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Modeling the Neurological and Physiological Effects of *Lactobacillus Rhamnosus* on Adult Mice

Zainab Suleiman
Department of Biology and Geology & The Interdisciplinary Neuroscience Program
Baldwin Wallace University
275 Eastland Road
Berea, Ohio 44017 USA

Faculty Advisor: Michael Kovach

Abstract

Probiotics are microorganisms that have beneficial properties for the host, and although probiotics have been used for generations to promote digestive health recent literature has suggested that these bacteria may provide more than just a localized effect at the level of the digestive track. Previous research demonstrated that mice consuming high doses of *Lactobacillus rhamnosus* had measurable differences compared to control mice in behavioral tests that measure stress and anxiety[6]. The previous studies did not identify what aspects of the microbial cell provided the positive, probiotic effect, so studies were initiated to determine whether this effect was due to direct interaction between the gut lining and the microbes or whether this effect was due to some factor produced by the microbes. To examine the probiotic effect a series of behavior tests that included an open field and forced swim test were performed on mice fed live *L. rhamnosus*, inactivated *L. rhamnosus*, cell lysates and *L. rhamnosus* conditioned media. Physiological factors including cortisol levels, fecal output, and mass of each mouse were collected. It was hypothesized that mice that were administered live strains of the bacteria would show a significant decrease in anxiety and depressive behaviors. A trend appears in the open field test where mice fed inactive bacteria are the least responsive in each component of the open field test with the lysate samples approaching the group that received no bacterial cells. The significance of this trend is unknown and suggests a molecule that is sensitive to heat and denaturation may play a role in the health benefits. The cortisol levels analyzed from fecal samples showed no significant differences between treatment groups with a negative trend shown in all groups, where the lysate treated group had elevated levels of cortisol. If probiotics or some component of probiotics can aid in decreasing stress-like disorders, it could augment the current chemotherapeutic options.

Keywords: Probiotics, Microbiome-Gut-Brain Axis, Behavior

1. Introduction

Probiotics are microorganisms that confer health benefits for the host when introduced via food or supplementation (in adequate amounts) and provide a resource for altering the composition of gut microbiota through the ingestion of live cultures[7]. Probiotics confer health benefits by preventing the colonization of pathogenic microorganisms by producing several antimicrobial peptides. Ng et al. demonstrated that the production of organic acids by probiotics lowered the intestinal pH which inhibited the growth of pathogenic microorganisms. Additionally, the human intestinal microbiota improves overall health by contributing to the maturation of the immune system as well as serving as a direct barrier against pathogen colonization[21]. Recent studies suggest that intestinal health may also be correlated with the brain and behavior, Bercik et al., suggested that alterations in the gut microbiome could in turn cause changes in the neurotransmitter function in the brain due to the bi-directional signaling associated with the gut-brain-axis. The underlying mechanism associated with probiotics and the brain is still under investigation. Garakani et al.,

demonstrated that 70-90% of patients with inflammatory bowel disease experience clinical depression and anxiety, and therefore the gut-brain axis has been used to describe the high co-morbidity between stress-like disorders and gastrointestinal disorders. The bidirectional signaling between the gastrointestinal tract and the brain is vital for maintaining homeostasis and is regulated at the neural hormonal and immunological levels[25].

The stress response system also termed the hypothalamic-pituitary-adrenal axis (HPA axis) is triggered by external stressors as well as alterations in the levels of cortisol in the bloodstream, this system is a negative feedback mechanism. Corticosterone is a glucocorticoid secreted by the adrenal gland, it is a major indicator of stress in non-human mammals[22]. Dysregulation of these systems leads to alterations in the stress response and overall behavior of the organism[25]. For example, germ-free mice have a boost in corticotrophin-releasing factor in the hypothalamus and adrenocorticotropic hormone in the anterior pituitary, which leads to elevated stress levels[8]. In the absence of certain microbes such as *Bifidobacterium* and *Bacillus*, a negative relationship between host and microbes is shown but when microbes are administered in adequate amounts (5×10^{11} colony forming units (CFU)/ gram of intestinal contents) recent findings show that gut microbiota influences mood and weight in the host in a beneficial symbiotic relationship[25]. In children diagnosed with autism spectrum disorders, Adams et al, found that these children had altered levels of gut flora compared to healthy children. Specifically, a decrease in species of *Bifidobacteria*, an increase in *Lactobacilli* and *Bacteroidetes*[1]. In studies utilizing germ-free rats, Desbonnet et al. demonstrated that when separated from the mother the rats possessed heightened levels of corticoptrophin-releasing hormone mRNA in the amygdala which is the area of the brain associated with stress and anxiety. The researchers also found that the germ-free rats displayed increased immobility during the forced swim test which is indicative of depressive-like behaviors[10]. The administration of a probiotic reversed the behavioral changes in the germ-free mice suggesting that the bacteria could mediate the stress effects[10].

Intestinal microbiota may mediate alterations in the central nervous system through the secretion of neuroactive molecules like neurotransmitters. Several bacterial genera found in the gut that have been shown to affect specific neurotransmitters including *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, and *Streptococcus*[16,17,28]. Interestingly, several molecules that are active in the brain and are affected by bacteria in the gut include gamma-aminobutyric acid (GABA), acetylcholine, dopamine, and serotonin[16,17,28]. The areas of the brain that are associated with behavioral changes include the striatum, amygdala, and hippocampus[8]. The microorganisms that are most commonly associated with probiotics is the genera *Lactobacillus*, which produces lactic acid through fermentation. This Gram-positive microorganism is commonly found in yogurt that advertises live cultures as active and is used to promote digestive health by re-establishing the composition of the intestinal flora[18]. *Lactobacillus rhamnosus* was found to cause changes to these molecules specifically by boosting GABA, which reduced the corticosterone levels in mice[6].

Collins et al., hypothesized that the mechanism by which *L. rhamnosus* causes this effect could be that signals between the gut and the brain move along the vagus nerve or are carried by chemical messengers. The vagus nerve is the longest cranial nerve that innervates major organs in the body, including the heart, lungs, pancreas, liver, stomach, and intestines and delivers information to the brain[12]. The vagus nerve is stimulated by both inhibitory and excitatory neurotransmitters. The sensory inputs from the vagus nerve first arrive in the nucleus of the solitary tract, which then get transmitted to extensive areas of the central nervous system[12]. Due to the range of neurotransmitters and the bacteria that could affect them, research into these interactions is critical. The mechanism by which *L. rhamnosus* stimulates the vagus nerve which, in turn sends signals to the brain is a key piece in understanding the interaction of the microbiome and the brain. One of the major areas of the brain associated with the stress response is the locus coeruleus[4]. Neurons of the rostral ventrolateral medulla oblongata (RVLM) provide one of the major sources of afferent inputs to the locus coeruleus, which in turn projects to areas of the cortex that are associated with stress-related behavior and affective disorders[4]. Repeated and chronic activation of the system between noradrenergic locus coeruleus neurons and areas of the forebrain that produce corticotropin-releasing factor (CRF) can lead to altered behavioral responses by causing changes in neuronal activity associated with anxiety, panic disorders, and depression[29]. The neurotransmitters associated with anxiety include GABA, serotonin, dopamine, and epinephrine, while the neurotransmitters associated with depression include serotonin, norepinephrine, and dopamine[11].

This role in communicating signals to the brain including areas associated with behavior could give insight into how gut bacteria and the central nervous system interact. For example, vagus nerve stimulation has been approved by the Food and Drug Administration (FDA) to treat intractable depression as well as epilepsy, which support the key role of the vagus nerve in behavior[27].

Probiotics are commonly prescribed and used to aid in digestive health, but recent literature has suggested that these bacteria could also change the way behavioral disorders are treated[6]. The literature has demonstrated that ingesting live *Lactobacillus rhamnosus* over a period of 28 continuous days alters anxiety, depression, and stress levels in adult mice[6]. The aim of this experiment is to determine if the documented behavioral changes are solely due to the presence

of the bacteria or due to some soluble factor synthesized by the bacteria. It is hypothesized that the mice that ingest live bacteria and conditioned media will show decreased signs of the stress like behaviors.

2. Materials and Methods

2.1. Bacterial Preparation

Lactobacillus rhamnosus strains were isolated from yogurt or purchased from the American Type Culture Collection (ATCC™ 7469). The bacteria were grown at 37°C with a 5% CO_2 enriched environment in Man-Rogosa-Sharpe liquid media (MRS broth; Difco Laboratories). Each treatment group received a mixture of both ATCC and BW isolated strains.

2.1.1. lysed bacteria

Lysed BW strain bacteria were prepared by inoculating five flasks of the BW strain in 200 mL MRS broth overnight (O/N) and transferring them to four sterile 500 mL centrifuge bottles. Once transferred the bacteria were centrifuged at 8,000 rpm (10,808 xg) for 30 minutes using the Sorvall SLA 3000 rotor. The supernatant was saved to be used for the conditioned media samples. The pellets were then washed one time with phosphate buffered saline (PBS) and resuspended in 20 milliliters (mL) PBS. Once resuspended and the bacteria was frozen at -80C. The total volume of cells was 80 mL. A sample of the prepared bacteria was plated on MRS agar at 37°C with 5% CO_2 O/N to determine the starting concentration, 1.65 x 10^{10} CFU/mL of bacteria before being lysed. The samples were lysed via French pressure in three treatments at 20,000 psi. The concentration after being lysed by French pressure was 5.36 X 10^8 CFU/mL. The samples were then lysed using a sonicator and spun at 13,000 rpm (27,247 xg) for 10 minutes which gave a final concentration of 9.00 X 10^6 CFU/mL.

Lysed ATCC strain bacteria were prepared by inoculating five flasks of the ATCC O/N and transferring them to 500 mL centrifuge bottles that were spun at 8,000 rpm (10,808 xg) for one hour using the SLA 3000 rotor. The supernatant was saved to be used for the conditioned media samples. The pellets were resuspended in 20 mL of PBS and centrifuged at 13,000 rpm (27,247 xg) for one hour. Once pelleted the supernatant was discarded and cells were stored at -20°C until being transferred to -80°C. The total volume of cells was 96 mL. A sample of the prepared bacteria was plated on MRS agar at 37°C with 5% CO_2 O/N to determine the starting concentration, 1.85 x 10^{10} CFU/mL of bacteria. The samples lysed via French pressure in three treatments at 20,000 psi. The concentration after being lysed was 3.73 X 10^7 CFU/mL. The samples were then lysed using a sonicator and spun at 13,000 rpm (27,247 xg) for 10 minutes. The final concentration of bacterial cells in the supernatant was less than 10 CFU/mL.

2.1.2. live bacteria

Live bacteria were prepared by inoculating five flasks of the BW strain O/N then transferred to 500 mL centrifuge bottles. The bacteria were centrifuged at 8,000 rpm (10,808 xg) for 30 minutes using the SLA 3000 rotor, once spun the supernatant was discarded and pellets were resuspended in 20 mL PBS. Suspensions were transferred to one sterile 250 mL centrifuge bottle and spun at 12,000 rpm (23,216 xg) for 30 minutes using the SLA 1500 rotor. Once centrifuged the pellet was resuspended in 25 mL PBS. The suspension was aliquoted into microcentrifuge tubes. A sample of prepared live bacteria was plated on MRS agar at 37°C with 5% CO_2 to determine the final concentration, 5.01 x 10^{10} CFU/mL of bacteria.

Live ATCC bacteria were prepared by inoculating five flasks of the ATCC strain O/N then transferred to sterile 500 mL centrifuge bottles and centrifuged at 8,000 rpm (10,808 xg) for 30 minutes. The pellets were then transferred to 250 mL centrifuge bottle and centrifuged at 13,000 rpm (27,247 xg) for 30 minutes. The supernatant was discarded and the pellet was centrifuged at 13,000 rpm (27,247 xg) for one hour. The supernatant was discarded and the pellet was aliquoted into microcentrifuge tubes. The samples were stored in -20°C until transferred to -80°C. A sample was plated in order to determine the concentration of live ATCC bacteria O/N in 37°C with 5% CO_2. The final concentration of live ATCC bacteria was 1.76 x 10^{10} CFU/mL.

2.1.3. conditioned media

The supernatant that was saved from the BW strain bacteria in the lysate preparation stage was transferred to a sterile 250 mL centrifuge bottle and spun at 12,000 rpm (23,216 xg) for 30 minutes using the SLA 1500 rotor. Once centrifuged the supernatant was transferred to 25 mL centrifuge bottles and spun at 13,000 (27,247 xg) rpm for 30 minutes. The sample was aliquoted into microcentrifuge tubes and stored at -80°C. A sample was taken to determine the concentration of bacteria in the conditioned media and was plated on MRS agar O/N in 37°C with 5% CO_2. The concentration of bacteria in the conditioned media was 5.00 x 10^3 CFU/mL.

The supernatant that was saved from the ATCC bacteria in the lysate preparation stage was transferred to two sterile 250 mL centrifuge bottles and spun at 13,000 rpm (27,247 xg) for 30 minutes using the SLA 1500 rotor. The supernatant was then aliquoted into microcentrifuge tubes and stored at -80°C. A sample was taken in order to determine the concentration of bacterial cells in the conditioned media and was plated on MRS agar in 37°C with 5% CO_2. The concentration of ATCC bacteria in the conditioned media was 4.33 x 10^3 CFU/mL.

2.1.4. inactive bacteria

Inactive bacterial samples were prepared using both ethanol and heat. The BW strain was inoculated O/N and transferred to 500 mL centrifuge bottles. The bacteria were spun at 8,000 rpm (10,808 xg) for 30 minutes using the SLA 3000 rotor. Once centrifuged the pellets were resuspended in 20 mL PBS and transferred to a sterile 250 mL centrifuge bottle. The suspension was centrifuged at 12,000 rpm (23,216 xg) for 30 minutes using the SLA 1500 rotor. Once pelleted the supernatant was discarded and the pellet was resuspended in 20 mL PBS. The suspension was then placed in 65°C water bath for 90 minutes to inactivate the bacterial cells. A sample of the suspension before heat inactivation was plated and the concentration was 1.52 x 10^{10} CFU/mL. After heat inactivation, the concentration of heat inactivated bacterial cells was less than 10 CFU/mL. The heat-inactivated cells were aliquoted into microcentrifuge tubes in -20°C until being transferred to -80°C. The ethanol inactivated BW cells were prepared by inoculating five flasks of 200 mL MRS broth with the BW strain in 37°C with 5% CO_2 O/N. The bacteria were transferred to four sterile 500 mL centrifuge bottles and spun at 8,000 rpm (10,808 xg) for 30 minutes using the SLA 3000 rotor. The pellets were resuspended in 20 mL PBS then transferred to sterile 250 mL centrifuge bottles and spun at 13,000 (27,247 xg) rpm for 30 minutes using the SLA 1500 rotor. The pellet was then resuspended in 18 mL PBS, 42 mL 100% ethanol was added after resuspension to achieve a final concentration of 70% ethanol. The culture remained in 70% ethanol for 60 minutes at room temperature. The ethanol was then removed from the culture by spinning the sample at 13,000 rpm (27,247 xg) for 30 minutes using the SLA 1500 rotor. The pellet was resuspended in 60 mL PBS after discarding the supernatant then centrifuged at 13,000 rpm (27,247 xg). The pellet was resuspended in 60 mL PBS and transferred to two 50 mL conical tubes. The cells were then spun for 30 minutes at 10,050 rpm (16,575 xg) using the SLA 600 TC rotor. A sample of bacteria was taken before ethanol inactivation and the concentration of bacteria was 4.65 x 10^{10} CFU/mL. The concentration after ethanol inactivation was less than 10 CFU/mL.

The ATCC strain was inoculated in five flasks of 200 mL MRS broth O/N and transferred to four sterile 500 mL centrifuge bottles. The bacteria were spun at 8,000 rpm (10,808 xg) for 30 minutes using the SLA 3000 rotor. Once centrifuged the pellets were transferred to a sterile 250 mL centrifuge bottle. The bacteria were centrifuged at 13,000 rpm (27,247 xg) for 30 minutes using the SLA 1500 rotor. Once pelleted the supernatant was discarded and the pellet was then placed in 65°C water bath for 90 minutes in order to inactivate the bacterial cells. A sample of the suspension before heat inactivation was plated and the concentration was 9.5 x 10^9 CFU/mL. After heat inactivation, the concentration of heat inactivated bacterial cells was less than 10 CFU/mL. The heat-inactivated cells were aliquoted into microcentrifuge tubes and stored at -80°C. The ethanol inactivated ATCC cells were prepared by inoculating five flasks of 200 mL MRS broth with the ATCC strain in 37°C with 5% CO_2 O/N. The bacteria were transferred to sterile 500 mL centrifuge bottles and spun at 9,000 rpm (13,679 xg) for 30 minutes using the SLA 3000 rotor. The pellets were transferred to sterile 250 mL centrifuge bottles and spun at 13,000 rpm (27,247 xg) for 30 minutes using the SLA 1500 rotor. The pellet did not form and was transferred to 25 mL centrifuge tubes and centrifuged at 15,000 rpm (31,465 xg) for 30 minutes. The pellet was first resuspended in 65 mL PBS then 280 mL of 100% ethanol was added in order to achieve a final concentration of 70% ethanol. The culture remained in 70% ethanol for 80 minutes at room temperature. The sample was washed with PBS in order to remove any trace of ethanol. The cells were aliquoted into microcentrifuge tubes. The bacterial sample that was taken before ethanol inactivation had a concentration of 6.15 x 10^{10} CFU/mL. The concentration after ethanol inactivation was less than 10 CFU/mL.

2.2. Animals

Adult female BALB/c mice (n = 50) were obtained from Harlan, a cage of six mice expired before initial testing began. All of the animals were allowed to acclimate for about one month in the housing facility before the experiment. Animals were group housed with five animals per cage except in the control group which had 4 mice in one cage and in the inactive group which had six animals in one cage. Animals were kept under standard conditions (room temperature of 21 °C, with a 12-h light–dark cycle, lights on at 06:00) with access to regular chow and water. Health checks were recorded daily. Mice were of comparable weight (19–20 g) and age (19 wks.) at the end of the experiment. All experimental procedures were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Baldwin Wallace University (CRF SU-14-01).

2.3. Treatment

Animals were randomly assigned to treatment groups and were treated with 0.1 mL of a sample according to the assigned treatment group by oral gavage over a 37-day period leading up to testing (broth control group n=4, lysate group n=10, conditioned group n=10, inactive group n=10, live group n=10). Oral gavage was carried out daily during the 37-day period. Toward the end of the treatment period mice underwent a forced swim test and an open field test in order to examine the anxiety and depressive behaviors of the mice. The behavioral tests took place two days apart. Fecal samples and mass of each mouse were collected on an individual basis in order to determine stress-induced corticosterone levels throughout the entire study.

2.4. Behavioral Analysis

Behavioral data was collected and analyzed using EthoVision XT-Noldus and Prism 6.0.

2.4.1. forced swim test

The forced swim test (FST) measures simple depression-like behaviors in rodents[20]. Mice were placed in a glass cylinder (50 x 20 cm) that contained 15 centimeters of room temperature (25-30°C) water. The sides were blocked to prevent the animals from seeing movements outside of the cylinder. The camera was aimed at the surface of the water in the cylinder as well as below the surface of the water. Mice were allowed to acclimate for two minutes, the mice were scored on mobility for **4** minutes. A mouse was judged to be immobile when making only those movements necessary to keep its head above water[20], the data was collected using EthoVision XT-Noldus software.

2.4.2. open field test

The open field test is a measure of anxiety and exploratory behavior[26]. Red lights were placed behind the enclosure to see the mice on the video used to analyze the selected variables. The white sound was played during the testing period and black foam boards were placed around the enclosure to ensure mice received no cues from the environment. A curtain separated the researcher from the mice being tested, which also protected against outside cues for the mice. A black foam board was placed under and around the enclosure to allow the camera to track the mice. Yellow tape was used to create each quadrant, which also aided in proper analysis of all variables. Mice were placed in the center zone (10 x 10 cm) individually in a 40.5 x 20.5 clear enclosure, the 10 minutes began when the mouse was placed in the center. After each animal was tested the arena was cleaned with 70% ethanol. The distance traveled, speed, mobility time and time spent in the center zone were measured using EthoVision XT-Noldus software.

2.5. corticosterone extraction

Corticosterone was extracted from fecal pellets using the extraction protocol described by Arbor Assays[22]. The fecal pellets were homogenized in ethyl acetate and the supernatant was dried using nitrogen and resuspended in 100% ethanol following the manufacturers recommendations. Samples were tested using a corticosterone solid enzyme immunoassay kit in which samples were placed in wells on the assay[22]. Nonspecific and maximum binding wells were used to normalize the data and corticosterone conjugates and antibodies were added to every well. To determine the corticosterone concentration samples were read for absorbance at 450 nm.

3. Results

Statistical analysis was performed using a one-way ANOVA[19] and Post hoc analysis was completed using a Student's t-test[23].

3.1. Forced Swim Test (FST)

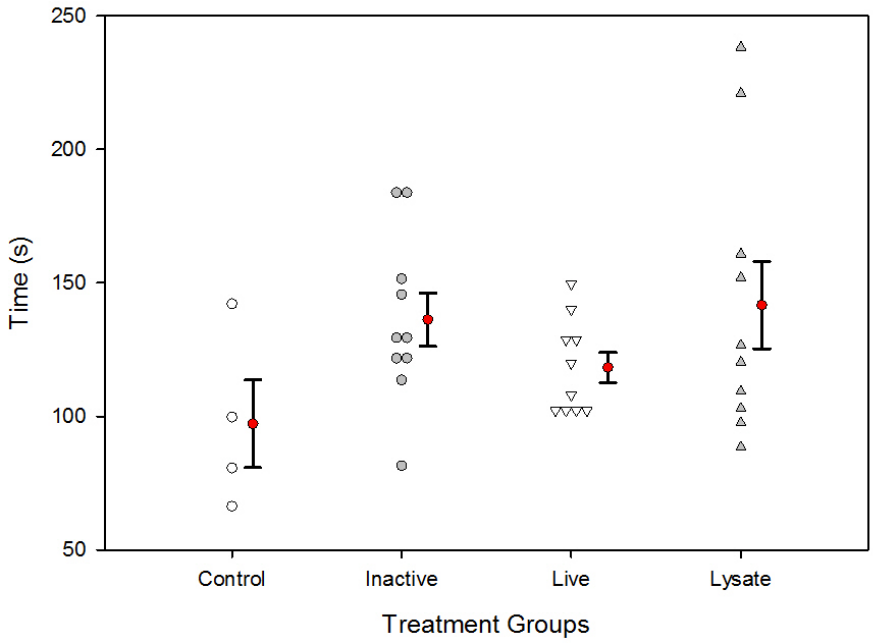

Figure 1. The time spent swimming for each mouse per treatment group in the forced swim test (FST) (n=34).

The mean and standard error of measurement are shown as a filled circle with error bars. Comparisons between the treatment groups showed no significant differences between the subjects, determined by one-way ANOVA ($F(3,30)=1.87$, $p=0.156$). The conditioned group (n=10) data was not available for analysis due to operator error resulting in the loss of data.

3.2. Open Field Test (OFT)

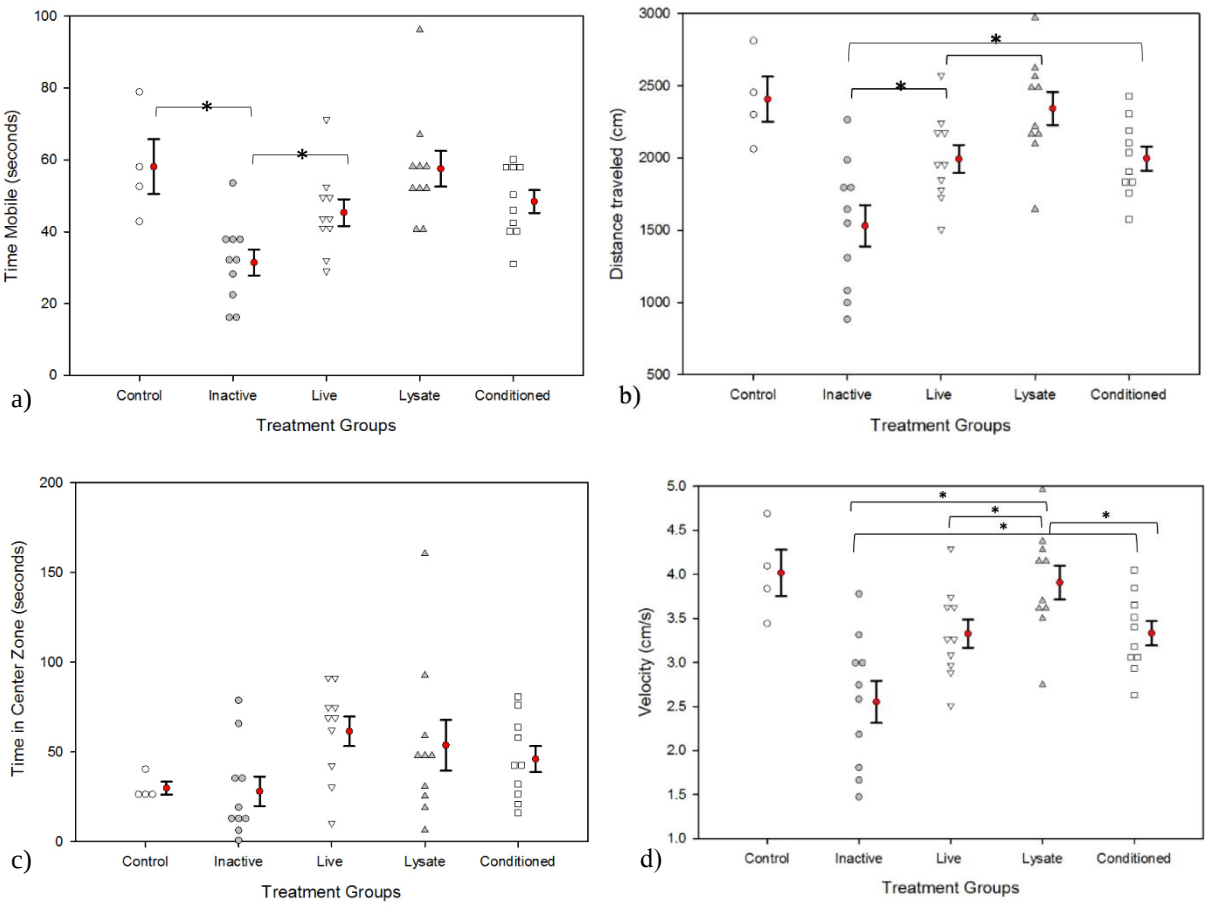

Figure 2. The effect of altered *L. rhamnosus* on anxiety as measured in the open field test.

(a) The time mobile for each mouse per treatment group in the open field test. The mean and standard error of measurement are shown as a filled circle with error bars. The animals treated with the lysate samples (n=10) showed mobility time approaching the animals fed control media. The animals treated with the inactive samples (n=10) spent less time mobile than all other groups. Comparisons between the treatment groups showed significant differences determined by a one-way ANOVA ($F(4,39)=6.333$, $p<0.001$). Post hoc analysis showed significant differences between control (n=4) and inactive (n=10) (p=0.004), and live (n=10) and inactive (n=10) (p=0.015) which are marked by an asterisk. (b) The distance traveled for each mouse per treatment group in the open field test. The mean and standard error of measurement are shown as a filled circle with error bars. The animals treated with the lysate samples (n=10) approached the control animals (n=4) in distance traveled. The animals treated with inactive cells (n=10) traveled less than all other groups. Comparisons between the treatment groups showed significant differences ($F(4,39)=8.195$, $p<0.001$). Post hoc analysis showed significant differences between the live and lysate (p=0.03), live and inactive (p=0.01), and conditioned and inactive (p=0.01). (c) The time spent in the center zone for each mouse per treatment group in the open field test. The mean and standard error of measurement are shown as a filled circle with error bars. The animals treated with live cells (n=10) spent more time than in the center zone than all other groups. The inactive group (n=10) spent less time in the center zone than all other groups. Comparisons between the treatment groups did not show significant differences as determined by a one-way ANOVA ($F(4,39)=2.029$, $p=0.109$). (d) The velocity for each mouse per treatment group in the open field test. The mean and standard error of measurement are

shown as a filled circle with error bars. The animals treated with the lysate samples (n=10) traveled faster in the open field arena than all other groups. The animals treated with inactive cells (n=10) traveled slower than all other groups. Comparisons between the treatment groups showed significant differences ($F(4,39)=8.196$, $p<0.001$). Post hoc analysis showed significant differences between, control and live (p=0.04), control and inactive (p=0.04), control and conditioned (p=0.04), inactive and conditioned (p=0.03), lysate and conditioned (p=0.03), live and lysate (p=0.01), and lysate and inactive (p=0.003).

3.3. cortisol extraction

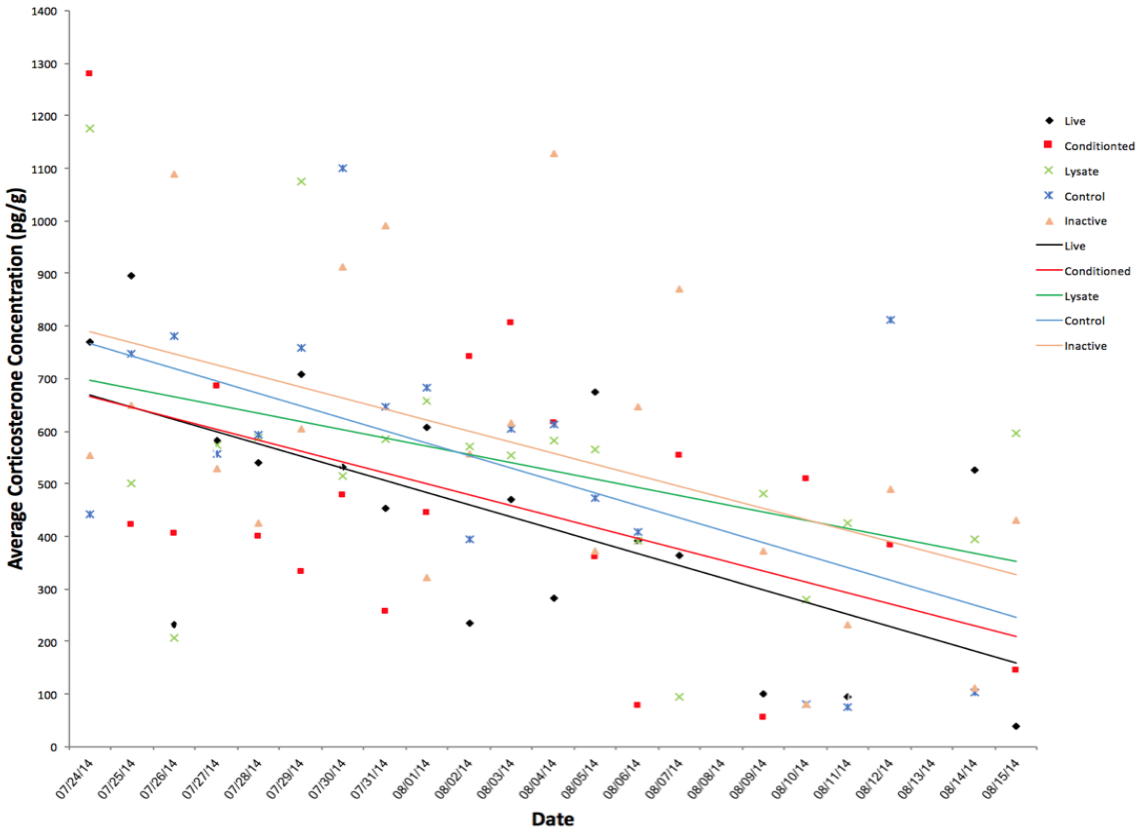

Figure 3. The average corticosterone concentration per treatment group over time.

The average corticosterone concentration extracted from fecal pellets of male mice collected per treatment group over time analyzed from the preliminary study. The trend line and data points for each group are shown. There was no statistical significance between groups as determined by one-way ANOVA ($F(4,96)=0.760$, $p=0.554$).

4. Discussion

The field of probiotics is becoming increasingly important in understanding the impact the microbiome has on human health. It was hypothesized that the subjects gavaged with live and conditioned cells would show decreased anxiety and depressive-like behaviors compared to the control and other groups in the behavior tests. The results of the forced swim test which assessed simple-depressive like behaviors between the treatment groups was not significant. The component of the bacteria that could cause an anti-depressant effect that was shown studies conducted by Bravo et al., could not be determined. *Lactobacillus rhanmosus* secretes GABA and elevations of this inhibitory neurotransmitter could inhibit anti-depressant effects, therefore analyzing the effects of microbes like *Candida*, *Enterococcus* and

Escherichia would be useful. These microbes have been found to secrete serotonin which is a neurotransmitter that regulates mood and is a pharmacological target for depression[11].

A trend appears in the open field test in which anxiety-like behavior was tested, where mice fed inactive bacteria are the least responsive to each component of the open field test with the animals treated with lysate samples approaching the control group. The significance of this trend is unknown and may suggest that a molecule that is sensitive to heat and denaturation may play a role in the health benefits as analyzed in this experiment by inhibiting an anxiolytic effect. The animals were treated with the inactive cells to determine if the presence of the bacterial cells alone would promote anti-depressant and anxiolytic effects. The inactive cells provided no benefit to the animal in terms of alleviating the stress-like behaviors, which may suggest that a molecule that is produced or synthesized by one of the bacterial species found in the gut was disrupted. The lysed cells may have caused the animals to be more responsive in the open field test where time mobile, distance traveled and velocity was measured. The lysed cells may be causing this positive effect because, although the cell is not intact, the molecules that the cells synthesized could be causing the effect seen in the open field test. These molecules include neuroactive products such as GABA and through fermentation, short chain fatty acids which can affect the nervous system through stimulation of the vagus nerve.

The ATCC strain used in this study produced a thick substance in the broth, and this should be evaluated in a future study to determine if the bacteria are producing a molecule that may be causing behavioral changes. The conditioned and live cells also caused similar positive effects and were hypothesized to be the most effective treatments suggesting that molecules secreted by the bacteria could contribute to the anxiolytic effect.

When looking at the physiological data the average corticosterone concentration in all groups slightly decreased over time, although comparisons of the treatment groups were not statistically significant. This decline in corticosterone concentration could be due to the mice becoming more acclimated to being held by the researchers and to the procedures involved, not due to factors of the bacteria. The physiological data (not shown) suggests that the mass of the mice and the mass of the fecal pellets was consistent in all the treatment groups over time.

The next steps necessary in continuing this research include assessing the effect that the estrous cycle may have had on components of the behaviors being analyzed because this study utilized female mice. Due to a small sample size in the control group from the loss of animals early in the study, the comparison between the control and other treatment groups was not appropriate and did not aid in determining whether the effects observed were due to the bacteria directly. Also, the time of day that the animals were tested for the open field behavior test may have influenced the results due to activity differences throughout the day. The mice who were tested early in the day could have had different activity levels than mice tested in the evening. These variables would need to be adjusted in future studies to clarify the results found in these experiments.

This study sought out to determine the essential characteristic of *L. rhamnosus* that may allow it to alter stress like behaviors. Understanding the mechanisms of these characteristics allows researchers to determine how these bacterial species could be manipulated to produce the desired effects. Prebiotics are non-digestible food ingredients that promote the growth of probiotics already located in the intestines. These components may offer another alternative to treatment methods used to combat the stress-like disorders and research to determine whether promoting the growth of these organisms would benefit the host could be essential additions to treatment with probiotics. As previously mentioned, several bacterial species found in the gut affect several neurotransmitters, therefore a future study should use a cocktail of these species and assess the behavioral effects. The microbes found at different point of the intestinal tract vary, the species that could provide benefits to a host need to be determined to be able to start clinical trials. The optimal dosage and treatment time are crucial for research into probiotics because these variables would whether significant results are found in future studies.

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