5-7-2020

The effects of antibiotics and probiotics on memory and depression across age groups

Amanda Powell

The Effects of Antibiotics and Probiotics on Memory and Depression Across Age Groups

Amanda Powell

A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Master of Arts

Department of Graduate Psychology

May 2020

FACULTY COMMITTEE:

Committee Chair: Melanie Shoup-Knox, Ph.D.

Committee Members/ Readers:

Corey Cleland, Ph.D.

Jeanne Horst, Ph.D.

Acknowledgements

I first must thank my advisor, Dr. Melanie Shoup-Knox, for her incredible support throughout my graduate and undergraduate experience. She has been a fundamental component in my academic, personal, and professional growth throughout my time at JMU, and I wouldn't change anything about my experience. Without your guidance, support, and feedback, I would not be the researcher that I am today.

I also would like to thank my other committee members, Dr. Jeanne Horst and Dr. Corey Cleland, for your assistance throughout this process and for your dedication to make me a better researcher and academic. You each brought unique perspectives to our one on one meetings and our committee meetings, and challenged me to think in a way that I hadn't yet considered.

I am extremely grateful for the faculty in both the Psychological Sciences and Biology departments for your flexibility and additional mentorship throughout my jumping around both departments, but in particular I am most thankful to my graduate cohort members, both past and present who have become a fundamental component of my life. There has never been a moment where I have not felt your support and encouragement behind me.

To all of my undergraduate research assistants – without you this entire project would not have been possible. To Samantha Moseley, Emily Zihal, Alyssa Kniffin, Robert Ford, Collin Gregg, Elizabeth Gott, Ryan Farmer, and Virginia Wright – thank you for your dedication to this project, to this laboratory, and for all of the hoops you jumped through during the development phase of this project – your effort did not go unnoticed.

Lastly, to Paulius – for your continuous support throughout this project and beyond. Thank you for your understanding of all of my early mornings, late nights, and busy weekends; from bringing me food and driving me around to assisting in brain tissue sectioning and prep, the days went much more smoothly because of your support and assistance. Thank you for believing in me in the moments I did not.

Table of Contents

List of Tables

List of Figures

Abstract

The gut-brain axis is a bidirectional pathway that acts as a connection between the gut and the brain. Bacterial changes in the gut alter this pathway, affecting organism's health, cognition, and behavior. Commensal bacteria in the gut can reduce inflammation and increase longevity while pathogenic bacteria can have opposite effects. Reduced commensal gut bacteria can result in an increase in stress activation, depression, and anxiety in both human and animal models. Increases in commensal bacteria and decreases in pathobiontic bacteria can decrease hypothalamic pituitary adrenal (HPA) system activation, anxious behavior, and depressive behavior. Alternatively, increases in pathobionts can lead to decreases in neurogenesis, synaptogenesis, and synaptic plasticity, and can be observed behaviorally through deficits in hippocampally-dependent tasks. Therefore, changes to gut microflora diversity due to diet, age, antibiotic use, or probiotic use can alter the functioning of the individual. Antibiotics reduce both harmful and commensal gut microflora, which can lead to reduced hippocampal neurogenesis, impaired spatial memory performance, and increased depressive-like behavior. Administration of antibiotics can have long-term consequences following the cessation of antibiotic treatment. Probiotics may rescue these effects by reinstating commensal gut bacteria. As people age, a decrease in gastrointestinal tract functioning occurs, altering the gut microbiome and resulting in decreased diversity of commensal bacteria and an increased diversity of harmful bacteria. These effects are associated with reduced cognitive performance. Antibiotic use also contributes to the loss of gut bacteria diversity, and may augment cognitive-related deficits in older adults. The current study examined the effects of antibiotic and probiotic administration on spatial memory

performance and depression across two age groups in Long-Evans rats. Spatial memory performance was assessed via object location task and depressive behavior was assessed via forced swim test. Baseline spatial memory performance and depressive behavior were compared to the same behaviors following antibiotic treatment and then again following probiotic treatment. Antibiotic consumption predicted forced swim test performance; however, no differences were observed between treatment groups and age. Probiotic consumption did not predict behavioral performance. Although not expected, results suggested that a possible dosage effect exists for the amount of treatment to yield an effect.

The Effects of Antibiotics and Probiotics on Memory and Depression Across Age Groups

Gut Microbiome Diversity

The primary purpose of gut microbiota is to extract nutrients from food eaten to benefit both organism and host (Kau et al., 2012). Additionally, these microbes have the ability to convert vitamins from food to byproducts, such as folate and cobalamin, which has been hypothesized to have a downstream effect on DNA methylation, chromatin structure, and gene transcription in the host (Kau et al., 2012). The ability of these microbes shows that the presence of gut microbiota may be essential to the health and survivability of the organism. Increased diversity in commensal strains, or beneficial bacteria, (e.g., Bifidobacteria) have been linked to reduced inflammation and increased longevity compared to individuals with lower commensal microflora diversity (Kato et al., 2017; Biagi et al., 2012). However, an increase in diversity of pathobiontic bacteria, organisms that originate as commensal bacteria that can become disease-causing over time (e.g., Proteobacteria species), can promote inflammation (Biagi et al., 2010) by triggering the innate immune system involved in the stress response (for review see Guigoz et al., 2008). In this competition for gut real estate, two types of diversity have been defined as important: gut species richness (i.e., the number of species present in the system) and functional response diversity (i.e., a functionally similar microbe "fills in" space created by the compromised former microbe); (Lozupone et al., 2013). Bacterial eutrophication, or the excessive colonization of a single species, negatively impacts both types of gut microbiome diversity because a single species overpopulates the area so that other, functionally different microbes cannot be established. It is this decrease in bacterial diversity that is linked to various disease states, including: obesity (Turnbaugh,

Bäckhed, Fulton, & Gordon, 2008), inflammatory bowel disease (Willing et al., 2010), and Clostridioides-difficile Associated Disease (Chang et al., 2008). Therefore, an introduction of commensal bacteria may have beneficial effects on the organism while continued existence of pathobiontic bacteria have long lasting, detrimental effects.

Increased pathobiontic species diversity can result in permeability of the gut as the result of increased inflammation (for review see Guigoz et al., 2008; Biagi et al., 2010). It has been proposed that adverse effects on an organism's health are caused by an increased permeability of the gut microbiome (for review see Guigoz et al., 2008), and are associated with the development of Alzheimer's Disease and other dementias (for review see Jiang, Li, Huang, Liu, & Zhao, 2017). In addition to affecting the permeability of the blood-brain barrier (BBB) (Braniste et al., 2015), increased permeability of the gut microbiome is also associated with increased proinflammatory cytokines (Biagi et al., 2010), diet (Claesson et a., 2011), mucosal adhesion (Ouwehand, Isolauri, Krijavainen, & Salminen, 1999; He et al., 2001), and antibiotics (Ceylani, Jakubowska-Dogru, Gurbanov, Teker, & Gozen, 2018; Leclercq et al., 2017; Moore & O'Keeffe, 1999). The increased permeability of the gut microbiome (Guigoz et al., 2008) has been hypothesized to subsequently trigger the inflammatory response, which, in turn, causes an increased permeability of the BBB triggering neuroinflammation, leading to neurodegeneration (for review see Quigley, 2017).

The importance of gut microbiome diversity is highlighted by studies examining germ-free (GF) mice. Early establishment of the gut microbiome is essential for proper development of the HPA axis, otherwise the system is not fully effective at suppressing the stress response under non-stressful conditions (Sudo et al., 2004). Early life stress is

linked to an increased permeability of the gut (Varghese et al., 2006), which allows essential nutrients to leak out of the gut (Guigoz et al., 2008) and pathogens to enter (Arrieta, Bistritz, & Meddings, 2006). This effect becomes especially problematic because bacterial-mucosal adhesion that is essential for the effectiveness of commensal bacterial strains decreases over time (Ouwehand, Isolauri, Krijavainen, & Salminen, 1999; He et al., 2001). Germ-free animals are often used to study the importance of gut microbiome diversity in animal models by rearing them without any contact with microorganisms. Therefore, they do not establish diverse microbiomes. Inducing stress in GF animals resulted in memory impairments and increased blood serum corticosterone, a hormone heavily involved in the stress response, compared to control animals with an established microbiome (Gareau et al., 2011). These studies highlight the importance of an established, diverse gut microbiome to serve as protection against the adverse effects of inflammation.

Stress and the Microbiome

The gut microbiome and the HPA axis communicate bidirectionally. Therefore, alterations to one system may result in alterations to the other system. Increased commensal gut flora diversity has been shown to have protective factors against stress. For example, using *Bifidobacterium* or *Lactobacilli* strains to establish the gut microbiome in GF mice, effects of stress were significantly reduced compared to GF animals with unestablished gut microbiomes (Sudo et al., 2004) and helped prevent the emergence of pathogens that target the gut (e.g., irritable bowel syndrome; Bailey & Coe, 1999), respectively. Alternatively, subjecting an animal to chronic stress at a young age can reduce the number of Lactobacilli present in the gut, which increased the likelihood

of pathogenic bacterial infection (Bailey & Coe, 1999). Probiotic administration can ameliorate these effects of stress (Eutamene & Bueno, 2007) by halting the reduction in established Lactobacilli caused by maternal separation in animals (Gareau, Jury, MacQueen, Sherman, & Perdue, 2007), effectively reducing corticosterone release in response to stress compared to controls (for review see Sarkar et al., 2016). Importantly, this evidence suggests that the administration of probiotics can reduce the activity of the HPA axis, further protecting the organism from the negative impacts of stress. Taken together, the observed effects of a diverse gut microbiome on stress reduction provide further evidence of the link between the brain and the gut.

Microbiome Diversity on Neurogenesis

Stress has also been shown to have a negative impact on neural functioning, and its effects can be exacerbated as the result of reduced gut microbiome diversity (Braniste et al., 2015). In response to an alteration in gut microbiome composition, GF mice exhibited a reduction in presynaptic neurotransmitter release and synaptophysin, a protein essential for synaptogenesis (Kwon & Chapman, 2011; Tarsa & Goda, 2002) in the striatum compared to control mice (for review see Tognini, 2017). In addition to affecting the creation of new synapses, GF mice exhibit reduced PSD95, a protein heavily involved in synaptic plasticity and maturation mice (Béïque & Andrade, 2003; El-Husseini et al., 2000). Both synaptophysin and PSD95 are essential for maintaining effective levels of neural activity in the brain. Without an established microbiome, new synapses can neither be created nor beneficially altered as effectively as an organism with an established gut microbiome, which has implications on how that organism learns new information and subsequently integrates the new into preexisting information.

Neurogenesis is the ability of the brain to produce new neurons. It occurs in the subgranular zone of the dentate gyrus within the hippocampus (Kempermann, 2008) and is the neural basis for learning and memory. Researchers found that neurogenesis in the subgranular zone of the adult hippocampus is regulated by gut microbiota (Fung, Olson, & Hsiao, 2017; Ogbonnaya et al., 2015). For example, researchers found that GF mice had significantly fewer cells in the subventricular zone (SVZ), a brain region believed to be the center for progenitor cells that guide neurodevelopment, compared to control mice (Sawada et al., 2018). However, after housing GF mice with control mice for a month, the number of new neurons in the SVZ was equivalent for both groups (Sawada et al., 2018), suggesting not only that having an established microbiome is important for neural progenation, but that the microbiome advantageously alter the brain later in development. Although an increased gut microbiome diversity is generally recognized as imperative for the health of the organism, an increased diversity of inflammation-causing pathobiontic bacteria resulted in a reduction in neurogenesis compared to GF mice that had no established gut microbiome (Luczynski et al., 2016). Therefore, the type of bacteria colonizing the gut is as important as high bacterial diversity.

The diversity of the gut microbiome has also been observed to alter the permeability of the BBB, in turn affecting the rate at which neurogenesis can occur (Braniste et al., 2015). Specifically, preventing the permeability of the BBB was found to promote neurogenesis, while increasing BBB permeability resulted in reduced neurogenesis (Braniste et al., 2015). These effects are hypothesized to be driven by the increased BBB permeability allowing glucocorticoids, a class of stress hormone, to then enter the brain more readily, and reduce neurogenesis by reducing brain-derived

neurotrophic factor (BDNF; Braniste et al., 2015). Reduced BDNF has been observed in both the hippocampus and the cortex of GF animals compared to controls (Sudo et al., 2004) as well as in adults that had their gut microbiota reduced during adolescence (Desbonnet et al., 2015). In addition to a reduction in BDNF, NMDA receptor subunit, NR2A, was also reduced in the hippocampus of GF mice compared to control mice (Sudo et al., 2004). The effects of a reduced gut microbiome on fully functional NMDA receptors demonstrate the importance of high commensal gut bacteria diversity, as these receptors are essential for the initiation of long-term potentiation.

Microbiome Diversity on Memory

Long-term potentiation is the neural basis for learning and memory by strengthening synaptic connection. Therefore, a reduction in NMDA receptors may result in an impairment of memory consolidation and retrieval. Cryan and O'Mahony (2011) argued that high diversity of commensal microflora is essential for improved performance on both spatial and working memory tasks. These types of tasks are hippocampally-dependent, and provide further evidence in support of observations made regarding the relationship between gut flora diversity and neurogenesis. Germ-free mice have exhibited memory deficits in non-spatial tasks, such as the novel object recognition task, and working memory tasks, such as the T-maze (Gareau et al., 2011). Differences in working memory and reference memory tasks, as measured by the hole-board test, have been shown to be manipulated by diet (Claesson et al., 2012; Li, Dowd, Scurlock, Acosta-Martinez, & Lyte, 2009), which largely effects gut microbiome diversity (Claesson et a., 2011). Specifically, animals that received a standard pellet-based diet with the addition of meat to increase diversity performed better on memory tasks

compared to animals that received the pellets alone (Li et al., 2009). Alternatively, a high sugar diet resulted in a significant decline on Morris Water Maze performance one-hour post training in Long Evans rats (Magnusson et al., 2015). This effect was exacerbated after receiving the high sugar diet for five weeks (Jurdak et al., 2008) as opposed to two weeks (Magnusson et al., 2015), suggesting that a long-term diet that consists of higher amounts of sugar compared to controls may increase the observed deficits in memory.

In addition to observed decreases in neural activity and memory due to alterations in the gut microbiome, a relationship between changes in gut composition and neurodegenerative diseases, disorders of both memory and neurogenesis, have been observed. As previously mentioned, chronic, low levels of inflammation have been hypothesized to be caused by an altered gut microbiome (Guigoz et al., 2008). The resulting chain reaction that ultimately results in neurodegeneration is thought to be the cause of β-Amyloid build up in the brain, the hallmark feature of Alzheimer's Disease (Quigley, 2017). In human studies, the composition of the gut microbiome has included decreases in anti-inflammatory commensal bacteria and an increase in proinflammatory bacteria (Quigley, 2017), which have been observed to more strongly activate the inflammatory response than do other gram-positive bacterial strains (Guigoz et al., 2008), and include: Enterobacteria (Guigoz et al., 2008), Proteobacteria (Biagi et al., 2010), and Enterococcaceae (Quigley, 2017). Additionally, early life stress created by maternal separation in rodents has been associated with increased levels of corticosterone and pro-inflammatory cytokines, which can have implications resulting in decreased memory performance, as the hippocampus has large numbers of glucocorticoid receptors, and depression later in life (O'Mahony et al., 2009). Taken together, factors that negatively

impact the gut microbiome can result in increased inflammation that can ultimately lead to memory disadvantage and depression.

Microbiome Diversity on Depression and Anxiety

While the link between gut microbiome diversity and memory has been well tested in animals, similar observations have been made in human studies of gut flora diversity and stress. Bacterial alterations have also been observed in disorders caused by excess inflammation, which suggests a link between variations in the gut microbiome and stress, anxiety, and depression (Zhou & Foster, 2015). A link between the microbiome and stress was hypothesized to increase the prevalence of depression, particularly in patients with increased proinflammatory cytokines (for review, see Collins and Bercik, 2009). More recently, diagnosed depression has been linked to specific gut bacteria, including reduced Bacteroidetes, increased proinflammatory Alistipes (Naseribafrouei et al., 2014), and increased pathobiontic bacteria, Proteobacteria and Actinobacteria (Jiang et al., 2015).

Anxiety-like behavior is largely affected by gut microbiome diversity. Studies using mice specifically colonized with commensal bacteria exhibited reduced anxiety-like behaviors (for review see Foster & McVey Neufeld, 2013; Zhou & Foster, 2015). Alternatively, colonizing the gut microbiome with pathogenic bacteria resulted in an increase in observed anxiety-like behavior in the elevated plus maze and the holeboard test (for review see Foster & McVey Neufeld, 2013). Animals that experienced gut microbiome insult from both parasitic infection and inflammation exhibited increased anxiety-like behavior; however, probiotic administration reversed this effect (for review see Foster & McVey Neufeld, 2013).

Antibiotics and Probiotics on Neurogenesis, Memory, and Depression

The hippocampus is largely involved with memory recall ability. Alterations in neurogenesis here can provide stronger evidence for observed changes in memory performance. Exposure to antibiotics has been shown to not only reduce levels of neurogenesis in the hippocampus in young rodents (2 to 4 months), but also mature neurons (labeled with NeuN), transient proliferating mitotic neuronal progenitor cells (labeled with doublecortin), and proliferating neurons (labeled with BrdU) were all actively reduced compared to control groups (Möhle et al., 2016). Therefore, antibiotic administration can have a negative impact on hippocampal neurogenesis. In addition to the adverse effects on neurogenesis, a reduction in gut flora diversity due to antibiotic administration resulted in a detriment to neural activity; specifically, a decreased firing rate, number of bursts, and percentage of spikes per burst in the dorsal CA3 hippocampal region in mice (Guida et al., 2018) have all been observed. Ampicillin, a beta-lactam antibiotic (Schliamser, Cars, & Norrby, 1991), resulted in a decrease in BrdU-positive cells in both the olfactory bulb and the subgranular zone of the hippocampus (Sawada et al., 2018), areas recognized to largely undergo neurogenesis. Interestingly, this effect was observed to be more potent in animals treated with ampicillin compared to other non-penicillin derived antibiotics, such as vancomycin (Sawada et al., 2018). The observed decrease in the rate of neurogenesis and memory performance due to antibiotic usage can have adverse effects on the health and behavior of the organism if left untreated.

Memory deficits in response to antibiotic administration have been observed in both humans and animals. Specifically, beta-lactam antibiotics, such as ampicillin, have been observed to have adverse effects such as memory difficulties, confusion, disorientation, and delirium in humans (Chow, Hui, & Szeto, 2005). In animals, repeated

administration of antibiotics (ampicillin, cefoperazone, and cefoperazone + ampicillin) resulted in reduced memory performance on the novel object recognition task compared to control groups (Ceylani, Jakubowska-Dogru, Gurbanov, Teker, & Gozen, 2018; Guida et al., 2018; Möhle et al., 2016). In addition to observed deficits on the novel object recognition task in animals, working memory tested via the Y maze, and social recognition memory were tested in animals treated with ampicillin, streptomycin, and clindamycin (Guida et al., 2018). Social recognition memory and novel object recognition were both found to be significantly impaired in the treatment group compared to controls (Guida et al., 2018); however, the researchers found no change in working memory in mice treated with antibiotics. It is likely due to the fact that working memory is not largely a hippocampally dependent task, therefore deficits to the hippocampus may not result in working memory impairment. In addition to behavioral observation, the effects of antibiotics on memory can also be observed at the neural level.

Although antibiotics have been observed to have negative effects on the gut microbiome, the brain, and behavior, strong evidence suggests that probiotic treatment can restore rates of neurogenesis back to baseline levels after experiencing prior deficits. For example, probiotic treatment fully restored rates of neurogenesis immediately following antibiotic treatment; however, probiotic treatment was unable to increase rates of neurogenesis above its baseline levels (Möhle et al., 2016). More colloquially, probiotic administration can attenuate the impact of antibiotics and restore that organism to its previous level of functioning, but cannot improve upon baseline levels of functioning. Interestingly, mice were protected from brain injury if they received probiotics (*Clostridium butyricum*) prior to sustaining the injury (Sun et al., 2016).

Additionally, pretreated, brain-injured animals had lower numbers of apoptotic cells (measured via Bax and Bcl-2) compared to brain-injured animals that did not receive pretreatment with probiotics (Sun et al., 2016). Probiotic pretreatment also had beneficial effects on neurogenesis by significantly increasing BDNF and decreasing microglial activation, part of the immune response (Ait-Belgnaoui et al., 2014). These findings provide evidence that probiotics may not be able to improve beyond baseline ability, but instead have the ability to restore neurogenesis effects following antibiotic insult or buffer the effects of adverse experiences before they happen.

Probiotic administration has been observed to be beneficial in restoring memory ability in organisms with previously observed deficits in memory performance. Probiotic treatment was observed to rescue performance on the novel object recognition task to baseline levels in animals previously treated with antibiotics (Möhle et al., 2016), as well as improve stress-induced memory dysfunction following *Citrobacter rodentium* infection (Gareau et al., 2011). Illnesses that slow down metabolism (e.g., diabetes) lead to an increase in inflammation, which results in decreased neurogenesis and memory impairment (Shalev & Arbuckle, 2017). Davari and colleagues (2013) observed a restoration in spatial memory performance, via the Morris Water Maze, in diabetic rats that received *Bifidobacteria* and *Lactobacillus* probiotic treatment compared to diabetic controls. Moreover, control rats that received probiotics performed better on the Morris Water Maze task than did the control group that did not receive probiotics (Davari et al., 2013). This suggests that the usage of probiotics may restore memory performance in disadvantaged rats, and bolster memory performance in experimentally naïve animals. Although evidence supports the benefits of probiotic administration on memory

performance, assessment of memory impairment at the neural level is required to strengthen this argument.

The reestablishment of gut bacteria via probiotics has also shown to be beneficial in the treatment and prevention of depressive- and anxiety-like behaviors, often defined as reduced movement and decreased exploration in an open field (O'Mahony et al., 2009; for review, see Sarkar et al., 2016). Specifically, the administration of the probiotic *Bifidobacterium infantis* in Sprague-Dawley rats aided performance on the forced swim task, decreased inflammation, and decreased corticotropin-releasing factor to levels comparable to rats that received an antidepressant medication (Sarkar et al., 2016). Similarly, fewer depressive-like (measured via the forced swim task) and anxious behaviors (measured via the elevated plus maze) were observed in mice treated with Lactobacilli (Sarkar et al., 2016). Matthews & Jenks (2013) found that mice that received a probiotic that contained *Mycobacterium vaccae* exhibited reduced anxiety-like behavior in a maze-learning task and exhibited improved performance on the task such that they completed the maze faster with fewer errors compared to mice that did not. Together these findings provide mounting evidence that animals treated with probiotics display reduced anxiety- and depressive-like behavior in conjunction with improved performance on these tasks. Similar effects have been observed in humans diagnosed with depression; specifically, the reestablishment of the gut microbiome via probiotic administration resulted in more participants rating themselves as feeling happy rather than depressed compared to individuals in the study that received a placebo (Benton, Williams, & Brown, 2007). Furthermore, healthy individuals administered probiotics rated themselves as having improved mood significantly more than healthy individuals

who received a placebo (Messaoudi et al., 2011). Importantly, the beneficial effects of probiotic treatment have been observed in both animal and human studies.

Increased gut flora diversity has been shown to protect against the negative effects of stress. After establishing the gut microbiome using *Bifidobacterium* strains in GF mice, their stress responses were significantly reduced compared to GF animals with unestablished gut microbiomes (Sudo et al., 2004). A similar effect was observed when pre-existing Lactobacilli populations prevented the emergence of pathobiontic bacteria (e.g., Proteobacteria) that target the gut (Bailey & Coe, 1999). Lactobacilli has been shown to be reduced by situations of chronic stress, including early life maternal separation (Gareau, Jury, MacQueen, Sherman, & Perdue, 2007), leading to an increased likelihood of pathogenic bacterial infection (Bailey & Coe, 1999). However, administering probiotics were found to ameliorate stress-induced changes in the gut (Eutamene & Bueno, 2007) as well as halt the reduction in established Lactobacilli (Gareau, Jury, MacQueen, Sherman, & Perdue, 2007). These observed behavioral effects of both enhancing and diminishing Lactobacilli presence highlight the importance of this bacterial genus. The effects of probiotics have also been observed at the hormonal level; animals that received probiotics exhibited a reduction in corticosterone release in response to stress compared to controls, which suggests that the administration of the probiotics reduces the activity of the HPA-axis (for review see Sarkar et al., 2016). In humans, healthy individuals administered a probiotic had reduced cortisol in their urine compared to healthy individuals who received a placebo (Messaoudi et al., 2011). This provides stronger evidence for the relationship between the reduction of stress and improved memory performance in both humans and animal models.

At the neural level, probiotics have been observed to aid in the prevention of neurodegeneration in addition to improving memory and neurogenesis, as previously discussed. For example, probiotics have been observed to influence the impact of neurodegenerative diseases by reducing HPA axis activation, resulting in a reduction of cytokines, thereby reducing inflammation (for review see Westfall et al., 2017). Additionally, treatment with a probiotic that contained *Bacteroides fragilis* was found to have neuroprotective effects of demyelination, and reduced the effects of experimental autoimmune encephalomyelitis, an animal model of neurodegenerative disorders (Kwon et al., 2013; Ochoa-Reparaz et al., 2010). These neuroprotective effects are believed to occur due to the reduction of pathogenic cytokines and increase in beneficial cytokines (Kwon et al., 2013). To reiterate, antibiotics, particularly beta-lactam antibiotics, have adverse effects on memory performance, neurogenesis and depressive-like behavior, while probiotics can ameliorate these effects, restoring the organism to their previous level of ability.

Microbiome Diversity and Aging

As people age, they experience a decrease in gastrointestinal tract (GIT) functioning, which leads to alterations in the composition of the gut microbiome (Kato et al., 2017). The decrease in GIT function may lead to a decreased host resistance, allowing some gut bacteria to initiate an immune response, infect, and attack other organs (Mitsuoka, 2014). Alterations to the gut microbiome composition are highlighted in studies analyzing the microbiome of older adults and centenarians (i.e., individuals aged 100+ years). When comparing the gut microbiome compositions of young adults, elderly individuals (i.e., aged 70+ years), and centenarians, the gut composition (Bacteroidetes,

Bifidobacteria, Betaproteobacteria, and Deltaproteobacteria) of individuals in the centenarian group was significantly different from younger adults and elderly (Actinobacteria and Clostridia); (Biagi et al., 2010; Odamaki et al., 2016). More specifically, it was observed that centenarians had a low microbiota diversity compared to the adults in the other age groups, and an increase in proteobacteria species, anaerobic bacteria known as pathobionts (Biagi et al., 2010). The decrease in commensal gut bacteria species over time are believed to contribute to increased inflammation that is observed in the elderly (Odamaki et al., 2016).

Although strong evidence exists for changes in gut bacterial diversity over the lifespan, the most common type of commensal bacteria, *Bifidobacteria*, decreases in both number and species over time (Kato et al., 2017). Specifically, *B. catenulatum* and *B. bifidum* populations were present in all age groups except for centenarians (Kato et al., 2017), *B. breve* populations were present in all individuals over the age of 50, and *B. dentium*, which actually increased in individuals over the age of 60 (Kato et al., 2017). The eradication of some species of the *Bifidobacterium* genus over time is likely due to the reduced adhesion of Bifidobacteria to mucus (Guigoz et al., 2008), as bacterial-mucus adhesion is lower in healthy elderly individuals compared to healthy adults (He et al., 2001; Mitsuoka, 2014). This decrease in commensal *Bifidobacterium* over time helps explain why many probiotics contain *Bifido-* species. Additionally, researchers noted that as people age, a general increase in Enterobacteria, due to the reduced effectiveness of gut microbiome barrier, is commonly observed (Guigoz et al., 2008; He et al., 2001; Ouwehand, Isolauri, Krijavainen, & Salminen, 1999). This change in gut bacteria composition is believed to be detected by the innate immune system, triggering

inflammation, regardless of whether the bacteria is considered “healthy” or not (Guigoz et al., 2008). In particular, nonpathogenic, gram-negative bacteria (e.g., Enterobacteria) have been shown to more strongly activate the inflammatory response than do other, gram-positive, bacterial strains (Guigoz et al., 2008). Although inflammation is a common occurrence in older adults, research has shown that certain prebiotics, probiotics, or synbiotics, the combination of pre- and probiotics, have shown anti-inflammatory effects in the elderly (Guigoz et al., 2008), which further decrease HPA axis effects on gut permeability.

In addition to changing gut microbiome composition over time, some bacterial species are associated with increased longevity (Mitsuoka, 2014). When comparing GF mice, conventional mice (GF mouse colonized with microbiome of non-GF mouse), and gnotobiotic mice (contain all possible microorganisms), conventional mice had an increased rate of pathobiontic bacteria, and therefore had a shorter lifespan compared to GF mice (Mitsuoka, 2014). This suggests that having an increased diversity of harmful bacteria can result in more deficits to the host compared to an organism with no established microbiome at all. Additionally, gnotobiotic mice with *B. longum* had a longer average lifespan than gnotobiotic mice without the presence of the *Bifidobacterium* strain (Mitsuoka, 2014). This highlights the importance of this strain of bacteria and organism longevity.

Diet has also been observed to effect gut microbiome diversity, and in turn, immunosenescence (i.e., gradual deterioration of immune functioning), health, and well-being. Researchers found that a more diverse diet was significantly positively correlated with gut microbiota diversity (Claesson et al., 2012). Specifically, diets low in fat and

high in fiber had the highest microflora diversity, while diets moderate in fat and high in fiber and diets high in fat and low in fiber had the lowest microflora diversity (Claesson et al., 2012), suggesting the gut microbiome is regulated by the individual's diet. One study showed that older adults living in long-stay facilities had reduced diet diversity, which led to a reduced gut microbiome diversity. These same adults had higher levels of inflammation, lower scores on the geriatric depression test (GDT), and lower scores on the mini-mental state exam (MMSE) compared to adults living in a community setting (Claesson et al., 2012). This highlights the importance of maintaining dietary diversity throughout the lifespan, given the natural gut microbiome changes that occur. These changes, compounded by a lack of dietary diversity, can have major effects on the brain and behavior.

Antibiotics and Probiotics on Aging

Beta-lactam (β) antibiotics are a class of antibiotics that consist of a beta-lactam ring structure (Abraham, 1981). This class of antibiotics includes the penicillins (Abraham, 1981) and any of its derivatives, including: cefazolin, imipenem, and ampicillin (Schliamser, Cars, & Norrby, 1991). In humans, these antibiotics have adverse effects such as nervous system hyperactivity, seizures, comas (Lerner et al., 1967), encephalopathies (Grill & Maganti, 2011), confusion, disorientation, and excessive drowsiness (Chow, Hui, & Szeto, 2005). Beta-lactam antibiotics have also been shown to have higher neurotoxic side effects compared to other compounds, particularly when applied directly to the brain (Schliamser et al., 1991). The risk of neurotoxic reactivity is increased among those with decreased renal function (Rodriguez-Julbe et al., 2004), blood-brain barrier damage, and old age (Chow et al., 2005;

Schliamser et al., 1991). This neurotoxic quality of beta-lactam antibiotics, combined with their frequent use to treat bacterial infection, may be a contributing factor to the increased cognitive deficits observed in old age. In addition to the observed effects of β-lactam antibiotics in central-nervous-system-compromised and elderly individuals, β-lactam antibiotics have been observed to affect young children (Grill & Maganti, 2011). More specifically, ampicillin has induced neurotoxic effects in newborn children likely due to the increased permeability of the blood-brain barrier (Grill & Maganti, 2011).

Adverse effects of antibiotic use have also been observed in animals and studies suggest that early administration can cause long-term effects on longevity, gut-bacteria diversity, and the brain and behavior. Repeated administration of antibiotics (ampicillin, cefoperazone, and cefoperazone + ampicillin) in juvenile mice resulted in both acute and long-term effects (Ceylani, Jakubowska-Dogru, Gurbanov, Teker, & Gozen, 2018). Acute effects included a significant decrease in locomotor activity and an increased immobility time in the forced swim task (Ceylani et al., 2018). Long-term effects of juvenile antibiotic administration resulted in reduced memory performance on the novel object recognition task, a decreased diversity in established gut bacteria, and an increase in new gut bacterial strains compared to control groups at two months of age (Ceylani et al., 2018). Long-term β-lactam antibiotic administration has been observed to increase the prevalence of obesity and reduce caloric excretion in mice compared to controls (Cho et al., 2012). Antibiotic administration to dams during late pregnancy has also been observed to have effects on pups' gut microbiota diversity at birth lasting to six weeks of age (Leclercq et al., 2017). When administered at the perinatal period, β-lactam antibiotics led to an observed increase in aggressive behavior, decrease in social novelty,

decrease in sociability, increased cytokine expression, and a decreased bacterial diversity (Bacteroidetes, Firmicutes) in the pups compared to control groups (Leclercq et al., 2017). Additionally, a decrease in bacterial diversity of the dams was observed (Cyanobacteria, Actinobacteria, Proteobacteria; Leclercq et al., 2017). In mice treated with antibiotics, Proteobacteria, pathobionts, were found to be significantly more abundant compared to controls (Leclercq et al., 2017). Proteobacteria are known to be positively correlated with pro-inflammatory cytokines in humans, which provides further evidence of increased inflammation that is a characteristic of old age (Biagi et al., 2010). More specifically, antibiotics increase proteobacteria, which exacerbates chronic inflammation that already occurs in old age. Although these studies provide strong evidence that antibiotic usage can have long term effects on the brain and behavior, even after the cessation of administration, the long-term effects of repeated antibiotic administration from post-weaning to old age has not yet been assessed.

There have been mixed reviews regarding the effects of antibiotics on health and behavior in older adults. In a review conducted by Moore and O'Keeffe (1999), antibiotics were found to be linked to delirium and psychiatric illness in older adults due to the body's decreased ability to maintain homeostasis. This antibiotic-illness link has been hypothesized to occur due to antibiotics' ability to inhibit GABA neurotransmission, as well as individual risk factors such as advanced age, decrease in blood-brain barrier effectiveness, and systemic administration (Moore & O'Keeffe, 1999). Interestingly, research suggests that diet is a more important indicator of microbiome diversity than past usage of antibiotics (Claesson et al., 2012). When comparing older adults prescribed long-term antibiotics to older adults without antibiotics, there were no observed

confounding effects of antibiotic usage on microbiota and health relationships or diet and health relationships (Claesson et al., 2012). However, antibiotics have been shown to alter gut flora diversity and also shift the pre-existing diversity in both older adults living in long-term care and in the community (for review see Buford, 2017). When comparing younger adults (20 to 59) to older adults (60 to 85), individuals in the older age group had higher blood-antibiotic levels across all antibiotics studied (Liu et al., 2017). This may occur as a result of an increased gut permeability due to both age and antibiotics (O'Toole & Claesson, 2010), and subsequently lead to the inability to metabolize and excrete bacterial byproducts (Woodmansey, McMurdo, Macfarlane, & Macfarlane, 2004). Further research is necessary to compare the effects of antibiotics on the gut microbiome, brain, and behavior across age groups.

Although there is evidence that antibiotics can have adverse effects on the gut microbiome and health across the lifespan, probiotic administration has been shown to help ameliorate these negative effects. Probiotics have been observed to reverse the effects of a reduced gut microbiome barrier that may occur due to antibiotic administration (Hsiao et al., 2013) or age (for review see Guigoz, Dore, & Schiffrin, 2008). Probiotic administration following antibiotic administration during the perinatal period prevented decreases in social novelty, sociability, *Enterobacteria* (a pathobiontic species), and anxiety-like behavior (Leclercq et al., 2017), and has been observed to increase the average lifespan and locomotor activity while reducing biological markers of aging (lipofuscin) of C. elegans treated with *Bifidobacteria* (Komura et al., 2013). Lastly, probiotics have been observed to have protective factors against the development of neurodegenerative diseases by reducing inflammation in the gut microbiome (Westfall

et al., 2017) that is a hallmark of aging (Guigoz et al., 2008). Research supports the benefits of probiotic administration across the lifespan, especially in conjunction with antibiotic treatment; however, further research is necessary to compare these benefits across age groups.

Overview of the Current Study

While the effects of antibiotics and probiotics on the rate of neurogenesis, memory task performance, and depression are well established, it is unknown whether or how these affect age groups differently. The current study seeks to investigate the effects of both antibiotics and probiotics on memory, hippocampal cell count, and depressive behavior in both young and old rats. If antibiotic usage substantially reduces the gut microbiome, it is expected that animals receiving antibiotics without probiotic recovery will perform worse on a memory task, have a reduced number of cells in the granule layer within the dentate gyrus of the hippocampus, and exhibit more depressive-like behavior compared to both naïve animals and animals that receive probiotics. Because probiotics have been shown to restore the gut flora diversity, rats that are treated with probiotics are expected to perform similarly to naïve animals on the behavioral tasks, and show similar hippocampal cell count. Although some age-related differences in performance on the object location task and forced swim task are expected, it is expected that antibiotic administration to older animals will exacerbate these age-related deficits more so than in the young animals. In addition, it is expected that older rats receiving antibiotic treatment will have a lower hippocampal cell density compared to younger rats in the same treatment group. Finally, it is hypothesized that older animals will be less

responsive to probiotic recovery after previously receiving antibiotic treatment, due to the decreased bacterial adhesion in older organisms.

Previous research has demonstrated that the use of antibiotics has adverse effects on hippocampal neurogenesis, memory performance, and measures of depression, while probiotics can rescue the adverse antibiotic effects. However, these effects have not yet been compared across age groups. The current study will attempt to replicate prior research regarding the gut-brain axis, while providing a novel, and important contribution by examining multiple age groups. Originally, the current study attempted to replicate previous findings of reduced neurogenesis in the subgranular zone within the dentate gyrus of the hippocampus by performing a count of nissl stained cells across all age and treatment groups. However, the fixation method used failed to effectively penetrate the region of interest and therefore nissl bodies could not be stained. These methods are examined further in the discussion. However, research on behavioral outcomes are important due to the increased use of prescription antibiotics and antibiotic usage in food products. In fact, the abundance of antibiotic exposure has led to an observed increase in antibiotic resistance (Karp & Engberg, 2004). The current study aims to provide a more complete picture of the connection between the brain and the gut by contributing to the growing body of literature assessing the influence of the gut microbiome on cognition. This is a step towards better understanding the role of the gut microbiome on the development of psychiatric illness (e.g., depression), and age-related illnesses (e.g., Parkinson's Disease and other dementias), illnesses recently speculated to have origins in the gut.

Method

Animals

Twenty-five young (2 months at acquisition) and twenty-five old (12-13 months at acquisition) male Long-Evans rats weighing between 300-800 grams were utilized in the study over a four-week period divided into two consecutive treatment periods that lasted three weeks and then one week respectively (see Figure 1). Young animals were obtained from Charles River, and old animals were obtained from Envigo. All animals were treated according to guidelines set forth by the IACUC and kept on a 12-hr reverse light/dark cycle (lights off at 0800) with free access to food and water.

Procedure

Prior to behavior testing, animals were randomly assigned to one of five possible treatment groups. Animals that did not receive either antibiotic or probiotic treatment (Naïve) served as one control group, while animals that did not receive antibiotic treatment but received probiotic treatment (No Treatment + Probiotics) served as the other. Additionally, animals assigned to first receive antibiotic treatment were assigned to later receive no treatment (Antibiotics + No Treatment), probiotic treatment (Antibiotics + Probiotics), or were sacrificed immediately following antibiotic treatment (Antibiotics Only). All animals underwent pre-test behavioral measures to establish baseline memory performance on the object location task and baseline depressive behavior as measured by the forced swim task. Behavior testing occurred over a period of three days. On day one, animals completed the "study" phase for the object location task. The following day, animals were tested on the object location task, allowed to rest for 5 minutes, and then endured forced swim habituation before they were returned to

their home cage. On the last day, animals underwent the forced swim test phase. All behavior test days began at 0900 and ended by 1200. Following baseline behavioral measures (B), animals assigned to receive antibiotic treatment began antibiotic (1.5 g/L ampicillin) administration via drinking water for a period of 3 weeks. Animals that did not receive antibiotics received regular tap water during this 3-week period. Immediately following three weeks, all animals underwent the same battery of behavioral tests (T1). Animals in the Antibiotics Only condition were sacrificed to undergo tissue staining immediately following behavior testing, in order to determine the immediate effect of antibiotic treatment without a recovery period. During the following week, animals randomly assigned to the probiotic treatment groups began probiotics (1×10^9 CFU/mL) administered via drinking water. Animals that did not receive probiotics received regular tap water during the 1-week period. Immediately following the 1-week treatment, all animals endured a final battery of behavioral testing before being sacrificed for tissue staining (T2). All animals' water intake was tracked for the duration of the study in order to determine the actual antibiotic and probiotic concentration consumed by each animal. See Figure 1. All procedures outlined in this protocol were approved by the IACUC.

Treatment

Ampicillin Sodium Salt (crystalline powder) antibiotics were purchased from Fischer Scientific (Fischer Scientific International, Inc., Hampton, NH) and stored at 4 °C. Antibiotic were dissolved in DI water to a concentration of 1.5 g/L and stored at 4 °C for up to one week at a time. Antibiotics were administered ad libitum via the drinking water rather than via oral gavage to minimize any effects of stress on the animals, and served as the only access to drinking water. To ensure animals still consumed a healthy

amount of water, daily water intake (g) was recorded for a period of three weeks. A dosage of 1.5 g/L was chosen as a theoretical equivalent of 500mg dosage twice daily in humans and were based on dosages from previous research (Möhle et al., 2016).

Primal Gut Powder probiotic blend (20+ billion Colony Forming Units (CFUs)) was purchased from Corganic and stored at 4 °C. Primal Gut Powder contains 12+ billion *Lactobacillus* species, including: *L. rhamnosus, L. casei, L. acidophilus, L. plantarum, L. paracasei, L. brevis, L. salivarius,* and *L. gasseri*. Additionally, Primal Gut Powder contains 8+ billion CFUs of *Bifidobacterium* species, including: *B. infantis, B. longum, B. bifidum, B. breve,* and *B. lactis*. Probiotics were dissolved in DI water to a concentration of 1 x 10^9 CFU/mL and stored at 4 °C for up to one week at a time. A dosage of 1 x 10^9 CFU/mL *Lactobacilli* and *Bifidobacteria* were chosen based on previous research (Savignac et al., 2015). Probiotic strains were chosen based on their frequency of occurrence in the gut microbiome and because ampicillin targets these strains. Similar to antibiotics, probiotics were administered ad libitum via the drinking water, and daily water intake (g) was recorded for a one-week period.

Object Location Task

The object location memory task is a task that specifically assesses spatial memory and discrimination in rodents. Training procedures were adapted from Vogel-Ciernia & Wood (2008), and adjusted for use with rats. Before baseline testing, animals were acclimated to an empty testing environment twice for five minutes each (83.8 cm x 51.5 cm x 34.3 cm). During testing, the open field was divided into four equal quadrants. Four objects of similar size and material but differing in shape were Velcroed to the floor of the open field chamber, one in each quadrant. Each individual animal was

allowed to explore the area and the objects for five minutes to serve as the learning phase. Twenty-four hours later, in the recall phase, each animal was returned to the open-field with the same four objects from the day before, but with two objects having swapped quadrants. The amount of time the animal spent with each object was video recorded. Object discrimination was assessed by two blinded scorers, and based on the amount of time each animal spent with an object in a novel quadrant compared to an object in a familiar quadrant. The field and objects were sanitized between each test in order to avoid possible influences on other animals.

Forced Swim Task

The forced swim test examines depressive-like behavior in rodents. Training procedures were adapted from Yankelevitch-Yahav, Franko, Huly, and Doron (2015). Plastic containers (12.00 in x 16.85 in x 14.24 in), used for juvenile animals, were filled with tap water and adjusted for the animal's size. Glass cylinders (57 cm x 20 cm), used for older animals due to their larger size, were filled with tap water, adjusted for the animal's size and so that its hind legs could not touch the bottom of the container. Water was maintained at a temperature range of 22-24 °C. Each animal underwent a 15-minute pretest that served as the habituation phase. Twenty-four hours later, each animal was placed in the container for a maximum duration of five minutes, and the amount of time spent immobile rather than actively trying to escape was recorded as a measure of depression. After the five-minute time limit was reached, the rat was removed from the container, dried off with a towel, placed in a drying cage with a heating pad under the cage, and monitored until fully dry prior to being returned to its home cage. Water was changed between each of the animals to avoid possible influence on other animals.

Histology

Brain tissue was extracted, immersion fixed in 4% paraformaldehyde solution, and sliced into 100 μm sections using a cryostat then mounted on slides to dry overnight. The following day, slides were rehydrated through 100%, 95%, and 70% alcohol distilled water in order to reduce background fat staining. A 0.1% cresyl violet solution was warmed between 37-50 degrees to improve penetration and enhance staining. Sections were submerged in cresyl violet solution for 2-10 minutes and then rinsed quickly with distilled water. Slides were then placed in 95% ethyl alcohol for 30 sec, dehydrated in 100% alcohol twice for 5 min each and cleared in xylene twice for 5 min each before being fixed with DPX permanent mounting medium. At this point, it was determined that immersion fixation in 4% paraformaldehyde was not sufficient to fix the medial dentate gyrus structures and is further highlighted in the discussion.

Statistical Analyses

Mixed ANOVAs explored the effects of age, treatment, and individual changes from baseline testing on variation in each behavioral variable (spatial memory measured by object location and depression measured by forced swim). Therefore, a 2 (age group: juvenile, old) x 5 (treatment: Antibiotics + No Treatment, No Treatment + Probiotics, Antibiotics + Probiotics, Naïve, and Antibiotics Only) x 3 (Testing Session: Baseline, Time 1 (following 3 weeks), Time 2 (following 1 week)) mixed ANOVA was conducted in SPSS for each behavioral dependent variable. Age and Treatment were treated as between-subject variables while Testing Session was treated as a within-subject variable. For all ANOVA analyses, main effects and interactions were examined. Due to *a priori* hypotheses, *t*-tests examined differences between antibiotic and probiotic treatment

groups and compared to controls, regardless of main effect significance to determine differences in task performance and water consumption.

Finally, simple linear regressions were used to determine if the amount of antibiotics consumed had an impact on object location performance or forced swim test performance following antibiotic treatment (T1). Additionally, simple linear regressions examined whether the amount of probiotics consumed by each animal predicted performance on the object location task or forced swim test following probiotic treatment (T2). Multiple regression analyses were conducted to determine whether adding the categorical treatment group variable would explain significantly more variance in both object location performance and forced swim performance above and beyond the continuous antibiotic and probiotic consumption variables.

Results

Upon completion of the study, only 48 of the 50 animals were used in the final object location analysis. Only 47 were used in the final forced swim analysis. Animals were excluded from analyses due to health complications. Data were analyzed in accordance with previously published studies (Möhle et al., 2016) that utilized the forced swim task and the novel object location task. For analyses of the object location task, assumptions were not violated, including: normality, homogeneity of variance, and sphericity ($\chi^2(2) = 1.10$, $p = .578$). When evaluating homoscedasticity of these data beyond homogeneity of variance testing, Hartley's Fmax was conducted, and ratios were found to be homogenous for both the object location task and the forced swim test. Additionally, QQ plots suggested that the data were homoscedastic. For the forced swim task, assumptions were not violated (sphericity: ($\chi^2(2) = 3.19$, $p = .203$) with the

exception of normality due to the presence of one outlier; however, all cases were left in the data set to provide a more accurate representation of the data. Descriptive statistics for each behavioral task are presented in Table 1 and 2.

Object Location Task

It was originally predicted that animals that received antibiotics would spend less time with objects in novel locations compared to animals that did not receive antibiotics. Object location performance did not differ between the Treatment Groups when collapsing across Testing Session and Age, $F(4, 38) = 1.58$, $p = .198$, $\eta_p^2 = .143$. Observed power was .44. However, this was a medium effect size, where 14.3% of the variance in object location performance could be explained by treatment group. Animals receiving antibiotics and probiotics ($M = 35.73$) performed better than those who only received antibiotics (Antibiotics + No Treatment group, $M = 16.70$; Antibiotics Only group, $M = 22.78$), only received probiotics ($M = 11.57$), and animals that did not receive treatment at all ($M = 21.50$).

Older animals were predicted to spend less time with the novel object locations compared to juveniles, especially if the animals additionally received antibiotics. Collapsing across treatment groups and testing session, performance on the object location task did not differ between the old and young animals, $F(1, 32) = 0.83$, $p = .368$, $\eta_p^2 = 0.03$, which is a small effect. Observed power was 0.03. When collapsing across treatment group and age, a significant effect of testing session was found $F(2, 64) = 5.145$, $p = .008$, $\eta_p^2 = 0.14$, such that time spent with novel locations at Baseline ($M = 27.24$, $SD = 30.85$) was significantly greater than the time spent with the novel object locations at Time 2 ($M = 4.13$, $SD = 37.91$), $p = .012$, 95% CI [4.34, 43.17]. The partial

eta squared effect size was medium, where 14% of the variance in object location performance could be explained by testing session. The observed power was .81. Bonferroni corrections were conducted for all post-hoc t-tests to correct for Type I error. Differences across testing sessions did not vary between Time 1 (M = 15.78, SD = 28.51) and Baseline, $t(47) = 2.11$, $p = .211$, 95% CI [-4.38, 29.41] or between Time 1 and Time 2, $t(39) = 1.39$, $p = .479$, 95% CI [-8.48, 30.95]. No significant interactions were found for Testing Session and Treatment Group ($p = .417$), Testing Session and Age ($p = .371$), or Testing Session, Treatment Group, and Age ($p = .203$), meaning that object location performance did not depend upon the age of the animal or treatment group membership (see Table 3).

In order to determine whether animals spent more time exploring any of the objects (i.e., novel or familiar) at any given time point, a repeated measures ANOVA was conducted. Mauchly's test of sphericity indicated that sphericity had been violated for total object location exploration time, so a Greenhouse-Geisser adjustment was used to adjust degrees of freedom ($\chi^2 = 38.42$, $p < .001$). Performance did not vary between the behavior tests, such that animals did not differ in their exploration of any object, novel or familiar ($F(1.22, 47.67) = 0.03$, $p = .170$, $\eta_p^2 = 0.05$), which is a small effect.

Forced Swim Test

For the forced swim test, it was predicted that animals that received antibiotics would spend more time inactive compared to animals that did not receive antibiotic treatment. Collapsing across testing session and age group, forced swim performance did not differ between the treatment groups, $F(3, 32) = 0.50$, $p = .684$, $\eta_p^2 = 0.05$, with an observed power of .14 (See Table 4). The partial eta squared effect size was very small,

such that 5% of the variance in forced swim task performance could be explained by treatment group. Moreover, older animals were predicted to spend more time inactive, particularly if they had previously received antibiotics. However, juveniles (M = 107.18, SD = 47.24) spent more time inactive compared to adults (M = 47.30, SD = 50.79), $F(1, 32) = 16.51$, $p < .001$, $\eta_p^2 = 0.34$, regardless of Treatment Group or Testing Session. This effect size was large, where age accounted for 34% of the variance in forced swim task performance. The observed power was 0.98. Differences across testing session did not vary when collapsing across age and treatment group, $F(2, 64) = 1.77$, $p = .178$, $\eta_p^2 = 0.05$, with an observed power of .36. Lastly, there were no significant interactions for Testing Session and Treatment Group ($p = .864$), Testing Session and Age ($p = .075$), or Testing Session, Treatment Group, and Age ($p = .206$), meaning that performance on the forced swim task did not depend upon the animals age or the treatment group to which they were assigned. Bonferroni corrections were used to control for Type I error.

Treatment Dosage

In order to determine whether the amount of water consumed differed between treatment groups and age during the three-week period of antibiotic treatment, a 2 (age) x 5 (treatment group) ANOVA was conducted. Importantly, even though treatment was administered via drinking water, the amount of water consumed did not differ between the treatment groups, $F(4, 39) = 1.24$, $p = .311$, when collapsing across age groups. Regardless of treatment group, juveniles (M = 53.53, SD = 7.18) consumed significantly more water during the three-week interval following baseline testing than the adult animals (M = 34.50, SD = 7.18), 95% CI [15.44, 22.95] ($F(1, 39) = 106.86$, $p < .001$, $\eta_p^2 = 0.73$). The observed power was greater than .999 (See Table 5). This was a large

effect, such that age accounted for 73% of the variability in water consumption. When analyzing the amount of water consumed between treatment groups during the three-week period between Baseline testing and Time 1, there was no significant interaction between Treatment Group and Age in the amount of water consumed ($F(4, 39) = 0.72$, $p = .581$).

To determine if there were differences between both age and treatment groups in the amount of water that contained probiotic treatment consumed compared to water that did not contain probiotic treatment, a 2 (age) x 4 (group; Antibiotics Only animals were sacrificed following Time 1 behavior testing) ANOVA was conducted. Unlike with antibiotic consumption, water consumption containing probiotics differed between the treatment groups, across all age groups, $F(3, 32) = 15.89$, $p < .001$, $\eta_p^2 = 0.03$. Specifically, animals drank more probiotic water following antibiotic treatment ($M = 48.54$, $SD = 16.46$) than animals that did not previously receive antibiotics ($M = 31.66$, $SD = 11.42$), $p < .001$, 95% CI [7.10, 26.67]. Moreover, animals in the Antibiotics + No Treatment group ($M = 54.90$, $SD = 13.87$) consumed significantly more compared to animals in the No Treatment + Probiotics Groups, $p < .001$, 95% CI [13.45, 33.02]. Lastly, animals in the Naïve Group ($M = 45.39$, $SD = 11.84$) consumed significantly more water compared to animals in the No Treatment + Probiotics Group, $p = .002$, 95% CI [3.94, 23.51]. There was no significant interaction found for Treatment Group (i.e., not including Antibiotics Only group as they were sacrificed prior to Time 2) and Age in the amount of water consumed between Time 1 and Time 2, $F(3, 32) = 2.173$, $p = .110$. Moreover, animals in the Antibiotics + No Treatment group ($M = 54.90$, $SD = 13.87$) consumed significantly more compared to animals in the No Treatment + Probiotics

Groups, $p < .001$, 95% CI [13.45, 33.02]. Lastly, animals in the Naïve Group ($M = 45.39$, $SD = 11.84$) consumed significantly more water compared to animals in the No Treatment + Probiotics Group, $p = .002$, 95% CI [3.94, 23.51]. A significant main effect (though small effect size) for age was also found, $F(1, 32) = 70.62$, $p < .001$, $\eta^2 = .05$, where animals in the Juvenile age group ($M = 55.46$, $SD = 14.55$) consumed significantly more water than animals in the Adult age group ($M = 34.78$, $SD = 7.92$), 95% CI [15.66, 25.67]. See Table 6.

Simple linear regression was used to determine the effects of antibiotic consumption on behavioral task performance. The average amount of antibiotics consumed per kilogram body weight significantly predicted forced swim inactivity time at Time 1, $\beta = 0.73$, $t(1) = 2.74$, $p = .011$, $R^2 = .23$ (See Figure 2). The average amount of antibiotics consumed per kilogram body weight did not predict object location discrimination performance at Time 1, $\beta = 0.13$, $t(1) = 0.85$, $p = .402$, $R^2 = .03$ (See Figure 3). To provide a deeper understanding, regression was used to determine if there were differences between age groups on antibiotic consumption predicting behavior performance. Age did not impact the effect of antibiotic consumption per kilogram of body weight on object location performance, such that juveniles ($\beta = 0.34$, $t(1) = 0.61$, $p = .555$, $R^2 = .03$) did not differ on object location performance compared to adults ($\beta = -1.03$, $t(1) = -0.95$, $p = .359$, $R^2 = .07$; See Figure 4). Additionally, age did not impact the effect of antibiotic consumption per kilogram of body weight on forced swim performance; juvenile animals ($\beta = .11$, $t(1) = 0.10$, $p = .924$, $R^2 = .00$) did not differ from adults ($\beta = -1.21$, $t(1) = -0.78$, $p = .452$, $R^2 = .05$) on forced swim test inactivity (See Figure 5). Multiple regression was conducted in order to determine whether the

categorical treatment variable explained significantly more variance in forced swim inactivity above and beyond antibiotic consumption alone, R^2_{change} = .14, F_{change} (1, 44) = 9.01, p = .004. Additionally, adding the categorical treatment variable did not explain significantly more variance in object location discriminability above and beyond antibiotic consumption alone, R^2_{change} = .00, F_{change} (1, 45) = .01, p = .92.

Because animals in different age groups varied in body size and growth rates, a 2 x 5 ANOVA was also conducted to determine if animals differed in treatment consumption (mL) per kilogram of body weight. This revealed that juvenile animals (M = 130.94) consumed significantly more antibiotic water per kilogram of body weight compared to adult animals (M = 60.03; 95% CI [64.04, 77.77]), F(1, 38) = 437.10, p < .001, η_p^2 = .92. The effect size was large, such that 92% of the variance in antibiotics consumed depended on the age of the animal. The observed power was greater than .999. This suggests that juvenile animals received more antibiotic treatment per kilogram of body weight compared to the adult animals.

Simple linear regression was estimated to determine whether probiotic consumption predicted Time 2 behavior task performance. Unlike with antibiotic consumption, the average amount of probiotics consumed did not significantly predict Forced Swim inactivity, β = 0.33, t(1) = 0.99, p = .334, R^2 = .05 (See. Figure 6). Additionally, the average amount of water consumed that contained probiotics did not significantly predict Object Location Task performance at Time 2, β = 0.15, t(1) = 0.90, p = .379, R^2 = .04 (See Figure 7). Lastly, regression was used to determine differences in age groups on probiotic consumption predicting behavior performance. Age did not differ when observing the effect of probiotic consumption on object location performance

(See Figure 8). Juveniles (β = -0.13, $t(1) = -0.71$, $p = .497$, $R^2 = .06$) did not differ compared to adult animals (β = 1.73, $t(1) = 1.39$, $p = .203$, $R^2 = .19$). Additionally, age did not have an impact on the amount of probiotics consumed predicting forced swim performance (See Figure 9). Juvenile animals (β = 0.15, $t(1) = 0.42$, $p = .683$, $R^2 = .02$) did not differ from adult animals (β = -3.73, $t(1) = -1.57$, $p = .154$, $R^2 = .24$) on forced swim performance following probiotic treatment. Multiple regression was conducted in order to determine whether adding in the categorical treatment variable would explain significantly more variance in forced swim inactivity at Time 2 above and beyond probiotic consumption alone. Adding treatment group did not explain significantly more variance in forced swim performance $R^2_{change} = .06$, $F_{change}(1, 17) = 1.20$, $p = .29$. Lastly, adding treatment variable into the model did not explain significantly more variance in object location discriminability at Time 2 than did probiotic consumption alone, $R^2_{change} = .06$, $F_{change}(1, 17) = 1.07$, $p = .316$.

Finally, to determine whether animals differed in the amount of probiotic water consumed (mL) per kilogram of body weight, a 2 x 5 ANOVA was conducted. Juvenile animals ($M = 120.93$) consumed significantly more probiotic water compared to adult animals ($M = 60.21$; 95% CI [49.64, 71.80]), $F(1, 32) = 124.56$, $p < .001$, $\eta_p^2 = .80$. The effect size shows that 80% of the variation in probiotic consumption could be explained by the age of the animal. The observed power was greater than .999. This indicates that juvenile animals received more probiotic treatment per kilogram of body weight compared to adult animals.

Discussion

The current study sought to accomplish two primary goals. The first goal was to replicate previously established evidence supporting a connection between the gut microbiome and behavior. Specifically, we examined previously established effects of gut microbiome diversity on memory performance, the hippocampus, and depressive-like behavior. The second goal was to compare these effects across age groups in order to provide a deeper understanding of how changes to the gut microbiome affect depressive-like behavior, memory performance, and hippocampal cell density at various points during the lifespan. In contrast to what was predicted, results obtained did not follow similar trends as those observed in previously published literature. However, our results do suggest that the amount of antibiotics consumed can impact behavior.

The amount of antibiotics consumed significantly impacted forced swim behavior. The more antibiotics the animal consumed, the more time that animal spent inactive during the task. Moreover, 23% percent of the variability in forced swim test performance could be explained by the amount of antibiotics consumed. Although the current finding did not replicate results obtained from previous studies that also administered antibiotics via passive administration (Guida et al., 2017; Möhle et al., 2016), this linear relationship suggests that a specific amount of antibiotic dosage is required in order to observe the detrimental effects on behavior performance. The amount of antibiotics consumed did not significantly predict performance on the object location task, and only explained 3% of the variability in task performance; therefore, it is possible that the antibiotic doses needed to observe depressive-like behavior are not the same doses required to observe memory impairment. Instead, larger or longer antibiotic

treatment may be necessary to observe a deficit in spatial memory performance. Antibiotic administration via oral gavage may help ensure that all animals receive enough of the treatment to observe an effect (Fröhlich et al., 2016). However, because of the possible effects of stress due to repeated gavaging, passive treatment administration was selected to prevent any adverse effects on the hippocampus and subsequent memory.

The amount of probiotic consumed did not significantly predict performance on either the forced swim task or the object location task. At first glance, this seems to be a negative effect in regards to using probiotics as a form of treatment. However, as previously mentioned, probiotic treatment is not able to improve above and beyond the organism's previous ability (Möhle et al., 2016); instead, it can be employed to reestablish and recover the organism's microbiome diversity, in turn regaining previous memory performance. Therefore, observing large differences in Time 2 performance in animals that received probiotic treatment is unlikely. It is important to keep in mind that a dose response relationship may also be observed in animals subjected to probiotic treatment; however, as with antibiotics, controlled dosing is necessary to fully examine that claim.

Antibiotic treatment did not significantly impair object location performance, and probiotic treatment did not rescue performance. Specifically, only the groups that received antibiotics were expected to significantly decrease from Baseline to Time 1. Although no significant effect was observed, the variability between the groups yielded a medium effect size, such that 14.3% of the variance in object location performance could be explained by treatment group. Animals that received probiotics following antibiotic administration continued to decrease in object location performance across the three

behavioral testing sessions, which highlights the necessity to consider a dose response relationship between both antibiotic treatment and probiotic treatment. Animals that received antibiotics alone (i.e., Antibiotics + No Treatment or Antibiotics Only groups) also decreased in object location performance over time, suggesting antibiotic treatment may have resulted in impaired performance. Interestingly, the animals that received sterile drinking water following antibiotic administration largely returned to their baseline performance levels in the last object location testing session, which suggests that no intervention following antibiotic administration alone may be salient enough to recover spatial memory ability. Although no significant differences were observed between the treatment groups via inferential statistical testing, it is important to examine effect sizes and group trends to determine practical significance (Cohen 1992).

Object location performance decreased across the testing sessions, though this was not originally predicted. Object location performance was expected to decrease only following antibiotic treatment and improve if the animals then received probiotic treatment. Instead, the ability to discriminate between the novel object locations and the familiar object locations decreased at each testing point regardless of both age and treatment group. Although objects were changed for each testing period, it is possible that the task became monotonous after each repetition, so the time spent interacting with each object decreased as the study progressed, resulting in poorer discriminability. Additionally, it is possible that the animals that received treatment did not have their gut microbiomes fully altered after consuming either antibiotics, probiotics, or both. Moreover, a therapeutic dose of probiotics has yet to be established, though a few studies recommend a daily dose of 10^9 to 10^{10} (1×10^9 CFU/mL was utilized for the current

study) to be therapeutically beneficial (for review see Kopp-Hoolihan, 2001; Sanders et al., 1996). Although previous research has typically utilized recognition memory tasks to evaluate whether antibiotic insult impairs memory performance, animals in the present study spent less time exploring objects in their novel locations compared to animals that explored a completely novel object (Hoban et al., 2016).

Adult animals did significantly outperform juvenile animals on spatial memory performance, which, again, was not predicted. Although possible that adult animals do not actually differ between juvenile animals on spatial memory discriminability, the obtained finding might result from research that suggests that Long Evans males do not differ in hippocampal spine density when comparing rats aged 19-22 months, 24-26 months, 12 months, and 3-5 months (Luine, Wallace, & Frankfurt, 2011). Because the ages of the animals utilized in the current study were 3-4 months and 13-14 months at study completion, it is possible that no differences in age between the groups were observed because age-related deficits were not yet evident. In fact, age-related deficits are not observed until 18 months in Long-Evans rats (Bizon & Gallagher, 2003); however, these age groups were utilized to ensure that any impairments observed between the age groups were due to the effects of treatment, rather than due to natural cognitive or physical aging.

Forced swim performance did not significantly differ between the treatment groups. No differences were found at any Testing Session or Treatment Group for the forced swim task, and the effect size was nominal; only 5% of the variability in forced swim performance could be explained by treatment group membership. Again, it is possible that the administered treatments did not alter the animals' gut microbiomes as

intended, which resulted in the probiotics unable to reestablish the gut microbiome with commensal bacteria. It is also possible that the antibiotics did have an effect on reducing the diversity of the gut microbiome, but the probiotic dosage was not strong enough to replenish the diversity of the gut microbiome back to the organism's baseline levels. Interestingly, when comparing inactivity time to previous studies that utilized the forced swim test in rats, animals in the present study spent much more time inactive compared inactivity time reported in other research, however different rat strains were used (Hoban et al., 2016).

Juvenile animals spent significantly more time inactive during the forced swim task compared to the animals in the adult group, which was not in the hypothesized direction. Age accounted for 34% of the variance in forced swim task performance. It is possible that differences observed here were due, in part, to container differences used during the task for young and old animals. Animals in the aged group were extensively larger than animals in the juvenile condition, and required a larger container during the forced swim task. Because of the size differences, older animals were forced to remain more upright in the container, which may have impacted their ability to float and remain immobile compared to the much smaller, juvenile age group.

Juvenile animals consumed significantly more water overall than the adult animals. Because treatment was administered via drinking water, this difference affects the dosage received by each age group. Consumption differences between age groups were found for both antibiotic treatment and probiotic treatment, though effect sizes were nominal (4% and 5% respectively), suggesting minimal practical significance in these statistically observed differences. When collapsing across age of the animals, no

significant differences were observed between animals that received antibiotic treatment and animals that received sterile drinking water, but did significantly differ between treatment groups for probiotic consumption. Animals that previously received prior antibiotic treatment drank significantly more than the animals that had previously received sterile drinking water, although the magnitude of the effect was nominal (3%). It is possible that differences in taste between antibiotic water, probiotic water, and sterile water affected the amount of consumption for some of the groups. Because the antibiotic powder was a sodium salt mixture, it likely resulted in a more alkaline, bitter taste compared to sterile drinking water. The difference in taste could account for the increase in water consumption following the antibiotic treatment phase for animals that previously received antibiotics compared to the animals that previously received sterile water.

Limitations

Although careful measures were taken to ensure design consistency and minimize error, several limitations arose that may have impacted the study. A potential limitation that may have contributed to the lack of effects is antibiotic and probiotic treatment time. In the current study, antibiotic treatment lasted three weeks, which was selected as the "middle ground," based on previous results that utilized differing treatment times. Specifically, behavioral effects following antibiotic administration were observed in as few as two weeks (Guida et al., 2018), three weeks (Ceylani et al., 2018), and as long as seven (Möhle et al., 2016) and 13 (Anukam, 2017) weeks. Behavioral effects following probiotic treatment were observed in as little as two days (Möhle et al., 2016) and as long as 11 weeks (Savignac et al., 2015). It is possible that the treatment times selected for the study were not long enough to fully alter the gut microbiome. Additionally, older

animals utilized in the study were not aged in the same facility where the study took place. Therefore, the adult animals had their gut microbiomes largely established at another facility, while the juvenile animals did not. It is possible that the differences between these groups served to better protect, or impair their gut microbiome in response to antibiotic insult.

In the originally proposed study, histological analyses were planned in order to determine if altering the gut microbiome had an effect on the number of cells within the subgranular zone of the dentate gyrus within the hippocampus. However, the cresyl violet acetate stain did not target the nissl bodies of the neural cells as expected. Upon deeper investigation, post-fixing a rat brain in 4% paraformaldehyde solution was insufficient to fix the tissue. Although some studies found no significant differences in tissue fixation and staining quality, these studies examined more superficial structures and smaller tissue samples (Hare et al., 2014; Kasukurthi et al., 2009). For deeper and/or larger structures, however, immersion fixation will not reach the internal structures of the tissue (e.g., the dentate gyrus within the hippocampus) before hypoxia begins to change tissue structure (Gage, Kipke, & Shain, 2012). These findings highlight the importance of transcardial perfusion prior to histological staining to ensure best fixation practices.

Another limitation that might have had an effect on behavior might be that all animals in the adult age condition were retired breeders, as that was the oldest age attainable from any approved vendor. Previous research suggests that the aggressive nature of Long Evans retired breeders is resultant from elevated corticosterone and altered catecholamine levels (Patki et al., 2014), both of which can have an impact on memory performance (Song, Che, Min-Wei, Murakami, & Matsumoto, 2006) and

depressive-like behavior (Zangen, Overstreet, & Yadid, 1999). However, previous studies comparing behavioral differences between retired breeders and aged virgin animals did not find significant differences in the open field test, tightrope test, and passive avoidance learning (Ingram, Spangler, & Vincent, 1983). Therefore, it is not likely that observed differences between the age groups were the result of one group previously used for breeding purposes. However, virgin adult rats would have been ideal to compare to virgin juvenile rats.

Lastly, the current study attempted to utilize a better measure of spatial memory performance in rats via the object location task. Previous literature that examined behavioral effects of an altered the gut microbiome largely utilized the novel object recognition task (Möhle et al., 2016; Magnusson et al., 2015); however, evidence suggests that the hippocampus is imperative for object location, but not explicitly for recognition memory (Barker & Waterburton, 2011; Broadbent, Squire, & Clark, 2004). Although the dependency on the hippocampus is apparent in the object location task, it is possible that the "study phase" was not long enough for the magnitude of the delay between the study and test phase. The present study based the timing of the study phase off of previous research that utilized a 5-minute study period (Gareau et al., 2011). However, Ozawa, Yamada, and Ichitani (2011) found that a study phase of 20-minutes, rather than 5-minute and 10-minute study sessions, yielded significant discrimination indices after a 24-hour delay.

Future Directions

Future research should include a measure of fecal analysis in order to determine whether antibiotics effectively eliminated the diversity, and probiotic administration

effectively reestablished the commensal diversity of the gut microbiome of the animal. This will ensure confidence that the gut microbiome was sufficiently altered, provide a stronger link between any observed differences in behavioral and neural data, and help aid future researchers in establishing an effective window for both antibiotic and probiotics treatment periods. Researchers should also conduct transcardial perfusions with paraformaldehyde in addition to 4% paraformaldehyde immersion fixation to examine the direct effects of antibiotic and probiotic treatment on the hippocampus. Perfusion fixation will prevent structural changes within the tissue following oxygen depletion and reduce the likelihood of artifact (e.g., erythrocytes remaining in vasculature) while staining. It is possible that treatment affected the hippocampus or other brain structures, however those effects did not have sufficient time to produce measurable behavioral changes. Future studies could include an additional age group to incorporate pre-pubertal animals to further elucidate the possible effects of altering the gut microbiome at various developmental periods throughout the lifespan. During the data collection phase of the present study, multiple research assistants noticed drastic behavior changes following antibiotic administration in only animals that received the treatment while conducting daily health checks. Aggressive behavior and agitation largely increased immediately following the start of antibiotic administration. Although it's anecdotal evidence, future projects that attempt to alter the gut microbiome using antibiotics should consider including a measure of home cage behavior. Lastly, future studies should attempt to determine whether a specific therapeutic dosage of both antibiotics and probiotics is necessary in order to sufficiently alter the gut microbiome.

These suggested lines of research will add to the rapidly growing body of literature determined to provide a more direct link between gut flora diversity, the brain, and age.

References

Abraham, E. P. (1981). The beta-lactam antibiotics. *Scientific American, 244*(6), 76-87.

Anukam, K. (2017). Effects of ampicillin on the gut microbiome of an adult male as determined by 16S rRNA V4 metagenomics sequencing and Greengenes Bioinformatics Suite. *Journal of Advances in Microbiology*, *7*(4), 1-18.

Arrieta, M. C., Bistritz, L., & Meddings, J. B. (2006). Alterations in intestinal permeability. *Gut*, *55*(10), 1512-20. doi: 10.1136/gut.2005.085373

Bailey, M. T. & Coe, C. L. (1999). Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. *Developmental Psychobiology, 35*(2), 146-155.

Barker, G. R. I., & Warburton, E. C. (2011). When is the hippocampus involved in recognition memory? *The Journal of Neuroscience, 31*(29), 10721-10731.

Béïque, J. C., and Andrade, R. (2003). PSD-95 regulates synaptic transmission and plasticity in rat cerebral cortex. *The Journal of Physiology, 546,* 859–867. doi: 10.1113/jphysiol.2002.031369

Benton, D., Williams, C., & Brown, A. (2007). Impact of consuming a milk drink containing a probiotic on mood and cognition. *European Journal of Clinical Nutrition*, *61*(3), 355-361. doi: 10.1038/sj.ejcn.1602546

Biagi, E., Candela, M., Fairweather-Tait, S., Franceschi, C., & Brigidi, P. (2012). Aging of the human metaorganism: the microbial counterpart. *Age*, *34*(1), 247-67. doi: 10.1007/s11357-011-9217-5

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., … De Vos, W. (2010). Through ageing, and beyond: Gut microbiota and inflammatory status in seniors and centenarians. *PLoS ONE, 5*(5), e10667. doi: 10.1371/journal.pone.0010667

Bizon, J. L., & Gallgher, M. (2003). Production of new cells in the rat dentate gyrus over the lifespan: relation to cognitive decline. *Eur J Neurosci, 18*(1).

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., Korecka, A., Bakocevic, N., Ng, L. G., Kundu, P., Gulyás, B., Halldin, C., Hultenby, K., Nilsson, H., Hebert, H., Volpe, B. T., Diamond, B., … Pettersson, S. (2014). The gut microbiota influences blood-brain barrier permeability in mice. *Science translational medicine, 6*(263), 263ra158. doi: 10.1126/scitranslmed.3009759.

Broadbent, N. J., Squire, L. R., & Clark, R. E. (2004). Spatial memory, recognition memory, and the hippocampus. *PNAS, 101*(40), 14515-14520. doi: 10.1073

Buford, T. W. (2017). Dis(trust) your gut: The gut microbiome in age-related inflammation, health, and disease. *Microbiome, 5*(80), 1-11. doi: 10.1186/s40168-017-0296-0

Ceylani, T., Jakubowska-Dogru, E., Gurbanov, R., Teker, H. T., & Gozen, A. G. (2018). The effects of repeated antibiotic administration to juvenile BALB/c mice on the microbiota status and animal behavior at the adult age. *Heliyon, 4*, 1-23. doi: https://doi.org/10.1016/j.heliyon.2018.e00644

Chang, J. Y., Antonopoulos, D. A., Kalra, A., Tonelli, A., Khalife, W. T., Schmidt, T. M., & Young, V. B. (2008). Decreased diversity of the fecal microbiome in

recurrent clostridium difficile-associated diarrhea. *The Journal of Infectious Diseases, 197*(3), 435-438.
doi: 10.1086/525047

Chow, K. M., Hui, A. C., Szeto, C. C. (2005). Neurotoxicity induced by beta-lactam antibiotics: From bench to bedside. *European Journal of Clinical Microbiology and Infectious Diseases, 24*(10), 649-653.

Cohen, J. (1992). A power primer. *Psychological Bulletin, 112*(1), 155-159.

Collins, S. M., & Bercik, P. (2009). The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. *Gastroenterology, 136,* 2003-2014.
doi: 10.1053/j.gastro.2009.01.075

Claesson, M. J., Jeffrey, I. B., Conde, S., Power, S. E., O'Conner, E. M., Cusack, S., … O'Toole, P. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature, 488,* 178-184.
doi: 10.1038/nature11319

Cryan, J. F. & O'Mahony, S. M. (2011). The microbiome-gut-brain axis: From bowel to behavior. *Neurogastroenterology & Motility, 23*(3). 187-192.
doi: 10.1111/j.1365-2982.2010.01664.x

Davari, S., Talaei, S. A., Alaei, H., & Salami, M. (2013). Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: Behavioral and eletrophysiological proofs for microbiome-gut-brain axis. *Neuroscience, 240,* 287-296.

Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R. D., …

Cryan, J. F. (2015). Gut microbiota depletion from early adolescence in mice: Implication for brain and behavior. *Brain Behavior & Immunity, 48,* 165-173. doi: 10.1016/j.bbi.2015.04.004

El-Husseini, A. E., Schnell, E., Chetkovich, D. M., Nicoll, R. A., and Bredt, D. S. (2000). PSD-95 involvement in maturation of excitatory synapses. *Science, 290,* 1364–1368.
doi: 10.1126/science.290.5495.1364

Eutamene, H. & Beuno, L. (2007). Role of probiotics in correcting abnormalities of colonic flora induced by stress. *Gut, 56,* 1495-1497.

Foster, J. A., & McVey Neufeld, K-A. (2013). Gut-brain axis: How the microbiome influences anxiety and depression. *Trends in Neurosciences, 36*(5), 305-312.

Fröhlich, E. E., Farzi, A., Mayerhofer, R., Reichmann, F., Jačan, A., Wagner, B., …Holzer, P. (2016). Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. *Brain, Behavior, and Immunity, 56,* 140-155.

Fung, T., Olson, A., Hsiao, E., (2017). Interactions between the microbiota immune and nervous systems in health and disease. *Nature Neuroscience 20,* 145-155.
doi: 10.1038/nn.4476

Gage, G. J., Kipke, D. R., & Shain, W. (2012). Whole animal perfusion fixation for rodents. *Journal of visualized experiments : JoVE*, (65), 3564.
https://doi.org/10.3791/3564

Gareau, M. G., Jury, J., MacQueen, G., Sherman, P. M., & Perdue, M. H. (2007). Probiotic treatment of rat pups normalizes corticosterone release and ameliorates

colonic dysfunction induced by maternal separation. *Gut, 56*, 1522-1528.

Gareau, M., G., Wine, E., Rodrigues, D. M., Cho, J. H., Whary, M. T., Philpott, D. J., … Sherman, P. M. (2011). Bacterial infection causes stress-induced memory dysfunction in mice. *Gut, 60*(3), 288-289. doi: 10.1136/gut.2010.226779

Grill, M. F., & Maganti, R. K. (2011). Neurotoxic effects associated with antibiotic use: Management considerations. *British Journal of Clinical Pharmacology, 72*(3), 381-393.

Guida, F., Turco, F., Iannotta, M., De Gregorio, D., Palumbo, I., Sarnelli, G., … Maione, S. (2018). Antibiotic-induced microbiota perturbation causes gut endonnabinoidome changes, hippocampal neuroglial reorganization and depression in mice. *Brain, Behavior, and Immunity, 67*, 230-245. doi: http://dx.doi.org/10.1016/j.bbi.2017.09.001

Guigoz, Y., Dore, J., & Schiffrin, E. J. (2008). The inflammatory status of old age can be nurtured from the intestinal environment. *Current Opinion in Clinical Nutrition and Metabolic Care, 11,* 13-20.

Hare, D. J., George, J. L., Bray, L., Volitakis, I., Vais, A., Ryan, T. M. …Finkelstein, D. I. (2014). The effect of paraformaldehyde fixation and sucrose cryoprotection on concentration in murine neurological tissue. *Journal of Analytical Atomic Spectrometry, 29*, 565-570. doi: 10.1039/c3ja50281c

He, F., Ouwehand, A. C., Isolauri, E., Hosoda, M., Benno, Y., & Salminen, S. (2001).

Differences in composition and mucosal adhesion of bifidobacterial isolated from healthy adults and healthy seniors. *Current Microbiology, 43,* 351-354. doi: 10.1007/s002840010315

Hoban, A. E., Moloney, R. D., Golubeva, A. V., Mcvey Neufeld, K. A., O'Sullivan, O., Patterson, E., … Cryan, J. F. (2016). Behavioral and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat. *Neuroscience, 339*, 463-477.

Ingram, D. K., Spangler, E. L., & Vincent, G. P. (1983). Behavioral comparison of aged virgin and retired breeder mice. *Experimental Aging Research, 9*(2), 111-113. doi: 10.1080/03610738308258436

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z. Yin, Y., … Ruan, B. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain, Behavior, and Immunity, 48*, 186-194. doi: https://doi.org/10.1016/j.bbi.2015.03.016

Jurdak N, Kanarek RB (2009) Sucrose-induced obesity impairs novel object recognition learning in young rats. *Physiology & Behavior, 96*, 1–5.

Karp, B. E. & Engberg, J. (2004). Does the use of antiobiotics in food animals pose a risk to human health? A critical review of published data. *Journal of Antimicrobial Chemotherapy, 54*(1), 273-274.

Kasukurthi, R., Brenner, M. J., Moore, A. M., Moradzadeh, A., Ray, W. Z., Santosa, K. B., Mackinnon, S. R., & Hunter, D. A. (2009). Transcardial perfusion versus immersion fixation for assessment of peripheral nerve regeneration. *Journal of Neuroscience Methods, 184*(2), 303 – 309.

doi: https://doi.org/10.1016/j.jneumeth.2009.08.019

Kato, K., Odamaki, T., Mitsuyama, E., Sugahara, H., Xiao, J., & Osawa, R. (2017). Age-related changes in the composition of gut *Bifidobacterium* species. *Current Microbiology*, *74*, 987-995.

doi: 10.1007/s00284-017-1272-4

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., & Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature*, *474*(7351), 327-36.

doi:10.1038/nature10213

Kempermann, G. (2008). The neurogeneic reserve hypothesis: What is adult hippocampal neurogenesis good for? *Trends in Neurosciences*, *31*(4), 163-169.

doi: 10.1016/j.tins.2008.01.002

Komura, T., Ikeda, T., Yasui, C., Saeki, S., & Nishikawa, Y. (2013). Mechanism underlying prolongevity induced by bifidobacterial in *Caenorhabditis elegans*. *Biogerontology*, *14*, 73-87.

doi: 10.1007/s10522-012-9411-6

Kopp-Hoolihan, L. (2001). Prophylactic and therapeutic uses of probiotics: A review. *Journal of the American Dietetic Association*, *101*(2), 229-241.

doi: https://doi.org/10.1016/S0002-8223(01)00060-8

Kuhn, H. G., Dickinson-Anson, H., & Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. *The Journal of Neuroscience*, *16*(6), 2027-2033.

Kwon, S. E., and Chapman, E. R. (2011). Synaptophysin regulates the kinetics of

synaptic vesicle endocytosis in central neurons. *Neuron, 70*, 847–854. doi: 10.1016/j.neuron.2011.04.001

Kwon, H. K., Kim, G. C., Kim, Y., Hwang, W., Jash, A., Sahoo, A., … Im, S. H. (2013). Amelioration of experimental autoimmune encephalomyelitis by probiotic mixture is mediated by a shift in T helper cell immune response. *Clinical Immunology*, *146*(3), 217-227. doi: https://doi.org/10.1016/j.clim.2013.01.001

Leclercq, S., Mian, F. M., Stanisz, A. M., Bindels, L. B., Cambier, E., Ben-Amram, H., … Bienenstock, J. (2017). Low-dose penicillin in early life induces long-term changes in murine gut microbiota, brain cytokines, and behavior. *Nature Communications*, 1-12. doi: 10.1038/ncomms15062

Lerner, P. I., Smith, H., & Weinstein, L. (1967). Penicillin neurotoxicity. *Annals of the New York Academy of Sciences*, *145*(2), 310-318.

Levy, S. B. (1998). The challenge of antibiotic resistance. *Scientific American*, *278*(3), 46-53.

Li, W., Dowd, S. E., Scurlock, B., Acosta-Martinez, V., & Lyte, M. (2009). Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria. *Physiology & Behavior*, *96*(4-5), 557-567. doi: https://doi.org/10.1016/j.physbeh.2008.12.004

Liu, S., Zhao, G., Zhao, H., Zhai, G., Chen, J., & Zhao, H. (2017). Antibiotics in a general population: Relations with gender, body mass index (BMI) and age and their human health risks. *Science of the Total Environment, 599-600*, 298-304.

doi: http://dx.doi.org/10.1016/j.scitotenv.2017.04.216

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gutmicrobiota. *Nature*, *489*(7415), 220–230.

doi: https://doi.org/10.1038/nature11550

Luczynski, P., McVey Neufeld, K. A., Oriach, C. S., Clarke, G., Dinan, T. G., & Cryan, J. F. (2016). Growing up in a bubble: Using germ-free animals to assess the influence of the gut microbiota on brain and behavior. *International Journal of Neuropsychopharmacology*, *19*(8), 1-17.

doi: 10.1093/ijnp/pyw020

Luine, V. N., Wallace, M. E., & Frankfurt, M. (2011). Age-related deficits in spatial memory and hippocampal spines in virgin, female Fischer 344 rats. *Current gerontology and geriatrics research, 2011*, 316386.

doi: https://doi.org/10.1155/2011/316386

Magnusson, K. R., Hauck, L., Jeffrey, B. M., Elias, V., Humphrey, A., Nath, R., … Bermudez, L. E. (2015). Relationships between diet-related changes in the gut microbiome and cognitive flexibility. *Neuroscience, 300,* 128-140.

doi: https://doi.org/10.1016/j.neuroscience.2015.05.016

Matthews, D. M. & Jenks, S. M. (2013). Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. *Behavioral Processes, 96,* 27-35.

Mitsuoka, T. (2014). Establishment of intestinal bacteriology. *Bioscience of Microbiota, Food and Health*, *33*(3), 99-116.

Möhle, L., Mattei, D., Heimesaat, M. M., Bereswill, S., Fischer, A., Alutis, M., … Wolf, S. A. (2016). Ly6C^{hi} monocytes proved a link between antibiotic-induced changes in gut microbiota and adult hippocampal neurogenesis. *Cell Reports, 15*(9), 1945-1956.
doi: https://doi.org/10.1016/j.celrep.2016.04.074

Moore, A. R., & O'Keeffe, S. T. (1999). Drug-induced cognitive impairment in the elderly. *Drugs and Aging, 15*(1), 15-28.

Naseribafrouei, A., Hestad, K., Avershina, E., Sekelja, M., Linlokken, A., Wilson, R., & Rudi, K. (2014). Correlation between the human fecal microbiota and depression. *Neurogastroenterology & Motility, 26*, 1155-1162.
doi: 10.1111/nmo.12378

Ochoa-Reparaz, J., Mielcarz, D. W., Wang, Y., Begum-Haque, S., Dasgupta, S., Kasper, D. L., & Kasper, L. H. (2010). A polysaccharide from the human commensal *Bacteroides fragilis* protects against CNS demyelinating disease. *Mucosal Immunology, 3*(5), 487-495.
doi: 10.1038/mi.2010.29

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J., … Osawa, R. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: A cross-sectional study. *BMC Microbiology, 16*(90), 1-12.
doi: 10.1186/s12866-016-0708-5

O'Mahony, S. M., Marchesi, J. R., Scully, P., Codling, C., Ceolho, A. M., Quigley, E. M. M., … Dinan, T. G. (2009). Early life stress alters behavior, immunity, and microbiota in rats: Implications for irritable bowel syndrome and psychiatric

illnesses. *Biological Psychiatry, 65*(3), 263-267. doi: https://doi.org/10.1016/j.biopsych.2008.06.026

Ogbonnaya, E. S., Clarke, G., Shanahan, F., Dinan, T. G., Cryan, J. F., & O'Leary, O. F. (2015). Adult hippocampal neurogenesis is regulated by the microbiome. *Biological Psychiatry, 78*(4), e7-e9. doi: https://doi.org/10.1016/j.biopsych.2014.12.023

O'Toole, P. W., & Claesson, M. J. (2010). Gut microbiota: Changes throughout the lifespan from infancy to elderly. *International Dairy Journal, 20*(4), 281-291. doi: https://doi.org/10.1016/j.idairyj.2009.11.010

Ouwehand, A. C., Isolauri, E., Kirjavainen, P. V., & Salminen, S. J. (1999). Adhesion of four Bifidobacterium strains to human intestinal mucus from subjects in different age groups. *FEMS Microbiology Letters, 172*, 61-64.

Ozawa, T., Yamada, K., & Ichitani, Y. (2011). Long-term object location memory in rats: Effects of sample phase and delay length in spontaneous place recognition test. *Neuroscience Letters, 497*, 37-41. doi: 10.1016/j.neulet.2011.04.022

Patki, G., Atrooz, F., Alkadhi, I., Solanki, N., & Salim, S. (2014). High aggression in rats is associated with elevated stress, anxiety-like behavior, and altered catecholamine content in the brain. *Neuroscience Letters, 584*, 308-313.

Quigley, E. M. M. (2017). Microbiota-brain-gut axis and neurodegenerative disease. *Current Neurology and Neuroscience Reports, 17*, 94. doi: https://doi.org/10.1007/s11910-017-0802-6

Rodriguez-Julbe, M., Ramirez-Ronda, C., Arroyo, E., Maldonado, G., Saavedra, S.,

Melendez, B., … & Figueroa, J. (2004). Antibiotics in older adults. *Puerto Rico Health Sciences Journal, 23*(1), 25-33.

Sanders, M. E., Walker, D. C., Walker, K. M., Aoyama, K. & Klaenhammer, T. R. (1996). Performance of commercial cultures in fluid milk applications. *Journal of Dairy Science, 79*(6), 943-955.
doi: 10.3168/jds.S0022-0302(96)76445-7

Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F., & Burnet, P. W. J. (2016). Psychobiotics and the manipulation of bacteria-gut-brain signals. *Trends in Neurosciences, 39*(11), 763-781.
doi: https://doi.org/10.1016/j.tins.2016.09.002

Savignac, H. M., Tramullas, M., Kiely, B., Dinan, T. G., & Cryan, J. F. (2015). Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behavioral Brain Research, 287*, 59-72.
doi: 10.1016/j.bbr.2015.02.044

Sawada, N., Kotani, T., Konno, T., Setiawan, J., Nishigaito, Y., Saito, Y., … Matozaki, T. (2018). Regulation by commensal bacteria of neurogenesis in the subventricular zone of adult mouse brain. *Biochemical and Biophysical Research Communications, 498*(4), 824-829.
Doi: https://doi.org/10.1016/j.bbrc.2018.03.064

Schliamser, S. E., Cars, O., & Norrby, S. R. (1991). Neurotoxicity of β-lactam antibiotics: Predisposing factors and pathogenesis. *Journal of Antimicrobial Chemotherapy*, *27*(4), 405-425.
doi: https://doi.org/10.1093/jac/27.4.405

Shalev, D., & Arbuckle, M. R. (2017). Metabolism and Memory: Obesity, Diabetes, and Dementia. *Biological psychiatry, 82*(11), e81–e83. doi:10.1016/j.biopsych.2017.09.025

Song, L., Che, W., Min-Wei, W., Murakami, Y. & Matsumoto, K. (2006). Impairment of the spatial learning and memory induced by learned helplessness and chronic mild stress. *Pharmacology, Biochemistry, and Behavior, 83,* 186-193.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X. N., et al. (2004). Postnatal microbial colonization programs the hypothalamic-pituitary adrenal system for stress response in mice. *The Journal of Physiology, 558*(1), 263–275. doi: 10.1113/jphysiol.2004.063388

Sun, J., Ling, Z., Wang, F., Chen, W., Li, H., Jin, J., … Liu, J. (2016). *Clostridium butyricum* pretreatment attenuates cerebral ischemia/reperfusion injury in mice via anti-oxidation and anti-apoptosis. *Neuroscience Letters, 613,* 30-35. doi: https://doi.org/10.1016/j.neulet.2015.12.047

Tarsa, L., and Goda, Y. (2002). Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America, 99,* 1012–1016. doi: 10.1073/pnas.022575999

Tognini, P. (2017). Gut microbiota: A potential regulator of neurodevelopment. *Frontiers in Cellular Neuroscience, 11*(25), 1-8. doi: 10.3389/fncel.2017.00025

Turbaugh, P. J., Bäckhed, F., Fulton, L., & Gordon, J. I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome.

Cell Host & Microbe, *21*(3), 278-281.

doi: https://doi.org/10.1016/j.chom.2008.02.015

Varghese, A. K., Verdu, E. F., Bercik, P., Khan, W. I., Blennerhassett, P. A., Szechtman, H., & Collins, S. M. (2006). Antidepressants attenuate increased susceptibility to colitis in a murine model of depression. *Gastroenterology, 130*(6), 1743-1753.

doi: https://doi.org/10.1053/j.gastro.2006.02.007

Vogel-Ciernia, A. & Wood, M. A. (2008). Examining object location and object recognition memory in mice. *Current Protocols in Neuroscience, 69*, 1-22.

Willing B.P., Dicksved, J., Halfvarson, J., Andersson, A. F., Lucio, M., Zheng, Z… Engstrand, L. (2010). A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. *Gastroenterology, 139*(6), 1844–1854.

doi: 10.1053/j.gastro.2010.08.049

Westfall, S., Lomis, N., Kahouli, I., Dia, S. Y., Singh, S. P., & Prakesh, S. (2017). Microbiome, probiotics, and neurodegenerative diseases: Deciphering the gut brain axis. *Cellular and Molecular Life Sciences*, *74*, 3769-3787.

doi: 10.1007/s00018-017-2550-9

Woodmansey, E. J., McMurdo, M. E. T., Macfarlane, G. T., & Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Applied and Environmental Microbiology*, *70*(10), 6113-6122.

doi: 10.1128/AEM.70.10.6113–6122.2004

Yankelevitch-Yahav, R., Franko, M., Huly, A., & Doron, R. (2015). The forced swim test

as a model of depressive-like behavior. *Journal of Visualized Experiments,* (97).

Zangen, A., Overstreet, D. H., & Yadid, G. (1999). Increased catecholamine levels in specific brain regions of a rat model of depression: Normalization by chronic antidepressant treatment. *Brain Research, 824*, 243-250.

Zhou, L. & Foster, J. A. (2015). Psychobiotics and the gut-brain axis: In the pursuit of happiness. *Neuropsychiatric Disease and Treatment, 11*, 715-723. doi: http://dx.doi.org/10.2147/NDT.S61997

Table 1.

Descriptive Statistics for Object Location Performance for Treatment Group and Age

		Baseline						Time 1						Time 2					
		Juvenile			Adult			Juvenile			Adult			Juvenile			Adult		
Group Name	*N*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*
Naïve No Treatment	10	12.75	21.40	19.75	40.26	35.15	27.73	22.35	12.78	17.03	10.64	22.09	17.65	-3.92	21.62	-10.71	11.30	59.24	7.86
+ Probiotics	10	18.38	9.39	18.95	14.19	52.41	3.24	0.32	19.71	3.07	13.36	23.72	3.88	6.81	21.11	-1.23	-31.29	48.53	0.00
Antibiotics + Probiotics	10	33.15	12.92	39.22	50.98	36.26	54.70	38.92	16.47	32.91	19.89	59.90	24.43	0.83	24.47	-1.13	-0.54	20.62	0.00
Antibiotics + No Treatment	10	25.55	16.08	16.98	27.80	49.08	50.04	3.20	39.93	-9.67	14.23	20.37	7.21	-0.67	15.95	-5.53	50.52	38.41	58.33
Antibiotics Only[a]	8	28.23	20.48	32.29	27.23	30.00	39.90	32.98	18.93	34.41	2.68	8.76	2.79	--	--	--	--	--	--

Note. Higher numbers indicate better object discrimination performance.

[a]Animals in this group were sacrificed prior to Time 2.

Table 2.

Descriptive Statistics for Object Location Performance for Treatment Group and Age

		Baseline						Time 1						Time 2					
		Juvenile			Adult			Juvenile			Adult			Juvenile			Adult		
Group Name	*N*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*	*M*	*SD*	*Mdn*
Naïve	10	105.29	42.64	134.58	65.61	52.44	49.27	130.60	54.62	154.40	46.06	46.61	21.09	123.12	46.05	145.66	42.20	44.37	32.44
No Treatment + Probiotics	10	93.27	37.50	101.25	80.75	58.14	87.55	126.84	45.36	117.10	65.68	73.39	41.81	90.26	10.60	92.30	65.46	98.63	22.41
Antibiotics + Probiotics	10	106.91	35.94	111.05	39.50	13.53	37.17	86.63	41.54	99.05	46.30	38.80	34.88	96.13	62.35	96.20	37.68	51.19	12.84
Antibiotics + No Treatment	10	106.63	59.09	126.47	42.33	52.52	21.05	122.63	59.80	100.56	20.12	13.50	15.35	97.90	71.18	70.08	16.01	14.37	11.78
Antibiotics Only	7	89.95	58.74	66.78	26.60	24.86	24.26	102.72	93.34	106.71	54.48	91.01	10.31	--	--	--	--	--	--

Note. Time is represented in seconds.

Table 3.

Mixed ANOVA for Age and Treatment Group on Object Location

	Type III Sum of Squares	*df*	Mean Square	*F*	Sig.	Eta Squared	Observed Power
Testing Session**	11296.13	2	5648.07	5.15	0.008	0.14	0.81
Time x Group**	6751.29	6	1125.22	1.03	0.417	0.09	0.38
Time x Age**	813.97	2	406.98	0.37	0.690	0.01	0.11
Time x Group x Age**	9686.15	6	1614.36	1.47	0.200	0.12	0.53
Error**	70262.79	64	1097.86				
Intercept	29930.11	1	29930.11	29.51	0.000	0.48	1.00
Treatment Group	6953.38	3	2317.79	2.29	0.098	0.18	0.52
Age	845.12	1	845.12	0.83	0.368	0.03	0.14
Group*Age	4140.91	3	1380.3	1.36	0.272	0.11	0.33
Error	32458.11	32	1014.32				

** Repeated Measures Analyses.

Table 4.

Mixed ANOVA for Age and Treatment Group on Forced Swim Test

	Type III Sum of Squares	*df*	Mean Square	*F*	Sig.	Eta Squared	Observed Power
Testing Session**	2274.66	2	1137.33	1.77	0.178	0.05	0.36
Time x Group**	1612.86	6	268.81	0.42	0.864	0.04	0.16
Time x Age**	3454.75	2	1727.38	2.70	0.075	0.08	0.52
Time x Group x Age**	5616.00	6	936.00	1.46	0.206	0.12	0.53
Error**	41024.22	64	641.00				
Intercept	715957.80	1	715957.80	109.89	0.000	0.774	1.00
Group	9805.04	3	3268.35	0.50	0.680	0.05	0.14
Age	107586.64	1	107586.64	16.51	0.000	0.34	0.98
Group*Age	10160.98	3	3386.99	0.52	0.672	0.05	0.14
Error	208495.32	32	6515.48				

** Repeated Measures Analyses.

Table 5.

Mixed ANOVA for Age and Treatment Group on Water Consumption at Time 1

	Type III Sum of Squares	*df*	Mean Square	*F*	Sig.	Eta Squared	Observed Power
Intercept	93937.38	1	93937.38	2252.18	0.000	0.98	1.00
Group	206.27	4	51.57	1.24	0.311	0.11	0.35
Age	4456.93	1	4456.93	106.86	0.000	0.73	1.00
Group*Age	120.63	4	30.16	0.72	0.581	0.07	0.21
Error	1626.67	39	41.71				
Total	102149.21	49					

Table 6.

Mixed ANOVA for Age and Treatment Group on Water Consumption at Time 2

	Type III Sum of Squares	*df*	Mean Square	*F*	Sig.	Eta Squared	Observed Power
Intercept	81431.29	1	81431.29	1345.37	0.000	0.98	1.00
Group	2885.74	3	961.91	15.89	0.000	0.60	1.00
Age	4274.56	1	4274.56	70.62	0.000	0.69	1.00
Group*Age	394.66	3	131.55	2.17	0.110	0.17	0.50
Error	1936.86	32	60.53				
Total	90923.11	40					

				3-Week Period		**1-Week Period**	
Group Name	# Rats Young	# Rats Old	B	Treatment	T1	Treatment	T2
Naïve	5	5	Baseline Behavior Testing	No Treatment	Behavior Testing	No Treatment	Behavior Testing
No TX + PBX	5	5		No Treatment		Probiotics	
ABX + No TX	5	5		Antibiotics		No Treatment	
ABX + PBX	4	5		Antibiotics		Probiotics	
ABX Only	5	4		Antibiotics			

Figure 1. Graphical depiction of the study timeline.

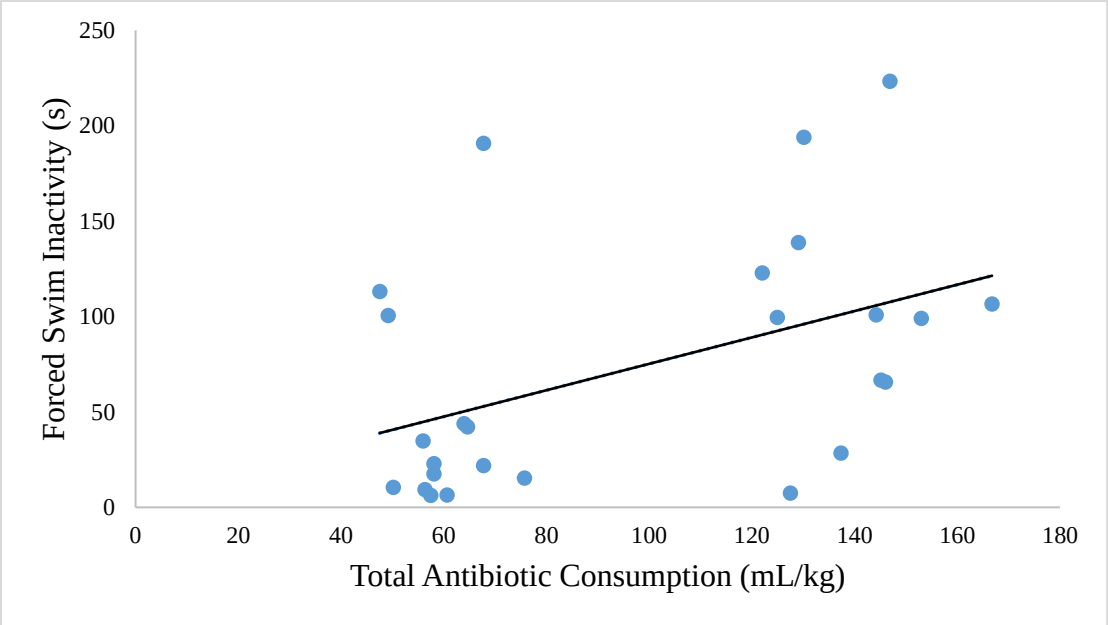

Figure 2. Average antibiotic consumption per kilogram weight and Time 1 Forced Swim Performance (N = 28). Antibiotic consumption spanned the three-week period between Baseline forced swim test performance and Time 1 performance, $\beta = 0.73$, $t(1) = 2.74$, $p = .011$, $R^2 = .23$.

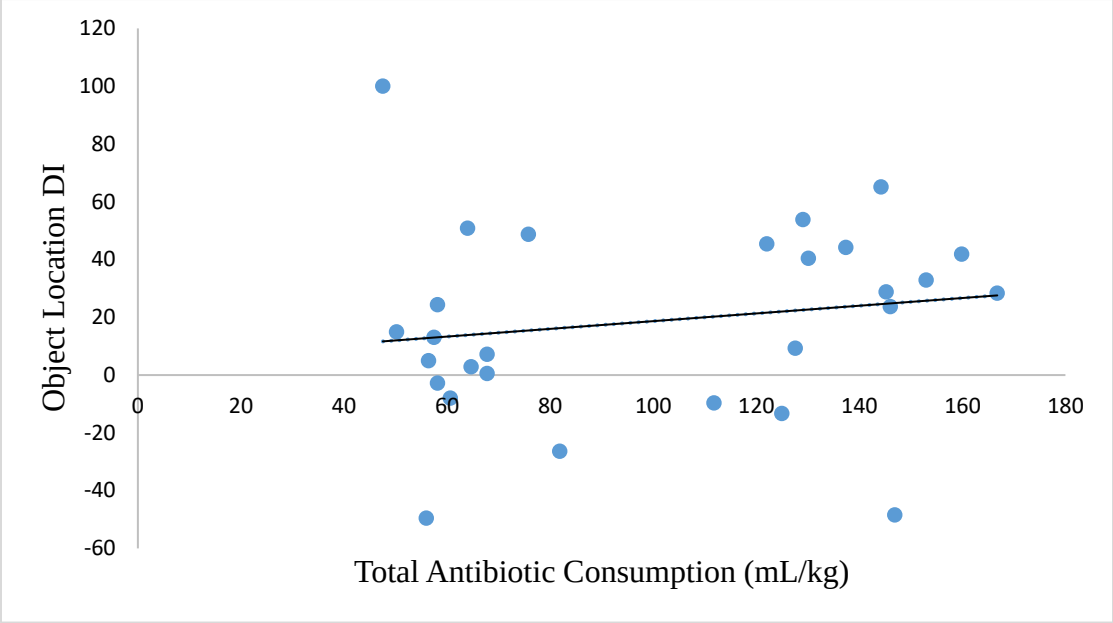

Figure 3. Average antibiotic consumption per kilogram weight and Time 1 Object Location Performance (N = 28). Antibiotic consumption spanned the three-week period between baseline object location performance and Time 1 performance, β = 0.13, $t(1)$ = 0.85, p = .402, R^2 = .03.

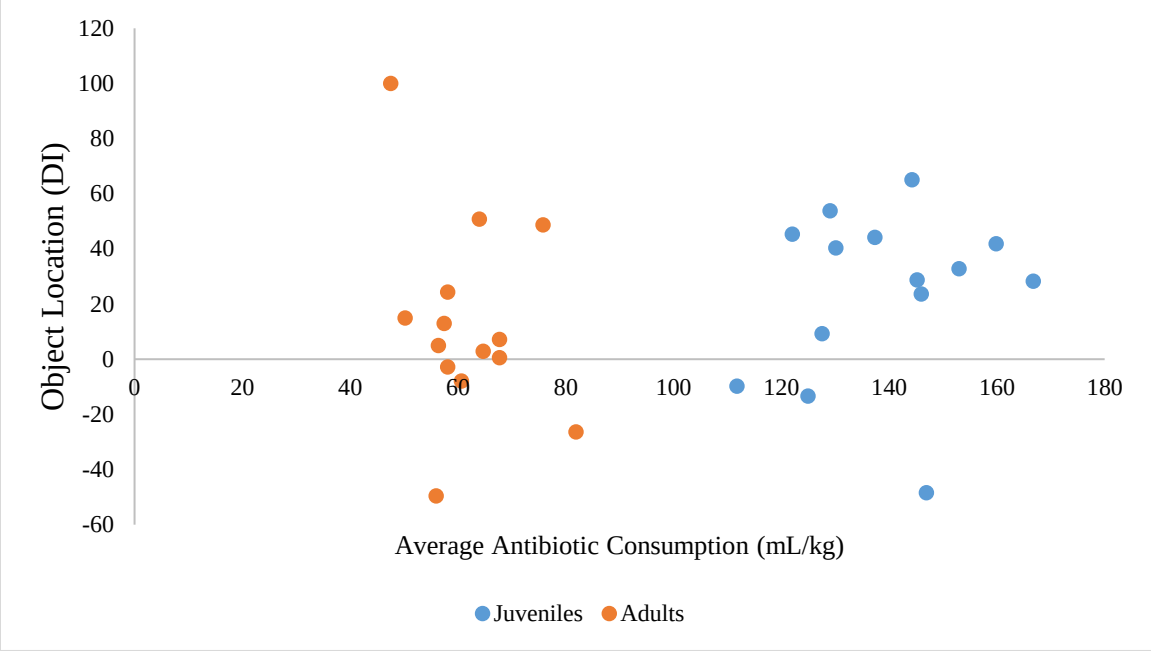

Figure 4. Age on antibiotic consumption per kilogram weight and Time 1 Object Location Performance (N = 28). Antibiotic consumption spanned the three-week period between baseline object location performance and Time 1 performance. Juveniles (β = 0.34, $t(1) = 0.61$, $p = .555$, $R^2 = .03$) did not differ on object location performance compared to adults (β = -1.03, $t(1) = -0.95$, $p = .359$, $R^2 = .07$

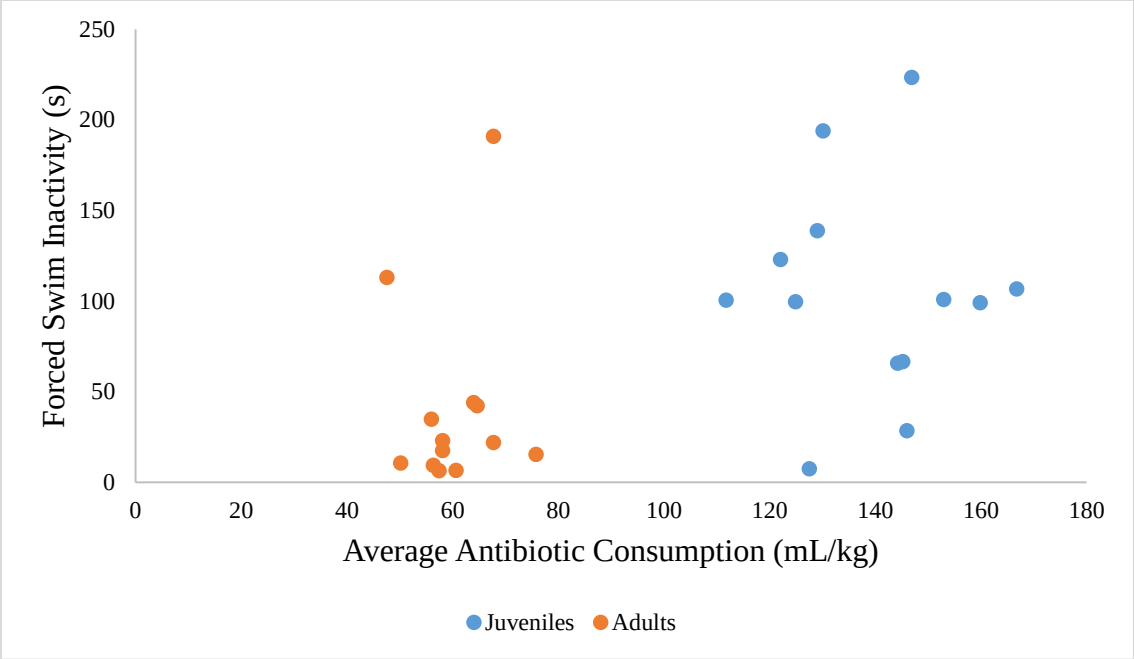

Figure 5. Age on antibiotic consumption per kilogram weight and Time 1 Forced Swim Performance (N = 28). Antibiotic consumption spanned the three-week period between Baseline forced swim test performance and Time 1 performance. Juvenile animals (β = .11, $t(1) = 0.10$, $p = .924$, $R^2 = .00$) did not differ from adults (β = -1.21, $t(1) = -0.78$, $p = .452$, $R^2 = .05$) on forced swim test inactivity.

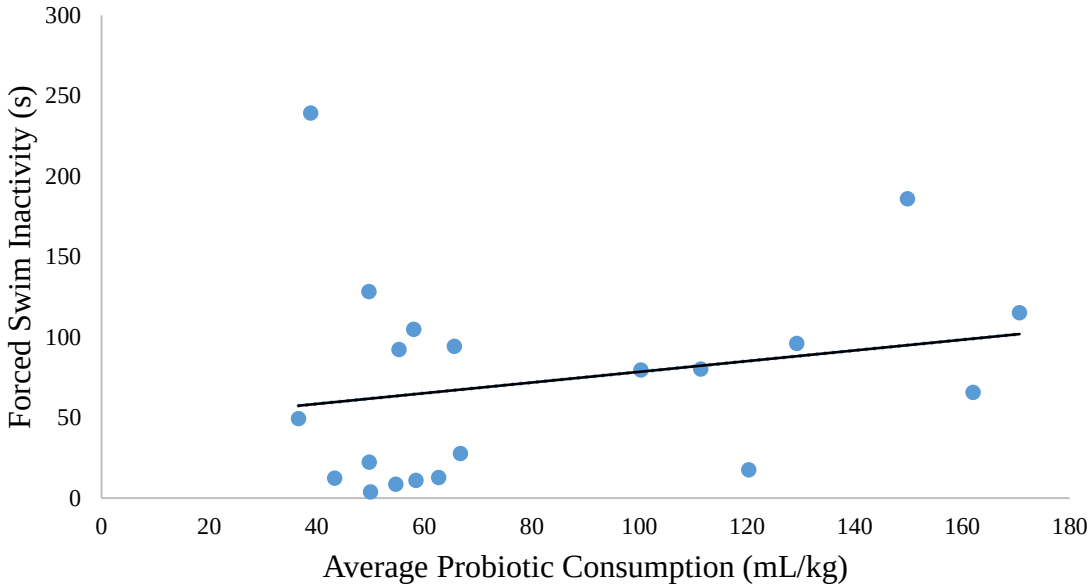

Figure 6. Average probiotic consumption per kilogram weight and Time 2 Forced Swim Performance (N = 20) . Probiotic consumption spanned the one-week period between Time 1 forced swim test and Time 2 forced swim test, $\beta = 0.33$, $t(1) = 0.99$, $p = .334$, $R^2 = .05$.

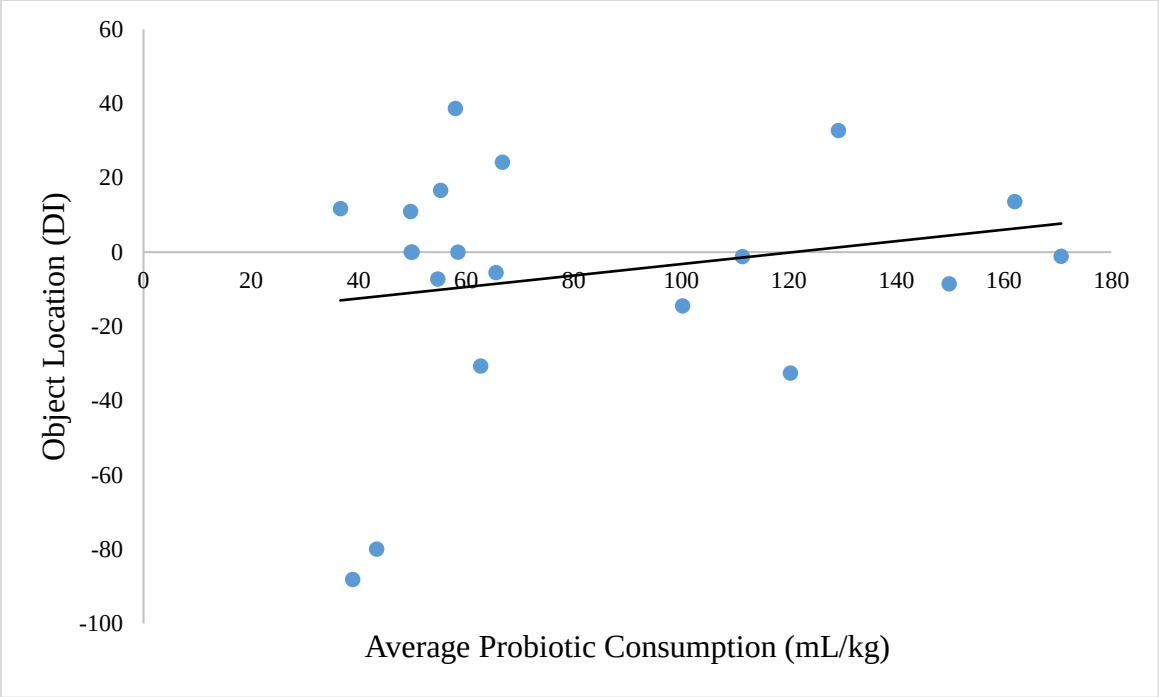

Figure 7. Average probiotic consumption per kilogram weight and Time 2 Object Location Performance (N = 20). Probiotic consumption spanned the one-week period between Time 1 object location test and Time 2 object location test, $\beta = 0.15$, $t(1) = 0.90$, $p = .379$, $R^2 = .04$.

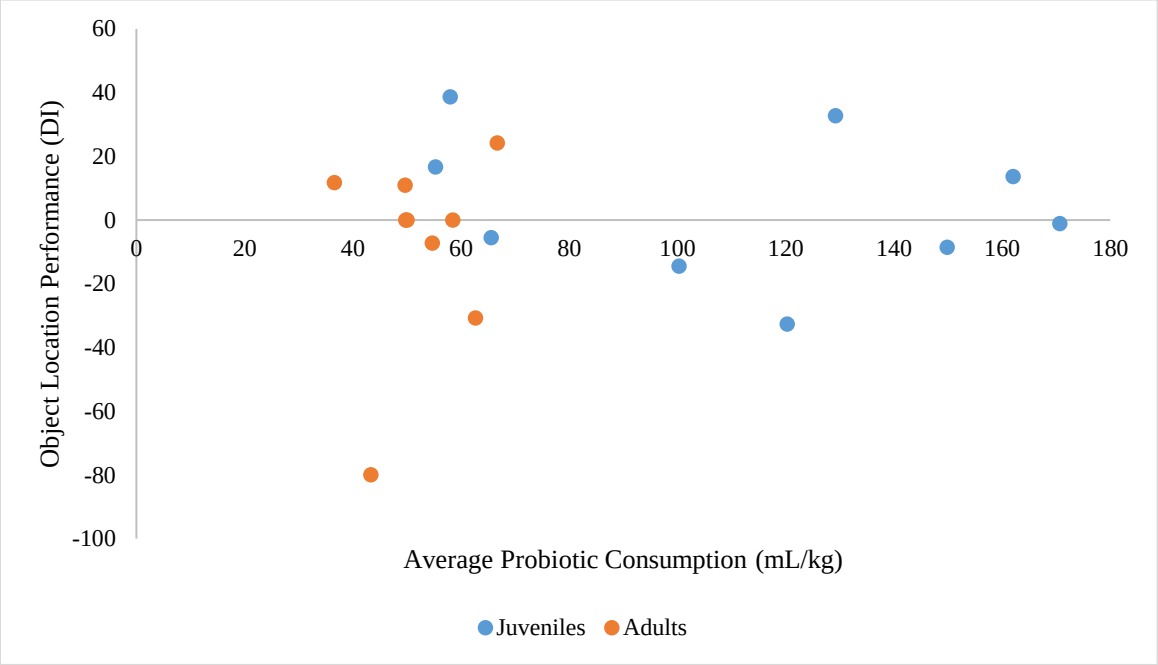

Figure 8. Age on probiotic consumption per kilogram weight and Time 2 Object Location Performance (N = 20). Probiotic consumption spanned the one-week period between Time 1 object location test and Time 2 object location test. Juveniles (β = -0.13, $t(1) = -0.71$, $p = .497$, $R^2 = .06$) did not differ compared to adult animals (β = 1.73, $t(1) = 1.39$, $p = .203$, $R^2 = .19$).

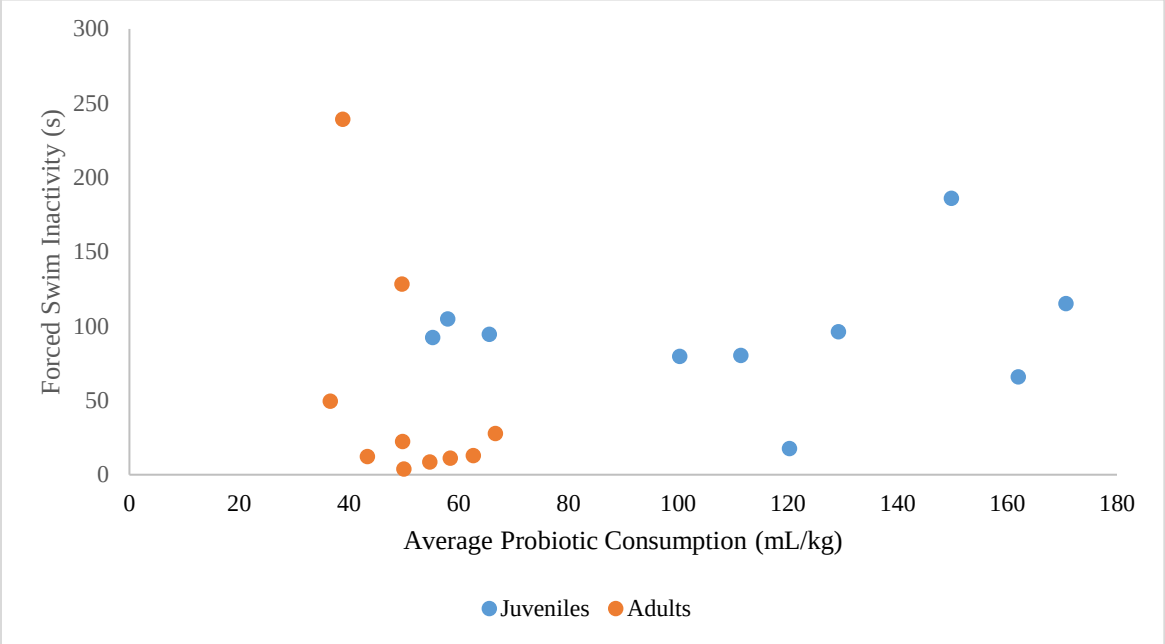

Figure 9. Age on probiotic consumption per kilogram weight and Time 2 Forced Swim Performance at (N = 20). Probiotic consumption spanned the one-week period between Time 1 forced swim test and Time 2 forced swim test. Juvenile animals (β = 0.15, $t(1)$ = 0.42, p = .683, R^2 = .02) did not differ from adult animals (β = -3.73, $t(1)$ = -1.57, p = .154, R^2 = .24) on forced swim performance following probiotic treatment.