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Abstract

Adolescent stress in humans has been correlated with an increased likelihood of an individual to develop an alcohol use disorder later in life. Literature has demonstrated that rats subjected to adolescent stress tend to show an increased preference and consumption of ethanol. Adolescence is a critical time of development. The link between adolescent stressors and alcohol use disorders is not fully understood yet. This study examined the relationship between adolescent stress and alcohol consumption and preference in rats. Probiotics are bacteria with potential health benefits and have been well accepted as a dietary supplement. Literature shows that probiotics could decrease rodent anxiety-like behaviors derived from adolescent stress, such as social isolation. This adolescent stress could lead to a subsequent increase in alcohol consumption in rats. An earlier study conducted at the University of Dayton showed that rats that received probiotics exhibited significantly higher anxiety-like behavior in comparison to the groups that did not. This finding contradicts the positive perception associated with probiotics, showing that some probiotics potentially have a negative impact on affective behaviors. In the current study we observed, while non-significant, the same trend that our lab previously showed for the rats that consumed probiotics. Moving forward, the positive and negative effects of probiotics should be further investigated.

Acknowledgements

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Introduction

Adolescent stress in humans correlates with an increased likelihood of an individual to develop addictive behaviors later in life (Keyes et al., 2011). Alcohol Use Disorders (AUD) are addictive behaviors that are classified as a chronic brain disease involving the inability to stop or control alcohol use in spite of the consequences (NIAAA, 2018). By 2015 6.2%, or 15.1 million, of Americans aged eighteen years or older had an AUD (NIAAA, 2018). Additionally, there were an estimated 88,000 alcohol-related deaths in America annually, making AUD the third leading cause of death in the United States (NIAAA, 2018). In an effort to decrease AUDs research has been aimed at studying adolescent stress. Adolescence is defined as a critical period of maturation (Doremus-Fitzwater et al., 2010). This is a critical time for any individual, rat or human, to develop behaviorally, neurologically, and physiologically (Spear, 2000).

Data suggests that neural alterations that occur throughout adolescence are similar across mammalian species, suggesting animal models are a viable mode to study adolescent stress (Spear, 2000). Animal models provide a preclinical model to study the brain and behavior. Researchers have the ability to gain more control over an animal's environment and diet among other variables during an experiment. Animals, such as rats, have a much shorter life span than humans. One day in a rat's life is equivalent to 34.8 human days (Sengupta, 2013). Therefore, researchers can observe changes that adolescent stress has on adulthood fairly quickly, creating a more cost effective and time efficient manner of studying brain and behavior processes. The information acquired about physiological, neural, and behavioral processes through studying a rat model can hopefully be translated into prevention methods and treatments for humans.

There have been many studies that have implemented various stressors on rats. Some of these involve physical and psychological stressors (Heilig et al., 2016). The model that we implement in our lab, social isolation, has been shown to be a potent model for neurological, physiological, and psychological changes. This experiment was designed to further explore adolescent stress on adult anxiety-like behaviors in rats and observe how the housing and diet would effect ethanol (EtOH) consumption. It has been shown that rats that are stressed during adolescence are likely to form behaviors different from rats that did not experience a stressor in adolescence (Spear, 2000). Rats have an adolescent period of post-natal date (PND) 28 to PND 70 (Veenema, 2009). During this time, rats have an increased sensitivity to stressors (Chappell et al., 2013). Additionally, rats that were socially isolated only as adults did not show the same effects on their behavior as the adolescent socially isolated rats did (McCool and Chappell, 2009). This shows that adolescent rats were more vulnerable to stress than adult rats. Typically in this model rats arrive on PND 21. Once the rats arrived after a period of acclimation, they were randomly divided into groups for housing. Half of the cohort was placed in social isolation (SI) and the other half was group housed (GH). Rats are social animals. Previous studies have shown that rats placed in SI during adolescence present with anxiety-like behavior in comparison to rats in GH (Chappell et al., 2013). We implemented this model in our study through randomly assigning the rats into SI and GH groups. Previous studies have shown that a variety of behavioral changes result from adolescent stress from social isolation (Butler and Weiner, 2016). Following social isolation, SI rats were more likely to have anxiety-like behavior on an Elevated Plus

Maze (EPM) and were more likely to show preference and self-administer EtOH than their GH counterparts (Butler and Weiner, 2016).

Anxiety-like behavior in rodents can be measured in multiple ways. One of the most common measurements is the Elevated Plus Maze (EPM). The EPM is designed with two open arms that are fully exposed to a light source, and two closed arms that have walls. Connecting the four walls is a central junction that is also exposed to the light. Non-stressed rats have been shown to spend more time in the open arms and central junction of the EPM (Pellow et al, 1985). Rats that were exposed to chronic social instability spent a significantly decreased amount of time in the open arms, which indicates an increase in anxiety-like behavior (Roeckner et al, 2017). These results have been reproduced in a multitude of studies, marking the EPM as a reliable test of anxiety-like behavior (Roeckner et al, 2017). Additionally, these results on the EPM have been pharmacologically validated (Pellow et al., 1985). Anxiolytic drugs (anxiety alleviating drugs), such as chlordiazepoxide, diazepam and phenobarbitone, increased the time the rats spent in the open arms of the EPM (Pellow et al., 1985). On the other hand, anxiogenic drugs (anxiety causing drugs); such as yohimbine, pentylenetetrazole, caffeine, amphetamine; caused the rats to spend less time in the open arms (Pellow et al., 1985). Another measure of anxiety-like behavior is the Light/Dark Box (L/D Box). The L/D Box includes two boxes, one is dark and covered and the other is light and uncovered. Decreased time spent in the light box indicates increased anxiety-like behavior in rats (Slawecki, 2005). Both the EPM and the L/D Box take advantage of a rat's natural predisposition to avoid bright areas (Slawecki, 2005). The L/D Box and EPM were implemented in this study as measures of anxiety-like behavior.

Individuals who experience adolescent stress or are diagnosed with an anxiety disorder are more likely to develop an alcohol use disorder (Keyes et al., 2011). There is a high rate of comorbidity between alcohol use disorders and mood and anxiety disorders. Of the individuals that suffer from an alcohol use disorder, 20 percent also suffer from a mood disorder and 18 percent suffer from an anxiety disorder (Grant et al., 2004). Addictive behaviors in humans are typically increased by the presence of stress during adolescence (Keyes et al., 2011). The adolescent GH/SI housing assay creates anxiety-like behaviors in SI rats that are correlated with an increase in ethanol (EtOH) consumption in SI male Long Evans rats (McCool and Chappell, 2009). This is parallel to humans that experience chronic adolescent stress and later develop alcohol use disorders. Male Long Evans rats have been shown to express this correlation between an increase in anxiety-like behavior and EtOH preference and consumption, however, other strains of rats and female Long Evans rats have not shown this phenotype. Male Long Evans rats are a strong model due to their consistency in this paradigm. Alcohol consumption is defined as the amount of alcohol a rat consumes during a given time period; whereas, alcohol preference is the rats increased intake of EtOH over water when both are present (Butler et al., 2014).

Models to date have shown the effects of social isolation as a form of adolescent stress. Our goal in this study was to implement an intervention during the stressor period that spans adolescence to attempt to prevent the effect of anxiety-like behavior in adulthood. It has been shown that probiotics may provide health benefits and assist in mood regulation (Rao et al., 2009). The Word Health Organization defines probiotics as living microorganisms that confer positive health benefits to the host when administered

in adequate amounts (FAO/WHO, 2002). Many probiotics are native to the gut microbiota of the mammalian host (Boumis et al., 2018), marking them safe for human consumption (FAO/WHO, 2002). The gut microflora needs a balance of a variety of bacteria and probiotics are a venue to maintain the optimum balance for positive health (Sarkar, 2013). Probiotics influence the gut microbiota, decrease the ability of pathogens that cause harm to the host, and interact with intestinal cells (Suzuki et al., 2017). How each strain effects the gut of a rat and a person are not fully understood due to the magnitude of strains that exist. *Lactobacillus rhamnosus* was determined to be a probiotic that reduced anxiety-like behavior in previous studies; and thus, was deemed to be a strong choice of probiotic for this study. In a study done on mice, *L. rhamnosus* was shown to provide an anxiolytic effect, as the mice spent an increased time in the open arms of the EPM (Bravo et al., 2011). This study showed that *L. rhamnosus* provides protective effects against stress. *L. rhamnosus* is one of the most extensively studied probiotic (Suzuki et al., 2017). Specifically, *L. rhamnosus* creates health benefits through its ability to modify the host immune response, increase the function of the epithelial barrier, and prevent the adherence of pathogens to epithelium (Suzuki et al., 2017).

Probiotics are believed to influence the gut-brain axis (Kelly et al., 2017). The gut-brain axis involves the communication directly between the brain and the gut. This is often influenced by the microbiome already in existence in the gut. If the microbiome is altered, then the brain will also be changed, whether positive or negative (Bravo et al., 2011). Probiotics alter the gut microflora (Kelly et al., 2017). This change is directly communicated to the brain through the vagus nerve and creates changes in the brain chemistry, such as changes in neurotransmitter receptors, mRNA expression, and HPA

axis response to stress (Bravo et al., 2011). *L. rhamnosus* has been shown to increase corticosterone production in certain areas of the brain in mice (Bravo et al., 2011). This same study showed that the mice that were not fed probiotics did not present with the increased corticosterone levels (Bravo et al., 2011). It has been shown that the vagus nerve is an integral part of the bidirectional communication pathway between the gut and brain (Bravo et al., 2011). The vagus nerve originates in the brain and goes to the gut, creating a direct pathway for communication. Vagotomized, chronically stressed mice did not present with the ameliorative effects against stress that chronically stressed mice with their vagus nerve intact did after repeat consumption of probiotics (Bravo et al., 2011). The rats that we used in the study had their vagus nerve and gut-brain axis intact. Thus, it was hypothesized that the probiotics would decrease the effects of the adolescent stress. If an individual undergoes a stressful event, then they have the potential of altering their gut microbiota (Rao et al., 2009). This shows that the gut-brain axis is bidirectional, and changes in the gut microbiota have effects on brain chemistry and vice versa. Patients with Chronic Fatigue Syndrome are more likely to develop Irritable Bowel Syndrome, and over half of these patients have an anxiety disorder (Rao et al., 2009). There is a high comorbidity between stress induced psychological disorders and gastrointestinal disorders (Rao et al., 2009). A study was conducted where the probiotic, *Lactobacillus casei* strain Shirota, was administered to individuals suffering from various degrees of depression and anxiety. Those with a low baseline for anxiety and depression showed significant mood improvement following the consumption of probiotics (Rao et al., 2009). This study demonstrates the connection between the gut and the brain and the ability of probiotics to change affect.

This study aims to observe the development, or lack thereof, of anxiety-like behavior in SI rats while consuming probiotics. Additionally, this study will investigate the effects of housing and diet during adolescence on future behaviors related to anxiety-like behavior and EtOH consumption and preference. Initially, we hypothesized that the SI rats taking probiotics would have a decreased anxiety-like behavior compared to SI rats on a non-probiotic diet. Following the studies that previously used the same model, we predicted that the SI rats would display increased EtOH consumption and preference (Butler et al., 2014). Previously our lab conducted this experimental design with the aforementioned hypotheses; however, the data generated did not support the hypotheses (Griff, 2018). Instead, our lab found that the rats on a probiotic diet displayed a significant increase in anxiety-like behavior compared to the non-probiotic groups (Griff, 2018). The current study was a replication in an attempt to add to the previous study's original findings.

Methods

Animals and Housing

Sixteen male Long Evans rats arrived on post-natal day (PND) 21 from Envigo, Indianapolis, IN. The cohort arrived in group housing and remained this way for six days post arrival (n = 4/cage) in order to acclimate to their new surroundings and researchers handling them. When the rats were received they were marked with specific colors of sharpie on their tail to distinguish between them. On the seventh day (PND 27), the rats were randomly assigned into housing groups of either social isolation (SI) or group

housed (GH). There were two cages of GH rats, four rats in each cage (56cm x 39cm). The other eight rats were SI and were placed in their individual cages (43cm x 22cm). The SI rats received the same environmental stimulation, including visual, olfactory, and auditory stimulation, as the GH rats, but the SI rats were deprived of all social interaction and physical contact with the other rats in the study. This is in accordance with the protocol used in previous studies (for example, Chappell et al., 2013). Half of GH and SI groups were randomly assigned to a diet: probiotic or non-probiotic. The four groups are Group Housed probiotic (GHp), Group Housed no probiotic (GH), Socially Isolated probiotic (SIp), and Socially Isolated no probiotic (SI) (Figure 1). The rats remained in this housing situation for the duration of the housing/diet protocol. After the first six weeks of the experiment (PND 69), the GH rats were separated into SI housing for the behavioral tests and drinking procedure. The rats had free access to water and food for the duration of the experiment. The vivarium kept lights on from 7:00 until 19:00 every day. Additionally, the rats were housed without enrichment. The Guide for Care and Use of Laboratory Animals (National Research Council, 2011) cites environmental enrichment as an independent variable and states that it may decrease anxiety and stress behaviors in rats. IACUC approved the removal of enrichment access to the rats as part of the experimental design. The animal protocol was approved by the University of Dayton Institutional Animal Care and Use Committee. The experimental protocols aligned with the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

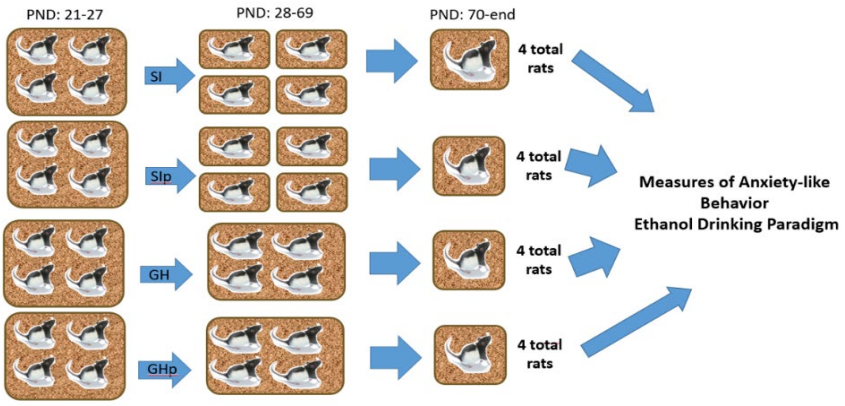

Figure 1: Housing and Diet Groups. The rats were separated into either SI or GH and received either a diet of probiotic or no probiotic.

Experimental Design

The probiotic and housing protocol were carried out for six weeks (PND 27 - 69). The week of PND 69 the cohort underwent two measures of anxiety-like behavior, the Light/Dark Box (L/D Box) and Elevated Plus Maze (EPM). The Light/Dark box was administered in the afternoon on Monday (PND 69) and the Elevated Plus Maze was performed in the morning of that Tuesday (PND 70). On PND 76 the rats began the EtOH drinking paradigm. Body weights were recorded every Monday throughout the experiment. Fecal samples were collected on PND 27, PND 48, PND 69, and PND 104. The rats were sacrificed on PND 105 and the cecum and colon were collected after decapitation. These events are outlined in the experimental timeline below (Figure 2).

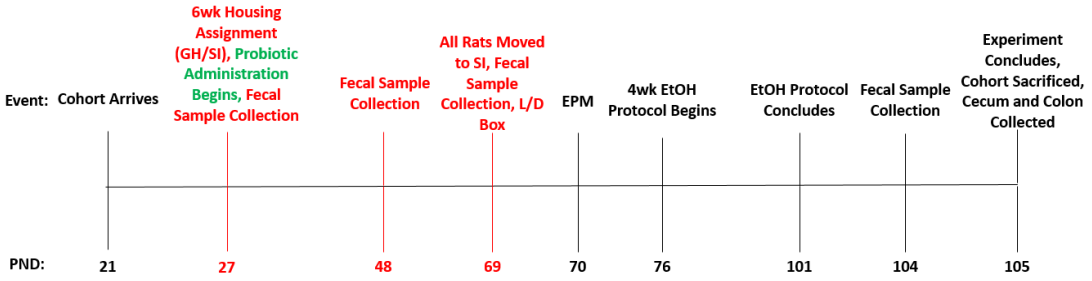

Figure 2: Experimental Timeline. GH - Group Housed; SI – Socially Isolated; L/D Box – Light/Dark Box; EPM – Elevated Plus Maze; EtOH – Ethanol; Red – Stressor Period; Green - Intervention

Probiotic Culturing and Administration

L. rhamnosus (strain GG, lab strain 3; 3/7/2017) was cultured each Friday before the Monday it was given to the rats. The cultures were started by streaking plates made of MRS media and left in an incubator overnight. The day before administration of probiotics (Sunday through Thursday each week), cultures were started by dotting a single *L. rhamnosus* colony into a tube of MRS media and placed into an incubator. The morning of probiotic administration 1 milliliter of culture was spun down and re-suspended in 100 microliters of MRS media. 10 microliters of the re-suspended culture were pipetted onto peanut butter placed on a food pellet. Rats that were not given the probiotic were just given peanut butter on a food pellet without the *L. rhamnosus*.

During probiotic administration, each rat was placed in their own clean cage. They were given a single food pellet with the peanut butter and probiotic per group designation. The rats were left alone in the room they were housed in for 20 minutes to eat the peanut butter. During this time, the experimenter left the room. After the 20 minutes allotted, the experimenter determined if the peanut butter was consumed and recorded this. Consumption of the peanut butter was recorded as consumed all, consumed some, or not consumed. The rats were returned to their respective cages. The probiotic protocol was continued over a six week time span (PND 27 - 69). Probiotics were administered every Monday, Wednesday, and Friday.

Fecal Sample Collection and Analysis

Fecal samples were collected before probiotic administration (PND 27), at the start of the fourth week of probiotic administration (PND 48), at the conclusion of probiotic administration (PND 69), and before sacrificing the cohort (PND 104). Each of these dates was a Monday. Each rat was placed in a clean, empty single house cage until two fecal samples were released. Once the samples were collected, the rats were returned to their respective cages. Metal tweezers were used to place the samples in two micro-centrifuge tubes per rat. The samples were stored in a -80 degree Celsius freezer for future analysis. Future analysis will involve extracting the DNA using a Fecal Sample DNA Extraction Kit from Fischer Scientific. The quantity of DNA will be measured using qPCR to determine the presence of probiotic in the GI tract. In a previous study at the University of Dayton fecal sample analysis yielded no significant difference between the probiotic and non-probiotic groups. A study conducted to evaluate strain specific quantification via qPCR of probiotics in human fecal samples found that there was an increased presence of the probiotics administered during the consumption period in comparison to the placebo (Karjalainen et al., 2012). An increase in *L. rhamnosus* would confirm the intake of the probiotic in the probiotic diet group.

Light/Dark Box

In the afternoon of PND 69, the rats were subjected to a Light/Dark (L/D) Box test as a measure of anxiety-like behavior. Four rats at a time were brought into the testing room and allowed to acclimate for ten minutes. The lights were turned off in the

room other than the lamp that illuminated the light box. The light intensity was measured to be 54 LUX. The dark box had an IR light placed on top of it to allow the camera to measure movement in the dark box. Ethovision software was used to track and record movement for five minutes per rat. The rats were given free reign between the two boxes, which were connected by an opening in the two boxes (10cm x 10cm). Both the light and dark box were 50 x 50cm with walls that were 35cm tall (Figure 3). Each rat was tested one-by-one. The rats were placed in the light box facing away from the dark box. The rats were tested in counterbalanced fashion, meaning one rat from each group was run. Then the second rat from each group was tested, and so on until all rats were tested. A shorter duration of time spent in the light box indicated greater anxiety-like behavior. The total distance a rat moved was measured as an indicator of general locomotion. After the five minutes were up, the rat was placed back in his personal cage. Both cages (floors, walls, and ceilings) were cleaned with soap and 70% ethanol before the next rat was tested.

Figure 3: Light/Dark Box. Consisting of a connected light and dark box. Decreased time in the light box indicates increased anxiety-like behavior. This figure shows two L/D Box set-ups.

Elevated Plus Maze

In the morning of PND 70 the rats were subjected to an Elevated Plus Maze (EPM) test as a measurement of anxiety-like behavior. Previous studies have shown that

an indication of increased anxiety-like behavior in rats is decreased time spent in open arms and central junction of the EPM (Butler et al., 2014). The rats were tested in a counterbalanced fashion as described above. The EPM consists of two closed arms and two open arms with an open central junction. The closed arms have three tall walls with a height of 32cm and an open ceiling. The open arms did not have any walls, allowing the rats to see over the edge of the structure and sit in the light. The closed arms were significantly darker than the open arms. The open arms are across from each other and the closed arms are across from each other. Each arm has the dimensions of 10.2cm x 50.8cm, and the central junction was 10cm x 10cm. The entire EPM was elevated 75cm above the ground (Figure 4). The rats were tested one at a time. They were placed in the center of the EPM facing the same open arm each time. Their movement was measured for 5 minutes using an overhead camera and EthoVision Software. Open arm time was measured. Less open arm time signified greater anxiety-like behavior. General locomotion was determined through the number of closed arm entries. Following the test, each rat was returned to their personal cage. The EPM was cleaned with soap and 70% ethanol before the next rat was tested.

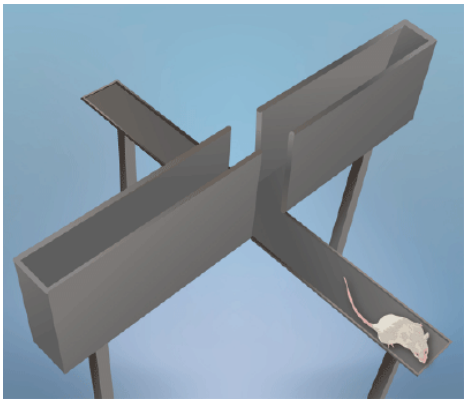

Figure 4: Elevated Plus Maze. Consisting of 2 open arms and an open central junction and 2 closed arms. Decreased time in the open arms and the open central junction indicates increased anxiety-like behavior

Drinking Procedure

The drinking paradigm was initiated on PND 76. The paradigm involved intermittent access and two-bottle choice with self-administration (Butler et al., 2014). The rats were given the choice between water and ethanol (EtOH) at each administration. Every Monday, Wednesday, and Friday the rats were given a bottle of water and a bottle of 20% EtOH in their home cage (Figure 5). To control for side preference, the bottle placement was alternated on each drinking day. The bottles were weighed before administration, at the 30 minute time point, and at the 24 hour time point. The 30 minute and 24 hour weights signified consumption amounts at those time points. Ethanol preference was calculated for each time point by dividing EtOH consumed by total fluid consumed. After 24 hour access, the two bottles were removed and a large water bottle was provided until the next administration. Free access to food pellets were provided through the duration of the drinking paradigm. The rats were weighed at the start of each drinking period throughout the four weeks of the drinking procedure. The drinking paradigm was designed to match previous studies (Butler et al., 2014) and the previous study conducted in our lab.

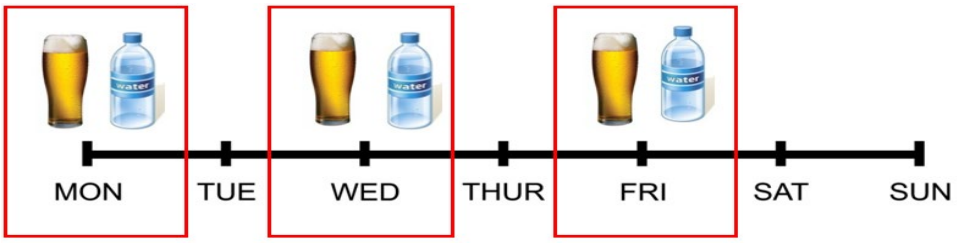

Figure 5: EtOH Paradigm. The rats were subjected to an intermittent access two-bottle choice, self-administration between water and 20% EtOH.

Cecum and Colon Procurement

At the conclusion of the study, the rats were sacrificed in-line with The Guide for Care and Use of Laboratory Animals (National Research Council, 2011). The cecum and colon were collected post-sacrifice and stored for future analysis. The small intestine has a number of factors; such as a biofilm, microvilli, special organelles that mediate adhesion, and bacteria that are already colonized there; that challenge the colonization of probiotics (Schrezenmeir and de Vrese, 2001). However, the colon does not have these factors and allows for colonization of probiotics (Schrezenmeir and de Vrese, 2001), providing a strong venue for analysis microbial gut population. Further analysis of the cecum and colon of the rats in this cohort could reveal how *L. rhamnosus* effected the cells and microbiota of the gut.

Data Analysis

The Light/Dark Box and Elevated Plus Maze test for anxiety-like behavior. This data was analyzed using a two-way ANOVA between housing and diet. The Ethanol data was analyzed via a three-way ANOVA between housing, diet, and time. The fecal sample *L. rhamnosus* DNA content will be quantified through qPCR and then compared through time point between probiotic and non-probiotic as well as between group housed and social isolation using a one-tailed t-tests. All statistics tests had a significance level set at $P<0.05$.

Results

Body Weight

A 3-way ANOVA between time, diet, and housing was performed on the bodyweights. There was a significant main effect for PND [$F(12, 156) = 617$, $p < 0.0001$] and housing [$F(1, 156) = 63.51$, $p < 0.0001$]. There was an interaction between housing x diet [$F(1, 156) = 18.7$, $p < 0.0001$] (Figure 6), thus we collapsed across PND and ran a two-way ANOVA (housing x diet) for body weight gain from PND 21 to PND 104. There was a main effect of housing [$F(1, 12) = 7.26$, $p < 0.05$] such that GH rats gained more weight than SI rats, but no main effect of diet or significant interaction. Sidak's multiple comparisons post hoc tests showed no significant differences for pairwise comparisons (Figure 7).

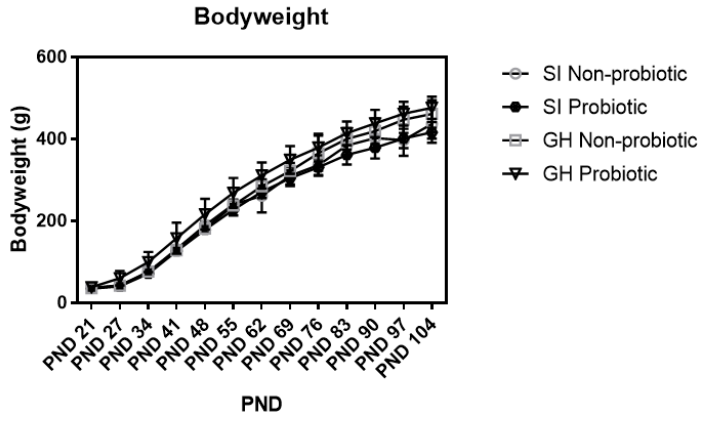

Figure 6: Bodyweight. There was a main effect for PND and housing. There was an interaction between housing x diet.

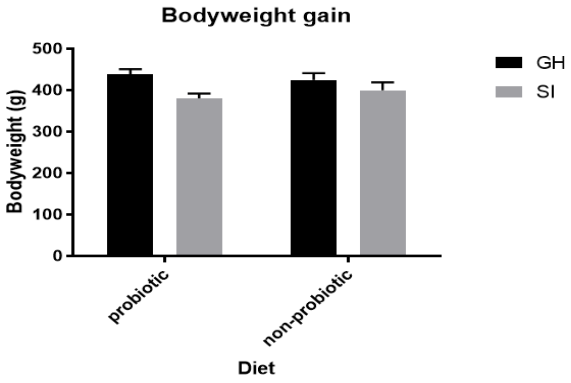

Figure 7: Bodyweight Gain. The GH rats gained more weight than the SI rats did.

Light Dark Box

The L/D box is a measure of anxiety-like behaviors in rats. We ran a 2-way ANOVA between time and housing to asses light box duration. Decreased time duration in the light box shows anxiety-like behavior. The L/D box test for anxiety-like behavior did not yield significant results for this cohort (Figure 8). There was no significant diet x housing interaction, [F (1, 12) = 0.01, p = 0.92]. There was no main effect of diet, [F (1, 12) = 0.11, p = 0.75], and no main effect of housing, [F (1, 12) = 0.005, p= 0.95].

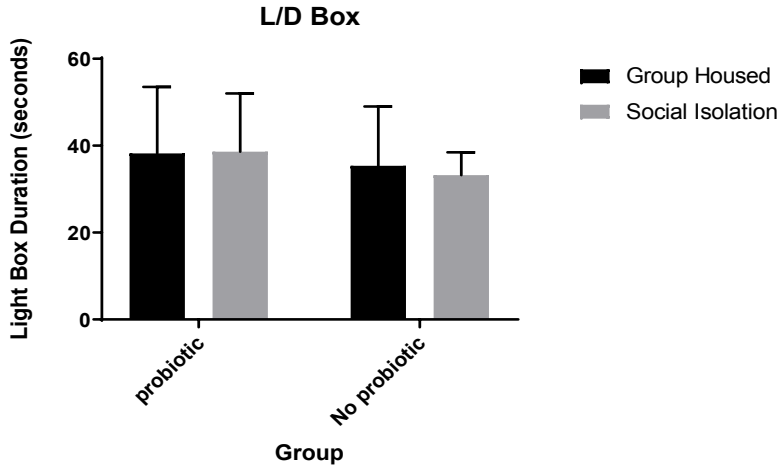

Figure 8: Light box duration time as a measure of anxiety-like behavior. The groups did not have a significant difference for light box time.

To measure total distance moved, a 2-way ANOVA was performed between total distance moved and housing. Total distance moved is a measure of locomotor activity. There was no significant interaction between diet x housing [F (1, 12) = 0.02, p = 0.89]. Additionally, there was no main effect of diet [F (1, 12) = 0.31, p = 7.60], or housing [F (1, 12) = 1.83, p = 0.20] (figure 9).

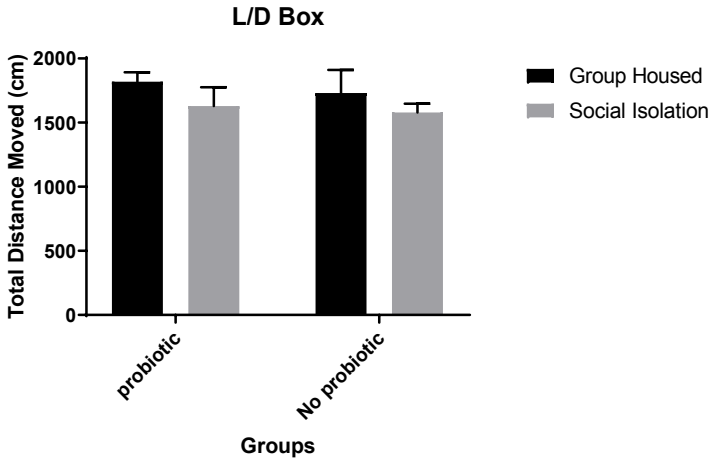

Figure 9: Total distance moved as a measure of general locomotion. The groups did not have a significant difference for general locomotor activity.

Elevated Plus Maze

Previous studies have shown that SI rats demonstrated a significantly higher anxiety-like behavior than GH rats following the six week housing protocol (McCool and Chappell, 2009; Chappell et al., 2013). The Elevated Plus Maze is a measure of anxiety-like behavior in rats. Increased open arm and central junction time shows less anxiety-like behavior in rats. A 2-way ANOVA between diet and time was performed for open arm + junction time in the Elevated Plus Maze. There was no significant diet x housing interaction, [F (1, 12) = 1.07, p = 0.32]. There was no main effect of diet, [F (1, 12) = 4.22, p = 0.06], or housing [F (1, 12) = 0.08 p = 0.78] (Figure 10).

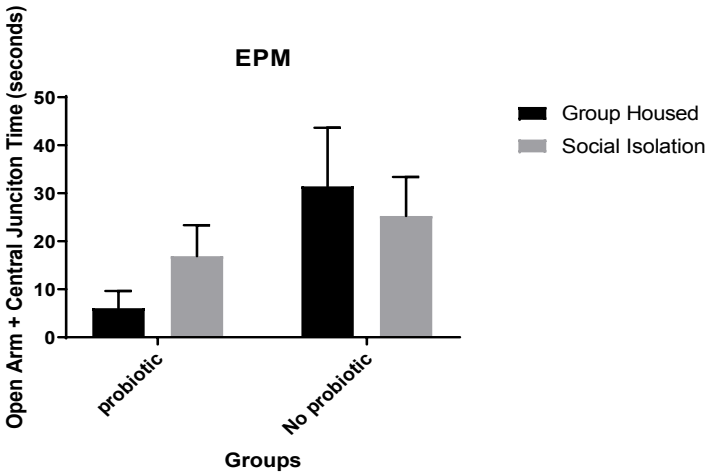

Figure 10: EPM open arm + junction time as a measure of anxiety-like behavior. The groups did not have a significant difference for EPM open arm + junction time.

There were no group differences in locomotor activity for this cohort as indicated by the number of closed arm entries. A 2-way ANOVA was performed for closed arm entrance frequency. There was no significant diet x housing interaction, [F (1, 12) = 0.21, p = 0.66]. There was no main effect for diet [F (1, 12) = 0.17, p = 0.22], or for housing [F (1, 12) = 1.21, p = 0.29] (Figure 11).

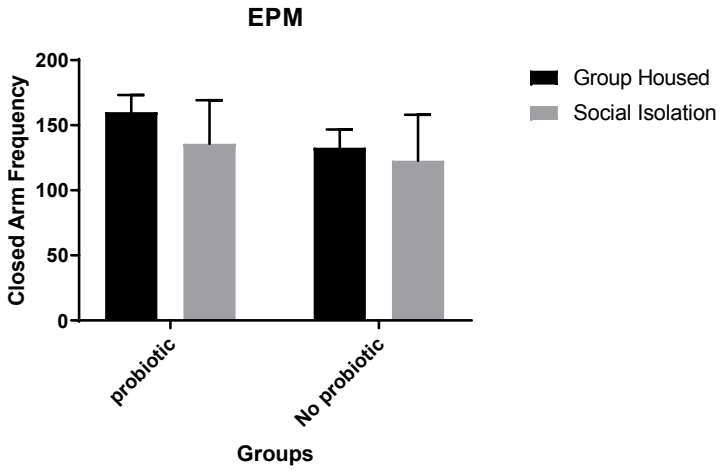

Figure 11: EPM closed arm entries as a measure of general locomotion. The groups did not have a significant difference for general locomotion.

Ethanol Paradigm

Ethanol data were averaged across week for the 30-minute ethanol intake time point. A 3-way ANOVA between housing, ethanol intake, and was performed for the averaged 30-minute ethanol intake time point. There was a main effect of week [F (1, 48) = 3.96, p = 0.01]. There was an interaction between week x diet [F (1, 48) = 1.50, p = 0.23] (Figure 12), so we collapsed the data and performed a two-way ANOVA to compare diet groups across weeks. There were no main effects found.

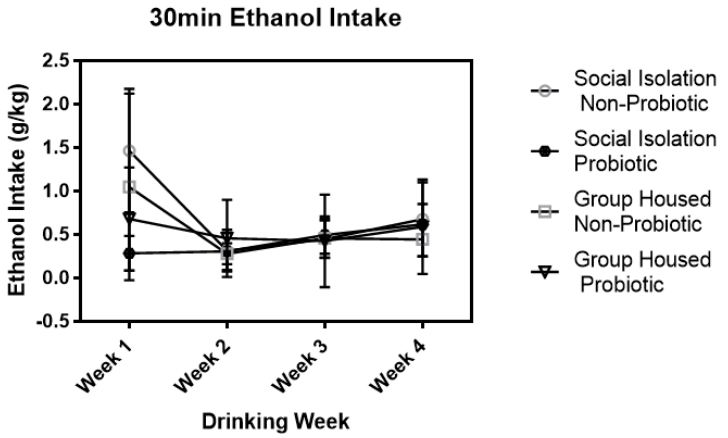

Figure 12: Ethanol intake for the 30 minute time point per week. There was a main effect of week and an interaction between week x diet.

A 3-way ANOVA was performed for the 30 minute time point ethanol preference per week. There were no significant interactions or main effects (Figure 13).

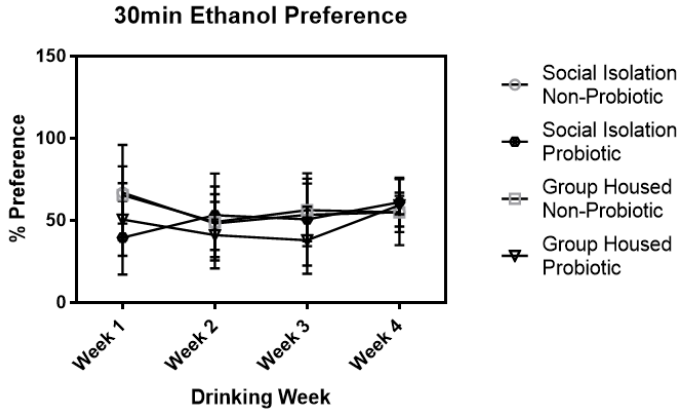

Figure 13: Ethanol preference for the 30 minute time point per week. The groups did not have a significant difference for the 30 minute time point ethanol preference.

A three-way ANOVA was performed for the 24-hour time point ethanol intake per week. There were no interactions. There was a main effect of time [$F(3, 48) = 20.04$, $p < 0.0001$] (Figure 14). We followed up on the main effect of time by collapsing within groups and comparing across week through a two-way ANOVA. There was a main effect of time [$F(3, 9) = 15.54$, $p = 0.0007$]. Sidak's multiple comparisons post hoc test indicated that Drinking Week 1 was significantly higher than Drinking Weeks 2, 3, and 4 (Figure 15).

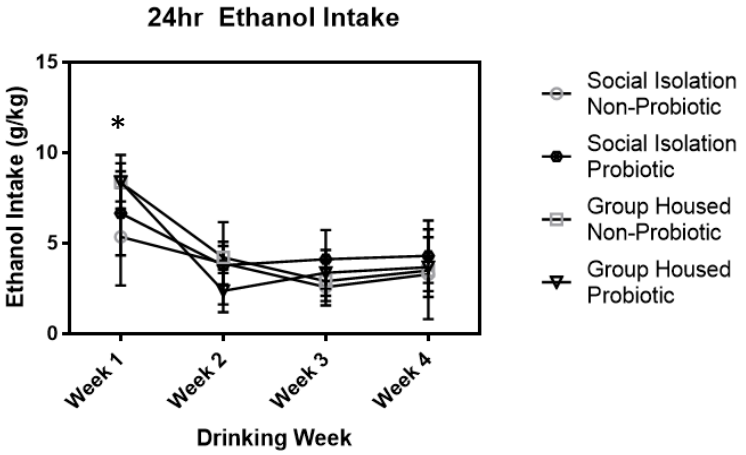

Figure 14: Ethanol intake for the 24 hour time point per week. A main effect was found for time.

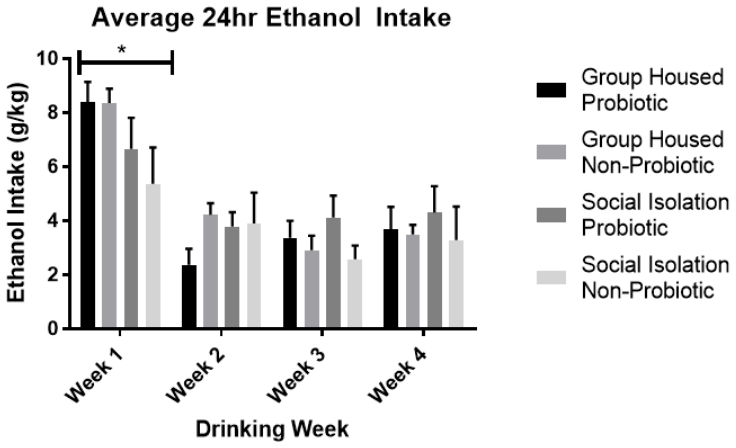

Figure 15: Avg. 24hr ethanol intake. Week 1 showed significantly higher EtOH intake across all groups in comparison to weeks 2, 3, and 4.

A three-way ANOVA was performed for 24-hour time point for ethanol preference per week. There were no interactions. There were no main effects (Figure 16).

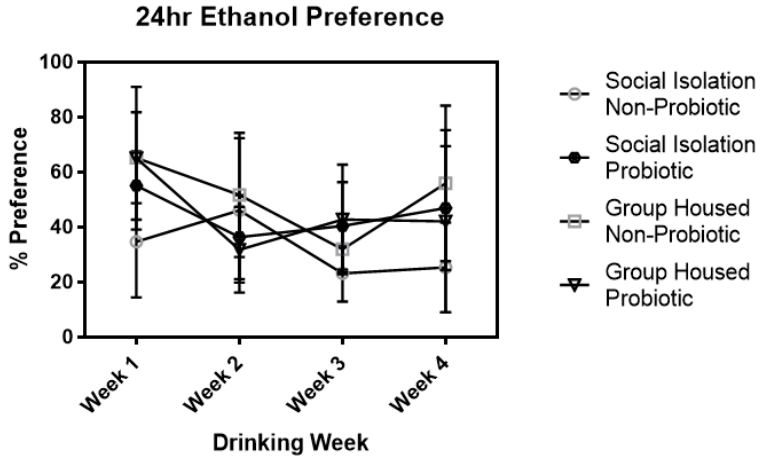

Figure 16: Ethanol preference for the 24 hour time point per week. The groups did not have a significant difference for the 24 hour time point ethanol preference.

Discussion

We set out to study the effects of adolescent stress in the form of social isolation and whether it preceded adult anxiety-like behavior and EtOH consumption and preference in male Long Evans rats. We used the probiotic, *L. rhamnosus*, in an attempt to protect against the adolescent stressor and prevent adult anxiety-like behavior. This study was a replication of a previous study conducted at the University of Dayton. We hypothesized that the probiotics would decrease anxiety-like behavior in SI rats. Additionally, it has been shown that SI rats consume and prefer EtOH more than GH rats do in the social isolation model (Butler et al., 2014). We hypothesized that this would be the same result that we would observe among our rats. However, the previous study found that rats that consumed the probiotic showed increased anxiety-like behavior

(Griff, 2018). This contradicted our original hypothesis. Thus, the current study was a replication and aimed to add to the findings our lab previously found.

We weighed the rats on each Monday of the study as a measure of their overall longitudinal health and to ensure that probiotic ingestion was not altering feeding behavior. There was an interaction for housing and diet though. After collapsing the data to observe the bodyweight gain during the housing and diet protocol (PND 21 to 104), we discovered that the main effect was due to the housing. The GH rats gained more weight than did the SI rats. This finding agrees with the literature that has demonstrated rats placed in social isolation to exhibit greater weight loss than rats that are group housed (Ness et al., 1995). However, we do not typically observe an effect of housing in the GH/SI model with Long Evans rats (Chappell et al., 2013). The probiotic did not affect the bodyweight gain of this cohort. In the previous study, the rats that consumed the probiotic weighed less than the non-probiotic group on PND 104. A study conducted to evaluate the effects of probiotics on obese Sprague-Dawley rats found that *Bifidobacterium longum* caused a weight reduction compared to controls (Karimi et al., 2017). More research should be conducted to fully understand how probiotics and housing affect bodyweight gain and loss.

This study implemented two measures of anxiety-like behavior, the EPM and L/D Box. Utilizing multiple measures of anxiety-like behavior provides a more thorough characterization of animal behavior. When measures of multiple tests support the presence or absence of anxiety-like behavior, the results are strengthened (Chappell et al., 2013). In the current study, neither test yielded significant results for anxiety-like behavior. Decreased time in the light box is an indicator of increased anxiety-like

behavior. There were no significant differences observed between the groups on the L/D Box. The previous study done in our lab did not observe a significant difference between the groups in the L/D Box either (Griff, 2018). Likewise, a previous study found that SI and GH rats did not show a difference in anxiety-like behavior in the L/D Box (McCool and Chappell, 2009). Another study found that the optimal LUX for the L/D Box is 30 LUX in comparison to 60 LUX (Slawecki, 2005). It has been found that with an increase in anxiogenic factors, rats show increased anxiety-like behavior (Martin, 1998). The LUX of the light is a type of anxiogenic factor as rats have been shown to avoid brightly lit areas (Slawecki, 2005). Slawecki found that with an increase in LUX the rats showed a decrease in time spent in the light box (Slawecki, 2005). In the current study we used 54 LUX during the L/D Box measure. One of the groups in Slawecki's study were restrained as a form of stressor prior to undergoing the L/D Box measure (Slawecki, 2005). These rats displayed an increase in anxiety-like behavior compared to the non-restrained rats (Slawecki, 2005). Therefore, even with a LUX close to 60, it is odd that our cohort did not display differences in terms of anxiety-like behavior on the L/D Box. Our lab should explore implementing a lower LUX when using the L/D Box in the future. Another study showed that Long Evans rats did not show a difference in anxiety-like behavior following the same GH/SI housing protocol implemented in the current study (McCool and Chappell, 2009). Perhaps Long Evans GH and SI groups are not different in terms of anxiety-like behavior on the L/D Box. Therefore, our lab should implement an alternative measure of anxiety-like behavior moving forward.

The second measure of anxiety-like behavior implemented in this study was the EPM. The EPM was by far the most common anxiety-like behavior measure utilized

throughout the literature (Roeckner et al., 2017). The EPM has been pharmacologically validated, demonstrating that the EPM was a reliable assay to examine anxiety-like behavior in rats (Pellow et al., 1985). In accordance with this literature, our lab has previously shown that the EPM has been a strong and consistent measure of anxiety-like behavior for SI rats (Butler et al., 2014). Increased open arm and central junction time has been shown to indicate an increased anxiety-like behavior in male Long Evans rats (McCool and Chappell, 2009; Chappell et al., 2013). However, though non-significant, we observed a trend for animals that consumed the probiotic to display increased anxiety-like behavior. This was the same trend that was observed in the previous study conducted in our lab. Previously, the probiotic groups were observed to have a significant increase in anxiety-like behavior when compared to the non-probiotic groups (Griff, 2018). This is contrary to other studies that have shown probiotics to have a protective effect against adolescent stress. The gut-brain axis provides a direct means of communication via the vagus nerve. Perhaps the increase in anxiety-like behavior can be attributed to the change in gut microflora causing anxiety-like behavior to manifest in the brain. Additionally, literature demonstrated that dimming the lights during an EPM test could encourage the rats to explore the maze (McCool and Chappell, 2009). In this study, we did not dim the lights in the room during the EPM tests. Our lab previously showed that the results were not altered if the lights were dimmed or not. Nonetheless, this could have been a factor that affected the results yielded from the EPM. On the other hand, since neither the L/D Box nor the EPM showed differences, perhaps probiotic/peanut butter administration altered anxiety-like behavior with this model.

Literature has shown a variability in health effects, both positive and negative, from probiotics. In a case study conducted on patients with infective endocarditis, it was determined that *L. rhamnosus* was the main bacteria present in the blood cultures (Boumis et al., 2018). The patients that were studied had previously been taking a probiotic supplement that included *L. rhamnosus* (Boumis et al., 2018). Another study involved a randomized group of healthy male humans that were given *L. rhamnosus* orally for eight weeks; however, at the conclusion of this study these individuals did not show a different score from placebo individuals on measures of acute stress (Kelly et al., 2017). A study conducted in adolescent Sprague-Dawley rats showed that the probiotic, *Lactobacillus casei* 54-2-33, did not show differences on the EPM in comparison to the control group (Barrera-Bugueño et al., 2017). The rats that were given *L. casei* 54-2-33 showed an increase in basal corticosterone in comparison to controls, possibly as a result of an inflammatory response in the gut produced by the probiotic (Barrera-Bugueño et al., 2017). Thus, more research should be done on the potential risks of taking a probiotic such as *L. rhamnosus*. Additionally, there have been multiple studies on the effects of individual probiotic strains, but very few studies comparing probiotics against each other (Suzuki et al., 2017). Comparing a multitude of probiotics would create a greater understanding of the microbiota in the gut and the benefits and risks associated with multiple probiotic strains.

Fecal samples were collected for later quantification of the *L. rhamnosus* content. Literature has demonstrated that humans who consume a probiotic exhibited a strain specific, significant increase in their fecal samples (Karjalainen et al., 2012). The presence of the administered probiotic increased by 2-3 log; whereas, the placebo group's

probiotic levels remained around the baseline for detection (Karjalainen et al., 2012). The increase in probiotic strain occurred only during the period of probiotic intervention, with levels returning to baseline after the probiotic administration concluded (Karjalainen et al., 2012). The previous study conducted at the University of Dayton did not find a significant difference between the probiotic and non-probiotic groups for *L. rhamnosus* content in the fecal sample (Griff, 2018). This shows that the probiotic group did not display an increase in *L. rhamnosus* following probiotic administration. This could be attributed to the *L. rhamnosus* colonizing the gut rather than being expelled in the fecal samples. Studies have shown that probiotics are capable of colonizing the gut, especially the colon (Schrezenmeir and de Vrese, 2001). Additionally, the time the fecal samples were collected was on Monday, which was 72 hours after the last probiotic administration the previous Friday (Griff, 2018). Therefore, the probiotic could have already been expelled prior to collecting the fecal sample (Griff, 2018).

We implemented the EtOH Paradigm in an effort to evaluate the behaviors of the rats following the stressor period that spanned adolescence. Increased EtOH consumption and preference has been shown to be correlated with increased anxiety-like behavior (McCool and Chappell, 2009). Studies that have previously used the SI/GH housing model have shown that SI rats consume significantly more EtOH than GH rats do. We assessed the effect of the housing and probiotic groups on the drinking measures of intake and preference through an EtOH paradigm. There are many forms of EtOH paradigms for rats. The previous study done at the University of Dayton implemented the two-bottle intermittent access with self-administration paradigm. Our lab has demonstrated an increase in SI EtOH consumption utilizing the two-bottle, intermittent access with self-

administration (Butler et al., 2014). This study did not show a significant difference between groups for the EtOH paradigm. There were no robust differences between the current and previous results found in our lab. Our results do not agree with previous literature that found a correlation between adolescent stress and EtOH preference and consumption in male Long Evans rats (Butler et al., 2014). This could suggest that the probiotic itself was a stressor parallel to the adolescence stressor of social isolation (Griff, 2018). Further research should evaluate the effect of probiotics on EtOH consumption and preference measures.

Conclusion

In conclusion, our lab showed that, though non-significant, there was a trend for animals consuming the probiotic to spend less time on the open arms of the EPM, which is indicative of anxiety-like behavior. This was a trend that was also demonstrated in previous Honors work conducted in our lab. The previous study found this trend with significant difference between the diet groups. In our other measure of anxiety-like behavior, the L/D Box, we did not find significant differences between our housing and diet groups. This mimics the previous study conducted in our lab. Additionally, we did not show significant differences for EtOH consumption or preference between our housing and diet groups. There were no robust differences observed between the current data or the data acquired in our lab previously. Moving forward our lab will need to quantify the *L. rhamnosus* DNA in the fecal samples. This will provide a more well-rounded understanding to our data. Additionally, the cecum and colon samples will need

to be analyzed as they will equip our lab with a better understanding of the effects *L. rhamnosus* had on the cohort. Lastly, the positive and negative effects of the various strains of probiotic should be further investigated. The recurring trend of an increased anxiety-like behavior in the probiotic groups suggests that *L. rhamnosus* may cause harm to male Long Evans rats. More research is necessary to further understand how probiotics effect an individual. This will increase our understanding of their use in humans.

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