

Assessment of Gut Microbiome Role in Diet-Related Changes in Cognition

By
Clarisa Caballero-Ignacio

An Undergraduate Thesis Submitted to

Oregon State University
College of Agricultural Sciences

In partial fulfillment of
the requirements for the degree of

Baccalaureate of Science in BioResource Research,
Toxicology Option

May 31, 2017

APPROVED:

Dr. Kathy R. Magnusson, Biomedical Sciences and CVM

Date

Carmen Wong, Public Health and Human Science

Date

Katharine G. Field, BRR Director

Date

© Copyright by Clarisa Caballero-Ignacio
May 31st, 2017
Student
All Rights Reserved

I understand that my project will become part of the permanent collection of Oregon State University Library, and will become part of the Scholars Archive collection for BioResource Research. My signature below authorizes release of my project and thesis to any reader upon request.

Clarisa Caballero-Ignacio

Date

TABLE OF CONTENTS

	Page
1. INTRODUCTION.....	9
2. MATERIALS AND METHOD.....	10
2.1 Animals.....	10
2.2 Behavioral Testing.....	13
2.2.1 Step-down latency.....	13
2.2.2 Novel object recognition.....	13
2.2.3 Marble burying.....	14
2.3.4 Morris water maze.....	15
2.2.4.1 Acclimation.....	16
2.2.4.2 Spatial long-term memory.....	16
2.2.4.3 Cognitive flexibility.....	17
2.2.4.4 Cued control task.....	17
2.2.5 Body weight and food consumption.....	18
2.2.6 Data Analysis.....	18
3. RESULTS	19
3.1 Behavioral Cognitive Testing.....	19
3.1.1 Step-down Latency differences.....	19
3.1.2 Open field exploration.....	20
3.1.3 Novel object recognition.....	21
3.1.4 Marble-burying differences in compulsiveness anxiety.....	23

3.1.5 Morris water maze differences in memory and cognitive flexibility.....	25
3.2 Body weight and average food consumption.....	27
4. DISCUSSION	28
5. ACKNOWLEDGMENTS.....	32
6. APPENDIX.....	32
7. REFERENCES	33

LIST OF FIGURES AND TABLES

Figures	Page
1. Step-down latency.....	19
2. Open field exploration.....	21
3. Novel object recognition testing.....	22
4. Marble burying task.....	24
5. Number of partial marbles buried with data collapsed across diet.....	24
6. Number of total marbles buried with data collapsed across diet.....	25
7. The effects of diet on Morris water maze for long term memory.....	27
8. Body weight, water and food consumption.....	28
Table	
1. Diet Composition.....	12
2. Timeline.....	12

Highlights

- Antibiotic treatment improved long-term memory in the Morris water maze, regardless of diet.
- On average, mice showed less anxiety in the step-down task, but more anxiety in the marble-burying task.
- On average, mice showed learning in all water maze tasks.
- No high-sucrose diet effects compared to a defined control diet for the behavioral tests conducted

Key words: Gut microbiome, Cognitive flexibility, High sucrose, Long-term memory,

Antibiotics

Clarisa Caballero-Ignacio^{1,2,3}, Michelle Flores^{2,3}, Valerie Elias², Michelle Bermudez^{2,3}, Cedric Boluda^{2,3}, Natalia Shulzhenko², Andriy Morgun², Luiz Bermudez², Kathy R. Magnusson^{2,3}
BioResource Research Interdisciplinary Program¹, Department of Biomedical Science and
College of Veterinary Medicine², Linus Pauling Institute³
Oregon State University, Corvallis, Oregon 97331

Abstract

Western diets can influence behavior and gut microbiome due to the excessive intake of high fat and sucrose. Altering the microbiome can also influence the brain and behavior. The hypothesis that was tested was that diet-induced changes in the microbiome cause changes in cognitive abilities. A previous study showed learning and cognitive flexibility deficits in mice fed a high sucrose diet. The present study was designed to investigate whether altering the microbiome, via antibiotic treatment, will change the behavioral results when animals are on a high sucrose diet. Eight week old, male mice were randomly assigned to either high-sucrose (12% Kcal fat, 18% protein, 70% CHO (primarily sucrose)) or control defined (13% Kcal fat, 25% protein, 62% CHO) diets and either water or a combination of 4 antibiotics (vancomycin, neomycin, metronidazole and ampicillin) in the water. The animals were tested during the study for memory, anxiety, impulsiveness, and cognitive flexibility. Step-down latency, novel object recognition and marble burying tasks were performed both pre- and 7 weeks post-diet change. The Morris water maze, which tested for long and short-term memory and cognitive flexibility, was conducted during week 8 post-diet change. We found a significant effect of the antibiotic treatment on long term memory. The mice on antibiotics performed better than those on water treatments, suggesting that animals with reduced gut bacteria are learning better than those with only water treatment. There were no effects of diet in this study, which may be due to the use of a defined control diet, rather than the chow used in the previous study. Fecal microbiome

analysis and Western blots, to examine effects on the brain, will be performed. These results suggest that the microbiome does play a role in learning.

1. Introduction

Western diets are unhealthy due to the excessive intake of sugar, carbohydrates, and saturated fats. Consuming inadequate fruits, vegetables, whole grains, and low-fat dairy products, while taking in excessive amounts of saturated fats and carbohydrates, can lead to health issues [1]. Western diets promote obesity, metabolic syndrome, and cardiovascular disease [1]. High-sugar diets may lead to cognitive impairment and a predisposition to disorders such as Alzheimer's disease [2]. Alzheimer's disease diminishes memory, thinking skills, cognitive functioning, and other brain functions; and is the sixth leading cause of death in the United States[3]. There is currently no cure for Alzheimer's disease. The treatments are only symptomatic [2]. Combating high-energy diets could impact the possibility of developing Alzheimer's disease.

The human gut is a microbial ecosystem containing 100 trillion microorganisms, including fungi, bacteria, and viruses, living in our intestines. These microorganisms contain 100-fold more genes than the entire human genome [4]. The microbiome is becoming recognized as a major player influencing the health status of the host [5]. In previous studies it has been shown that high sucrose diets are associated with alterations in the microbiome [6].

The gut microbiome can influence host metabolism and contributes to obesity and metabolic disease [7]. The gut microbiota has also recently been demonstrated to play an important role in the maintenance of cognitive function [8]. Changes in the composition of the microbiota following bacterial infection, administration of antibiotics or probiotics, or in germ-free mice show that modification in the microbiota can impact behavior and cognition [8]. The gut-brain axis also consists of bidirectional communication network that monitors and integrates gut functions and link them to cognitive and emotional centers of the brain [9]. There is evidence

showing that the microbiome can be altered by a high sucrose diet within two weeks. High sucrose diets can also influence early memory, cognitive flexibility, and working memory within four-to-six weeks [6]. Bacteroidales and Clostridiales have shown changes across diets that show relationships to reductions in cognitive flexibility[6]. Since Bacteroidales and Clostridiales are the major bacterial population components seen in the human and mouse gut microbiome, it seems likely that these bacteria may be involved. However, it is not known whether these effects of the high sucrose diet are due to the bacteria or due to effects of the diet independent of the microbiome. Determining the role that diet-related changes in the gut microbiome may contribute to alterations in cognitive flexibility, spatial memory, cognitive abilities, and anxiety could expand our understanding of the impact of the gut microbiome on brain health and lead to new therapies.

One way to test the role of the microbiome is to alter the gut microbiota by having animals consume a high sucrose diet. Antibiotic treatment is expected to alter the microbiome in mice separate from the effects of the diet, by eliminating microbes. Ampicillin and neomycin in combination are commonly used to target the commensal microbes and achieve a significant reduction in bacteria [10]. An antibiotic cocktail including ampicillin, neomycin trisulfate, vancomycin, and metronidazole has been used as a substitute for germ-free mice [11]. In the present study, we addressed the hypothesis that the diet-induced changes in the microbiome can cause changes in cognitive abilities, by administering a cocktail of antibiotics and examining whether behavioral alterations induced by a high sugar diet was altered.

2. MATERIALS AND METHODS:

2.1 Animals

In this study, there was a 2X2 factorial design with twenty-four male C57BL/6 mice that were eight weeks of age. Mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Animals were housed in a twelve-hour light and twelve-hour dark cycle. The mice were assigned to a treatment group based on pre-treatment behavior in the step-down latency and 24-hour novel object recognition tasks to equalize performance between groups prior to treatment. Sets of 4 male sibling animals were housed together prior to the antibiotic treatment starting. All mice in the study were exposed to each other's feces by rotating the sets between each of the eight cages for two weeks before the actual experiment began. The cage rotations were performed to obtain a microbiome baseline where all the mice were exposed similarly. All the animals were fed with defined diet and water before the antibiotic and diet treatments were begun. Mice within each set of 4 were randomly assigned to two different diets and two different antibiotic treatments, with 6 mice per diet/treatment group/repetition. The diets were high sucrose (12% Kcal fat, 18% protein, 70% CHO (primarily sucrose)) or control defined (13% Kcal fat, 25% protein, 62% CHO) diets, as shown in Table 1, and the antibiotic treatments were a combination of four antibiotics in water or normal drinking water. The antibiotics were vancomycin (.5 gm/L), neomycin (1 gm/L), ampicillin (1 gm/L), and metronidazole (1 gm/L), as shown in Table 2. Antibiotics were purchased from Sigma-Aldrich, St Louis, MO. The antibiotic cocktail was made and refreshed every two days. Control water was refreshed every four days. The mice were fed and weighed every seven days and observed for any major changes or concerns with weight and food consumption differences. Fecal collections were done prior to antibiotic treatment onset, one-week post-antibiotic treatment onset, and two and six weeks post-diet change (Table 2). The step-down latency, marble burying, and novel object tasks were performed prior to antibiotic treatment onset and week seven post diet change. Water maze

testing was performed week eight post-diet change. At the end of the study, all animals were euthanized via CO_2 exposure, followed by decapitation. The brains were frozen on dry ice and stored at $-80^\circ C$ in the freezer. The brains will be assessed in the future for brain-derived neurotrophic factor and NMDA (N-methyl-D-aspartate) receptor protein expression via Western blots to identify specific amino-acid sequences from a complex mixture of proteins extracted from cells. Statistical analysis of behavior was performed by analysis of variance.

Table 1: Diet composition. The composition of fat, carbohydrates, protein and sucrose in control diet versus high-sucrose diet.

	Control defined diet TD.160121	High-sucrose diet TD. 98090 ^a
Kcal/g diet	3.6	4.0
Percent of kcal provided by:		
Fat	13.0	11.8
Protein	25.0	17.7
Carbohydrates (CHO)	61.9	70.4
Sucrose, g/kg diet	100.00	645.6

^a Purchased from Envigo Teklad Diets (Madison, WI).

Table 2: Timeline. Behavioral testing and feces collection timeline for twenty-four mice

	Control Diet	High Sucrose	Antibiotics	Behavior Testing	Fecal Collection
Week -3	Start on control diet				
Week -2				Step down latency, novel object, and marble burying	Swap feces end of week
Week -1			Start antibiotics cocktail		Antibiotic feces end of week

Week 0		Start high sucrose			
Week 2					2 week post-diet change end of week
Week 5					Collect feces end of week
Week 7				Step down latency, novel object, and marble burying	
Week 8				Morris water maze	

2.2 Behavioral Testing

2.2.1 Step-down latency

The step-down latency task was conducted as previously described one week prior to onset of antibiotic treatment and on week seven post-diet change [12]. The mice were placed on a 12.5 x 8.5 cm platform elevated 4 cm from a metal surgical table in a dimly-lit room. The latency for the mouse to step down off the platform with all four paws on the surface was recorded. Three trials with a five-minute maximum were conducted with a one-minute intertrial interval. A block design was utilized for animals to be tested in two different rooms for pre- and post-diet change. The purpose of the step-down latency was to record the total time it takes for the mouse to step down and place all four feet on the surface. The duration of time, compared to control animals, indicates anxiety (increased latency) or impulsiveness (decreased latency) within the mouse.

2.2.2 Novel object recognition

The novel object recognition task was based on procedures used by Hohmann and coworkers, with some modifications [13]. These tasks were conducted both pre-and post-diet

change called testing sessions. The arenas were plastic boxes (12''W x 18''D x 10''H) and the objects were either glass or plastic. Between each session, the arenas and objects were cleaned with 70% ethanol. The novel object tasks included a habituation trial, familiarization trial, one-hour delay test, and twenty-four-hour delay test. These tests were conducted within two days. Each session was five-minutes in duration. Session 1: consisted of habituation to an empty arena, Session 2: exploration of two similar objects, one-hour break, Session 3: Novel object test (one of the two objects was replaced by a novel object), 24-hour break, Session 4: Novel object test (the previous novel object was replaced by a new object). The mice were timed each time they approached an object, but only if the nose was pointing toward or touching the object. If the mice were on top of the object, the time was excluded due to the likelihood of escape intentions instead of curiosity of the object. The habituation trial was also used to assess open field behavior. The trial was tracked and analyzed for percentage time in the center zone (10 centimeters from each wall), latency to enter the center zone, center zone entries, exploration distance traveled, and speed with the use of SMART tracking system (San Diego Instruments, San Diego, CA). The habituation trial was analyzed to measure anxiety. Less time in and longer latency to enter the center zone indicated anxiety [14]. For novel object tasks, exploration time for each object was obtained by manual timing and a discrimination index was calculated by the following formula: $((\text{exploration time for novel object} - \text{exploration time for familiar object}) / \text{total exploration time})$ for a single subject.

2.2.3 Marble burying

The marble-burying test is a useful model of anxiety and defensive burying. The marbles provide an effective unconditioned stimulus that provokes burying, indicating anxiety [15,16].

The marble burying method consisted of a cage with 2 inches of bedding depth lightly tamped down to make a flat and even surface. There were fifteen glass marbles arranged in an array of 3 x 5 on flattened bedding. The cages were inside the plastic box arenas, so that the mice were not able to see each other during the study. Individual cages were used for each mouse. Between each session, the marbles were cleaned with 70% ethanol. The mouse was placed inside the cage for thirty minutes and then removed. The number of marbles displaced, partially buried, and fully buried were recorded. The marble-burying method demonstrates anxiety when the mice bury the marbles in their bedding [16].

2.2.4 Morris water maze

Spatial long-term memory, cognitive flexibility and associative memory were assessed using the Morris water maze, as described previously with some modifications[17,18]. A 4-foot diameter plastic tank was filled with water (16-18 °C) that was made opaque with non-toxic paint. The cold water was necessary to motivate the mice. Inside the pool, a removable, circular platform was positioned at the appropriate quadrant for specific trials or removed for probe trials. The water was filled to 1 cm above the level of the platform in the hidden platform trials. Spatial cues consisted of geometric shapes, including a star, circle, and square; pieces of cloth, and toys (Appendix I). The cues were either on the walls or on the edge of the tank. The behavioral testing trials were videotaped using a CCD camera placed on the ceiling and centered over the tank. The trials were analyzed by using the SMART video tracking system (San Diego Instruments, San Diego, CA, USA). The mice were placed in the water, facing the tank wall, from different entry points for each trial.

2.2.4.1 Acclimation

The goal of acclimation was to give the mice experience with swimming for 1 minute and to train them to stay on the platform for 30 seconds. The platform used during acclimation was placed in a location not used for memory testing. The mice went through acclimation twice, two days prior to place training. The procedure was repeated for the second day of acclimation. During acclimation, there were no cues on the walls or on the tank. The mice were placed inside the water tank facing the wall, where there was no platform present. After all mice have completed their swim the platform was placed in the center of the tank. Each mouse was then placed in the platform and had to remain on the platform for 30 seconds. This training was important because, during the actual spatial memory tasks, the mice would need to remain on the platform for 30 seconds to observe their surroundings to make a spatial map.

2.2.4.2 Spatial long-term memory

The following day after acclimation, mice underwent place trials for spatial reference (long-term) memory for eight trials per day for four days, with a 2-min inter-trial interval. The place trials were performed in two, four-trial blocks with a 90-minute rest period between blocks and a one-hour rest period after the eighth trial of each day. The platform remained in the same southeast (SE) quadrant for all the place trials. During place trials, the mice were placed in the water, facing the wall of the tank, and were allowed to search for the platform for 60 seconds. The mice remained on the platform for 30 seconds before being removed. Random entry points varied between trials within each day and were along the wall in the middle of the quadrants (NW, SW, NE), excluding the quadrant where the platform was located.

Probe trials were also performed in order to determine whether a spatial bias for the platform location was developed across days [19]. The platform was not present during probe trials and the animal could search for 30 seconds. The first day of long-term memory task started with an initial probe trial to detect a bias for a specific quadrant and to better see improvements across days, if present. The rest of the probe trials occurred after the second set of four place trials each day.

2.2.4.3 Cognitive flexibility

Reversal trials were used to assess cognitive flexibility [20]. For one day following long-term memory training, the platform was moved to the opposite quadrant (NW) and eight reversal trials and one reversal probe trial were conducted as described above. The entry points were randomly assigned (SE, NE, and SW). The mice were allowed to search for the platform for 60 seconds followed by a 30 second period on the platform and a 2 min intertrial interval in the reversal trials. The reversal probe trials were as described above.

2.2.4.4 Cued control task

An associative (control) memory task was employed to test motivation, visual acuity, and physical ability for memory testing [20]. The platform was slightly above the level of the water. The white platform had a black flag on top of the platform. The platform position was changed to a different position for each trial for 6 trials and the mice were placed into the tank facing the wall at entry points across from the platform location. The mice were allowed to search for the platform for 60 seconds. All mice were tested at one platform position before the platform was moved to a new position.

2.2.5 Body weight and food consumption

The effects of high-sucrose diet and antibiotic treatments on weight gain and food consumption were monitored throughout the experiment. The mice were given 28g of the appropriate diet each week. The amount of food given and leftover was weighed. This allowed us to keep track of how much food was consumed. The antibiotic cocktail was made and switched every two days. The filtered water was given and changed every four days. The amount of water leftover was measured with a graduated cylinder when water was added. The antibiotic group was given 18ml every 2 days and the control water group was given 25 ml every 4 days.

2.2.6 Data Analysis

Cumulative proximity was used to measure the performance of the Morris Water Maze trials including place, probe, reversal, and cued trials. The cumulative proximity was obtained from the SMART tracking program method [19]. The computer measured the animal's distance from the platform or proximity measure, for the duration of the animals swim every 0.2 seconds. Cumulative proximity was calculated by adding together the distance calculated at each 0.2 s interval. Correction for start position was performed using a macro in Excel software (Microsoft Corp., Seattle, WA, USA). The proximity measures were corrected for start position by calculating the cumulative proximity for the ideal path based on the average swim speed and platform position, and subtracting this from the cumulative proximity measurement from the tracking system. The average proximity to the platform was used to measure the performance on the probe trials [19]. The cumulative proximity measures were used to assess the performances because they are less influenced by swim speed differences than more traditional measures such as latency to reach the platform [19]. The measures used are also sensitive to some of the

alternative strategies that animals can use to find the platform that may not involve place learning [19]. The statistics used to analyze the behavioral testing was two-way analysis of variance or three-way ANOVA and the Fisher's PLSD post hoc analysis was also conducted. The novel object tasks exploration time for each object was obtained by manual timing and a discrimination index as previously mentioned ((exploration time for novel object - exploration time for familiar object) / total exploration time) for a single subject.

3. RESULTS

3.1 Behavioral cognitive testing

3.1.1 Step-down latency differences

There was a significant effect of pre- versus post-diet change testing session ($F(1, 40) = 6.657, p = .01$) on step-down latency, but no significant main effect of diet ($F(1, 20) = 0.007, p = .93$) or antibiotic ($F(1, 20) = 0.04, p = .85$) treatments. Mice stepped down faster when tested post-diet change than pre-change, when data was collapsed across treatments.

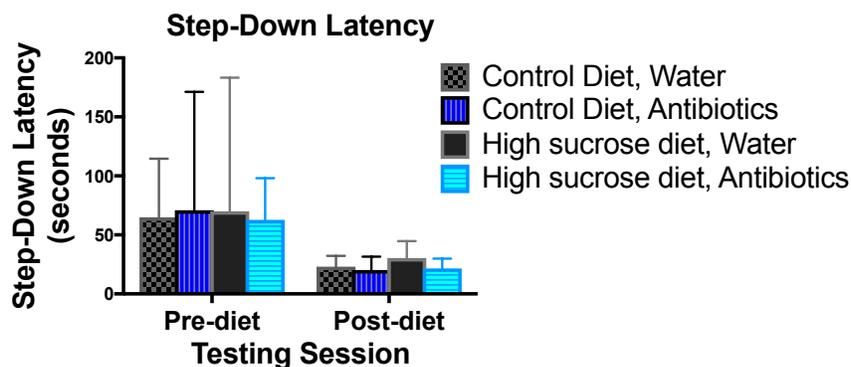


Fig. 1. Step-down latency. There was no effect of high sucrose diet or antibiotic treatment on step-down latency. On average, all treatments stepped down more quickly in the post-diet change session. Means \pm SEM. N= 6.

3.1.2 Open field exploration

The habituation session for novel object recognition was analyzed for open field exploration by measuring the entries into the center zone and the latency to enter the center zone. There was a significant main effect of testing session on entries into the center zone ($F(1,20) = 8.39$, $p < 0.01$) (Fig. 2A). There were no significant main effects of diet ($F(1,20) = 0.87$, $p = 0.36$) or antibiotic ($F(1,20) = 0.01$, $p = 0.92$) treatments on entries into the center zone (Fig. 2A). Mice entered the center zone less often when tested after the diet change than before (Fig. 2A). The distance traveled in center zone had a significant main effect between pre- versus post diet testing session ($F(1,20) = 48.64$, $p < 0.01$) (Fig. 2B). There were no significant main effects of diet ($F(1,20) = 2.27$, $p = 0.15$) or antibiotics ($F(1,20) = 1.20$, $p = 0.29$) on the distance traveled in center zone. (Fig. 2B). There was a near significant main effect of testing session change for pre-versus post-diet in latency to enter the center zone ($F(1,20) = 4.01$, $p = 0.06$), but no significant main effect of diet ($F(1,20) = 0.93$, $p = 0.35$) or antibiotic ($F(1,20) = 0.86$, $p = 0.37$) treatments (Fig. 2C). There was a near significant interaction between diet and water treatments ($F(1,20) = 3.69$, $p = 0.07$) in latency to enter the center zone (Fig. 2C). The speed of exploration time in an open field had no main effect on diet ($F(1,20) = 2.28$, $p = 0.15$) or antibiotic ($F(1,20) = 1.21$, $p = 0.28$) treatments (Fig. 2D). The testing session for the average speed ($F(1,20) = 49.22$, $p < 0.01$) showed a significant difference in pre-versus post-diet, with slower speeds seen in the post-diet change session (Fig. 2D).

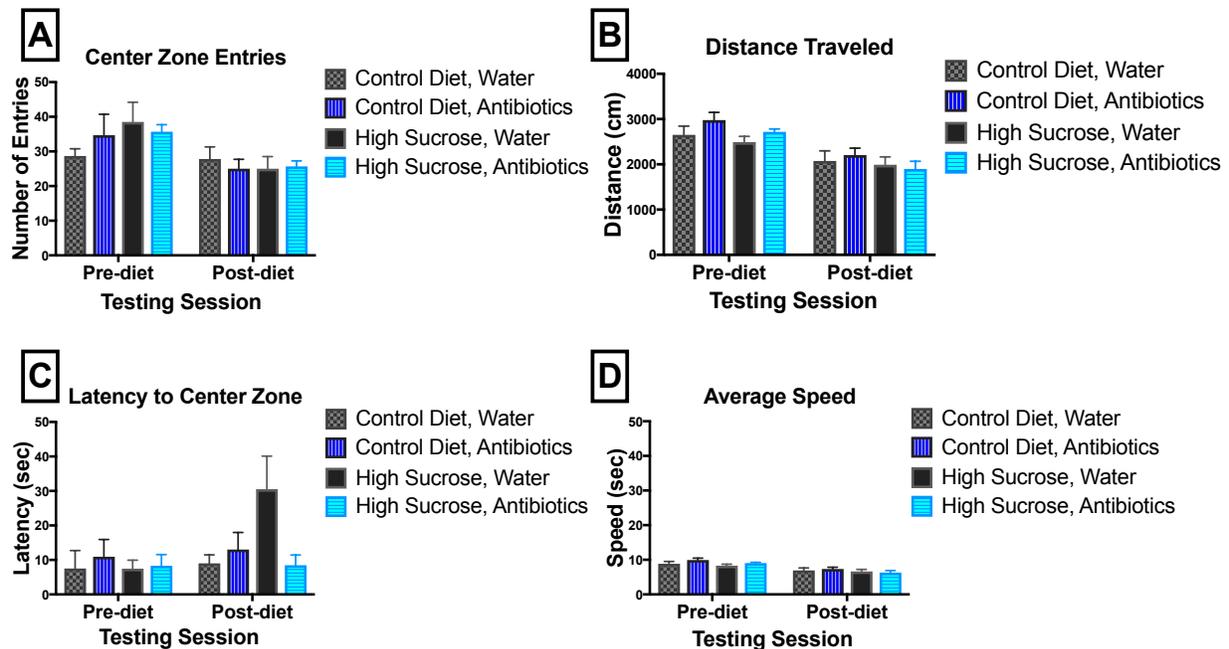


Fig. 2. Open field exploration. The number of entries to center zone (A), the distance traveled in center zone (B), latency to enter the center zone (C), and the speed of exploration in an open field (D). Mean \pm SEM. N= 6.

3.1.3 Novel object recognition

There was a significant main effect of pre- versus post-diet change testing session on the discrimination index between exploration of right and left similar objects in the familiarization trial ($F(1,20) = 8.23, p = 0.01$) (Fig 3A). Mice showed more preference for the one side before the diet change and the opposite side after the diet change. There was no main effect of diet ($F(1,20) = 0.42, p = 0.52$) or antibiotic ($F(1,20) = 0.87, p = 0.36$) on the discrimination index during the familiarization trial, but there was a significant interaction between diet, water treatment, and testing session ($F(1,20) = 5.46, p = 0.03$) (Fig 3A).

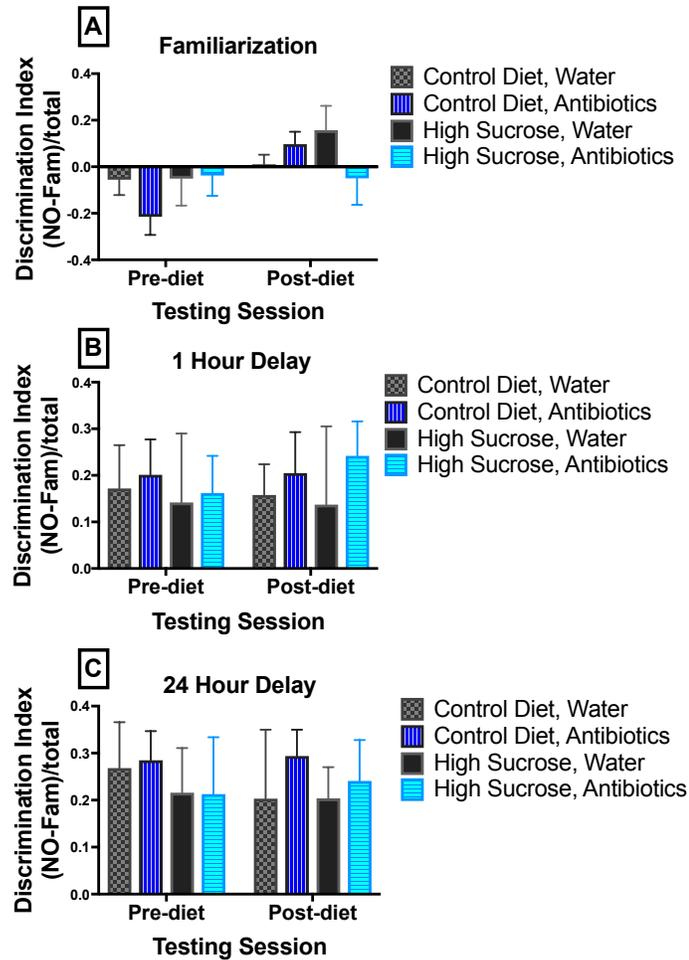


Fig. 3. Novel object recognition testing. Discrimination Index for familiarization (A) and 1 hour delay (B) and 24 hour delay (C) novel object testing. ANOVA & Fisher's PLSD post hoc test was used. Mean \pm SEM. N= 6.

The novel object one-hour test had no significant main effects of diet ($F(1,20) = 0.02$, $p = 0.89$) or antibiotic ($F(1,20) = 0.31$, $p = 0.59$) treatments on discrimination index (Fig. 3B). There was also no main effect of pre-versus post diet testing session ($F(1,20) = 0.09$, $p = 0.76$) on discrimination index in the one-hour test (Fig. 3B). The twenty-four hour test showed no main effects of diet ($F(1,20) = 0.28$, $p = 0.60$) or antibiotic ($F(1,20) = 0.18$, $p = 0.67$) treatments on discrimination index (Fig. 3C). No main effect of pre-versus post-diet change testing session

$F(1,20) = 0.05, p = 0.83$) was seen in the discrimination index on the twenty-four hour delay test (Fig.3C).

3.1.4 Marble-burying differences in compulsiveness and anxiety

There was a main effect of pre- versus post-diet change testing session on the number of fully buried marbles ($F(1,20) = 36.48, p = <0.01$). The number of marbles fully buried increased in post-diet compared to pre-diet change testing (Fig. 4A). There was no significant main diet treatment effect ($F(1,20) <.01, p = 0.97$) or antibiotic ($F(1,20) = 2.9, p = .10$) treatment effect on fully buried marbles (Fig. 4A). The partially buried marbles (Fig.4B) showed no main effect of diet ($F(1,20) = 0.48, p = 0.50$) or antibiotic ($F(1,20) = 0.04, p = 0.85$) treatments. The number of partially buried marbles showed a significant interaction between pre- versus post-diet testing sessions and antibiotic treatments ($F(1,20) = 4.83, p = 0.04$) (Fig. 4B). The interaction between antibiotic treatment and testing session was due to the antibiotics group partially burying more during pre-diet change testing session ($F(1,22) = 4.03, p = 0.06$) (Fig. 5A) than post-diet change testing session ($F(1,22) = 1.18, p = 0.29$) (Fig. 5B). The total number of buried marbles showed a significant main effect of antibiotic treatments ($F(1,20) = 4.83, p = 0.04$), but no significant diet effect ($F(1,20) = 0.58, p = 0.46$) (Fig. 4C). Mice in the antibiotic treatment group buried more total number of marbles than those on water (Fig 6C), but this appeared to be due to mice assigned to the antibiotic group burying more marbles in the pre-diet change session than those assigned to water treatment ($F(1,22) = 5.07, p = 0.03$) (Fig. 6A). There was no difference between antibiotic treatments in the post-diet change session ($F(1,22) = 0.08, p = 0.78$) (Fig. 6B). There was a main effect of pre- versus post diet testing session on total number of buried marbles ($F(1,20) = 38.23, p = <0.01$) and displaced marbles ($F(1,20) = 29, p < .0001$) (Fig. 4C). More

total marbles were buried or displaced in the post-diet change session than before the diet change. There was no main effect of diet ($F(1,20) = 0.03$, $p = 0.86$) or antibiotic ($F(1,20) = 0.07$, $p = 0.79$) treatments on displaced marbles (Fig. 4D). The number of marbles displaced showed a significant interaction between pre-versus post-diet testing session and diet and antibiotic treatments ($F(1,20) = 4.61$, $p = .04$) (Fig. 4D).

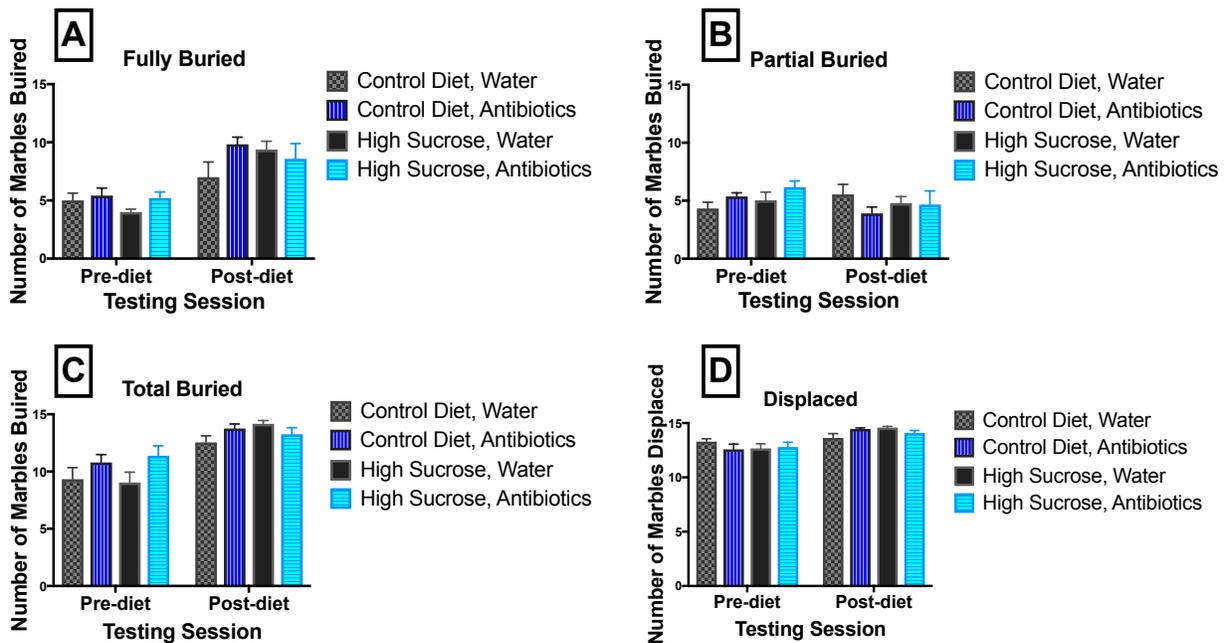


Fig. 4. Marble burying task. Testing session and/or antibiotics impacted the number of fully buried marbles (A), number of partially buried marbles (B), number of total buried marbles (C), and the number of displaced marbles (D). Mean \pm SEM. N= 6.

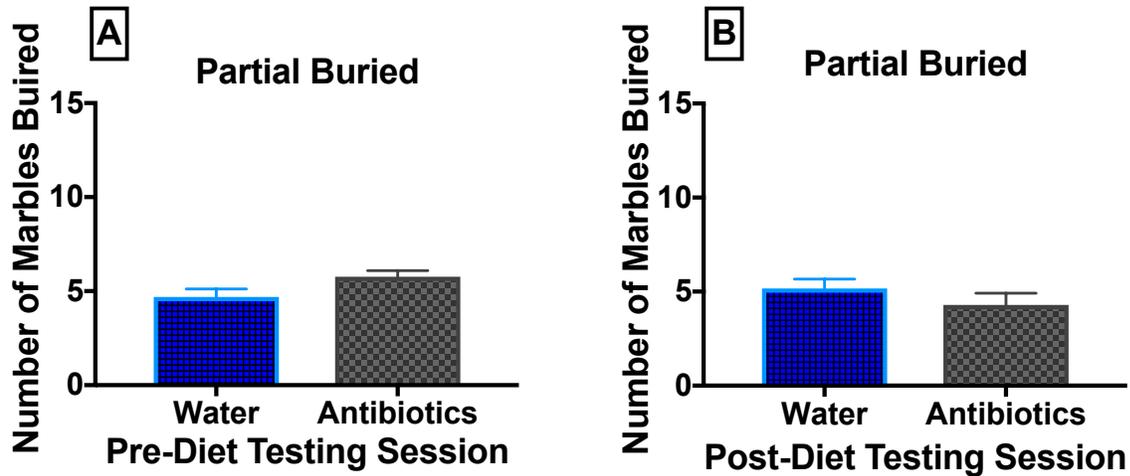


Fig. 5. Number of partial marbles buried with data collapsed across diet. Pre-diet testing session for partial number of marbles buried in antibiotics and water treatments (A), post-diet testing session for partial number of marbles buried (B). Mean \pm SEM. N= 6.

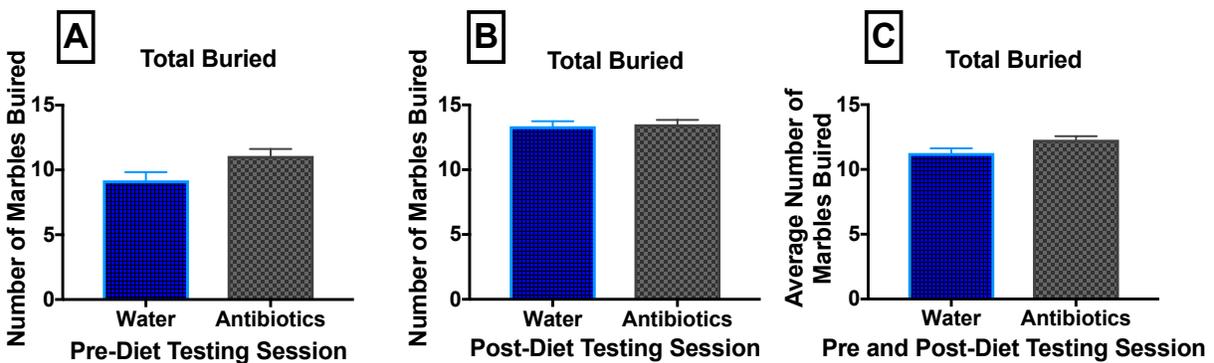


Fig. 6. Number of total marbles buried with data collapsed across diet. Pre-diet testing session for total number of marbles buried in antibiotics and water treatments (A), post-diet testing session for total number of marbles buried (B), number of total marbles buried averaged across pre-diet and post-diet testing session for average (C). Mean \pm SEM. N= 6.

3.1.5 Morris water maze differences in memory and cognitive flexibility

There was a main effect of trial on corrected average proximity in the probe trials ($F(4,80) = 32.04, p = < 0.01$) (Fig. 7A). Mice showed significantly lower corrected average proximity in the last probe trials, as compared to the first, when data was collapsed across diet and antibiotic treatments. There was no main effect of diet ($F(1,20) = 0.19, p = 0.67$) or antibiotic ($F(1,20) = 2.76, p = 0.11$) treatment in the probe trials (Fig. 7A). The place trials were separated into blocks of 4 trials. In the blocks of 4 place trials, there was a significant main effect of blocks of place trials on corrected cumulative proximity ($F(7,140) = 23.68, p = < 0.01$) (Fig. 7B). Mice showed significantly lower corrected cumulative proximity on the last block of trials than the first, when data was collapsed across diet and antibiotic treatments. There was also a significant main effect of antibiotic treatment ($F(1,20) = 4.6, p = .04$) (Fig. 7B). Mice treated with antibiotics had lower corrected cumulative proximities than those treated with water, as shown in figure 6B. In the reversal trials there was a main effect of trial on corrected cumulative proximity in the reversal trials ($F(7,140) = 8.45, p = < 0.01$) (Fig. 7C). There was a near significant main effect of antibiotic treatment ($F(1,20) = 3.76, p = 0.07$) (Fig. 7C). There were no significant effects of diet treatment ($p = .48-.77$) on water maze tasks. For the cued trials there was a main effect of trial on corrected cumulative proximity ($F(5,100) = 12.82, p = < 0.01$), but no significant effect of diet ($F(1,20) = .09, p = 0.77$) or antibiotic ($F(1,20) = .15, p = 0.70$) treatments (Fig. 7D). For reversal and cued trials, mice showed significantly lower corrected cumulative proximity on the last trial than the first, when data was collapsed across diet and antibiotic treatments.

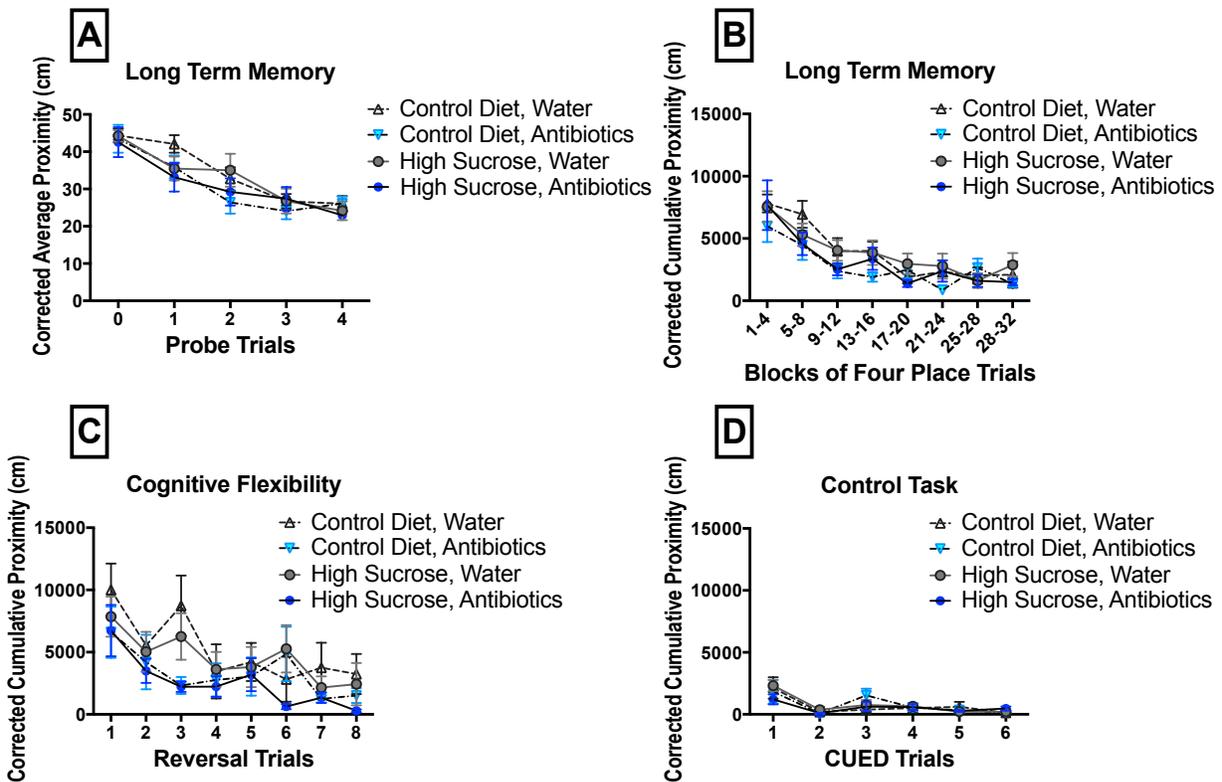


Fig. 7. The effects of diet on Morris water maze for long-term memory. Corrected cumulative proximity for long term memory (A,B), cognitive flexibility (C), and cued trials (D).

Mean \pm SEM. N=8. ANOVA and Fisher's PLSD post hoc test.

3.2 Body weight and average food consumption

Body weight, food consumption, and water consumption were monitored, but statistics were not performed. The results could not be accurately quantitated because of losses due to food falling into the cage, water loss while clearing the drinking tubes, and water leaks (Fig.8 A-D). During the study, it was noted that the antibiotic treated animals appeared to drink less than the control mice, but body weight and consumption appeared to be similar.

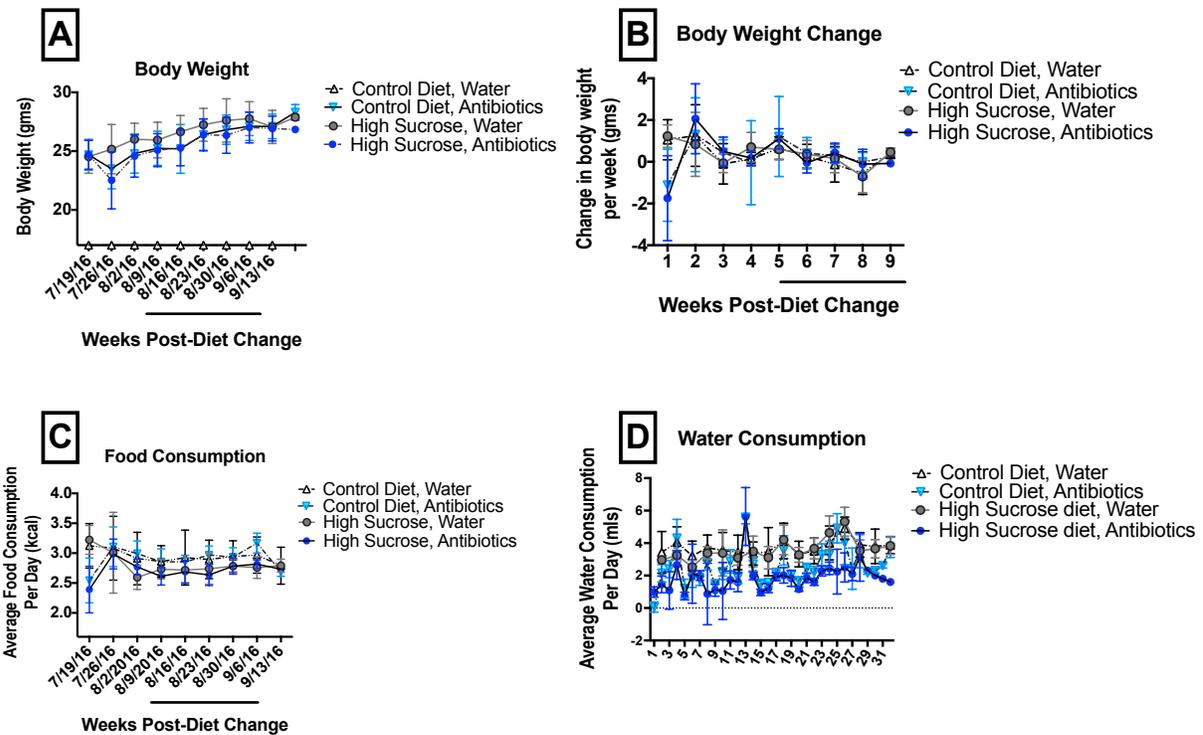


Fig. 8. Body weight, water and food consumption. Body weight (A), Body weight change (B), Average food consumption (C), Average water consumption (D).

DISCUSSION

The goal of this study was to investigate whether altering the gut bacteria via antibiotics, will change the behavioral results when animals are on a high sucrose diet. Although, there were no significant diet effects on any of the behavioral tests conducted, there were antibiotic effects on long-term memory.

On the Blocks of four place trials for the Morris water maze behavioral task showed a significant main effect on the antibiotic treatment. The antibiotic treatment improved long term memory performance throughout the trials. These results indicate that the reduced gut bacteria mice are performing better than the control group, thus suggesting that the microbiome plays an

important role in cognitive function. The trial effects also indicated that the mice, in general, were learning the platform position.

The reversal trials from the Morris water maze measure cognitive flexibility. Cognitive flexibility in this study was assessed as the ability to learn a new platform position (NW) after the mice had learned the original (SE) quadrant position in the long-term memory trials. The task showed a near significant trend for improvement in cognitive flexibility in the mice treated with antibiotics. There was also a significant improvement throughout the trials, showing that all the mice, on average, were learning.

The cued trials in the Morris water maze was set up as a control task where it can show any problems that the mice might have during the behavioral testing. During the cued trials, there was a visible flag on top of the platform. The mice showed improved performance between the first and last trial. This improvement indicated that the mice were motivated, had physical ability, and good vision to conduct the Morris water maze control task.

The step-down latency behavioral test had a significant testing session effect, but no treatment effect. The step-down latency reduction in post-diet was expected. The mice took less time stepping down in the post-diet session, as compared to pre-diet. The mice may have stepped down sooner in post-diet change testing session compared to pre-diet test session because of familiarization with the task and environment. During the pre-diet test session, the mice had not experienced the test before. This suggests that the mice were less anxious on the step-down latency task in the post-diet change session.

In the marble burying task, the number of marbles buried is proportional to signs of anxiety and compulsiveness. The results showed a significant antibiotic treatment group effect on the marble burying task for the number of total marbles buried across both testing sessions,

but this appeared to be primarily due to antibiotic treatment group differences in the pre-treatment session. During pre-diet change testing the control and antibiotic treatment groups were all on water, as well as control diet. Theoretically both treatments should have had similar results, but we normalized the treatment assignments based on step-down latency and 24-hour novel object task performances and not marble burying. Thus, this effect of antibiotic treatment assignment on total marbles buried cannot be ascribed to the antibiotic treatment itself.

The number of partially buried marbles showed no main effect for diet or water treatment, but there was a significant water treatment interaction between pre-diet versus post-diet testing session. The significant interaction led to conducting the post-hoc test for these results. The interaction between antibiotic treatment and testing session appeared to be due to the antibiotic treatment group mice showing a trend for partially burying more marbles during pre-diet (and pre-antibiotics) change testing session compared to post diet. As with the total marbles buried, the effects of antibiotic treatment group on partially buried marbles were mostly due to the pre-diet change assignment groups not performing equally in this task.

All mice on average, buried more marbles fully and in total during the post-diet change testing session. This suggests that all mice showed more anxiety in the marble burying task in the second testing session. It is not clear why this measure of anxiety is opposite to the step-down latency task.

The non-significant high sucrose diet effect may be due to using a non-chow, defined diet as the control. A similar study was conducted between high-sucrose, normal diet, and high-fat [6]. In this study, it was found that animals on the high-sucrose diet showed more significant alterations. The normal (chow) used in the previous study was different than the control defined diet used in this study. The control defined diet contained diet by kcal by .2% less fat, .2% less

carbohydrates, and .3% more protein compared to the normal (chow) [6]. There is a possibility that the difference between the previous normal diet and the control defined diet used in this study could have affected the results. The microbiome analyzes will demonstrate whether the bacteria present in the control defined diet are similar to the high-sucrose diet.

The use of antibiotics has been researched and proven to decrease bacteria in the microbiome [11]. Psychiatric side effects of antibiotics indicate that there is a link between antibiotic exposure and altered brain functions, ranging from anxiety and panic to major depression, psychosis and delirium [21] [9]. In the article by Sternbach and State indicate that these side effects were influenced by risk factors such as prior psychopathology, medical conditions, antibiotic dosage, intrathecal or intravenous administration [21]. The mice used in this study did not have previous medical conditions nor were at risk for anxiety and depression side effects from antibiotics administration. The mice were healthy and the antibiotic cocktail used has been proven to be effective for this study purpose, to reduce bacteria in the gut microbiome [11]. A study showed the effects of antibiotics on the mouse gut, revealing microbiome-dependent and independent mechanism. Both direct effects of the antibiotics and proliferation of antibiotic-resistant microbes induce mitochondrial-dependent cell death in the intestine [11].

The findings from this study of significant antibiotic treatment effect on long-term memory suggest that the gut microbiota influences behavioral outcomes. These results can be further investigated through microbiome analysis that is still to be conducted in the future. The bacterial content from fecal collection will demonstrate the bacteria present and or reduced from antibiotics and any differences between diets.

5. Acknowledgements

I want to thank Foundation Grants, Louis Stokes Alliance for Minority Participation (LSAMP), and United States Department of Agriculture NIFA Multicultural Scholar Program for the financial support. Special thanks to Dr. Fikru Nigussie for the support in this project.

6. Appendix A. Supplementary Data



Appendix I. Morris Water Maze Tank used for this study.

7. References

- [1] A. Manzel, D.N. Muller, D.A. Hafler, S.E. Erdman, R.A. Linker, M. Kleinewietfeld, Role of “Western Diet” in Inflammatory Autoimmune Diseases, *Curr. Allergy Asthma Rep.* 14 (2014) 404. doi:10.1007/s11882-013-0404-6.
- [2] K.G. Yiannopoulou, S.G. Papageorgiou, Current and future treatments for Alzheimer’s disease, *Ther. Adv. Neurol. Disord.* 6 (2013) 19–33. doi:10.1177/1756285612461679.
- [3] N.I. on Aging, Alzheimer’s Disease Fact Sheet, *Natl. Inst. Aging.* (2011). <https://www.nia.nih.gov/alzheimers/publication/alzheimers-disease-fact-sheet> (accessed April 17, 2017).
- [4] The Microbiome and Obesity- Is Obesity Linked to Our Gut Flora.pdf, (n.d.). <http://www.healthy-outcomes.com/sites/default/files/The%20Microbiome%20and%20Obesity-%20Is%20Obesity%20Linked%20to%20Our%20Gut%20Flora.pdf> (accessed April 17, 2017).
- [5] S. Ghaisas, J. Maher, A. Kanthasamy, Gut microbiome in health and disease: Linking the microbiome-gut-brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases, *Pharmacol. Ther.* 158 (2016) 52–62. doi:10.1016/j.pharmthera.2015.11.012.
- [6] K.R. Magnusson, L. Hauck, B.M. Jeffrey, V. Elias, A. Humphrey, R. Nath, A. Perrone, L.E. Bermudez, Relationships between diet-related changes in the gut microbiome and cognitive flexibility, *Neuroscience.* 300 (2015) 128–140. doi:10.1016/j.neuroscience.2015.05.016.
- [7] A. Zarrinpar, A. Chaix, S. Yooseph, S. Panda, Diet and Feeding Pattern Affect the Diurnal Dynamics of the Gut Microbiome, *Cell Metab.* 20 (2014) 1006–1017. doi:10.1016/j.cmet.2014.11.008.
- [8] M.G. Gareau, Microbiota-gut-brain axis and cognitive function, *Adv. Exp. Med. Biol.* 817 (2014) 357–371. doi:10.1007/978-1-4939-0897-4_16.
- [9] G.B. Rogers, D.J. Keating, R.L. Young, M.-L. Wong, J. Licinio, S. Wesselingh, From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways, *Mol. Psychiatry.* 21 (2016) 738–748. doi:10.1038/mp.2016.50.
- [10] M.R. Bomhof, H.A. Paul, M.B. Geuking, L.K. Eller, R.A. Reimer, Improvement in adiposity with oligofructose is modified by antibiotics in obese rats, *FASEB J.* (2016) fj.201600151R. doi:10.1096/fj.201600151R.
- [11] A. Morgun, A. Dzutsev, X. Dong, R.L. Greer, D.J. Sexton, J. Ravel, M. Schuster, W. Hsiao, P. Matzinger, N. Shulzhenko, Uncovering effects of antibiotics on the host and microbiota using transkingdom gene networks, *Gut.* 64 (2015) 1732–1743. doi:10.1136/gutjnl-2014-308820.
- [12] H. Anisman, S. Hayley, O. Kelly, T. Borowski, Z. Merali, Psychogenic, neurogenic, and systemic stressor effects on plasma corticosterone and behavior: Mouse strain-dependent outcomes, *Behav. Neurosci.* 115 (2001) 443–454. doi:10.1037/0735-7044.115.2.443.
- [13] C.F. Hohmann, E.M. Walker, C.B. Boylan, M.E. Blue, Neonatal serotonin depletion alters behavioral responses to spatial change and novelty, *Brain Res.* 1139 (2007) 163–177. doi:10.1016/j.brainres.2006.12.095.
- [14] J. Simpson, J.P. Kelly, The impact of environmental enrichment in laboratory rats—Behavioural and neurochemical aspects, *Behav. Brain Res.* 222 (2011) 246–264. doi:10.1016/j.bbr.2011.04.002.

- [15] J.P. Pinel, D. Treit, Burying as a defensive response in rats, *J. Comp. Physiol. Psychol.* 92 (1978) 708–712.
- [16] 2002_Individual_Differences_Behav_Brain_Res.pdf, (n.d.).
http://w3.csmu.edu.tw/~yjho/Publication/2002_Individual_Differences_Behav_Brain_Res.pdf (accessed April 17, 2017).
- [17] K.R. Magnusson, B. Scruggs, J. Aniya, K.C. Wright, T. Ontl, Y. Xing, L. Bai, Age-related deficits in mice performing working memory tasks in a water maze, *Behav. Neurosci.* 117 (2003) 485–495.
- [18] PubMed Central Full Text PDF, (n.d.).
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3355246/pdf/nihms-363726.pdf> (accessed April 17, 2017).
- [19] Gallagher-Burwell-Burchinal-1993.pdf, (n.d.).
<https://www.brown.edu/research/labs/burwell/sites/brown.edu.research.labs.burwell/files/uploads/Gallagher-Burwell-Burchinal-1993.pdf> (accessed April 17, 2017).
- [20] S.R. Das, R. Jensen, R. Kelsay, M. Shumaker, R. Bochart, B. Brim, D. Zamzow, K.R. Magnusson, Reducing expression of GluN10XX subunit splice variants of the NMDA receptor interferes with spatial reference memory, *Behav. Brain Res.* 230 (2012) 317–324. doi:10.1016/j.bbr.2012.02.014.
- [21] H. Sternbach, R. State, Antibiotics: neuropsychiatric effects and psychotropic interactions, *Harv. Rev. Psychiatry.* 5 (1997) 214–226.