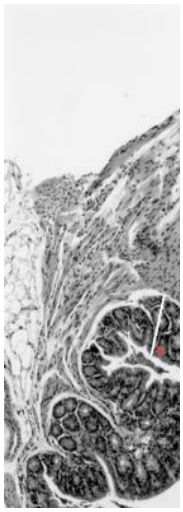
B5128M Male 3

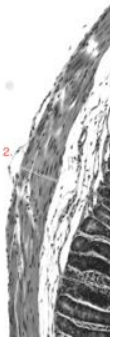


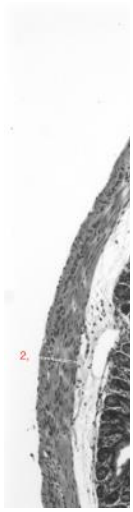
B5129M Male 1

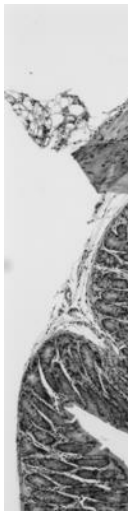
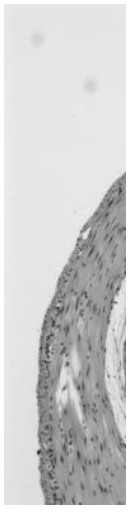


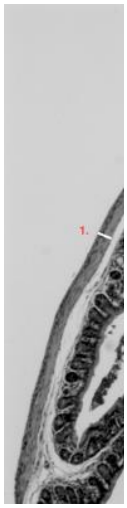
B5130M Male 5

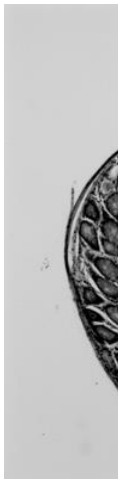
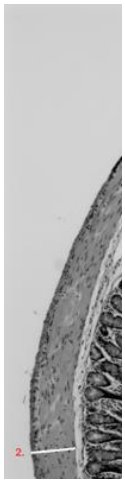


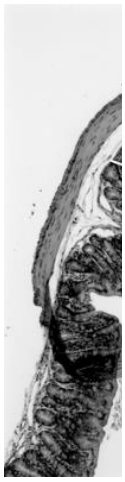
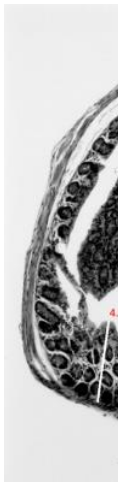
C95F	Female	1	
C97F	Female	2	
C98F	Female	1	

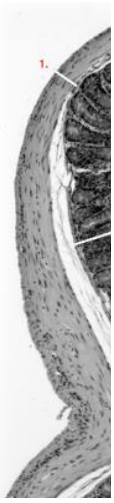
C100F	Female	2	
C125F	Female	3	
C126F	Female	1	

C127F	Female	1	
C128F	Female	1	
C2378F	Female	2	

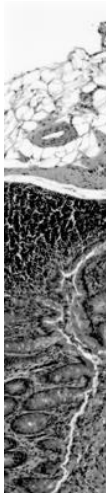
C2379F	Female	4	
V105F	Female	3	
V106F	Female	1	

V107F	Female	5	
V108F	Female	6	
V110F	Female	4	

V111F	Female	3	
V113F	Female	2	
V129F	Female	5	

V133F	Female	1	
V637F	Female	1	
B115F	Female	2	

B116F	Female	4	
B118F	Female	6	
B119F	Female	3	

B121F	Female	5	
B122F	Female	6	
B135F	Female	7	

B136F	Female	5	
B137F	Female	7	
B5122F	Female	5