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Gut Pathophysiology in Mouse Models of Social Behavior Deficits

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By

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ABSTRACT

Autism spectrum disorders (ASD) encompass neurodevelopment disorders characterized by atypical patterns of development that impact multiple areas of functioning beginning in early childhood. The etiology of ASD is unknown and there are currently no preventative treatment options. Gastrointestinal symptoms are commonly associated comorbidities. The microbiota-gut-brain axis is a multidirectional communication chain that connects the central and enteric nervous system that relates brain function to peripheral intestinal functions. Changes within this axis have been postulated in ASD. For example, the "leaky gut theory" proposes that chronic inflammation is linked to alterations in the bacterial profiles of the gut microbiome and subsequent shifts in the amount and type of short-chain fatty acids produced can affect downstream neuronal development. Short-chain fatty acids are signaling molecules produced by bacteria that can trigger nerve afferents in the gut. Dysbiosis causes altered signaling patterns that can be identified by altered intestinal morphology. In this study, C57BL/6J control mice and three mouse models of social behavioral deficits were used to investigate markers of intestinal pathophysiology. Fecal and intestinal samples were collected from adult wild type control mice and the social deficit groups of BTBR genetic knockout mice, C57BL/6J mice injected with valproic acid, and C57BL/6J mice injected with polyinosinic–polycytidylic acid. Short-chain fatty acid profiles that included acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids were obtained from fecal samples to determine differences between the models and control mice. The profiles of the BTBR genetic knockout and valproic acid models were found to be significantly different from control mice. Additionally, postmortem intestinal ileum samples underwent hematoxylin and eosin identification procedures to determine the thickness of the tunica muscularis and tunica mucosa. The thickness of the tunica muscularis was reduced in the

valproic acid group compared to the wild type control mice in early stages of development ($p=0.0279$). This research may illuminate developmental cues that attribute to autism spectrum disorders and may provide markers to assess future therapeutic treatments.

INTRODUCTION

Characteristics of ASD

Autism Spectrum Disorders (ASD) encompass neurodevelopment disorders characterized by atypical patterns of development that impact multiple areas of functioning beginning in early childhood. There are two traditional patterns of the onset of autism: onset prototype, where children show slight abnormalities in social and communicative development in the first year of life and get progressively worse, and regressive autism, where children show normal developmental cues for the first one or two years of life and then show regression in communication and social abilities (Ozonoff *et al,* 2008). Evidence suggests that the most common time of onset is around the second year of life (Martínez-Pedraza & Carter, 2009). The diagnostic criteria for ASD requires a child to have symptoms that fall in at least one of the following social behavior domains. The first domain is that the child must have qualitative impairment in social interaction which can include impairment in nonverbal behaviors such as limiting eye contact or abnormal gaze, abnormal facial expression, or abnormal body posturing; or difficulties in forming relationships and social/emotional reciprocity. The second domain is qualitative impairment in communication such as being on a nonverbal spectrum, using stereotyped or repetitive language, or lack of spontaneous play. The third domain is the presence is restrictive receptive and stereotyped patterns of behavior, interests, and activities such as a fixation on a specific topic that permeates most of their interests or repetitive hand motions (Martínez-Pedraza & Carter, 2009).

Autism disorders have a wide range of severity; therefore, they are called "autism spectrum disorders" or "ASD". They are lifelong disorders that exist in all ethnic, racial, and economic groups. However, they are more common in white populations than Hispanic or

African American populations (Biao *et al,* 2018). Although debatable, ASD is believed to be around 4 times more prevalent in males (Werling & Geschwind, 2013). ASD may present differently between the sexes, with girls having more internalized personality-related symptoms while boys have more externalized behavioral symptoms that are easier to diagnose (Werling & Geschwind, 2013). Genetic and hormonal differences could also play a role. For example, higher gestational testosterone levels are linked to the increased prevalence in males (Ruta *et al,* 2011). Overall, 1 in 59 children in the United States has been diagnosed with ASD by age 8. The prevalence in the United States has been increasing. In 2000, the prevalence of autism in eight-year-old populations was 6.7 diagnoses per 1000 children, but in 2014 it was 16.8 in 1000 (Baio *et al,* 2014). The etiology of ASD is unknown, but rates are increasing in the United States as well as globally. It is thought that increased awareness among medical professionals has contributed to an increased ASD diagnosis. Less stringent criteria for diagnosing ASD in early versions of the Diagnostic and Statistical Manual of Mental Disorders versions may have elevated the likelihood of an ASD being diagnosed. Support of this theory exists from research that shows irregularities in prevalence between states with increased awareness despite the population being fairly homologous (Wright, 2017). It is even shown that states with better resources and awareness campaigns are more likely to have higher calculated prevalence (Wright, 2017). However, overall it is still likely that ASD is underdiagnosed, even with the numbers increasing (Charman, 2002). Changes in diagnostic methodology do not fully explain the dramatic increase in prevalence, and it is likely that external environmental factors are behind the unexplained increase (Van den Hazel *et al*, 2008).

Associated total cost to diagnose and treat autistic patients has increased with the increasing prevalence of the disorder. Compared to adolescents without ASD, ASD children's

medical expenditures are 4.1 to 6.2 times greater (Qi *et al,* 2016; Shimbukuro *et al,* 2008). Intensive behavioral interventions for a child with ASD range from $40,000 to $60,000 per year per child on average (Rogge & Janssen *et al,* 2019). The direct and indirect costs including the cost of medical care, special education, and lost parental productivity totaled between $11.5 billion and $60.9 billion in 2011 (Lavelle *et al,* 2014).

The etiology or cause of ASD has yet to be defined. Because the etiology is unknown and there are no known measures that can be taken to prevent a child from developing autism. There are only therapeutic or behavioral treatments that focus on managing symptoms. ASD is believed to be caused by a combination of both environmental and genetic factors. Monozygotic twins do not always both present with ASD. In a recent study of 40 twin pairs, the probandwise concordance for male monozygotic twins was 0.58 while the concordance between dizygotic pairs was 0.27. Female twin pairs had similar results. The results show that ASD has moderate genetic heritability, but not complete heritability (Hallmayer *et al,* 2011), and no single gene contributes to the onset (Zeidán-Chuliá *et al*, 2013; Buxbaum, 2009). The relationship between genetics, person, and environment must be collectively taken into account to fully understand the etiology of ASD. Mouse models are often used in experimentation to help demonstrate the difference between environmental and genetic contributing factors (Ellegood & Crawley, 2015; Ellegood *et al,* 2015*)*.

Gut Microbiome

The possible environmental component of autism spectrum disorder may be illuminated through the connection between the gut microbiome, intestines, and neurodevelopment. Within the human gut lives trillions of microorganisms that can be broken down into tens of thousands of bacterial species with more than three million different genes that outnumber the entire human

genome. Gut bacteria can be beneficial to the body and are fundamental to digestive system function, but pathogenic bacteria or an unbalanced microbiome can be detrimental to human health (Sender *et al,* 2016). The gut's function is tied closely with the limbic system which is responsible for emotional control (Jones *et al,* 2006). Changes in the microbiome have been identified in neuropsychiatric conditions like depression, anxiety, and Parkinson's disease. Likewise, microbiome differences have been reported in animals that have exposed to environmental alterations during gestation that later display behavioral differences. It is likely that there are critical developmental windows in the prenatal environment and early postnatal stages of development for colonization by the correct ratio of beneficial bacteria (Warner, 2019; Dinan & Cryan, 2017). While the "microbiota fingerprint" can vary up to 50% between individuals, patients with similar disorders like autism tend to have more similarities to each other than to unaffected individuals (Clemente *et al,* 2012).

Advancing research has shown that the intrauterine environment during gestation is not likely sterile like previously thought. Recently, a diverse pattern of bacteria has been cultured from meconium, an infant's first stool, and is thought to represent the bacterial profile of the intrauterine environment. Also, epidemiological studies have shown a correlation between early colonization of the gut and pre-term delivery with worse neonatal outcomes. The largest and most important sources of bacterial colonization of infants happens during vaginal or cesarean birth. The fetal gut is nearly sterile until immediately after birth when the gut is colonized and within hours after birth the stool will contain bacteria. Gut colonization begins with primarily facultative anaerobes such as *Escherichia* and *Enterococcus* because the gut has positive oxidation/reduction potential, meaning that there is oxygen available for the bacteria to use. However, as the oxygen is consumed over time, the environment is more reduced and strict

anaerobes including Firmicutes such as *Clostridia* and Bacteroidetes such as *Bifodobacteria* that do not require oxygen begin colonizing (Penders *et al,* 2006; Houghteling & Walker, 2015). The early microbiome is called the pioneer microbiome and it is important because it establishes the gut and microbiome's relationship with the developing immune system and the future colonization of the gut (Houghteling & Walker, 2015). It has been suggested that changes in a single species profile during infancy can have lasting, permanent effects a child's microbiome health and immune system development causing chronic inflammation, allergies, and inflammatory bowel disease (Gaufin *et al,* 2018), and the development of diseases such as Type II Diabetes and obesity (Vael & Desager, 2009; Gaufin *et al,* 2018; Cox *et al,* 2014). Maternal genetics and environmental exposure to a diverse outside microbiome are also incredibly important for the development of a healthy and robust gut microbiome (Tiihonen *et al,* 2010; Neggers, 2014). Maternal genetics influence fetal exposure in-vitro, during birth, through breast milk, and through offspring genetics that can determine the development of a specific gut bacterial profile. Additionally, the environment participates in the development of the immune system, determines food diversity and other components that impact the digestive system and gut response in a fetus or child (Lazar *et al,* 2018).

Microbiome-Gut-Brain Axis

The relationship between the microbiome, gut, and brain is called the Microbiome-Gut-Brain axis. It is an integrative system of communication that monitors and facilitates interrelated changes. It connects the central nervous system to the enteric nervous system (Israelyan & Margolis, 2018). The microorganisms that live in the gut are contained by a barrier consisting of the microbiota, a mucous layer, and epithelial cells that are connected by regulatory gap junctions that divide the cytoplasm of adjacent cells that allow for controlled molecule diffusion.

In a healthy gut, nutrients and ions are allowed to pass through the tight junctions while toxins and antigens are prevented from spreading through the body (Ulluwishewa *et al,* 2011).

The brain influences the enteric microbiota directly and indirectly. The autonomic nervous system, which includes the sympathetic and parasympathetic nervous systems, mediates communication between the CNS and the gut. The autonomic nervous system is responsible for modulating gut functions that are vital to the survival of gut microbiota. Changes in villi motility, secretion of bile and acid, bicarbonates, mucous, and intestinal immune response change the intestinal environment and therefore directly impact microbiota's ability to survive, which can change the profile and diversity of the bacteria present in the gut. For example, the majority of bacteria live within the mucosal layer. Mucous secretion is controlled by the ANS. If the ANS reduces mucous secretion then the gut's environment becomes less hospitable to anaerobic bacteria, leading to a species shift that includes more facultative anaerobes. Stress responses that are controlled by the ANS can also cause the gut to become more permeable and activate immune responses that lead to decreases in microbial diversity (Rhee *et al,* 2009).

Bacteria can induce changes to physiology of the gut wall. *Bifidobacterium bifidum* and *Lactobacillus acidophilus* are both able to promote gut motility, while Escherichia species can inhibit gut motility affecting the host's nutritional uptake (Sang *et al*, 2009). Studies of germ-free mice have shown that morphological consequences including enlarged cecums, reduced intestinal surface area, increased enterochromaffin cell area, smaller Peyer's Patches and smaller villous thickness are present in germ-free mice when compared to wild-type mice (Grenham *et al,* 2011). Microbiota trigger toll-like receptors (TLRs) that maintain mucosal thickness and stimulate repair the intestinal epithelial after injury. TLRs are critical for maintaining inflammation response. A study done with mice that were deficient in TLR2, TLR4, and MyD88

which is an adaptor molecule that is fundamental to TLR-mediated induction of inflammatory cytokines showed that the deficient mice were significantly more likely to die when they were treated with a toxin that kills colonic epithelium compared to wild type mice. No wild type mice died while all of the TLR2, TLR4, and MyD88 deficient mice died. (Rakoff-Nahoum *et al*, 2004). Additionally, inflammation or stress increase intestinal permeability thus making it more likely that microbial signaling molecules can interact with afferent nerve terminals or cause signaling within the enterochromaffin cells, the transducer cells in the epithelial layer. More specifically, cells in the gut secrete signaling peptides such as serotonin when triggered by physiological or pathological luminal changes including changes in microbial factors or bacterial toxins (Rhee *et al,* 2013).

Although serotonin is most well-known as a neurotransmitter in the brain, the majority of serotonin is actually produced in the gut where it is synthesized from tryptophan, an essential amino acid, by enterochromaffin cells and in neurons in the enteric nervous system (O'Mahony *et al,* 2015 and Israelyan & Margolis*,* 2018). Interestingly, abnormalities in serotonin levels during development can cause anatomical changes in the brain that result in delayed sensory map formation (Muller *et al,* 2016). It has been hypothesized that serotonin is a key communicator between the enteric nervous system and the central nervous system. It is critical for neuronal differentiation and migration, axonal outgrowth, myelination, and synapse formation (Israelyan & Margolis*,* 2018). Studies of germ-free mice have shown that without proper gut colonization during early development, the central serotonergic system does not form properly. Gut bacteria, especially lactic acid producing bacteria such as *Lactobacillus johnsonii*, influence tryptophan availability and therefore serotonin synthesis. Lactobacilli that produce hydrogen peroxide can inhibit enzymes important to synthesis (O'Mahony *et al,* 2015). Changes in the concentration of

intestinal metabolites produced by gut bacteria can increase serotonin levels without creating more serotonin re-uptake transporters (SERT) (Israelyan & Margolis, 2018). When germ-free mice are colonized by using microbes rich in Clostridium spp., serum and colon serotonin are restored (Yano et a, 2016).

Thirty percent of individuals with ASD have high blood-serotonin levels and the high levels correlate with symptoms of constipation (Marler *et al*, 2016). In polyinosinic:polycytidylic acid (Poly(I:C)) mouse model experimentation, the Poly(I:C) mice expressed changes in behavior, CNS development, intestinal permeability, and microbiome changes similar to patients with ASD and that correlated with measured serotonin levels. (Israelyan & Margolis, 2018). Poly(I:C) are a maternal immune-activation model. When there is maternal immune activation, fetuses are exposed to pro-inflammatory cytokines that can disrupt brain development related lead to ASD-like conditions (Reisinger *et al*, 2015). The Poly(I:C) models have been shown to express increased levels of serotonin that accumulates without being properly broken down. Neuroanatomical differences are present: the serotonin axons are blunted within the forebrain (Goeden *et al*, 2016). Curiously, *B. fragilis* has shown some ability to regulate serotonin levels in the Poly(I:C) mice and mitigate the ASD-like symptoms (Israelyan & Margolis, 2018).

The vagus nerve is the vital neuronal conduit between the microbiome and the central nervous system. Vagal afferents can relay sensory information from the gastrointestinal system and is a primary neural regulator of the gut. The interoceptive awareness of the vagal nerve allows it to sense changes within the microbiome, convey the information from the enteric nervous system to the central nervous system, integrate the information, and trigger a response (Bonaz *et al,* 2018). Vagal afferent stimulation signals lower motor neurons in the brainstem that signal other brain regions that are involved in physiological responses such as heart rate or

cognitive responses that regulate the social engagement system. Social deficits are a defining symptom of autism, while deregulation of the heartrate is a common comorbidity. It is hypothesized that stereotyped behavior like rocking or swinging might be ways for people with autism to regulate an inefficient vagal system by purposefully stimulating peripheral baroreceptors that regulate blood pressure (Porges, 2005).

The vagal afferent fibers are found in every layer of the digestive wall except the epithelium of the mucosa where bacteria live. Therefore, these fibers can respond to bacterial products that can increase or decrease firing frequency of the vagus nerve. Serotonin and other bacterial products in the gut such as short-chain fatty acids can activate the vagus nerve (Bonaz *et al,* 2018). It is possible that unique bacterial species can influence human behavior via the vagus nerve. Animal studies have shown that colonization of the gut with *Lactobacillus reuteri* in Shank3B-/-, VPA, and BTBR mouse models which exhibit social deficits cause marked improvement in social behavioral symptoms (Sgritta *et al,* 2019).

It is tempting to speculate that changes in either the gut, microbiome, or brain can result in alterations to the downstream signaling pathways that display as ASD-related social behaviors (Mayer *et al,* 2015). Epidemiology studies show that ASD children are more likely to have gastrointestinal differences when compared to typically developing children. For example, children with ASD have been shown to have significantly elevated numbers of Paneth's cells which release antimicrobials in duodenal crypts (Horvath *et al,* 1999). Children with ASD are more likely to have gut dysbiosis and less diverse microbiomes than typically developing age-matched children (Li *et al*, 2017). They are also more likely to display gastrointestinal symptoms like constipation, diarrhea, bloating, abdominal pain, reflux, vomiting, gaseousness, foul smelling stools and food allergies than found in typically developing children (Fattorusso *et al,*

2019 & Li *et al*, 2017). It is interesting to note that the severity of the gastrointestinal symptoms is correlated with the severity of the ASD symptoms (Fattorusso *et al*, 2019).

The intestinal mucosa must be able to respond to constant challenges such as foreign antigens, environmental microorganisms, antigens from digesting food, and pathogens. For example, *E. coli* bacteria can invade intestinal cells, corrupt the immune response and tight junctions, and compromise the intestinal barrier and increase intestinal permeability. Proper regulation maintains mucosal immune function and prevents inflammation (Samsam *et al,* 2014). The association in ASD between changes in the intestinal permeability and the worsening regulatory potential of the gap junctions is referred to as the "leaky gut theory." Inflammation, especially chronic inflammation, of the gastrointestinal tract leads to weakening of the gap junctions and increased permeability of the gut barrier. A higher percentage of intestinal permeability has been recorded in children with autism compared to control children (Li *et al,* 2017). If the gut becomes more permeable, stress signals and metabolites from bacteria can spread and penetrate the blood-brain barrier which protects the brain from environmental dangers. The blood-brain- barrier and the gut-blood barrier show homology (Mahajan *et al,* 2019). Proper regulation maintains mucosal immune function and prevents inflammation (Samsam *et al,* 2014). Inflammation of the gut-blood barrier due to inflammatory mediators can lead to brain inflammation (Mahajan *et al,* 2019). Germ-free mice show elevated permeability in their blood-brain-barriers (Braniste *et al,* 2014). An intact, well-functioning blood-brain-barrier is critical for neurodevelopment.

Changes to the immune cells, including increased permeability, could cause systemic changes. Mucosal immune cells within the intestines account for 70% of all the immune cells in the body (Waslilewska & Klukowski, 2015). Specifically, bacterial products known as short

chain fatty acids (SCFA) can induce localized inflammation to permeate through leaky guts to eventually cross the blood brain barrier (Quigley, 2013; Dalile *et al,* 2019; Westfall *et al,* 2017; van de Wouw *et al,* 2017). They can also induce specific changes in the brain such as the activation of microglia. Dysbiosis or abnormalities of the gut microbiome and the subsequent changes in intestinal permeability have been linked to changes in brain pathology (De Palma, 2014).

The largest percentages of short chain fatty acids produced in the human gut are acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate. Each of the short chain fatty acids is capable of causing physiological changes in the human body. They are a major signaling molecules produced by bacteria aiding in digestion (Morrison & Preston, 2016). SCFAs are capable of modifying the colonic epithelial cells through phenotypic alteration, serving as major energy sources, modulating the enteric neuroendocrine system, and functioning as inflammatory factors (MacFabe, 2015). They are fundamental in balancing redox equivalent production in the gut and maintaining homeostasis (den Besten *et al,* 2013). Poor diet, genetic disposition, exposure to different bacteria in formative years, and competitive populating all contribute to changing and maintaining proper bacterial ratios. Bacterial species changes will alter the short chain fatty acid profiles and the physiological responses in populations (MacFabe, 2015).

SCFAs have been shown to be immunomodulatory. They are signals for G-protein coupled receptors that activate signaling cascades, controlling inflammation, and have been linked to inflammatory diseases like inflammatory bowel disease, ulcerative colitis, and Crohn's Disease which are all characterized by chronic gastrointestinal inflammation. Children with ASD are significantly more likely than children without ASD to suffer from gastrointestinal distress and chronic bowel inflammation (Waslilewska & Klukowski, 2015). The SCFA butyrate is most

closely linked to being anti-inflammatory (Venegas *et al,* 2019). Butyrate is also capable of altering the activity of cells within the blood-brain-barrier, which controls what can substances cross into the brain and has been linked to major depressive disorders (Huang *et al,* 2019). Likewise, in animal models that have been treated with propionate, the animals begin to show behavioral abnormalities similar to what is expected with ASD (Choi *et al,* 2018).

Several studies have identified that the bacterial profiles of autistic children are different from typically developing children. *Clostridiales*, *Betaproteobacteria*, and *Lactobacillus* are all higher in proportion in the ASD microbiome (Srikantha & Mohajeri, 2019). In a 2005 study, experimentation showed that autistic children were more likely to have toxin-producing Clostridium subtypes that can cause gut disfunction when compared to their siblings (Parracho *et al,* 2005). Late-onset autistic patients that were treated with the antibiotic vancomycin demonstrated an improvement in behavioral and gastrointestinal symptoms that was associated with a reduction of the toxin-producing Clostridium bacterial species in those patients; however, the symptoms returned upon stopping the medication (Finegold *et al,* 2002). *Sutterella* and *Desulfovibrio* populations tend also to be disproportionately larger in ASD populations (Benach *et al,* 2012). It is unknown what role these differences might play in the etiology of ASD, but it is suggestive that gut microbiota might be useful as a marker and lead to subsequent studies of downstream inflammatory events (Midtvedt, 2012). Interestingly, the severity of overall changes in the microbiota are correlated with the severity of the location of the patient on the spectrum of the disorder (Vuong & Hsiao, 2017).

Microbes colonize sections of the digestive tract differently. In the small intestine, food digestion and absorption of nutrients occurs. The small intestine can be subdivided into three main parts: the duodenum where food chime enters from the stomach, and the jejunum which is

characterized by muscular flaps called “plicae circulares” and villi that aid in absorption of digested food, and ilium that absorbs nutrients including bile acids and B12. The ileum connects to the large intestine (Collins & Badireddy, 2019). The large intestine is responsible for water and mineral absorption and can be subdivided into four sections: the ascending colon with the cecum, transverse colon, descending colon, and sigmoid colon. Complex foods that cannot be digested by the human gut may be digested by the microbes that the intestines host (Hillman *et al,* 2017). Stomach acidity, bile and gastric acid, and peristalsis likely result in fewer bacteria colonizing the proximal small intestines. More microbes, with a shift from aerobic to anaerobic, can be found as the body progresses from the small intestines to large intestines (Quigley, 2013). Anatomically, the small intestine sections can be identified by a glandular layer. The duodenum is characterized by the presence of Brunner's glands, the jejunum has varying glands and larger numbers of lymph nodes, and the ileum is characterized by the presence of Peyer's glands (Kim *et al,* 2013).

The wall of the gastrointestinal tract can be subdivided into four main layers: the tunica mucosa, tunica submucosa, tunica muscularis, and serosa or adventitia. Atrophy and inflammation are common in gastrointestinal disorders like Celiac disease, which is positively associated with ASD (Ludvigsson *et al,* 2013). The tunica mucosa lines the lumen and serves to protect the small intestine from contamination from the gut microbiome and antigens and pathogens from the external environment. It also is responsible for secretion and absorption during digestion. The tunica submucosa is highly vascularized and contains the submucosal nerve plexus and glandular tissue. Vagal villi afferents terminate in the villus, while vagal crypt afferent endings encircle the luminal end of crypts. The mucosal afferents can detect stretching and tension, as well as chemical absorption and transmit the information to the CNS. The

afferents are sensitive to bacterially produced short-chain fatty acids as well as cytokines, nutrients, and hormones, allowing for bacteria to effect change on the gastrointestinal system (Fülling *et al,* 2019). The submucosal nerve plexus is largely responsible for controlling glandular secretion. The tunica muscularis is made of two layers of smooth muscles with the myenteric nerve plexus in between the muscular layers. The myenteric nerve plexus is responsible mainly for motility. Within the muscular layer, vagal afferents form intraganglionic laminar endings and intramuscular arrays and synapse onto neurons from the ENS (Fülling *et al,* 2019). The tunica serosa covers organs in the peritoneal cavity (Nováková & Blanková, 2018).

Mouse Models

Mouse models can help show insight into the pathophysiology behind ASD symptoms. Mice are very social creatures that can exhibit social behavioral deficits and have homologous digestive tract anatomy with humans so that they can be used in studies. The following study compared intestinal samples from wild-type control mice, C57BL/6J, to mice with social behavioral deficits including the BTBR genetic knockouts, valproic acid injected C57BL/6J, and polycytidylic acid injected C57BL/6J mice.

C57BL/6J mice are commonly used as control mice in ASD studies. The mice demonstrate extreme genetically similarity between individuals (Song & Hwang, 2017). Compared to other inbred mice groups, C57BL/6J mice show good ability for complex learning, including testing for contextualized fear conditioning. In prepulse testing, in which a loud noise triggers the mice's startle reflex and the mouse, these animals can become desensitized to the noise to demonstrate a lower prepulse inhibition than other inbred strains (Crawley *et al,* 1997). They are highly social, as measured in Social Approach Tests. However, sociability scores change throughout their development, with sociability being variable in male mice more so than

female mice during puberty around days 25 to 45 of life. The changing of behavior during development mirrors the progression of ASD. Similarly, larger litters and litters with more female mice showed increased sociability. The sociability link with high female to male ratios demonstrates that prenatal hormonal exposure could be a variant in sociability. Likewise, male mice who gestate next to female mice are exposed to more estradiol and less testosterone than male mice who develop next to male mice. The hormone-gestation link is similar to the hypothesis that exposure to high prenatal testosterone makes it more likely that an individual may develop ASD (Fairless *et al,* 2012).

BTBR genetic knockout mice have been used for studies of autism-like behaviors including reduced social interactions, impaired play, unusual vocalizations, and repetitive behaviors such as obsessive self-grooming that causes balding. They are known for having a markedly reduced or absent corpus callosum and impaired synaptogenesis and neurogenesis (Meyza *et al*, 2013). They have reduced social motivation processes and do not form conditioned place preferences for contexts related to social stimuli and may be due to problems with retention of memory of social-specific cues (Careaga *et al,* 2015). Neuroanatomical similarities have been noted between the BTBR mice and subpopulations of ASD patients such as the changes in the hippocampus and amygdala shape and location, the presence of Probst bundles that fail to cross the corpus callosum, and the distance between ventricles. BTBR mice have marked sensorimotor balance difficulties. Studies have shown that administering ketogenic diets can restore sensorimotor skills and improve sociability implicating the gut. Administering serotonin (5HT) agonists to these mice has been shown to increase the mice's social approach possibly implicating the gut. BTBR mice have an altered stress response and tend to show more anxiety than other inbred mice groups. Additionally, they are known to have gut dysbiosis similar to that

of patients with ASD and increased gut permeability in the large intestine. The dysbiosis is different between male and female mice, just ASD presents differently in male and female patients (Coretti *et al,* 2017).

The valproic acid model is created by injecting pregnant C57BL/6J dams at gestational day 11 with valproic acid. Valproic acid is a long-standing drug for epilepsy and a mood stabilizer (Bath & Pimentel, 2017). It is known to have gastrointestinal side effects (Jahromi *et al,* 2011). In the United States, it is prescribed to around 22% of children with epilepsy. Children who take the drug during adolescence are more likely to have problems with cognitive functioning and lower IQ scores and reduced motor performance and visuospatial functioning (Bath & Pimentel, 2017). During gestation, if a pregnant mother takes valproic acid, there is increased incidence of ASD for the children. Pups who were prenatally exposed to valproic acid also show social behavioral deficits. They have been shown to have developmental delays in self-righting, eye opening tests, and impaired social recognition (Yang *et al,* 2016). The mice also show gut dysbiosis between males and females. Valproic-acid exposed males show significantly higher levels of Bacteroidetes when compared to wild-type control male mice (Liu *et al,* 2018). Rats prenatally exposed to valproic acid show decreased gastrointestinal mobility and decreased thickness of tunica mucosa and tunica muscularis in the stomach, duodenum, jejunum, ileum, and colon (Kim *et al,* 2013).

The maternal immune activated model consists of pregnant C57BL/6J dams injected with polycytidylic acid (Poly I:C) at gestational day 11. Maternal immune activation can increase the severity of ASD-like symptoms, increasing the number of ultrasonic calls in young pups and marble burying tendencies (Meyza & Blanchard, 2017). The immune activation causes inflammatory response that results in social behavior deficits (Chow *et al,* 2016). Poly(I:C) is a

synthetic analogue of double-stranded RNA that is similar to molecules synthesized during viral replication. The severity of the long-term brain and behavioral changes are positively correlated with the intensity of the immune reaction and inflammation response caused by the Poly I:C (Meyer, 2014). Poly(I:C) pups show delays in growth and sensorimotor development. They have delays in righting ability, geotaxis, and problematic grasping reflexes (Arsenault *et al,* 2014). They show reduced social interaction and decreased memory and learning ability in juveniles. Male Poly(I:C) mice are more likely to have reduced desire to interact with novel mice than female mice and the behavioral qualities show sooner, just as with ASD (Ratnayake *et al,* 2014). Neuroinflammatory responses and increased cytokine levels suggest brain inflammation from an early age, which could explain the complications in neurodevelopment and the developmental delays (Arsenault *et al,* 2014). There are few studies involving the gut microbiome in Poly (I:C) mice, but there is evidence that acute exposure to Poly(I:C) after birth reduces colon permeability to macromolecules but increases ileum permeability to electrolytes and small molecules (Moyano-Porcile *et al,* 2015). Unfortunately, the current Poly(I:C) analogues available on the market are unstable and can result in variable responses in the animals.

Hypothesis

The following study is crucial in identifying gastrointestinal markers in mouse models of social behavioral deficits, a core symptom of ASD. The gut-brain-microbiota axis is bidirectional and dependent on bacterial colonization and short chain fatty acid production that can impact the digestive tract physiology. It is expected that each mouse will demonstrate a unique SCFA profile. Additionally, it is hypothesized that significant morphological differences will exist in one or all of the different mouse groups when compared to wild-type control mice. Specifically, it is expected that the tunica muscularis and tunica mucosa will be significantly

different in the BTBR genetic knockouts, valproic acid model, or polycytidylic acid model when compared to wild-type control mice.

METHODS

Animal Care

All experiments that used animals were approved by the University Committee on Animal Care at East Tennessee State University. Animals were housed in plastic cages in a standard 12-hour light and dark cycle with unlimited access to food and water. The mouse strain, C57BL/6J, was obtained from Jaxson Laboratories. C57BL/6J mice were bred in the ETSU animal facility and reared in the same conditions. Pregnant C57BL/6J female dams were either given intraperitoneal injections of valproic acid 600 mg/kg (PXXXX, Sigma, St. Louis, MO) or with polycytidylic acid (PXXXX, Sigma, St. Louis, MO) 5mg/kg dissolved in 0.9% sodium chloride solution at gestational day eleven. Pups were reared until postnatal day 21 (P21) or postnatal day 105 (P105). The genetic inbred strain, BTBRT+Itpr3^{tf}/J (BTBR), mice were obtained from Jaxson labs and reared on site in the same conditions for experimentation. Only male mice were used in the experimental studies.

Marble Burying

Marble burying was performed to test for repetitive behaviors in the mice. Rat cages were filled with 2 to 3 inches of bedding. A clear lid was placed on each of the cages. Mice were put in individual cages for 20 minutes in the dark in order to acclimate to testing conditions. Mice were removed and twenty marbles were placed in five symmetrical rows. The mice were returned to the cages with marbles for 20 minutes. The mice were then removed and then the number of marbles buried was counted. A marble was counted as buried if it was more than 2/3 concealed under the bedding. Three independent counters were used, and the median count was calculated for each mouse.

Social Testing

The sociability test was performed using a box with three chambers of identical size with openings into each chamber. A small wire cage is placed in chambers one and three. Mice were habituated with free movement for an initial five-minute period in which the test mouse was allowed to roam while both of the interior cages were empty. In the second phase, one familiar mouse was placed into an interior cage and the test mouse was allowed to roam. In the third phase, an unfamiliar mouse was placed in the remaining interior cage and the test mouse was allowed to roam. Each phase was video recorded and time spent in each chamber was calculated using Anymaze tracking software.

Fecal Collection

Fecal samples were collected at approximately P105 for the adult male mice. Mice were placed in sterile, bed-free cages for thirty-five minutes. Initially, the abdomen of the mouse was massaged and the mouse was lifted by the tail to encourage defecation. Feces was collected in pre-weighed 1.5 ml Eppendorf tubes. Fecal mass was determined and stored at -80°C until use. Approximately 2 mg of feces was collected for each mouse.

Short-Chain Fatty Acid Assay

The SCFA extraction procedures were modeled after research done by Schwiertz *et al* and modified. One milliliter of the SCFA solution, which contained oxalic acid (0.1 mol/L), sodium azide (40 mmol/L), and caproic acid (0.1 mmol/L) was added to approximately 450 milligrams (wet-weight) or 80 mg (dry-weight) of feces that had been freeze-dried and stored -

81°C in a 16 x 100 mm disposable culture tube. Samples were vortexed for 30 seconds and placed on a horizontal shaker for one hour. After the incubation, samples were centrifuged for twenty minutes at 4000 revolutions per minute or rpms. The supernatant was removed and placed in a 1.5 mL polypropylene micro-centrifuge tube. Solution was followed with a centrifugation step at 12,000 rpm for 15 minutes. The supernatant was removed and put into a 2 mL amber vial to be stored at -81°C until it was analyzed with a Shimandzu GC2010 gas chromatograph with a Sigma-Aldrich ZB-Wax Plus capillary column. The samples were run using the SCFA.standard.run.gcm method.

Statistical Analysis of Short Chain Fatty Acid Data

Statistical Analysis was done using an ANOVA: One-Factor Without Replication test to compare the average grams of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate per 80 milligrams of sample in the wildtype group compared to the valproic acid, BTBR, and Poly(I:C) behavior models. Dunnett's multiple comparison's test was used as a post-hoc test. The calculated p-values were considered significant <0.05.

Intestinal Preparation

At P21 or P105 days, mouse small and large intestines were obtained and put into 4% paraformaldehyde. After 24 hours, the intestines were washed with 70% ethanol and stored at 4^{o}C until embedding. In brief, samples of the ileum were then embedded in wax, sectioned, and prepared for staining. Ileum sections were placed into 0.5 cm samples in length and placing the section tissue into metal cages for dehydration. Dehydration was done by placing the cages in 80% ethanol, 90% ethanol, and 95% ethanol in order for 30 minutes per solution. The cages were

then soaked in 100% ethanol for 30 minutes twice. The cages were then placed in 50:50 100% ethanol:Citrisolv concentrate for 30 minutes. Next, the cages were placed in 100% Citrisolv for 30 minutes. Following steps included incubating tissues in two changes of Paraplast embedding medium for one hour each. Finally, the tissue was embedded in fresh Paraplast vertically to allow for cross sectioning and stored at room temperature.

Hematoxylin and Eosin Staining

Previously prepared hematoxylin was filtered through a coffee-filter filtration system to remove impurities. The slides were simultaneously placed in different solutions using a strict time regimen. The first phase was rehydration. Slides with tissue were placed in hemo-de 1, hemo-de 2, 100% ETOH, 100% ETOH, 95% ETOH, 80 % ETOH, and 75% ETOH for three minutes each. The slides were then rinsed in distilled water for two minutes three times. Once rinsed, the slides were incubated in the hematoxylin for exactly three minutes, washed in distilled water for five minutes, and then dipped in 0.3% acid alcohol 8-12 times. Afterwards, the slides were washed for one minute twice in tap water and then for two minutes in distilled water. Once rinsed, the slides were placed in eosin for 30-45 seconds. During the dehydration phase, the soaking steps from the rehydration phase were repeated but in reverse, except that the ethanol steps were timed for 2 minutes each. The two hematotoxin soaks were performed for 3 minutes each. The slides were left to dry for at least 24 hours, and then covered with a coverslip using permount and left to dry for at least another 24 hours.

Image Acquisition and Tissue Measurements

Using a Zeiss Axioskop 40 microscope with a Cannon Powershot A640 camera, sections of ileum from each model were taken at 10X magnification. The samples were confirmed to be ileum using standardized image comparison to available ileum tissue. The thickness of the tunica muscularis and tunica mucosa were measured using standardized measuring scaling of pixels/nm from the Axios software. The tunica mucosa was measured from the bottom of the glandular tissue to the top of the villi six times per sample in equal intervals given that the tissue layers were clearly defined and had not been stripped during staining. The tunica muscularis excluded the submucosa in all measurements. Tunica muscularis measurements were only taken if the tissue was fully intact and not in ribbons. Tissue samples were excluded if the tunica muscularis or tunica mucosa were not clearly intact.

Statistical Analysis of Tissue Measurements

The tunica mucosa and tunica muscularis thicknesses in the mouse models of social deficits for P21 and P105 were compared to the wild type at P21 and P105 accordingly. A one-way ANOVA with Bonferroni post-hoc test with calculated significance of $p<0.05$ was used.

RESULTS

Behavioral Studies

Sociability testing and marble burying measurements were used to confirm that the mouse models of social deficits were showing the expected social deficits. Figure 1 shows that both polycytidylic acid injected C57BL/6J offspring and wild type mice were more likely to prefer empty cages than cages with familiar mice. Valproic acid injected C57BL/6J offspring mice and BTBR genetic knockout mice did not show a preference in choosing to interact with a mouse over an object. A p-value of <0.05 was considered significant.

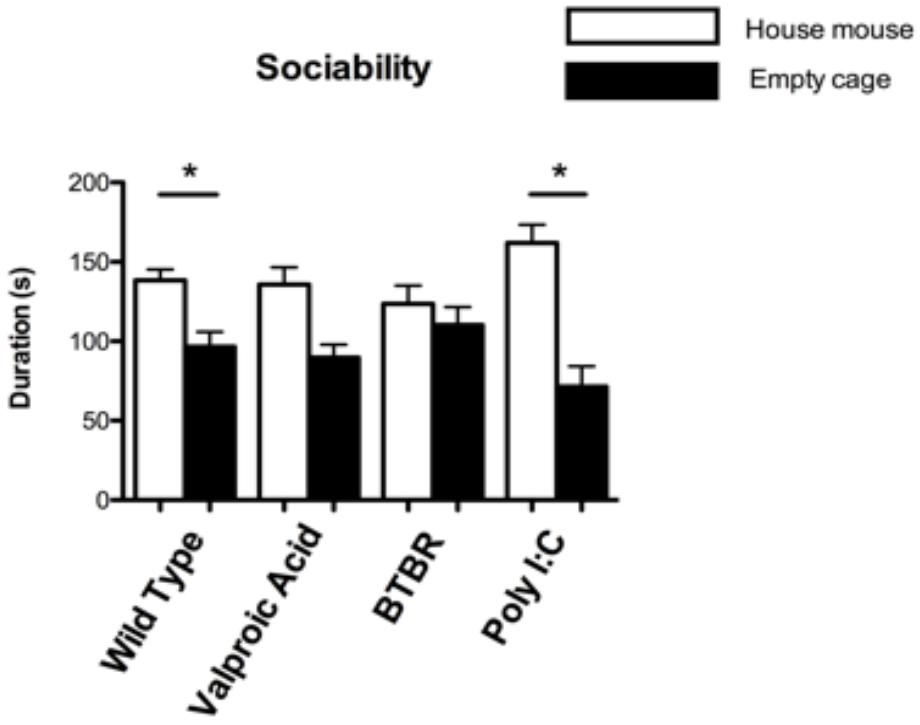


Figure 1. Sociability of Mouse Models of Social Behavior. Mice were evaluated for time spent with novel object or live animal with "duration" referring to the average time. Significant differences with p-values<0.05 are denoted by asterisks (Wild type N=6, Valproic acid N=10, BTBR N=9, and Poly(I:C) Males: N=8).

A second measure of behavior was performed to assess for repetitive behaviors in the animals. Repetitive behaviors must be displayed for an ASD diagnosis in human patients. The marble burying assay is used as a measure of mouse behavior when the animal is presented with multiple, novel objects in a novel environment. Our findings demonstrate that the wild type mice were significantly more likely to bury more marbles than the valproic acid injected C57BL/6J offspring and BTBR genetic knockout mice. BTBR genetic knockout mice were more likely to bury more marbles than valproic acid injected C57BL/6J offspring, although it was not statistically significant. Figure 2 shows the overall average number of marbles buried by each male group and the significance. There were no significant differences between the polycytidylic acid inject C57BL/6J mice and any other group. The numbers of marbles buried by each group is shown in Charts 1-8 in the Appendix. A p-value of <0.05 was considered significant.

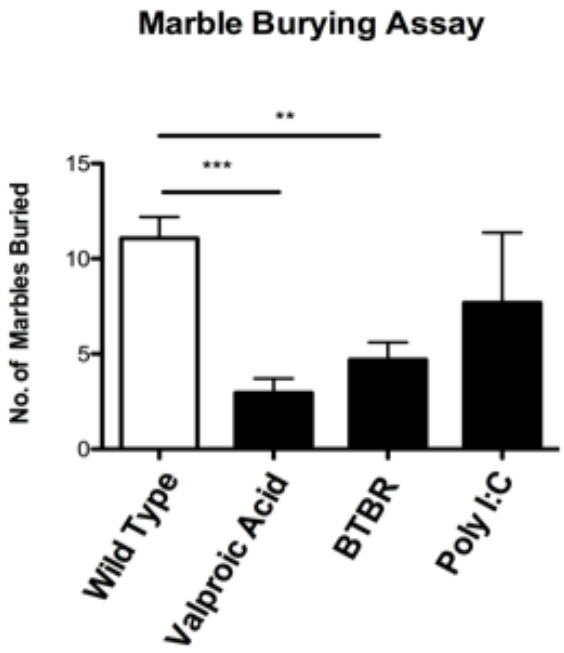


Figure 2. Marble Burying Assay of Mouse Models of Social Behavior. Mice were evaluated for repetitive behaviors with "No. of Marbles Buried" referring to the number of marbles that

were at least 2/3 buried. Significance is denoted by p-values of ** <0.01 and ***<.0001(control N=6, VA Males N=7, BTBR Males N=8, Poly(I:C) Males N=8).

Short Chain Fatty Acid Assays

Short-chain fatty assays were performed on the fecal samples to determine profile differences between the mouse models of social behavioral deficits. Table-1 shows the results of an ANOVA one- way statistical analysis comparing all mouse groups. P-values less than 0.05 are considered significant. Significant p-values are shown with asterisks: *denotes $p<0.05$, **denotes that $p<0.01$, and *** denotes that $p<0.001$. Offspring from valproic acid injected C57BL/6J mice and BTBR genetic knockout mice have significantly different levels of acetate, propionate, isobutyrate, butyrate, and valerate in their short-chain fatty acid profiles when compared to wild-type control mice. The polycytidylic acid injected C57BL/6J offspring did not have any measured significant differences compared to the control mice. It should be noted that only two animals were used in the polycytidylic acid injected C57BL/6J offspring group. Lastly, isovalerate levels were not significantly different in any of the groups compared to the wild type

Significance levels in SCFA per 80 mg of the Sample			
SCFA (% Area Under the Curve)	Wild Type vs Valproic Acid p-value	Wild Type vs BTBR p-value	Wild Type vs Poly I:C P-Value
Acetate	* 0.0007	** 0.0038	0.7954
Propionate	*** <0.0001	*** <0.0001	0.4253
Isobutyrate	** 0.0023	* 0.0174	0.2801
Butyrate	** 0.0042	* 0.0123	0.9429
Isovalerate	0.5957	0.9792	0.9244
Valerate	** 0.0044	* 0.0133	0.2321

Table 1. Significance Levels in SCFA per 80 mg of Sample in Mouse Models of Social Behavior (C57BL/6J males: N=6, VA males: N=7, BTBR males: N=8, Poly(I:C) males: N=2).

Levels of SCFA in graph form are from ach group are shown in Figures 4 and 5. Short chain fatty acids that were measured included: acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate (Figure 4). They are the most common SCFA that are expressed in mice. Acetate is shown independently due to the disproportionately larger amounts of this SCFA as compared to the other SCFA levels in mice (Figure 5). All SCFA data are shown in the Appendix Supplemental Table 9.

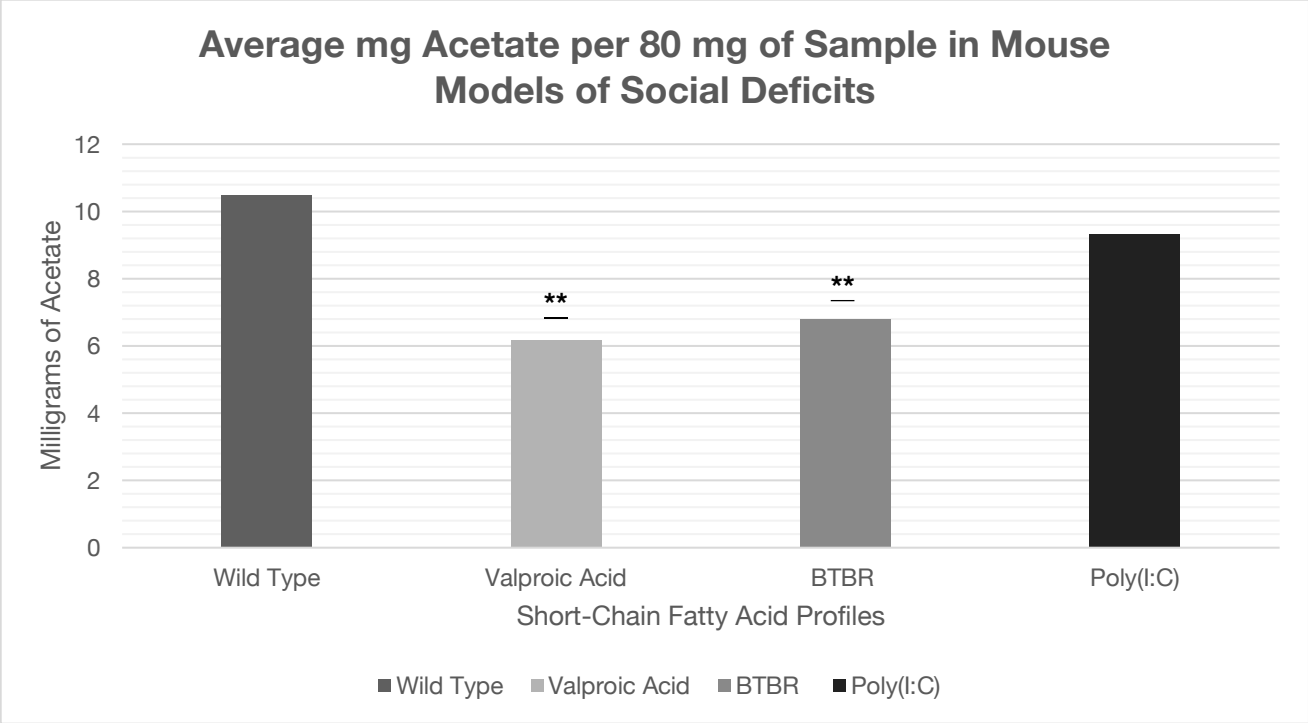


Figure 4. Average milligrams of acetate per 80 milligrams of sample in mouse models of social deficits (C57BL/6J males: N=6, VA males: N=7, BTBR males: N=8, Poly(I:C) males: N=2). Significance is denoted by p-values** <0.01.

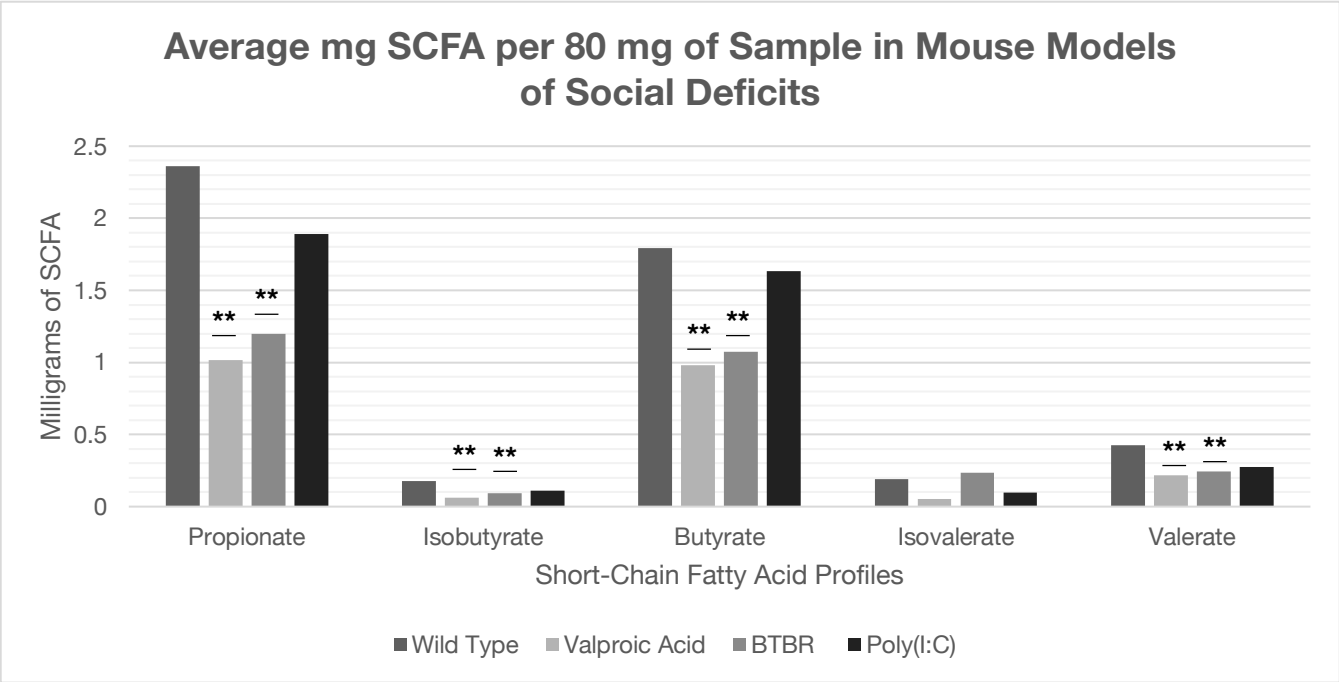


Figure 5. Average milligrams of propionate, isobutyrate, butyrate, isovalerate, and valerate per 80 milligrams of sample in mouse models of social behavioral deficits (C57BL/6J males: N=6, VA males: N=7, BTBR males: N=8, Poly(I:C) males: N=2). Significance is denoted by p-values** <0.01.

Intestinal Morphology Measurements

The lengths of the tunica muscularis and tunica mucosa were measured in postnatal day 21 (P21) and postnatal day 105 (P105) mice in order to show if there was a significant difference in the measured thickness between the wild type and the mouse models of social behavioral deficits. Figure 6 demonstrates the length of the tunica muscularis in mice from the wild type group as compared to the mouse models of social deficits at P21, and Figure 7 demonstrates the lengths of the tunica muscularis at P105. Valproic acid injected offspring were significantly different than control mice ($p=0.0463$) at P21. A p-value of <0.05 was considered significant. At postnatal day 105, the tunica muscularis appeared to be trending toward a reduction but was not

significantly different between any the groups. Overall, it was noted that the tunica layers were longer in the adult group than in the immature group. Figures 8 and 9 compare the lengths of the tunica mucosa of the wild type mice compared to the mouse models of social deficits at P21 and P105 accordingly. Reductions in the tunica mucosa length in the valproic acid group when compared to wild type control mice were significantly different ($p=0.0279$) in the 21-day old group (Figure 8). For the 105-day old group, no significant differences were not found when comparing groups (Figure 9). It should be noted that only one valproic acid gave reliable results; therefore, more animals will have to be used in the future to obtain official results. For Figures 6 through 9, the mice used for imaging are listed in Appendix Supplemental Table 10. Images used for measurements for each animal can be seen in the Appendix Supplemental Images 1-8. The different ages are representative of an immature mouse at P21 days and a fully developed adult mouse at P105 days. A one-way ANOVA was used to measure differences between groups.

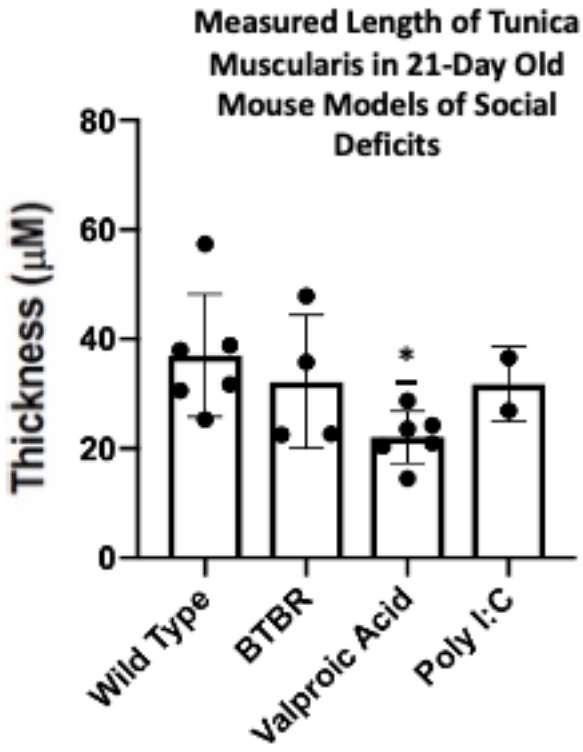


Figure 6. Length of the tunica muscularis in uM at 21 days of development in mouse models of social deficits. Dots denote the number of animals used in the study (Wild type N=6, BTBR N=4, Valproic Acid N=6, and Poly IC N=2). Significance is denoted with asterisks* using a one-way ANOVA with p-values of <0.05.

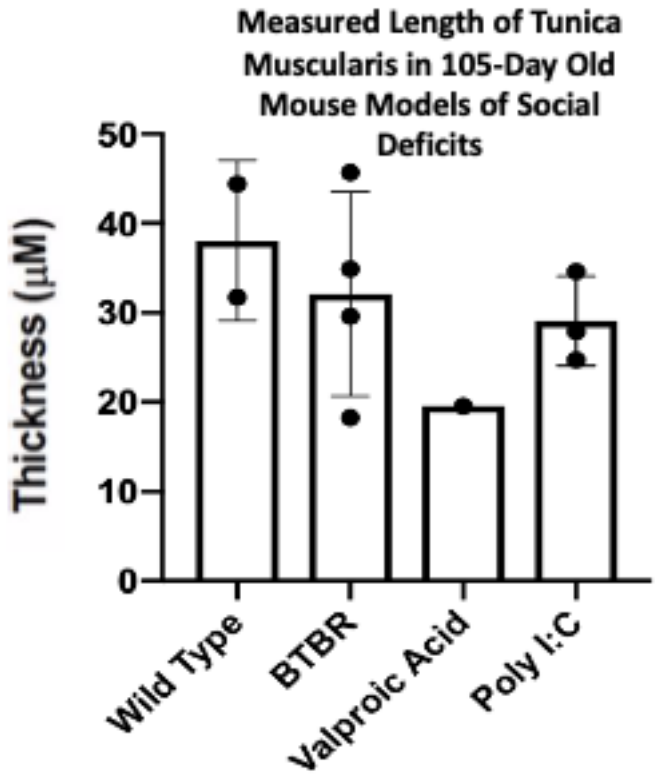


Figure 7. Length of the tunica muscularis in uM at 105 days of development in mouse models of social deficits. Dots denote the number of animals used in the study (Wild type N=2, BTBR N=4, Valproic Acid N=1, and Poly IC N=3). Significance was not observed using a one-way ANOVA with p-values of <0.05.

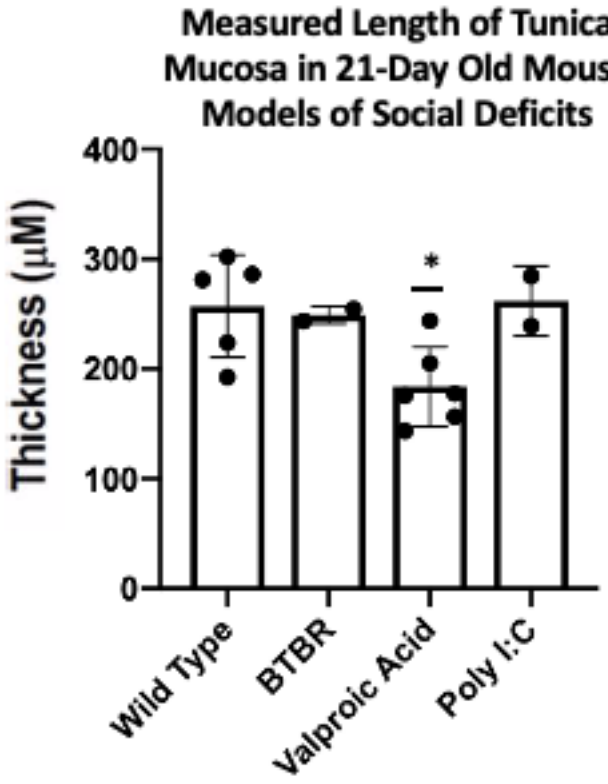


Figure 8. Length of the tunica mucosa in uM at 21 days of development in mouse models of social deficits. Dots denote the number of animals used in the study (Wild type N=5, BTBR N=2, Valproic Acid N=6, and Poly IC N=2). Significance using a one-way ANOVA is denoted with asterisks* of p-values of <0.05.

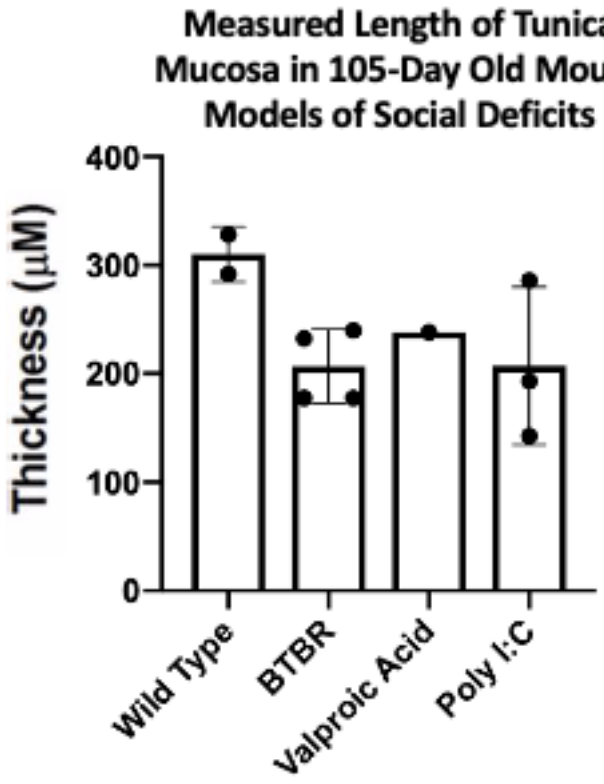


Figure 9. Length of the tunica mucosa in uM at 105 days of development in mouse models of social deficits. Dots denote the number of animals used in the study (Wild type N=2, BTBR N=4, Valproic Acid N=1, and Poly IC N=3). Significance was not observed using a one-way ANOVA with p-values of <0.05.

DISCUSSION

Behavioral Studies

There is currently no accepted animal model for autism spectrum disorder, but there are many models that display independent features. The behavioral studies were performed to ensure that the mice used in the studies demonstrated behaviors that have been identified in previous studies for these particular models. The results did support that the mice exhibited social behavioral deficits using the sociability test. However, the marble burying test for repetitive behaviors displayed conflicting results when compared to the literature. The marble burying test for the symptomatic increase in anxiety and repetitive behavior is associated with ASD. Mice show "defensive burying" when stressed, and therefore it is believed that mice with unregulated anxiety who are prone to more repetitive behaviors will bury more marbles. Research done by Thomas *et al* suggests that marble burying may not be indicative of novelty anxiety inasmuch as genetic predisposition for repetitive behavior. Therefore, it was expected that the wildtype group would bury the smallest amount of marbles. The results in this study showed the opposite with control mice burying the largest number of marbles. It is likely that the results were confounded by differences in the mice's burying and scurrying techniques, as even though the number of marbles buried was unexpected, the mice showed different behaviors during the trials. Additionally, rater behavior may have been different for the control group. The number of people who counted marbles and the number of marbles counted for each mouse is shown in supplemental tables 1-8 in the appendix. It was found that two of the three raters for the wild type control mice had elevated numbers when compared to a third rater. Marbles were only to be counted if they were two thirds buried in bedding. Mice burying the marbles were video recorded to determine if differences in their burying behavior could be identified by

viewing the animals. Burying behavior was different among the groups. For example, BTBR mice showed more active burying but fewer marbles, while C57 mice were more likely to dig in the corners of the cages with marbles then falling into holes as a consequence of the techniques. Additionally, there was variation among the individual counters who counted if the marbles were 2/3 buried or not, which can be seen in the Appendix. These studies will be repeated in the future. Although in the wrong direction, it is observed that the wild type control mice and the Poly(I:C) mice are significantly different from the valproic acid injected offspring and the BTBR mice. It should be noted that the Poly (I:C) mice had a very small study number, and given that the Poly(I:C) group's data was continually similar to the wildtype group, then it is highly likely that the dams did not receive the correct ratio of long versus short polyinosinic–polycytidylic acid molecules that are required for animals to display behavior.

A defining characteristic of autism spectrum disorders is an aversion to interacting with other people. It was expected in the sociability test that the wildtype mice would show more curiosity towards the cage with a mouse in it than towards an inanimate object while the social deficit mice would not show any inclination towards the house mouse with whom they were familiar or would prefer the inanimate cage (Moy *et al,* 2004) The mice in these studies simply did not have a preference. It has been noted that a confounding variable may exist in the social testing. For example, testing by Pearson *et al* showed that C57BL/6J mice may fail to exhibit social novelty in non-novel situations, meaning if the mice have acclimated to the three-chamber maze, then they will not show preference. The mice these studies may have been acclimated too early, especially because they were used as house mice before they were tested for novelty in some situations. It should be noted that the models used in these studies are highly reliable in demonstrating the social behaviors in the literature and coupled with differences among the

marble burying raters it appears they are valid study animals. Also, the valproic injected pregnant dams would demonstrate loss of consciousness after the valproic acid injection indicating that they did receive the required dose.

Despite the lack of robust behavioral results, it is still likely that the models still are reliable representations of ASD because of their historical use and the display of behavioral differences after injection. In the future, the trials should be repeated with several changes. For sociability testing, more consideration should be given to ensuring that the maze remains novel for the mice. Additionally, more specific measures i.e. time that mice are in the center around the object or the time in corner may be analyzed rather than just simply chamber time. As for marble burying, the same raters should be used for all trials to demonstrate consistency in determining the marble depth buried and the mice should always be video recorded so that unusual behavior can be better measured.

Short Chain Fatty Acid Assays

It was hypothesized that each mouse group would have a unique SCFA profile. This was supported by the significant differences in acetate, propionate, butyrate, and valerate amounts in feces from wild type controls and mice with social behavioral deficits. There were no significant differences in the amounts of isovalerate. Given that bacteria are what are producing the SCFA, the data suggests different bacterial profiles are different in the social deficit groups. Acetate is utilized to prevent enteropathogenic infection which can harm gut integrity; therefore, lower levels of acetate in the valproic acid and BTBR models may be indicative of a weakening intestinal barrier and increasing exposure of the vagal afferents (Fukuda *et al,* 2011). Acetate is used by bacteria in the synthesis of butyrate, which could be a factor in the lower amounts of

butyrate (Skonieczna-Żydecka *et al,* 2018). Likewise, butyrate has also been cited as having an important role in maintaining barrier integrity the small intestine (Chambers *et al,* 2018), regulating transepithelial fluid transport, preventing or managing mucosal inflammation and oxidative stress, and modulating visceral sensitivity and intestinal motility (Canani *et al,* 2011). Propionate, which was seen in lower levels in the valproic acid and BTBR groups, modulates immune response in the gut by reducing the cytokine load and stimulates *Bifidobacterium* which can aid in restoring proper gut permeability (Skonieczna-Żydecka *et al,* 2018). Like valerate, which is not well studied, accumulation or overexposure to propionate can lead to developmental delay and gastrointestinal disorders (Nankova *et al,* 2014) There was no significant difference in isovalerate levels. Very little research has been done on isovalerate (Stieb & Schink, 1986) and further study of it to see why the levels remain the same could be done.

The mouse data collected is concurrent with previous findings in humans with ASD that there are fewer SCFA produced overall. There are significantly reduced amounts of acetate, butyrate, propionate, and valerate in human fecal matter in patients with ASD compared to those without ASD with a 27% reduction of SCFA overall (Adams *et al,* 2011). Interestingly, some human studies have shown that there is an increase in valerate in human populations with ASD (Liu *et al,* 2019). Additionally, the valproic acid and BTBR groups have profiles more similar to each other than the wildtype, supporting that while there is a range of profiles between different severities of ASD, ASD groups are more similar to each other than non-ASD comparisons. Human data can be plagued with biased results due to an individual's diet; but the same diet was used in these mouse studies. Additionally, the valproic acid and poly IC offspring came from a wild-type dam.

Intestinal Morphology

The intestinal morphology data did not demonstrate robust significant differences between the wild type control mice and the mice with social behavior deficits. It must be noted that extremely small samples were eventually investigated. Offspring from the valproic acid treated dam did show significant differences in the lengths of the tunica muscularis and tunica mucosa for P21. Future studies could include exploration of the intestinal morphology relationship to vagus nerve stimulation. The tunica mucosa protects the underlying submucosal nerve plexus from toxic short-chain fatty acid products and bacterial toxins that ultimately weaken the intestinal barrier. The valproic acid model showed evidence of dysbiosis in the short-chain fatty acid expression and irregular patterns of SCFA signaling molecules. Changes in the intestinal barrier could amplify the effects of the dysbiosis making the vagal afferents more apt to generate an action potential. Vagal sensitivity is thought to be a common symptom of ASD. Additionally, the tunica muscularis contains the intrinsic myenteric nerve plexus that controls intestinal motility. Damage to the myenteric plexus can cause other gastrointestinal issues demonstrating a possible relationship to the gastrointestinal comorbidities associated with ASD.

The BTBR group did not show significant differences in the sizes of the tunica muscularis or tunica mucosa. The tunica mucosa and tunica muscularis were smaller, but not significantly different. More samples are needed to make a definitive conclusion. It is possible that the differences between the valproic acid and BTBR mice may be representative of the ASD spectrum. For example, not all patients with ASD exhibit gastrointestinal symptoms or serotonin dysregulation from improper gut functioning. Therefore, changes in intestinal morphology may be part of a subgroup of ASD presentations. The identification of subpopulations with similar pathophysiology is a major need in ASD research.

Limitations

Limitations of this experimentation were likely present. It is highly likely that the Poly(I:C) models were not successfully injected and were more similar to C57BL/6J than to the behavioral deficit model that they were supposed to represent. The sample size for Poly(I:C) was only 2 for both behavioral and SCFA assay testing, so it needs to be increased in future studies. We opted to discontinue the Poly(I:C) studies until a better chemical is available but wanted to show the data due to the similarity to the control group. Additionally, the wildtype possessed an outlier in aggression (supplemental table 1). The mouse was removed prior to the study and was not included in any part of the study.

During the intestine morphology study, there were issues with the tissue being properly embedded and some tissue samples were stripped or destroyed reducing sample sizes and making it more difficult to ascertain results from some samples. In future trials, it is recommended that only the ileum near the cecum is used for the necessity of having an anatomical landmark due to the difficulty of differentiating the duodenum, jejunum, and ileum. Future studies may look into the large intestine due to its continuing, more reliable anatomy. Sample size will need to be increased prior to publication.

Conclusions

The microbiome-gut-brain axis is an integrative system of communication that monitors and facilitates interrelated changes. It connects the central nervous system to the enteric nervous system. These studies identified differences in SCFA production in the gut of mouse models of social behavior deficits. Short-chain fatty acid profile differences due to dysbiosis in the microbiome likely causes altered signaling patterns which negatively impact intestinal

morphology and signaling to the brain. The vagus nerve serves as the pathway connecting the enteric nervous system to the central nervous system. Exposed vagal nerve afferents and increase intestinal permeability facilitate systematic changes. In ASD, the changes could exacerbate behavioral symptoms and gastrointestinal distress. The gut microbiome and intestinal morphology are both affected by genetics and the environment. Using mouse models of social deficits, it become apparent that the microbiome is fundamental to health. Future research should look at gut dysbiosis, short-chain fatty acid profiles, and intestinal morphology in mouse models to elucidate serotonin influences on the production in the gut that facilitate change in the brains. The prevalence of dysbiosis and altered signaling pathways in mouse models point to possible therapeutics for ASD treatment in human patients to alleviate gastrointestinal distress, overwhelmed vagal systems, and negative behavioral patterns.

APPENDIX

C57BL/6J Marble Burying Performed 10-18-18				
	Researcher 1	Researcher 2	Researcher 3	Average
Group 1				
M0	7	5	4	5.3
M1	10	9	7	8.7
M2	12	11	18	13.7
Group 2	******Group 2 was housed with violent mouse. Mice had wounded tails.			
M3	17	14	16	15.7
M4	10	12	12	11.3
M5	15	18	12	15
			Total Average:	11.6

Supplemental Table 1. Marbles buried during marble burying protocol by Group 1 and 2 of male C57BL/6J mice. Three independent researchers were trained on marble burying protocol and asked to count the number of marbles buried once the mice had been removed from the testing enclosures. Group 2 was housed with an unusually violent mouse that was removed and not included in the study. The remaining mice had wounded tails.

C57BL/6J Marble Burying Performed 10-20-18							
	Researcher 1			Researcher 2			
Group 3	Trial 1	Trail 2	Trial 3	Trial 1	Trail 2	Trial 3	Average
M0	0	0	0	0	0	0	0.0
M1	0	0	0	5	5	5	2.5
M2	0	0	0	1	1	1	0.5
M3	0	0	0	1	1	1	0.5
						Total Average:	0.88

Supplemental Table 2. Marbles buried during marble burying protocol by Group 3 of male C57BL/6J mice. Due to the availability of researchers, only two trained researchers participated, but each person counted the number of marbles buried 3 times.

C57BL/6J FEMALES Performed:2-17-20				
	Researcher 1	Researcher 2	Researcher 3	AVERAGE
F1	0	1	2	1.0
F2	0	0	0	0.0
F3	5	13	10	9.3
F4	4	1	1	2.0
F5	8	14	12	11.3
F6	0	1	1	0.7
F7	1	4	3	2.7
F8	0	0	0	0.0
F9	1	3	2	2.0
F10	2	4	4	3.3
			TOTAL AVERAGAE	3.2

Supplemental Table 3. Marbles buried during marble burying protocol by Group 1 of female C57BL/6J mice. The methods from Table 1 were repeated.

Valproic Acid Performed: 11-13-18				
	Researcher 1	Researcher 2	Researcher 3	AVERAGE
M1	0	0	2	0.7
M2	3	7	8	6.0
M3	0	1	4	1.7
M4	4	6	5	5.0
M5	0	2	2	1.3
M6	1	3	7	3.7
M7	1	1	5	2.3
			TOTAL AVERAGAE	2.95

Supplemental Table 4. Marbles buried during marble burying protocol by valproic acid males.

Methods from Table 2 were repeated.

BTBR Performed: 11-30-18				
Group 1	Researcher 1	Researcher 2	Researcher 3	AVERAGE
M1	7	7	6	6.7
M2	0	2	0	0.7
M3 ***had 24 total marbles***	6	7	7	6.7
M4	9	9	8	8.7
M5	0	0	0	0.0
			TOTAL AVERAGAE	4.5

Supplemental Table 5. Marbles buried during marble burying protocol by Group 1 of BTBR males. Methods from Table 2 were repeated. M3 erroneously had 24 marbles placed during the test instead of 20 marbles.

BTBR Performed:9-16-19				
Group 2	Trial 1	Trial 2	Trial 3	AVERAGE
M0	12	12	12	12
M1	10	11	11	10.7
M2	16	16	16	16
M3	5	5	5	5
M4	18	18	18	18
			TOTAL AVERAGE	12.3

Supplemental Table 6. Marbles buried during marble burying protocol by Group 2 of BTBR males. Methods from Table 2 were repeated. M3 erroneously had 24 marbles placed during the test instead of 20 marbles.

Poly(I:C) Performed: 3-21-19				
Group 2	Researcher 1	Researcher 2	Researcher 3	AVERAGE
P5	12	13	13	12.7
P6	16	15	15	15.3
			TOTAL AVERAGE	12.3

Supplemental Table 7. Marbles buried during marble burying protocol by Group 2 of Poly(I:C) males. Methods from Table 2 were repeated.

Poly(I:C) Performed: 4-30-19				
Group 3	Researcher 1	Researcher 2	Researcher 3	AVERAGE
p10	3	6	4	4.3
p11	7	8	8	7.7
p12	0	0	0	0.0
p13	4	6	6	5.3
			TOTAL AVERAGE	4.3

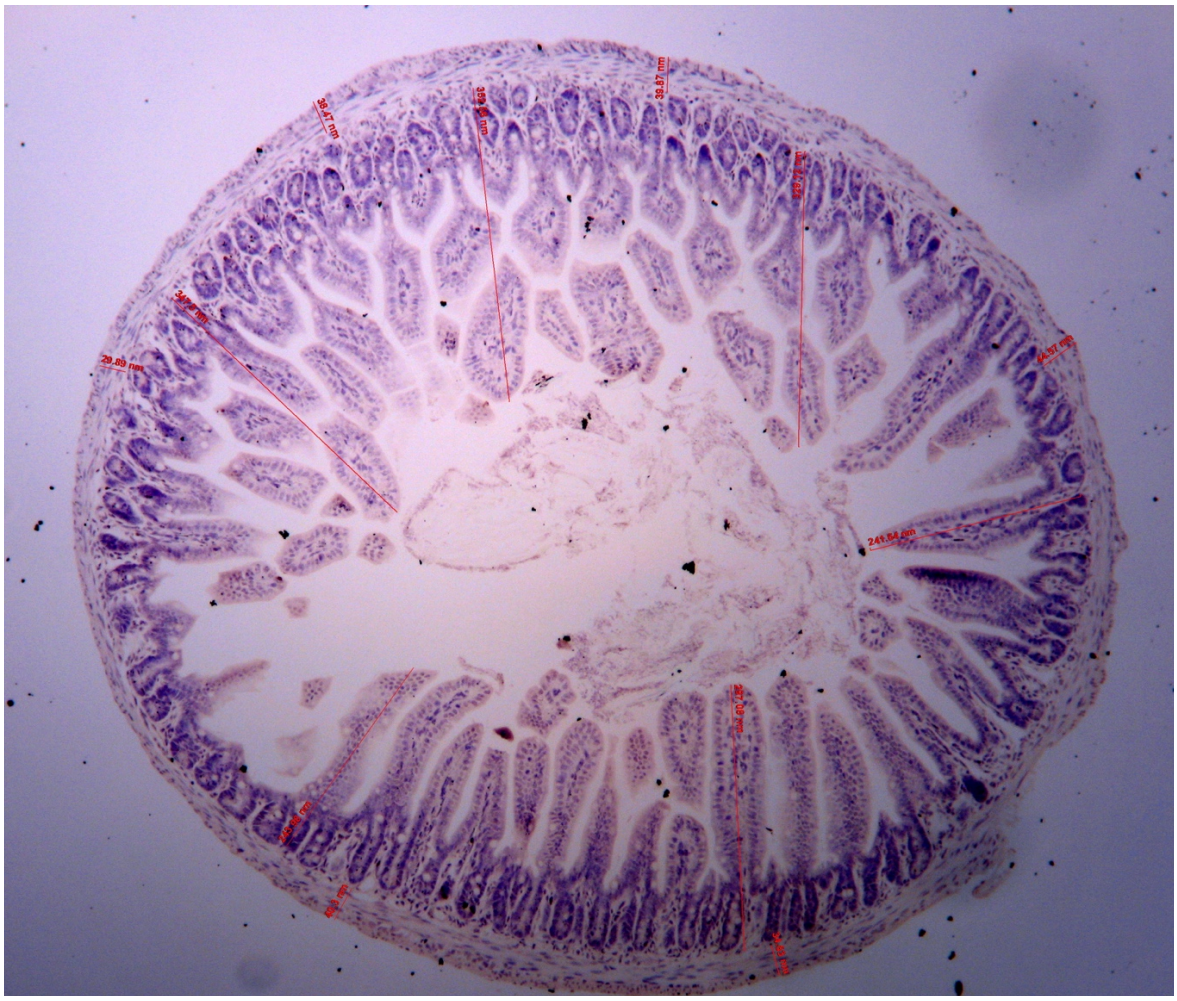
Supplemental Table 8. Marbles buried during marble burying protocol by Group 2 of Poly(I:C) males. Methods from Table 2 were repeated.

Average SCFA per 80 mg of Sample				
SCFA (% Area Under the Curve)	Wild Type Averages (mg)	Valproic Acid Averages (mg)	BTBR Averages (mg)	Poly (I:C) Averages (mg)
Acetate	10.49±1.295	6.180±0.340	6.81±0.415	9.31±0.206
Propionate	2.360±0.313	1.016±0.0317	1.198±0.077	1.892±0.021
Isobutyrate	0.178±0.034	0.0640±0.002	0.094±0.009	0.112±0.006
Butyrate	1.792±0.251	0.980±0.091	1.075±0.151	1.634±0.079
Isovalerate	0.192±0.039	0.054±0.003	0.234±0.155	0.096±0.004
Valerate	0.427±0.081	0.219±0.005	0.244±0.009	0.276±0.006

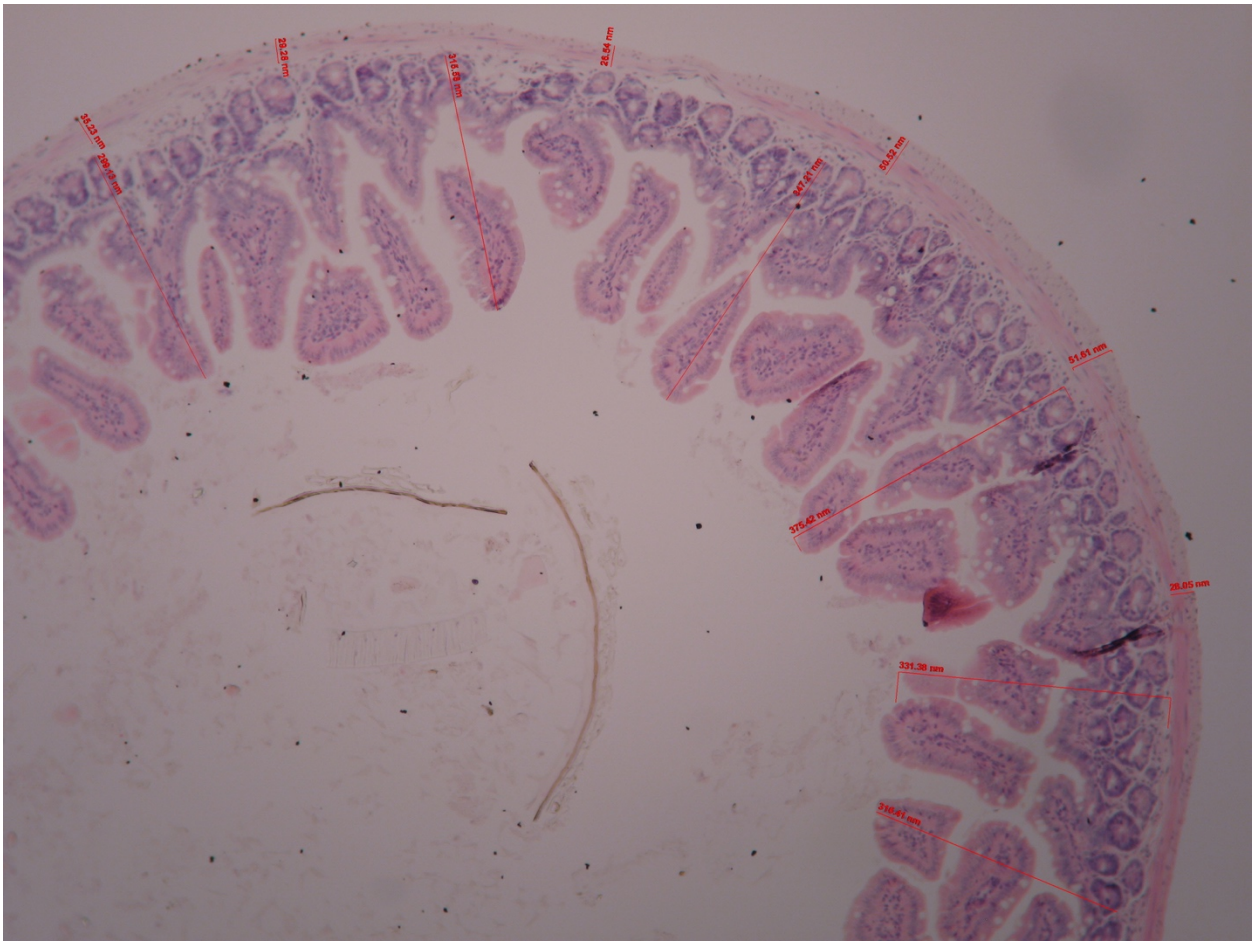
Supplemental Table 9. Average SCFA per 80 mg of Sample in Mouse Models of Social Behavior

105 Day Imaging (N=10)	21 Day Imaging (N=16)
C2206	C2208
C2209	C2211
B5003	C2212
B5004	C2213
B5005	C2215
B5022	C2229
V437	B5013
P2192	B5015
P3040	B5023
P3041	B5024
	V425
	V426
	V427
	V424
	V446
	P2188

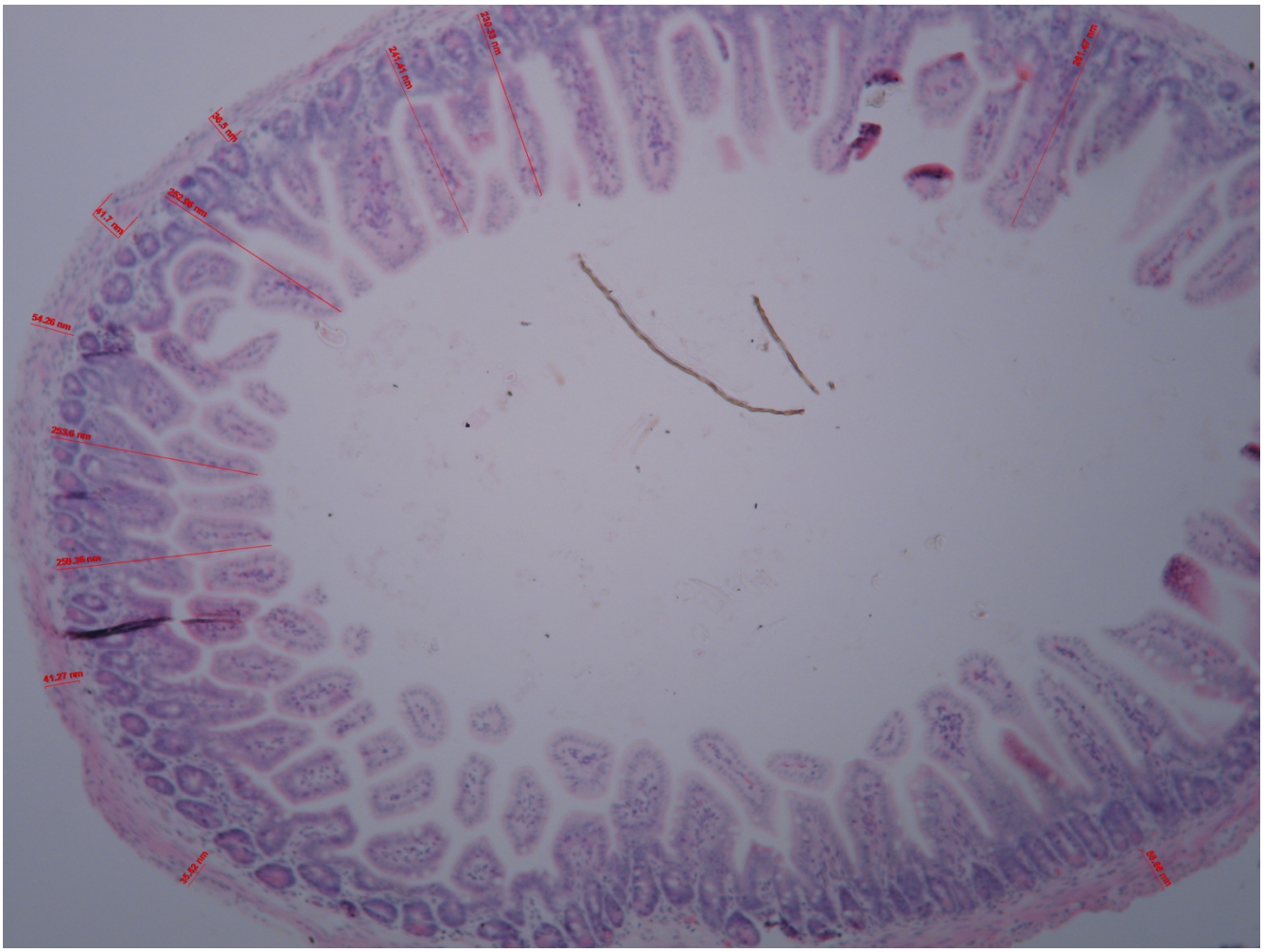
Supplemental Table 10. Mice used for imaging.



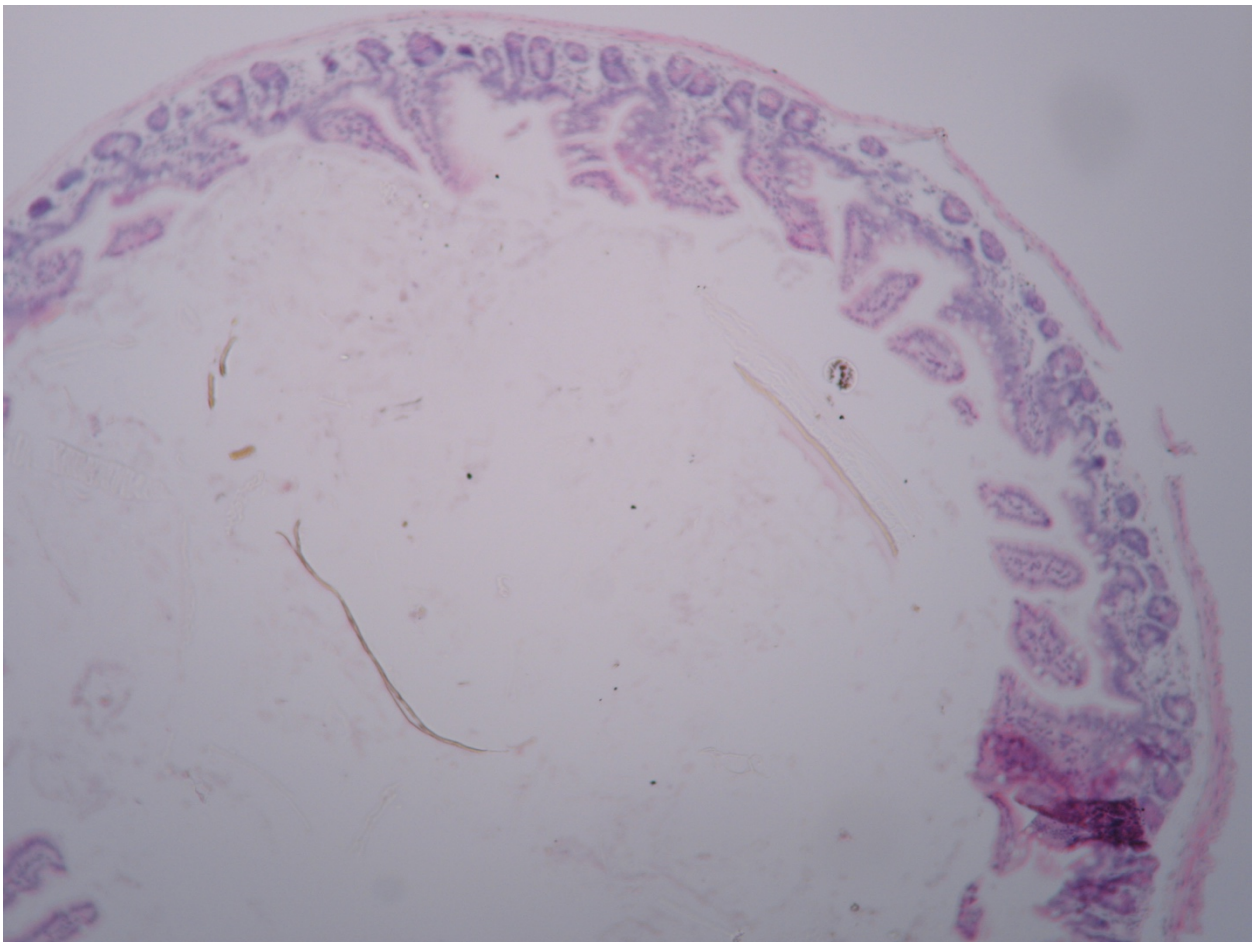
Supplemental Image 1. Measured tunica mucosa and tunica muscularis for C2213 at P21. (N=7)



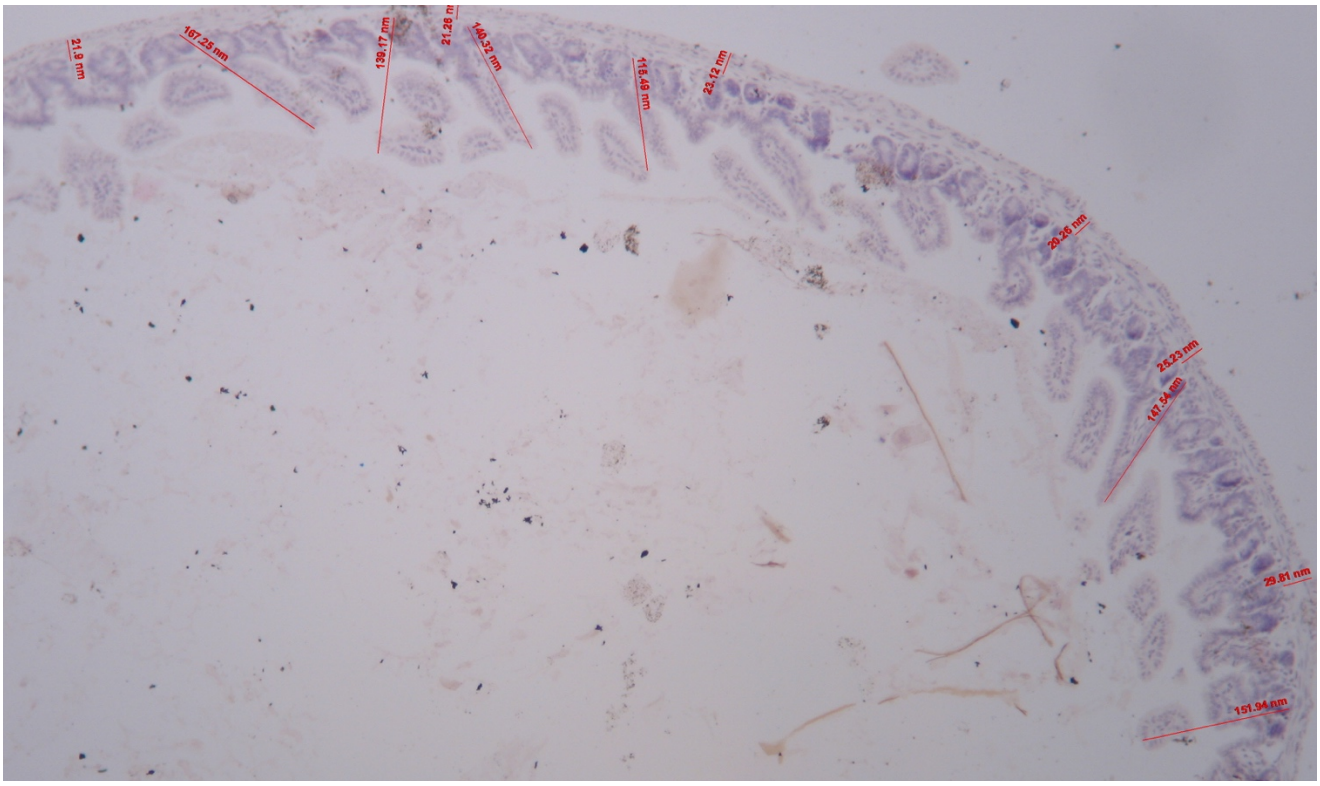
Supplemental Image 2. Measured tunica mucosa and tunica muscularis for C57BL/6J P105 C2209. (N=2)



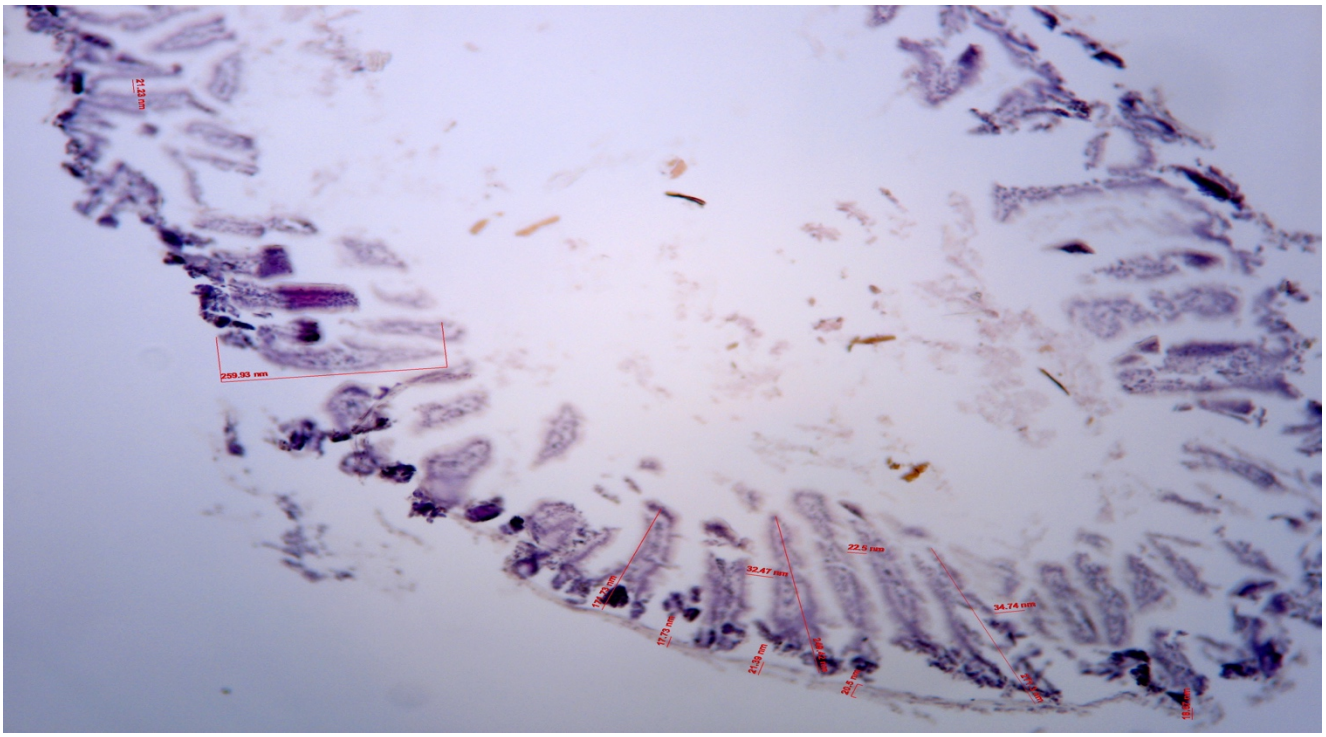
Supplemental Image 3. Measured tunica mucosa and tunica muscularis for BTBR P21 B5013. (N=4)



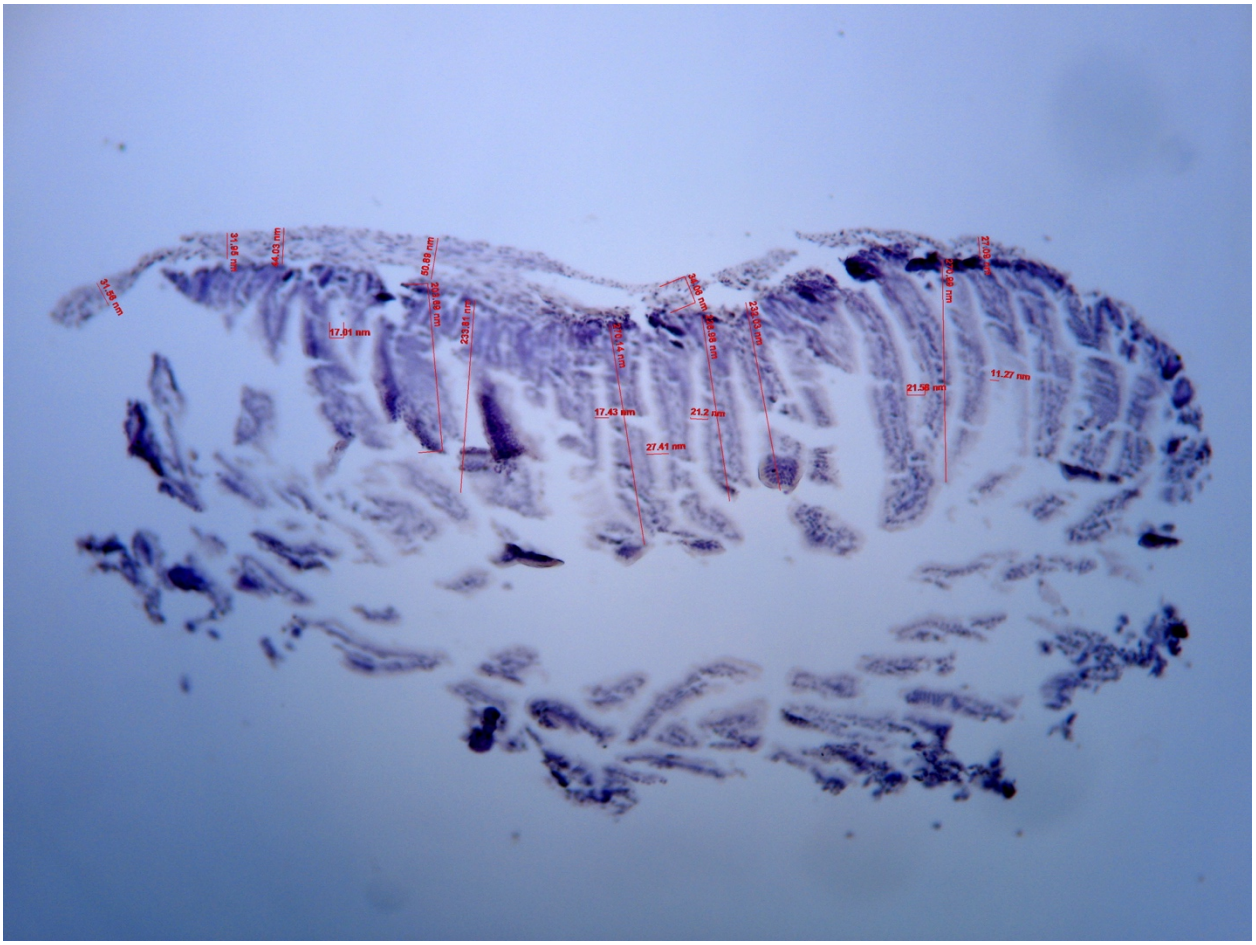
Supplemental Image 4. Measured tunica mucosa and tunica muscularis for BTBR P105 B5005. (N=4)



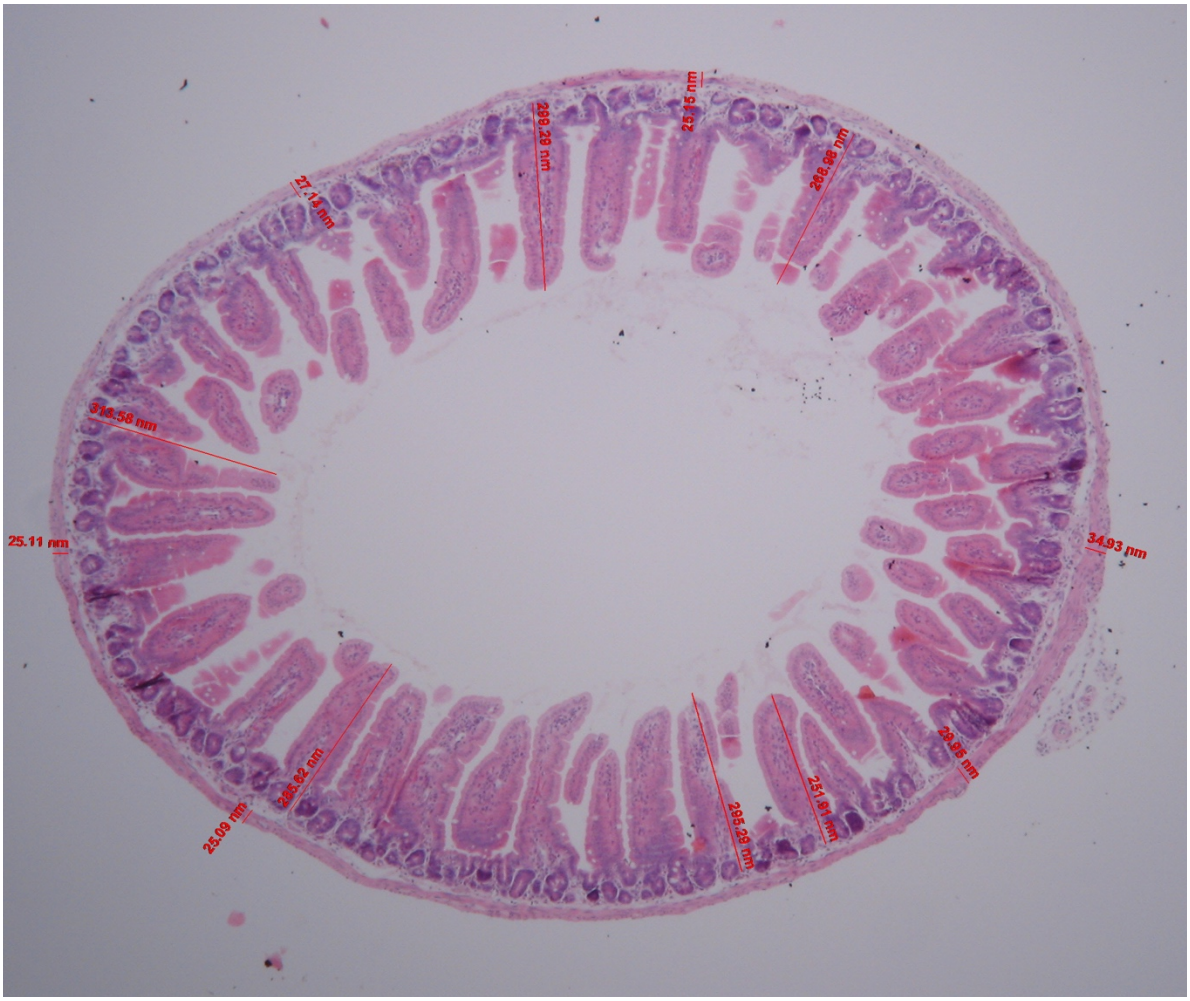
Supplemental Image 5. Measured tunica mucosa and tunica muscularis for valproic acid P21 V246. Image has been cropped for clarity. (N=6)



Supplemental Image 6. Measured tunica mucosa and tunica muscularis for valproic acid P105 V437. This was the only sample for valproic acid P105. (N=1)



Supplemental Image 7. Measured tunica mucosa and tunica muscularis for Poly(I:C) P21 2188. The tissue shows desiccation. In future studies, if Poly(I:C) is included again, the tests will need to be repeated given the unreliability of the samples. (N=2).



Supplemental Image 8. Measured tunica mucosa and tunica muscularis for Poly(I:C) P105 P2192. (N=3)

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