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Parker Maddison Griff

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Department: Psychology and Biology

Advisor: Tracy Butler, Ph.D. and Yvonne Sun, Ph.D.

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## Abstract

Early life stress is a risk factor for later development of alcohol use disorders and anxiety disorders in humans. Using rodent experimental models, we know that rats experiencing social isolation as early-life stress exhibit greater anxiety-like behavior and alcohol consumption than rats housed in groups. Examining potential preventive strategies, we investigated the effects of probiotics, which have previously been shown to decrease rodent anxiety-like behavior, on the relationship between early-life stress and anxiety-like behavior in rats. We hypothesized that probiotics consumption would decrease anxiety-like behavior in socially isolated rats, as well as in rats housed in groups. To our surprise, we found that the probiotics had no significant effect on anxiety-like behavior for socially isolated rats but significantly increased anxiety-like behavior in rats housed in groups. Our results suggest probiotics do not have a positive benefit to alleviate consequences of early life stress and raise caution for their therapeutic use.

## Acknowledgements

I would like to thank Dr. Butler and Dr. Sun for their constant help and support. I would like to thank Caroline Lynch and Hanna Petersen for their help in the lab. I would also like to thank Amanda Schleper for continuing the research and replicating the study. Finally, thank you to the University Honors Program for the opportunity and funding to carry out this research.



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## Introduction

According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), 6.2% of Americans (15.1 million) aged 18 and older had an alcohol use disorder as of 2015. The NIAAA estimates that 88,000 people die from alcohol-related causes each year, making alcohol the third leading cause of preventable death in the United States (NIAAA, 2017). Studies have shown that stress, especially early life stress such as childhood maltreatment, is correlated with later increased incidence of alcohol use disorders (Keyes et al., 2011). In male Long Evans rats, early life stress can be modeled through social isolation, which can induce greater anxiety-like behaviors as well as increased alcohol (ethanol) intake and preference when compared to group housed animals (Butler et al., 2014).

Research on the gut-brain axis in both humans and rodents has highlighted the importance of gut functions to neurological health. For example, studies have shown that gastrointestinal (GI) microbiota and GI status are strongly correlated with autism severity, suggesting a relationship between the central nervous system and the gut (Adams et al., 2011). Additionally, it has been shown that reconstituting germ free (GF) mice with microbes at an early developmental stage reverses the exaggerated hypothalamic-pituitary-adrenal stress response exhibited by GF mice (Sudo et al., 2004). The importance of the gut microbiota has stimulated the emergence of probiotics as a way to maintain a healthy gut microbiome. Potential mechanisms by which probiotics may exhibit health benefits include displacement of pathogens, competition with hostile bacteria, inhibition of bacterial translocation, enhancement of mucosal barrier function, effects on intestinal sensory neurons as well as receptors in intestinal epithelial cells, and modulation of the immune system (Bravo et al., 2012).

Multiple studies have demonstrated anxiolytic effects of probiotics in rodents and humans. A probiotic formulation (PF) of *L. helveticus* and *B. longum* were found to have a similar anxiolytic effect as the drug diazepam, producing lower scores of anxiety in the defensive burying test compared to a control group in rats (Messaoudi et al., 2011). Administration of the probiotic *L. rhamnosus* was found to have anxiolytic effects in mice on the elevated plus maze, producing significantly more entries into the open arms

than control animals (Bravo et al., 2011). While one study revealed that *L. rhamnosus* was not more effective than a placebo in modifying stress-related measures in healthy male humans (Kelly et al., 2016), a different study reveals that treatment with a PF significantly reduced scores on multiple measure of anxiety in normal human volunteers (Messaoudi et al., 2011). This evidence suggests the relationship between the gut microbiome and stress/anxiety in both humans and rodents and that the health benefits of probiotics have not been conclusively established, suggesting that further investigations are needed.

The model used in the present study simulates early life stress in rodents through utilization of the importance of social interaction during adolescence. The model compares rats raised in groups of four (group housed, GH) to rats raised in social isolation (SI) for a 6-week period on behavioral tests for anxiety-like behavior as well as on the two-bottle choice intermittent access ethanol drinking paradigm. Behavioral tests used include the elevated plus maze (EPM) and the light/ dark (L/D) box. An EPM consists of 2 open arms and 2 closed arms with an open roof, and the closed arms opposite each other. Rats prefer the closed arms of the maze to the open arms, with greater open arm time indicating less anxiety-like behavior in the rat, while number of closed arm entries is a measure of general locomotion (Pellow et al., 1985). The L/D box consists of a 2-chambered box, with one light and one dark chamber, uncovered and covered, respectively. Rodents prefer to avoid brightly lit areas, so measures of anxiety-like behavior in this test include decreased time spent in the light box, while locomotor activity is measured by total distance moved (Slawecki, 2005). The EtOH two bottle choice intermittent access procedure was carried out to measure voluntary drinking in the rats. Studies have shown that intermittent access to 20% EtOH with a 2-bottle choice leads Long Evans rats to increase their EtOH intake as well as EtOH preference, with escalation in EtOH intake over time (Simms et al., 2008). In male Long Evans rats, isolated housing conditions during adolescence predispose rats for greater anxiety-like behaviors and increased EtOH intake and preference compared to animals housed in groups (Butler et al., 2014).

Probiotics were administered to the rats to investigate the relationship between the gut-brain axis and anxiety-like behavior. Probiotics have been shown to have anxiolytic effects without creating any adverse side effects in rodent models (Messaoudi et al., 2011). The probiotic *Lactobacillus rhamnosus*, procured from American Type Culture Collection, was administered by placing a small dose on a standardized amount of peanut butter. Nut spreads have been shown to be a reliable method of drug administration in rodents (Ingberg et al., 2012). Administration occurred during the 6-week GH/SI protocol, with fecal samples taken for quantitative PCR analysis in order to determine the abundance of *L. rhamnosus* in the fecal sample as a positive confirmation for the probiotic administration. Though adverse effects of probiotics were not anticipated, rat health was monitored by regular measurements of body weight, as well as food and water intake during probiotic administration.

The current study investigated the impact of probiotic administration on the relationship between early life stress and anxiety-like behavior. We assessed the relationship between probiotics administration and anxiety-like behavior using the light/dark box (L/D box) and the elevated plus maze (EPM). We also assessed the relationship between probiotics administration and EtOH intake/ preference. We hypothesized that anxiety-like and EtOH intake behavior would be lower in the GH groups than the socially isolated SI groups in keeping with other studies using the same model (Butler et al., 2014). We also hypothesized that anxiety-like behavior and EtOH intake would be lower in the SI probiotic (SIp) group than the SI group, as well as lower in the GH probiotic (GHp) group than the GH group.

## Methods

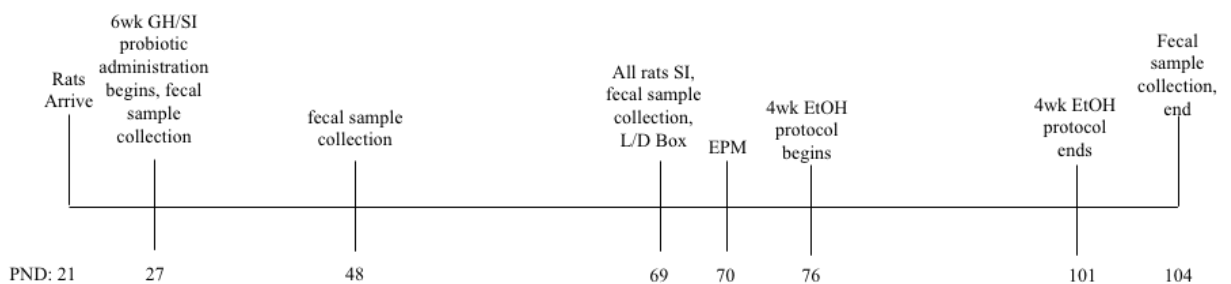
### Animals and Housing

Sixteen male Long-Evans rats (Envigo Laboratories, Indianapolis, IN) arrived immediately post-weaning on post-natal day 21 (PND 21) and were randomly placed into four groups (n=4): group-housed probiotic (GHp), group-housed (GH), socially isolated probiotic (SIp), and socially isolated (SI). They were put in group-housed (GH) cages (55cm x 38cm) with tail markings to differentiate each rat from their GH comrades. After

a 6-day adaptation period, the rats were weighed and placed in group-housed cages or socially isolated (SI) cages (2cm x 21cm; standard laboratory sized cages) based on group assignment (PND 27) for the first 6 weeks of the experiment. The rats had free access to food pellets and water. The lights were on a 12-hour light/ dark schedule (light 7:00-19:00). The experimental protocol was approved by the University of Dayton Institutional Animal Care and Use Committee. All procedures were in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

## Experimental Design

There were 6 weeks of probiotic feeding where the rats were in GH/ SI housing. After the housing protocol, all rats were placed in single housed cages (PND 69). The behavioral tests Light/Dark Box (PND 69) and Elevated Plus Maze (PND 70) were performed to test anxiety-like behavior over the course of 1 week. The rats were then kept in SI housing for the remaining 4 weeks of the experiment for the ethanol drinking paradigm.



**Figure 1- Experimental design.** GH, group-housed; SI, socially isolated; L/D Box, light/ dark box; EtOH, ethanol; PND, post-natal day.

## Probiotic Administration

Probiotics were prepared each day from 1 milliliter of *L. rhamnosus* (American Type Culture Collection) culture that was spun down and re-suspended in 100 microliters of MRS media. The rats receiving probiotics got peanut butter on a food pellet with 10 microliters of suspended *L. rhamnosus* pipetted onto it. The rats in the control groups received peanut butter on a food pellet without probiotics.

The rats were placed in separate, clean cages with the peanut butter on a food pellet for twenty minutes with the experimenter leaving the room. After twenty minutes,



the experimenter returned and determined if the peanut butter had been consumed or not and recorded the behaviors. The rats were then placed back in their normal cages.

Probiotic administration was conducted each day Monday through Friday for 6 weeks. A fresh culture was made each day Sunday through Thursday to be prepared the next day. A new plate was streaked every Friday to be used in making cultures for the following week.

### Fecal Sample Collection and Analysis

Fecal samples were collected on four Monday mornings: before probiotic administration (PND 27), after 3 weeks of administration (PND 48), after 6 weeks of administration (PND 69), and again at the end of the experiment (PND 104). The rats were placed in separate clean cages until samples were produced and collected, then rats were placed back in their individual cages. The fecal samples were stored in a -80°C freezer until prepared for analysis. A fecal sample DNA extraction kit (Fischer Scientific) was used to extract the DNA from the fecal samples. The DNA was then analyzed for *L. rhamnosus* content using qPCR analysis to test for presence of the probiotic in the GI tract.

### Light/ Dark Box

The Light/ Dark (LD) Box was done on PND 69 in the afternoon to test for anxiety-like behavior. The rats were brought into the procedure room 4 at a time to acclimate to the room conditions. Order was controlled for by having rat 1, 5, 9, 12 in the room first, and then the next rat from each group until all rats were tested. The lights in the room were turned off, with a lamp over the light box. The light intensity in the light box was measured to be 47 Lux. Each box was 50x50cm with an IR light placed on top of the dark box for detection of the rats with an overhead camera. The rats had free access to the light and dark boxes through an opening in the boxes. The rats were placed in the light box and monitored for 5 minutes with EthoVision software. Time in the light box was measured, with decreased time indicating greater anxiety-like behavior. Total distance moved was also measured as an indicator of general locomotion. After 5 minutes, the rats were removed from the L/D box and placed back in their cage. The L/D box was then cleaned with soap and ethanol before the next rat was placed in.

## Elevated Plus Maze

The EPM was done on PND 70 in the morning. The rats were brought into the procedure room and allowed ten minutes to acclimate before testing. Order was controlled for. Each arm of the EPM is 50cm, and the maze is elevated 75cm above the ground. The rats were placed one at a time in the middle of the maze and monitored for 5 minutes using EthoVision software to track movement. Open arm time was measured with greater open arm time indicating less anxiety-like behavior in the rat, while number of closed arm entries was measured as an indicator of general locomotion. After 5 minutes, the rats were placed back in their cage. The EPM was cleaned with soap and 70% ethanol before the next rat was placed on.

## Drinking Procedure

On PND 76 intermittent access, two-bottle choice home cage drinking paradigm began (Butler et al., 2014), during which all animals were single housed. The animals had recurrent access to two bottles in their home cage containing 20% EtOH and water on Mondays, Wednesdays, and Fridays. Consumption was measured after thirty minutes and twenty-four-hour access. An EtOH preference (EtOH consumed/ total fluid consumed) was calculated from each time point. Bottle position was alternated on each drinking day to control for side preference. This procedure was carried out for four weeks with rats weighed on each drinking day. The rats had free access to food pellets throughout the drinking procedure.

## Data Analysis

Two measures of anxiety-like behavior, the EPM and the L/D box, were analyzed using a repeated measures two-way ANOVA (housing x diet). For a comparison of fecal *L. rhamnosus* DNA content, differences in time points were computed for probiotic and non-probiotic rats as well as between housing groups across diet and compared using 1-tailed t-tests. The ethanol data were analyzed using a three-way ANOVA (housing x diet x time point).

## Results

## Body Weight

A three-way ANOVA was performed for the bodyweights. There was significant interaction [F (12, 156) = 569.8,  $p < 0.0001$ ] (Figure 2). We collapsed across housing groups and ran a two-way ANOVA and again found a significant interaction [F (12, 168) = 3.395,  $p = 0.002$ ]. Sidak's multiple comparisons post hoc test indicates a significant difference between rats receiving probiotics and rats not receiving probiotics on PND 104 ( $p < 0.05$ ), but not on any other day (Figure 3).

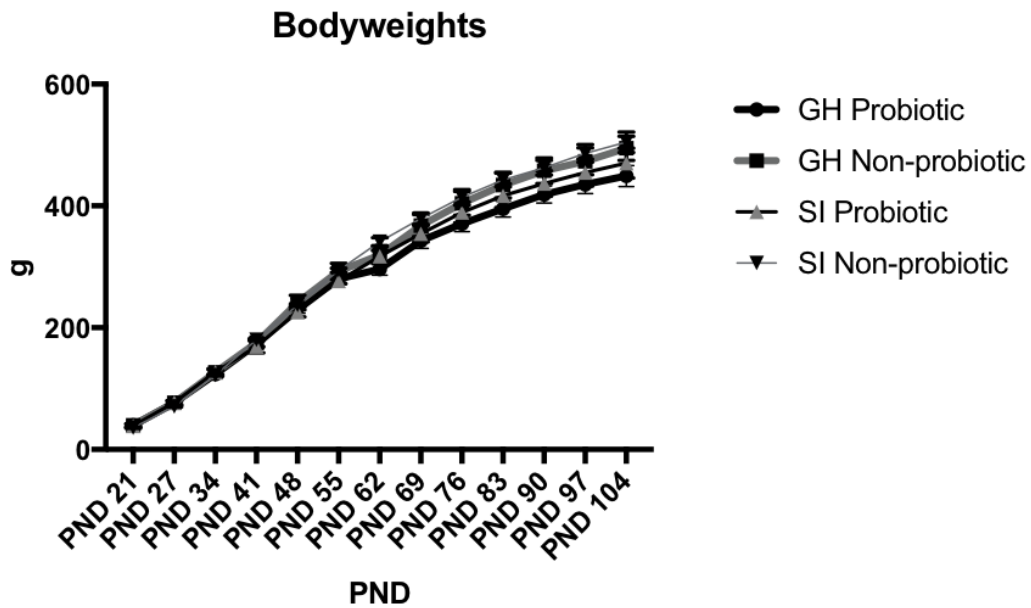
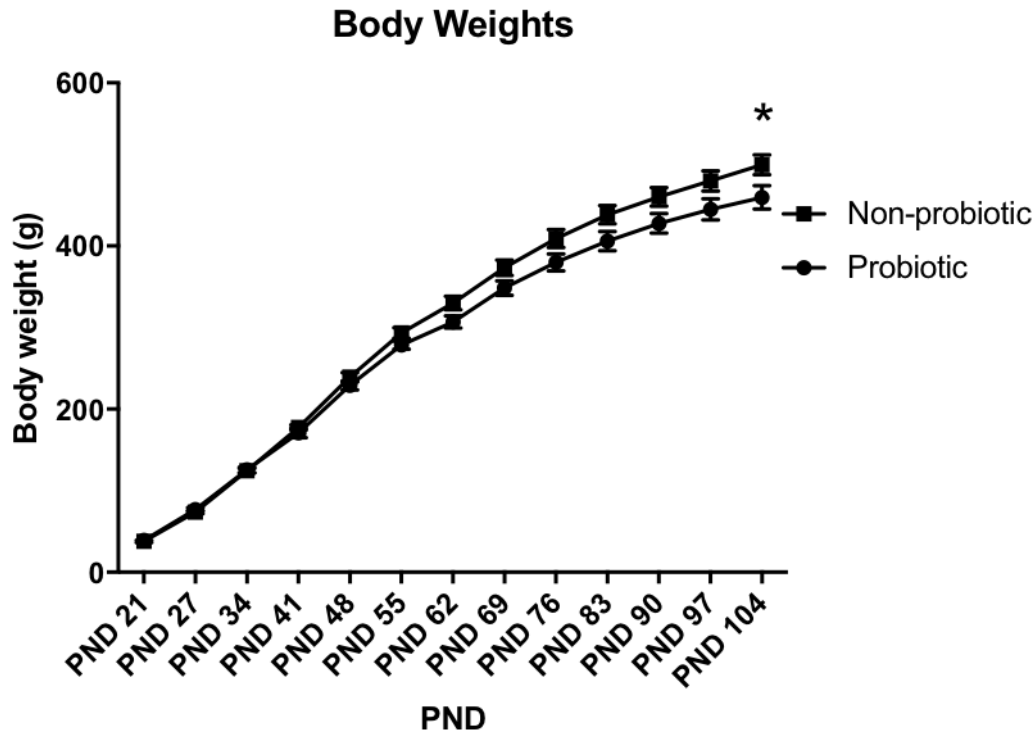


Figure 2- Bodyweights. There was an interaction for diet x housing x time.



**Figure 3- Bodyweights by diet group.** The probiotic rats weighed less than the non-probiotic rats on PND 104.

### Fecal Samples

One-tailed t-tests were performed to investigate *L. rhamnosus* DNA content in fecal samples for differences in time point across diet groups. The p-values as well as degrees of freedom (df) and the t-values are reported. No significant differences were found between the probiotic and non-probiotic groups (Table 1).

Weeks Being Compared	t(df) = t-value	p-value
3wk to before feeding	t(7.348) = 0.9775	0.1797
6wk to before feeding	t(11.97) = 0.2959	0.3862
5wk after feeding to before feeding	t(6.76) = 1.297	0.1215
3wk to 6wk	t(7.505) = 0.685	0.2570
3wk to 5wk after feeding	t(12.57) = 0.7177	0.2430
6wk to 5wk after feeding	t(5.072) = 1.162	0.1484

**Table 1- Fecal sample analysis.** T-tests were performed to analyze differences between the rats receiving probiotics and the rats not receiving probiotics. No significant differences were found. df, degrees of freedom.

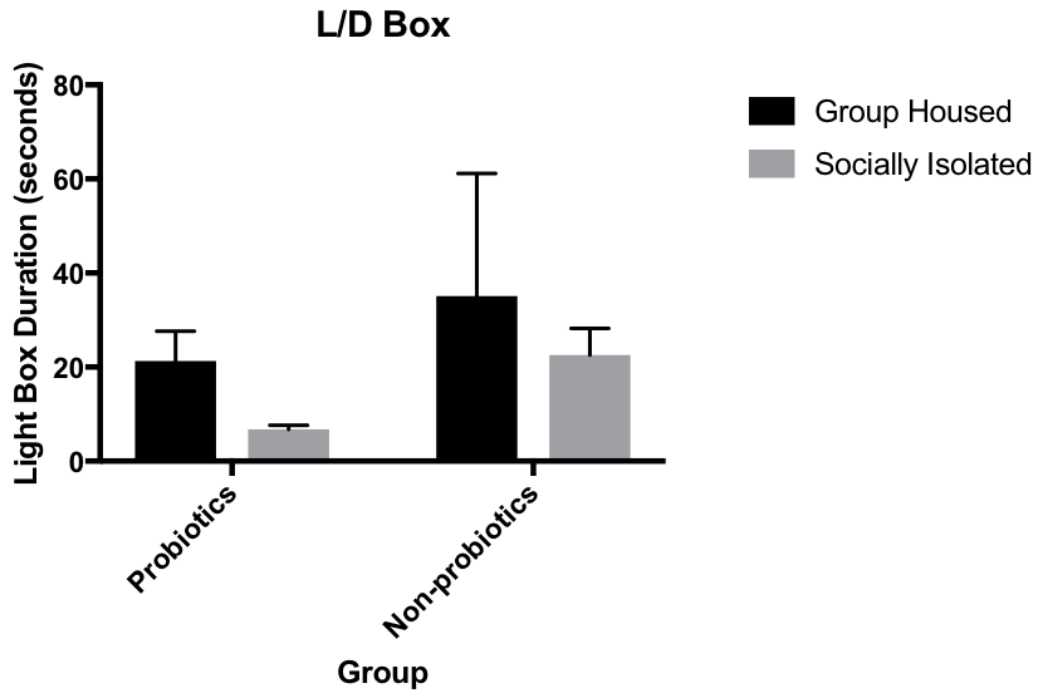
One-tailed t-tests were also performed to investigate *L. rhamnosus* DNA content in fecal samples for differences in time point across housing groups. No significant differences were found between the probiotic and non-probiotic groups (Table 2).

Weeks Being Compared	GHp vs SIp p-value	GH vs SI p-value
3wk to before feeding	0.1953	0.1996
6wk to before feeding	0.1642	0.2063
5wk after feeding to before feeding	0.0906	0.2478
3wk to 6wk	0.1360	0.0968
3wk to 5wk after feeding	0.0550	0.0993
6wk to 5wk after feeding	0.1732	0.1507

**Table 2- Fecal sample analysis across housing groups.** T-tests were performed to analyze differences between the GH and SI rats across diet groups. No significant differences were found.

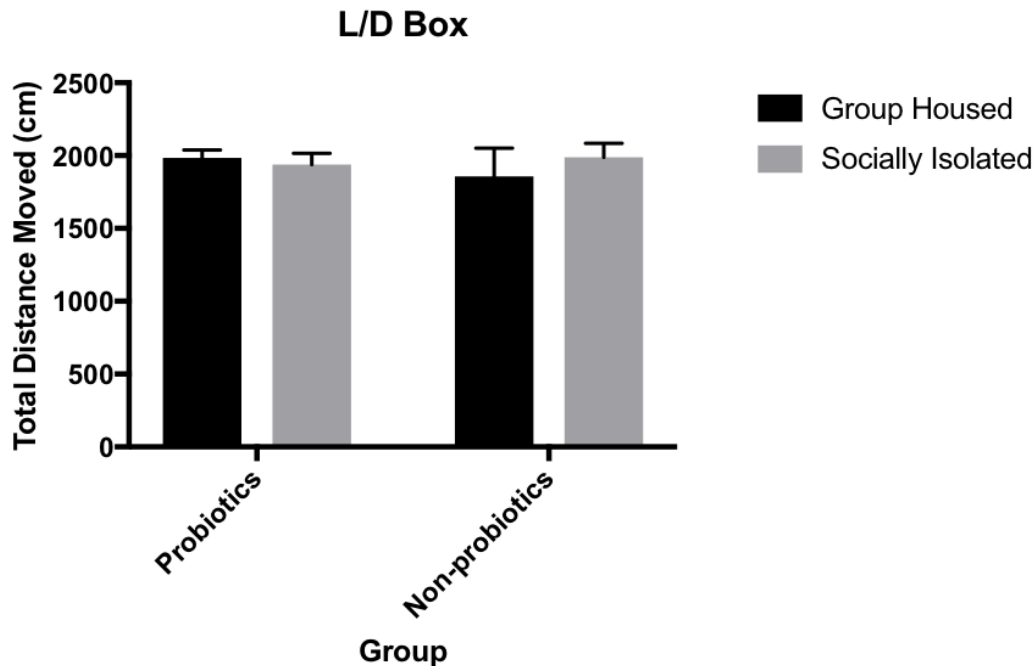
### Light Dark Box

Previous studies have shown decreased anxiety-like behavior for rodents receiving probiotics (Messaoudi et al., 2011). In this cohort, no significant results were found between groups for the L/D box test for anxiety-like behavior (Figure 4). A 2-way ANOVA was performed for light box duration. There was no significant diet x housing interaction, [F (1, 12) = 0.00537, p = 0.9428]. There was no main effect of diet, [F (1, 12) = 1.167, p = 0.3013], and no main effect of housing, [F (1, 12) = 0.9711, p = 0.3439].



**Figure 4- Light box time as a measure of anxiety-like behavior.** There were no significant differences between groups for light box time.

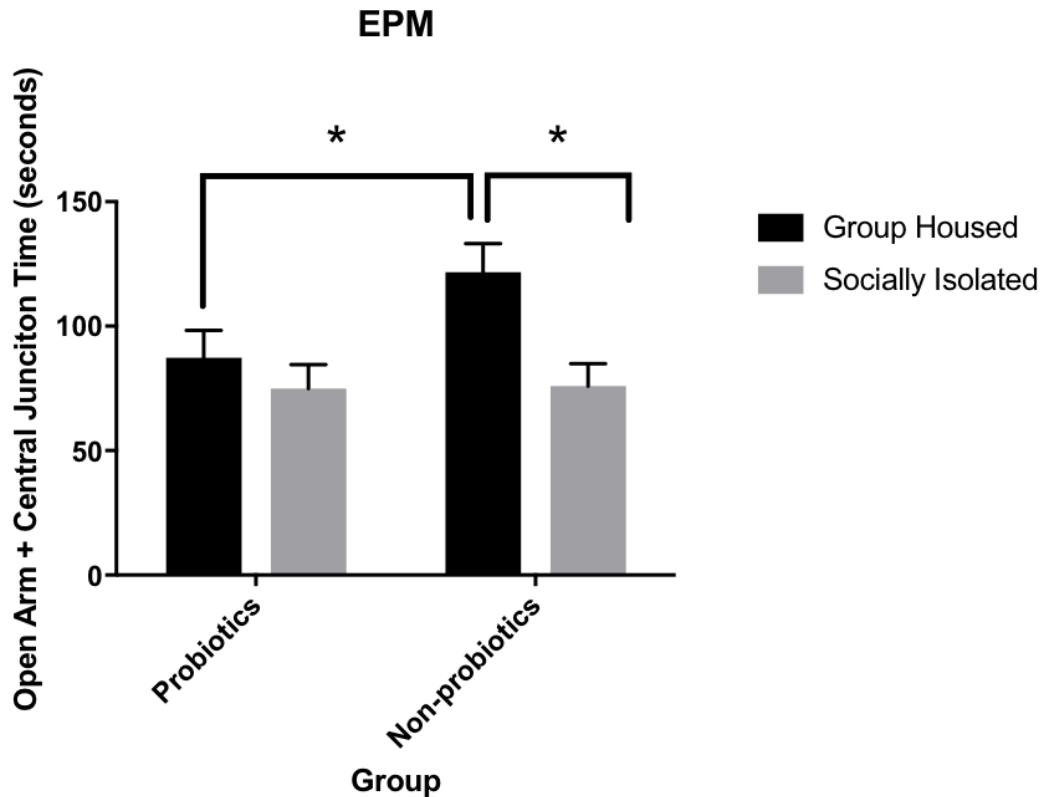
A two-way ANOVA was performed for total distance moved. There was no significant interaction of diet x housing, [F (1, 12) = 0.5553, p = 0.4705]. There was also no main effect of diet [F (1, 12) = 0.1017, p = 7.553], and no main effect of housing [F (1, 12) = 0.1339, p = 0.7207] (Figure 5).



**Figure 5-** Total distance moved as a measure of locomotor activity. There were no group differences in locomotor activity.

### Elevated Plus Maze

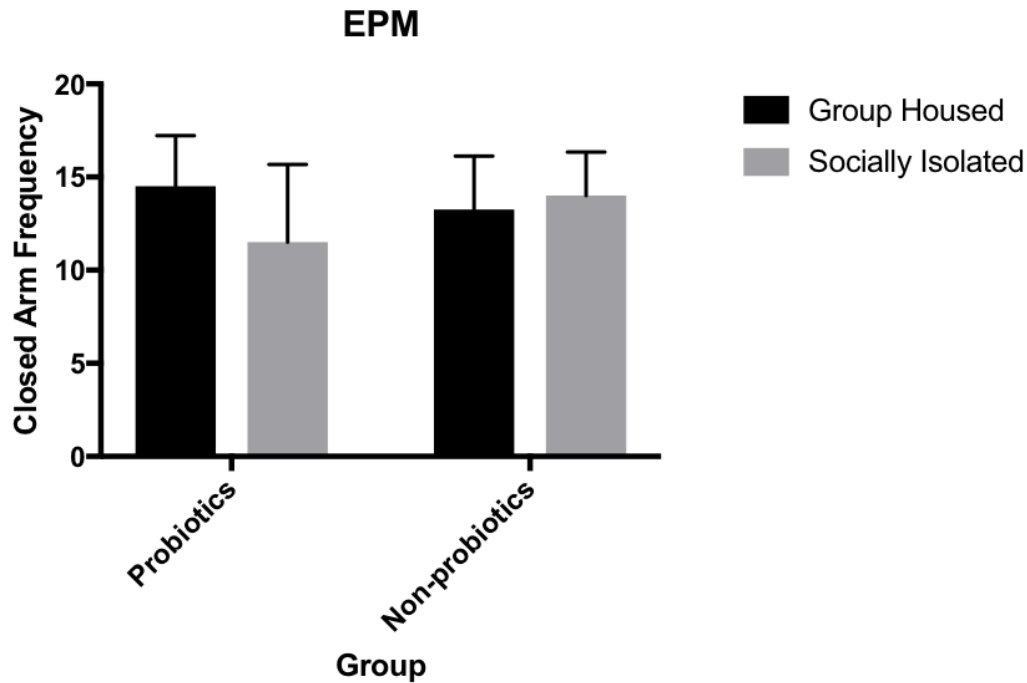
Previous experiments have shown that SI rats show significantly greater anxiety-like behavior than GH rats after the 6-week housing protocol (Butler, et al., 2014). A two-way ANOVA was performed for open arm + junction time. There was no significant diet x housing interaction, [F (1, 12) = 2.621,  $p = 0.1314$ ]. There was no main effect of diet, [F (1, 12) = 2.921,  $p = 0.1132$ ], but there was a main effect of housing [F (1, 12) = 7.929,  $p = 0.0156$ ]. Follow up post hoc uncorrected Fisher's LSD test based on a priori hypotheses indicate a significant difference between the GH and GHp groups ( $p = 0.0365$ ) and a significant difference between the GH and SI groups ( $p = 0.0086$ ) (Figure 6).



**Figure 6- EPM open arm + junction time as a measure of anxiety-like behavior.** SI rats showed significantly more anxiety-like behavior than GH rats. GHp rats showed significantly more anxiety-like behavior than GH rats.

In this cohort, there were no group differences in locomotor activity as indicated by the number of closed arm entries. A two-way ANOVA was performed for closed arm frequency. There was no significant diet x housing interaction, [F (1, 12) = 0.3647, p = 0.5572]. There was also no main effect of diet [F (1, 12) = 0.04052, p = 0.8438], and no main effect of housing [F (1, 12) = 0.1313, p = 0.7234] (Figure 7).





**Figure 7- Closed arm entries as a measure of locomotor activity.** There were no group differences in general locomotor activity

## Ethanol Paradigm

A three-way ANOVA was performed for the 30-minute ethanol intake per week. There were no interactions. There was a main effect of housing [ $F(1, 48) = 4.164, p = 0.0468$ ] so we collapsed the data and performed a two-way ANOVA to compare housing groups across weeks. This showed a main effect of drinking week [ $F(3, 42) = 3.343, p = 0.0280$ ]. Sidak's multiple comparisons post hoc test showed that housing groups were not significantly different on any week for drinking (Figure 8).

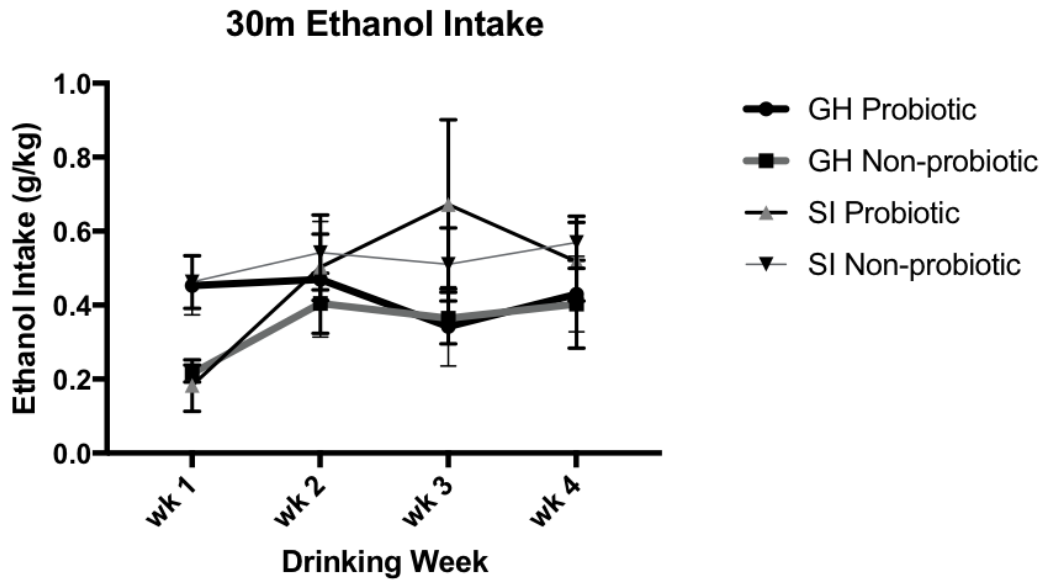


Figure 8- Ethanol intake for 30-minute time point. No significant differences between groups were observed.

A three-way ANOVA was performed for the 30-minute ethanol preference per week. There were no significant interactions or main effects (Figure 9).

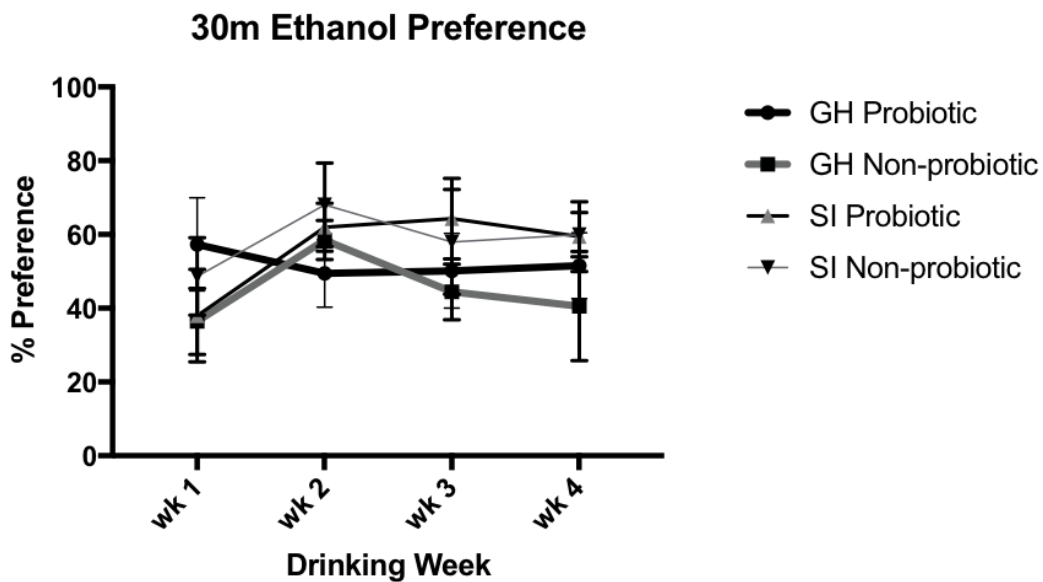
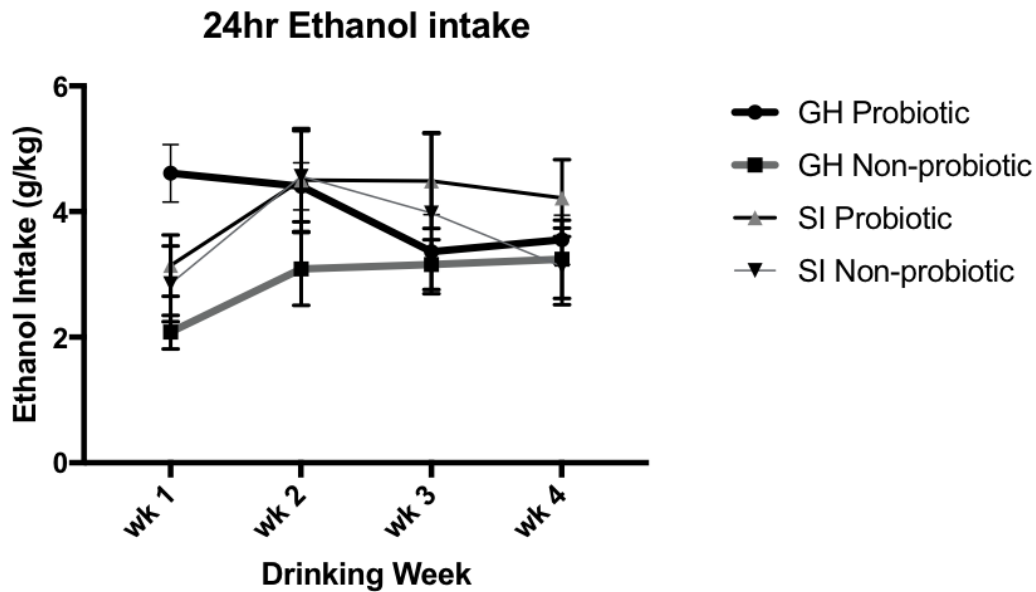


Figure 9- Ethanol preference for 30-minute time point. No significant differences between groups were observed.

Previous studies have shown that SI rats have significantly greater ethanol intake than GH rats (Butler et al., 2014). In the current study, no significant differences between housing groups were found for intake. A three-way ANOVA was performed for the 24-hour ethanol intake per week. There were no interactions. There was a main effect of diet

[F (1, 48) = 5.834,  $p = 0.0196$ ]. We consolidated the data and did a two-way ANOVA to compare diet groups across weeks. There was a main effect of diet [F (1, 56) = 5.739,  $p = 0.0200$ ]. Sidak's multiple comparisons post hoc test indicated that the diet groups were not different on any week for drinking. An unpaired t-test was performed to compare the average 24-hour ethanol intake for the probiotic and non-probiotic groups. There was no significant difference, [ $t(14) = 1.528$ ,  $p = 0.1488$ ] (Figure 10).



**Figure 10-** Ethanol intake for 24-hour time point. No significant differences between groups were observed.

Previous studies have shown that SI rats have significantly greater ethanol preference than GH rats (Butler et al., 2014). In the current study, no significant differences between housing groups were found for intake. A three-way ANOVA was performed for 24-hour ethanol preference per week. There were no interactions. There was a main effect of diet [F (1, 48) = 8.097,  $p = 0.0065$ ]. We consolidated the data and performed a two-way ANOVA to compare diet groups across weeks. There was still a main effect of diet [F (1, 56) = 8.476,  $p = 0.0052$ ]. Sidak's multiple comparisons post hoc test indicated that the diet groups were not different on any week for drinking (Figure 11).

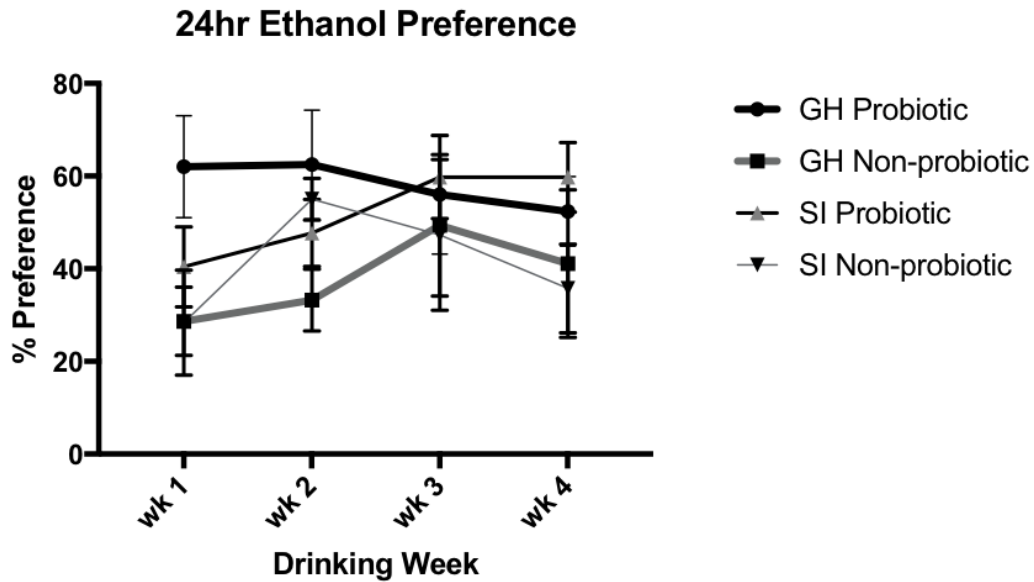


Figure 11- Ethanol preference for the 24-hour time point. A main effect of diet was observed.

A t-test was performed to compare the average 24-hour ethanol preference for the probiotic and non-probiotic groups. There was a significant difference between the groups [ $t(14) = 2.277$ ,  $p = 0.0390$ ], which indicates that the probiotic rats had a greater preference for ethanol than the non-probiotic rats (Figure 12).

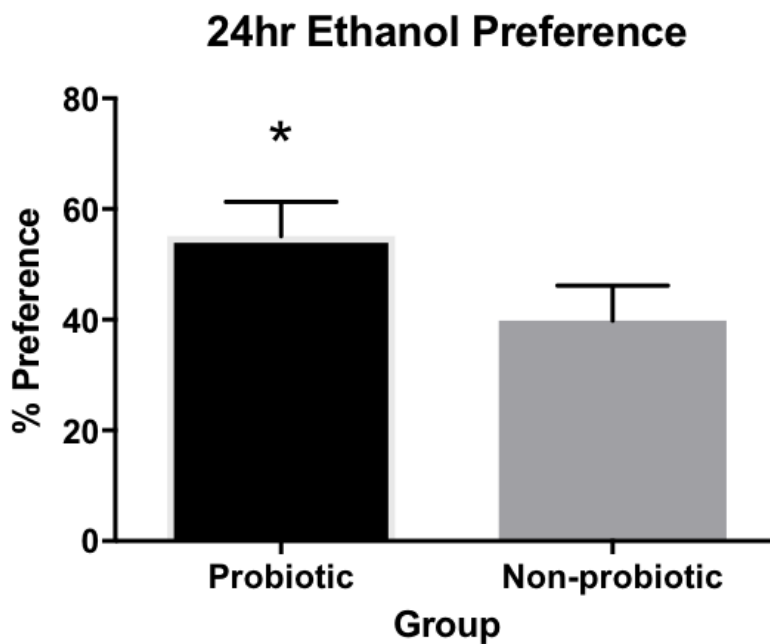


Figure 12- 24-hour ethanol preference for probiotic versus non-probiotic rats. Rats receiving probiotic had significantly greater preference for ethanol than the rats that did not receive probiotic

## Discussion

The current data suggest that probiotic administration results in increased anxiety-like behavior in GH rats and increased EtOH preference regardless of housing condition. Results on the EPM indicate that the control GH rats had significantly reduced anxiety-like behavior than the control SI rats, which indicated that the model effects were replicated. This study took the novel approach of investigating a potential preventative measure for the chronic stress phenotype seen in SI rats in this model. It is the second study that we know of to investigate prevention, specifically looking at the effects of probiotics. The current study found that the GHp rats had significantly greater anxiety-like behavior than the GH rats. These data suggest that the probiotic *L. rhamnosus* may have an anxiety-inducing effect in adolescent rats that typically show less anxiety-like behavior.

Anxiety-like behavior was assessed using the EPM. Previous studies using this model have found that in male Long-Evans rats, SI rats have significantly greater anxiety-like behavior than GH rats (Butler et al., 2014). This cohort replicated the model for increased anxiety-like behavior in SI rats following the early-life stress of social isolation, with the SI rats having significantly greater anxiety-like behavior than the GH rats.

Previous studies have shown variable outcomes with probiotics and anxiety-like behavior in rodents, with mice receiving probiotic *L. rhamnosus* having less anxiety-like behavior on the EPM than mice not receiving the probiotic (Bravo et al., 2011) and rats receiving a PF having reduced anxiety-like behavior than control rats on the defensive burying test (Messaoudi et al., 2011). Previous studies on probiotics with humans have also had variable outcomes, with healthy male humans receiving *L. rhamnosus* not having modified scores on stress related measures compared to the placebo group (Kelly et al., 2016), and normal human volunteers who were treated with a PF having significantly reduced scores on multiple measure of anxiety (Messaoudi et al., 2011). Despite the reports indicating decreased anxiety-like behavior in rodents receiving probiotics, this current cohort showed that GHp rats had significantly greater anxiety-like behavior on the EPM compared to the GH rats. This difference may be due to increased

intestinal inflammation in the probiotic rats, which could be a result of the differences in the microbiome of adults and adolescents, however it is not fully understood how *L. rhamnosus* worked to increase anxiety-like behavior in the current study.

It has been shown that *Lactobacillus* can completely displace *Clostridium*, a pathogenic gut bacterium that can cause gut inflammation that may be directly influencing the brain (Rao et al., 2009). The displacement of pathogens by probiotics is a potential explanation for their apparent anxiolytic effects in previous studies (Messaoudi et al., 2011). The findings of the present study suggest that probiotic administration had an anxiety-inducing effect in the group housed animals. This difference in findings could be due to the age at which rats were administered probiotics. One study has shown that in humans, the gut microbiome is different in adolescents than it is in adults with adolescents having a significantly higher abundance of *Bifidobacterium* and *Clostridium* than adults (Agans et al., 2011). A previous study showed anxiolytic effects of a PF in male rats who weighed 200g upon arrival (Messaoudi et al., 2011). The rats used in the present study were adolescents, arriving on PND 21. The differences in the gut microbiome at different developmental stages could be a reason that the findings of the current study differ from the findings of previous studies.

Anxiety-like behavior was also assessed using the L/D box. Previous studies using this model did not produce significantly different anxiety-like behaviors in the GH versus SI animals in the L/D box (McCool & Chappell, 2009). This cohort similarly lacked significant results for anxiety-like behavior on the L/D box measure. Previous studies have found reduced anxiety-like behavior on the EPM and defensive burying test with probiotic administration (Bravo et al., 2011; Messaoudi et al., 2011). In the current cohort, rats receiving probiotics showed a nonsignificant trend for increased anxiety-like behavior on the L/D box compared to the control animals in the same housing groups. This discrepancy may be due to age differences or a perhaps there is a potential inflammatory effect of *L. rhamnosus* in the intestines of the adolescent rats.

EtOH intake and preference were measured at the 30-minute time point and at 24 hours. Previous studies indicate that SI rats drink significantly more alcohol and have a significantly greater preference for ETOH than the GH rats (Butler et al., 2014). In the

current study, no significant differences between housing groups were found for intake or preference at either time point, but there was a nonsignificant trend for the SI rats to have greater EtOH intake and preference at the 30-minute time point compared to GH rats. The current cohort also showed that probiotic rats had significantly increased EtOH preference at the 24-hour time point than rats not given probiotics. Previous studies have not investigated the impact of probiotic administration on EtOH intake or preference. The current results indicate that probiotics could increase EtOH preference in male Long-Evans rats. Perhaps this increased preference is related to the increased anxiety-like behavior observed on the EPM for group-housed rats receiving probiotics. It is possible that the probiotics are inducing a stress response in the rats, thus causing them to later increase preference for the EtOH over rats not receiving the probiotics. Perhaps a different probiotic or a probiotic formulation would be more effective in preventing the chronic stress phenotype observed in SI rats in this model.

Fecal samples were taken to determine *L. rhamnosus* content using qPCR. A previous study has shown that in humans receiving probiotics, the treatment group had significant increases in total fecal *Bifidobacterium* and *Lactobacillus* compared to the placebo group, which had smaller increases in total fecal *Bifidobacterium* and *Lactobacillus* (Rao et al., 2009). In the current cohort, there were no significant changes in total fecal *Lactobacillus* for the probiotic or non-probiotic rats. This indicates that the fecal sample probiotic content was not increased following probiotic administration. This could indicate that the probiotics were colonizing the gut and were not being expelled in the feces. This result could also be due to the time between probiotic administration and fecal sample collection; fecal samples were taken on Monday mornings, which was 72 hours after the most recent probiotic administration on the previous Friday morning. Perhaps the probiotics had already been expelled in the feces previous to fecal collection.

There is increasing evidence to suggest an important link between the gut microbiome and the brain. Earlier studies have indicated that the probiotic *Lactobacillus rhamnosus* has an anxiolytic effect in mice (Bravo et al., 2011). GABA is the primary inhibitory neurotransmitter in the brain and is important in anxiety and depression. This study found that in mice receiving *L. rhamnosus*, there were changes in GABA<sub>B1b</sub> mRNA, with reductions in expression in the hippocampus, amygdala, and *locus*

*coeruleus* compared to control mice. This effect was not found in mice that were vagotomized, indicating the vagus nerve as a major part of the pathway between the gut and the brain (Bravo et al., 2011). Other studies indicate that chronic intestinal inflammation due to the presence of pathogenic bacteria can directly influence brain centers (Bercik et al., 2010). Probiotics such as *L. rhamnosus* and *B. longum* can displace pathogenic bacteria to reduce the intestinal inflammation, which can help to explain the anxiolytic effects of the probiotic seen in previous studies.

## Conclusions

One limitation of the current study is the small sample size of 16 rats with 4 rats in each experimental group. The study is currently being replicated in order to investigate if these effects can be recreated. Future directions for this line of questioning may include testing different types of probiotics and their effect on anxiety-like behavior in the current model. The aim of this project was to add to the body of knowledge about the gut-brain axis and to investigate the potential effects of probiotics on behavior. What we found was different than what the current literature predicts, with probiotics increasing anxiety-like behavior in the GH rats. The current results indicate a need for future studies to investigate the mechanism by which *L. rhamnosus* increases anxiety-like behavior in group-housed adolescent male rats. Further studies will also be needed to investigate how the gut microbiota influences the central nervous system to alter behavior and to determine the microbiome's mechanism of action on the brain.

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