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Temperament and its Relationship to Gut Microbiome Diversity in Male Sprague-Dawley Rats

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ABSTRACT

Temperament refers to a profile of behavioral tendencies and certain temperaments such as Behavioral Inhibition and Social Boldness have been implicated in impaired social behavior, stress coping, and mental health outcomes (Fox & Pine, 2012; Kabelick et al., 2021). To understand why specific temperaments are connected to adverse mental health outcomes, we look to the gut microbiome. Growing evidence suggests that the gut microbiome may play a role in the development of mental and behavioral health disorders. Given that both temperament and gut microbiome are associated with mental health and behavior, the relationship between temperament and gut microbial diversity may be key to understanding if the gut microbiome is a mechanism by which temperament affects the onset of psychological and neurodevelopmental disorders. To examine this, male Sprague-Dawley rats were subjected to a series of behavior tests, the results of which were used to classify the rats based on temperament and determine associations with gut microbial species richness. The findings indicate no significant relationship exists between gut microbial species richness and rat temperament. Although a consensus exists in the literature that the gut microbiome is associated with temperament, future studies should examine specific microbial compositions and their relationship to temperament to further refute or confirm that the gut microbiome explains the association between temperament and mental/behavioral health outcomes.

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Chapter 1

Introduction

Purpose of Study

The current study aims to understand how individual differences in the gut microbiome relate to individual differences in temperament in male Sprague-Dawley rats. Temperament is a strong predictor of mental and behavioral health outcomes (Fox et al., 2005; Konstantareas & Stewart, 2006) and one potential mechanism underlying this relationship may be gut microbial diversity. The gut microbiome is the collection of microorganisms inhabiting the intestines playing a role in numerous physiological and psychological processes. Gut dysbiosis is an imbalance in microbial composition and diversity that can lead to adverse health outcomes (Clapp et al., 2017). Given that both temperament and the gut microbiome are implicated in mental and behavioral health disorders, gut microbial diversity may explain why certain temperaments predict these disorders. Animal models are commonly used to examine the effects of gut microbial diversity on behavior and brain functionality. By determining if an association between temperament and gut microbial diversity exists in a rat model, we can translate these findings to humans and further our understanding of the development of psychological and behavioral disorders.

Social Temperament and Mental Health

Temperament refers to the differences in behavior displayed by individuals that are consistent over time and occur in numerous contexts. It is exhibited across many species and can predict mental health outcomes and has been associated with behavioral disorders such as autism spectrum disorder (Fox & Pine, 2005; Konstantareas & Stewart, 2006). Social Boldness is a temperament studied in animals and characterized by a quick willingness to engage with a novel social partner (Kabelik et al., 2021). Social boldness has also been found to be consistent across time and context and is associated with other traits such as active versus passive stress coping (Kabelik et al., 2021). Behavioral Inhibition is a temperament characterized by hesitancy to interact with novel environments or social partners. This temperament has been identified in children as a potential risk factor for anxiety disorders later in life (Fox & Pine, 2012; Schwartz et al., 1999; Kozlova et al., 2020).

Gut-Brain-Axis

The mechanism through which temperament relates to the gut microbiome is thought to be the gut-brain axis, the system of bidirectional communication between the gastrointestinal tract and the central nervous system. Although the extent of our knowledge on the concrete mechanisms behind this connection is still quite limited, there are several suspected means by which the microbiota and the brain are thought to influence each other. Research has demonstrated that vagal activation is required for the gut microbiome to affect aspects of brain function (Cryan & Dinan, 2012). The vagus nerve relays information from the intestinal lumen to the brain including information related to intestinal permeability. Research evidence shows a

connection between the intestinal barrier and stress regulating systems such as the Hypothalamic-Pituitary-Adrenal (HPA) axis and the autonomic nervous system (Carabotti et al., 2015). Furthermore, multiple neurotransmitters are known to be produced in the gut and influence enteric nervous system (ENS) activity including serotonin, GABA, and dopamine, all of which are implicated in anxiety and mood disorders. Another means by which the gut microbiome might influence the brain is through the production of short-chain fatty acids (SCFAs). Dietary fibers are broken down into SCFAs by microbes in the gut. SCFAs can then stimulate the sympathetic nervous system, serotonin release in the gut, and can influence learning and memory mechanisms (Carabotti et al., 2015). Additionally, galanin, a neuropeptide concentrated primarily in the brain and gut, stimulates the release of corticotropin releasing factor and adrenocorticotropic hormone by the HPA axis. Galanin can also directly stimulate cortisol and norepinephrine release (Carabotti et al., 2015). Many of the ways in which the gut microbiome and the brain interact directly implicate the stress response whose impairment is associated with the onset of disorders such as anxiety and depression. This connection demonstrates why the gut microbiome may play a role in the relationship between temperament and mental and behavioral health.

Human studies

The role of the gut microbiome's impact on brain functionality and its implications for mental health are demonstrated as early as infancy. In newborns, increased gut taxa diversity was linked to fronto-parietal connectivity, a brain network previously associated with later positive mental health outcomes (Kelsey et al., 2021).

Human research over the past several years has found evidence of a connection between gut microbiome composition and personality, mood, cognition, and psychiatric disorders. For example, stress and anxiety have been associated with lower levels of species richness indicated by the Shannon Index metric of alpha diversity (Johnson, 2020). Interestingly, lower levels of richness were also associated with agreeableness, a beneficial personality trait, which demonstrates the complexity of microbial diversity's relationship with human behavior (Johnson, 2020). Further illustrating the relationship between gut microbial diversity and temperament is a paper from Aatsinki and colleagues (2019) who observed that increased alpha diversity was associated with reduced negative emotionality and fear reactivity in infants. This is a substantial finding considering that elevated levels of negative emotionality have been linked to increased risk for anxiety, depressive symptoms, and autism spectrum disorder later in life (Aatsinki et al., 2019).

Experimental studies of humans typically involve administering probiotics to increase gut microbial diversity and monitoring the physiological or psychological effects. Results have been inconsistent when evaluating the relationship between probiotics and psychological benefits. A study of males 18-40 years old did not find any significant effects of probiotic treatment on levels of anxiety, mood, or stress (Kelly et al., 2017). However, a review paper on gut microbiome and social behavior discussed several studies which found that self-reported negative mood decreased with administration of psychobiotics, a class of probiotics thought to provide mental health benefits (Sarkar et al., 2020). The mood-altering effects were accompanied by a reduction in cortisol levels, demonstrating psychological and physiological benefits of psychobiotics (Sarkar et al., 2020).

Gut dysbiosis has been found in those with major depressive disorder (MDD) and autism spectrum disorder. In a study of patients 18-40 years old with active MDD, the depressed patients exhibited marked differences in gut microbiome composition. The researchers found elevated levels of *Acidaminococcaceae*, *Enterobacteriaceae*, *Fusobacteriaceae*, *Porphyromonadaceae*, and *Rikenellaceae* families in depressed patients compared to healthy controls, and reduced levels of *Bacteroidaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae*, and *Veillonellaceae* (Jiang et al., 2015). In patients with autism spectrum disorder, researchers found a significant increase in *Lachnospiraceae, Clostridiales, Erysipelotrichaceae, Dorea, Collinsella, and Lachnoclostridium* genera along with a decrease in *Bacteroides*, *Faecalibacterium*, *Parasutterella*, and *Paraprevotella* compared to controls (Ding et al., 2020). It should be noted that there is no standardized gut microbial profile for patients with depression or autism spectrum disorder. Compositions can vary greatly, and the literature often presents contradictory findings between studies. Nonetheless, a consensus exists in human studies that there is a connection between gut microbial composition and psychiatric and neurodevelopmental disorders.

To further understand the processes that underlie these disorders in humans, animal research should be evaluated for an association between temperament and gut microbial diversity.

Animal studies

The effects of gut microbial manipulation on the brain, behavior, and disease can be more thoroughly examined through animal studies. For example, studies have shown that germ-free

(GF) status in rodents impairs regions of the brain associated with social behaviors such as the amygdala, prefrontal cortex, and hippocampus (Sarkar et al., 2020). In the amygdala, a brain region closely tied to anxiety and fear responses, GF mice had altered expression of genes involved in synaptic activity, neural transmission, and nervous system development (Hoban et al., 2017). These results suggest that gut dysbiosis may play a role in neuronal functioning. These structural brain transformations represent a potential mechanism for why mental health can be altered from gut dysbiosis. There is also evidence of GF status impacting other regions of the brain involved in the stress response. In a hallmark study, Sudo and colleagues (2004) discovered a heightened HPA axis response to restraint stress in GF mice. Interestingly, this effect was reversed after colonizing the GF mice with *Bifidobacterium infantis* (Sudo et al., 2004). This is one of the major studies demonstrating a connection between gut microbial imbalance and stress-related endocrine signaling. These changes in brain structure and functionality have potential downstream effects on mental health. The disruption of typical social behaviors was demonstrated in a study conducted on rats where GF status impaired social investigation and was associated with greater anxiety-like behaviors in the Open Field Test (OFT) (Crumeyrolle-Arias, 2014). In contrast to GF rats, mice display anti-anxiety and anti-depressive behaviors when deprived of a gut microbiome (Luo et al., 2018). Not as many studies are conducted on wild rodents, but consistent with patterns in other studies, when the wild mouse gut microbiome was depleted with antibiotics, exploratory behaviors in the OFT increased, indicating lower anxiety-like behaviors (Jameson et al., 2020).

In addition to effects on brain and behavior, animal studies have also shown associations between psychiatric diseases and gut microbial diversity. Fecal microbiota transplant (FMT) from human patients with depression to GF rats induced depressive-like symptoms such as

anhedonia along with anxiety-like behaviors in those animals (Kelly et al., 2016). Regarding behavioral disorders such as autism, when administered antibiotics in adulthood, rats exhibited autistic-like behaviors through social behavioral abnormalities (Mintal et al., 2022). FMT has also been used in the murine model of autism where a fecal transplant from autistic-like mice induced autistic-like phenotypes in neurotypical mice (Sarkar et al., 2020).

The output of animal studies must keep pace with that of human gut microbiome research as they offer benefits not found in human studies such as larger sample sizes and a high level of experimental control.

Framework and Utility of Current Study

The subjects of this study were male Sprague-Dawley rats. This species is known for its sociability which makes them prime candidates for the study of social behavior. Two additional characteristics are the stability of their within-individual behaviors and the demonstrable behavioral variability among individuals. This enables us to document clear differences in behavioral profiles among rats that are consistent over time. Although studying both males and females would increase the generalizability of the study, focusing solely on males eliminates the need to control for variations in female hormone levels.

There are several ways in which the current study can contribute to the literature in this field. Firstly, this study presents a rare opportunity to determine if gut microbial diversity is related to social temperament even in animals that live in isolation. Since studies have shown that larger social networks contribute to increased gut microbial alpha diversity (Johnson et al., 2020), isolation will reduce the influence of social interaction on the gut microbiome. Isolated

housing will also limit exploratory behaviors which have been associated with gut microbial dynamics in previous research (Jameson et al., 2020). An additional benefit of the study is that although the germ-free model of rodent studies offers a high degree of experimental control for variability in individual microbiome profiles, it possesses a low degree of generalizability given that this does not occur in nature. Finally, the current study will contribute to the literature on socially bold and behaviorally inhibited temperaments in rats, which in its current state is quite sparse.

Given these deficits in the literature, the primary question driving this study is: Does there exist a relationship between gut microbial species richness in rats and temperaments associated with mental health outcomes in humans? The two phenotypes we analyzed were Social Boldness and Behavioral Inhibition. We hypothesize that Social Boldness is associated with increased species richness, and conversely, that Behavioral Inhibition is associated with reduced species richness. Based on data from the novel social, partner preference, and novel physical behavioral tests, we assigned each rat to temperament category indicative of their level of Social Boldness and then assigned them according to their level of Behavioral Inhibition. The data and temperament assignments were used to create correlations and ANOVAs that examined their relationship to gut microbial alpha diversity. Alpha diversity refers to the mean diversity within a single sample. Species richness is the measure of alpha diversity analyzed in the current study that represents the number of different species present in a sample. In the current study, Shannon Index and Faith's Phylogenetic Diversity were the two metrics used to assess species richness from rat fecal samples.

Chapter 2

Methods

Animals

A total of 54 adult, male Sprague-Dawley rats were tested, all of whom were 60 days of age (onset of adulthood) at the beginning of the experiment. Rats were housed individually in plastic cages with dimensions of 43.5 x 23.5 x 20.5 cm. They were maintained on a 12:12 h light:dark schedule with lights off at 10:00 AM and on at 10:00 PM Eastern Standard Time. The colony room temperature was set at 22 ℃ with 50% humidity. Each rat's cage was supplied with a red tube and wooden chew stick for enrichment. Food and water were provided continuously. After arrival in the lab, two weeks were allotted to allow the rats to adjust to housing conditions. During this time, the rats were handled daily to prepare for the experiment. All methods were approved by the Institutional Animal Care and Use Committee of Pennsylvania State University.

Behavior Tests

Three behavior tests were conducted to characterize temperaments in the study rats, including the novel social, novel physical, and partner preference tests. There were three rounds of testing with each test repeated at each round. Each individual test occurred over the course of three days to test all animals. Several measures were taken to minimize order effects. First, rats were tested in a random order. Second, those that were tested on the last day for one test were not tested on the first day of the next test. And third, the order in which the three tests were administered was randomized for each round of testing. The second round of testing was

administered three weeks after the initial round. The third and final round was conducted 4 months later. The rats were approximately 85, 100 and 220 days of age at each respective round. For the current study, we only accounted for data from the first two rounds of testing given that the third round occurred relatively late in the experimental timeline.

All tests lasted for 5 minutes and were administered in a separate testing room during the dark phase of the light cycle when the rats were most active, 3-5 hours after the lights had been turned off in the colony room. The arena that was used for all behavior tests was 120 x 120 cm with 46 cm high walls and the floor was covered with sawdust bedding. The rats' enrichment tubes were used to transport them from their home cage to the testing arena as this method has been shown to reduce anxiety-like behavior (Hurst & West, 2010). Behaviors were recorded immediately upon placement of the rat in the arena. After the test was completed, the rats were returned to their home cage and the arena was cleaned of any fecal pellets. The behavior coding software, AnyMaze was used to analyze behaviors from the novel social, novel physical and partner preference tests. The behaviors measured in each of the three tests are documented in Table 1 below.

Table 1. List of Behaviors Recorded in Each Test

Behavior Test:	**Behaviors Recorded:**	
Novel Social	Social Cage:	Number of approaches
		Time in proximity (s)
		Latency to approach
	Empty Cage:	Number of approaches

		Time in proximity (s)
		Latency to approach (s)
	Center of arena:	Distance traveled
		Number of entries
		Time spent (s)
		Latency to enter (s)
Novel Physical	Objects:	Number of approaches
		Time in proximity (s)
		Latency to approach first object (s)
	Center of arena:	Distance traveled
		Number of entries
		Time spent (s)
		Latency to enter (s)
	Periphery of arena:	Distance traveled
		Number of entries
		Time spent (s)
		Latency to exit (s)
Partner Preference	Familiar rat cage:	Number of approaches
		Time in proximity (s)

		Latency to approach
	Center section of arena:	Number of entries
		Time spent (s)
		Latency to exit (s)
	Unfamiliar rat cage:	Number of approaches
		Time in proximity (s)
		Latency to approach

Novel Social Test

This test helped assess both Social Boldness and Behavioral Inhibition in the study rats. The study rat was placed into the arena which contained two cages that were placed in opposite corners of the arena. A novel social partner was housed in one cage and the second cage was empty. Each study rat was moved from its home cage into an open corner of the arena while still inside its enrichment tube. The number of approaches, latency to approach, and time spent in proximity to the empty cage and the social cage were recorded for each rat. When evaluating behaviors in the center of the arena, the distance traveled, the number of entries, time spent, and latency to enter were recorded.

Novel Physical Test

This was the second test used to assess Behavioral Inhibition. Three novel physical objects were placed in three corners of the arena. The study rat was moved from its home cage to the open corner of the arena inside its enrichment tube. The number of approaches to novel objects, time spent in proximity to them, and latency to approach the first object were recorded. Behaviors measured in the center of the arena were the distance traveled, number of entries made, time spent, and latency to enter. In the periphery of the arena, the distance traveled, number of entries, time spent, and latency to exit were recorded.

Partner Preference Test

This was the second test used to measure Social Boldness. The arena contained two cages, one containing an unfamiliar social partner and the other containing a familiar social partner. The arena was divided into three sections with passage available for the study rat to travel between sections. The two cages were placed in two different side sections of the arena. The study rat was placed into the center section of the arena inside its enrichment tube. The number of approaches, time spent in proximity, and latency to approach the familiar and unfamiliar cages were assessed. The number of entries, time spent, and latency to exit the center of the arena were measured as well.

Factor Analysis

Because there is a plethora of behaviors associated with socially bold temperament, we used factor analyses to find behaviors that regularly covaried within each test and reduce the number of variables in the study. For instance, in the novel social test, one of the behaviors indicative of socially bold temperament is the number of approaches made to the novel partner. This behavior covaries with the amount of time the rat spent in the center of the arena. The data for both behaviors along with several others are compiled into a social boldness factor score for each test. A graduate student in the lab conducted factor analyses at each of the three time points to determine whether the same behaviors covaried within a test over time. Factor analyses were used in the quantification of social boldness, but not behavioral inhibition. Latency to approach on the novel physical and novel social tests was used instead to quantify this temperament.

Categorizing Behaviors

Using data from the novel social and partner preference tests, each rat was categorized as being socially bold, mixed, or socially un-bold. Social boldness factor scores were used from timepoints 1 and 2 of these two behavior tests. This made for a total of four scores which were used to categorize rats based on social boldness. The median score for each of the four tests was calculated. If an individual's factor score for the novel social test from time point 1 was greater than the median, then that rat was deemed to exhibit socially bold behavior for that test. If the rat's score was lower than the median, then it was deemed socially un-bold on that test. If the rat was deemed as socially bold for at least three out of four tests, then it was categorized as socially bold in terms of temperament. The same process was repeated to categorize socially un-bold rats.

If a rat was deemed as socially bold on two tests and socially un-bold on the other two tests, then it was categorized as "mixed".

A similar method was used to categorize the rats as being behaviorally inhibited, mixed, or not behaviorally inhibited. To categorize the rats based on this temperament, data from the first two time points of the novel social and novel physical tests were used. Factor analyses were not conducted for behavioral inhibition, so latency values were used instead. Latency is defined as the amount of time it takes a rat to approach a novel social partner or a novel object once placed in the arena (Cavigelli et al., 2007). Once again, data were evaluated from four tests and the median score for each test was calculated. If an individual's latency was greater than the median, it was deemed behaviorally inhibited for that test. If their latency was lower than the median, the individual was designated as not behaviorally inhibited for that test. If a rat was identified as behaviorally inhibited or not behaviorally inhibited for at least three of the four tests, it was categorized under that respective temperament. If a rat was deemed as behaviorally inhibited on two tests and not behaviorally inhibited on the other two tests, it was categorized as mixed.

Fecal Sample Collection

To analyze gut microbial diversity, fresh fecal samples were collected from rats and stored at -80º C until analysis. Samples were sent to the Lamendella Lab at Juniata College for 16S RNA analysis. The Shannon index was calculated at the family level and is a measure of biodiversity that accounts for both richness and evenness, but gives more weight to species richness. Richness is indicative of the number of species in a given sample. Faith's Phylogeny

values were also calculated by the Lamendella Lab. Faith's Phylogenetic Diversity measures biodiversity based on the sum of the branch lengths on the phylogenetic tree that a set of species covers. Higher values for the two metrics indicate a higher level of species richness. Accounting for these two metrics allowed for a more complex measure of microbial diversity for each individual.

Data Analysis

To analyze the data, 4 correlations and 4 one-way ANOVAs were conducted in SPSS. To determine the relationship between gut microbial diversity and Social Boldness, the average Social Boldness score across the four tests was calculated for each individual. A correlation was conducted between the Shannon Index and the average Social Boldness score, and another correlation was run between the Faith's Phylogenetic Diversity value and the average Social Boldness score. Scatterplots for each correlation were also generated.

In running the correlations between the gut microbiome diversity and Behavioral Inhibition, the average latency values across the four tests were calculated for each individual. When initially running the correlations, it was found that the average latency scores were not normally distributed (See Appendix). To counteract this, the natural log of the average latency scores were used in the correlations for Behavioral Inhibition versus biodiversity metrics. One correlation was conducted between Shannon Index values and the natural log of the average latency while another correlation was conducted between Faith's Phylogenetic Diversity values and the natural log of the average latency. Scatterplots were then generated for both correlations.

To determine if temperament was related to gut microbiome in a non-linear fashion, 4 one-way ANOVAs were conducted on the relationship between Social Boldness categories and Shannon index values, Social Boldness categories and Faith's Phylogenetic Diversity values, Behavioral Inhibition categories and Shannon Index values, and Behavioral Inhibition categories and Faith's Phylogenetic Diversity values.

Chapter 3

Results

Social Boldness

In Figure 1 comparing the average Social Boldness score to the Shannon Index, the slope of the line of best fit is 0.06. Although this demonstrates a minute positive trend in the data, the correlation was not statistically significant as the p-value of the correlation was 0.236 which is greater than the alpha level threshold of 0.05 (R_{52} = 0.167; p-value = 0.236). The relationship between Faith's Phylogenetic Diversity values and the average Social Boldness score was also not statistically significant as evidenced by Figure 2. The slope of the line of best fit was -0.03 and the p-value of the correlation was 0.927 (R_{52} = -0.013; p-value = 0.927). The p-value of the ANOVA for the comparison of Social Boldness temperament categories and Shannon Index values was 0.390, indicating no significant relationship between the level of Social Boldness and species richness ($F_{2,51}$ = 0.961; p-value = 0.390). Figure 3 is a graph of the Shannon Index values classified by the Social Boldness category. The graph shows that the socially bold rats had a slightly higher mean Shannon Index value, but as previously stated, the results were not statistically significant. The results of the ANOVA for the comparison between Faith's Phylogenetic Diversity values and the level of Social Boldness show that the p-value was 0.956, suggesting that there is no relationship between the level of Social Boldness and species richness as demonstrated by Faith's Phylogenetic Diversity values ($F_{2,51}$= 0.961; p-value = 0.956). The graph for this relationship shows that the average Faith's Phylogenetic Diversity values were nearly identical for all three levels of Social Boldness temperament (Figure 4).

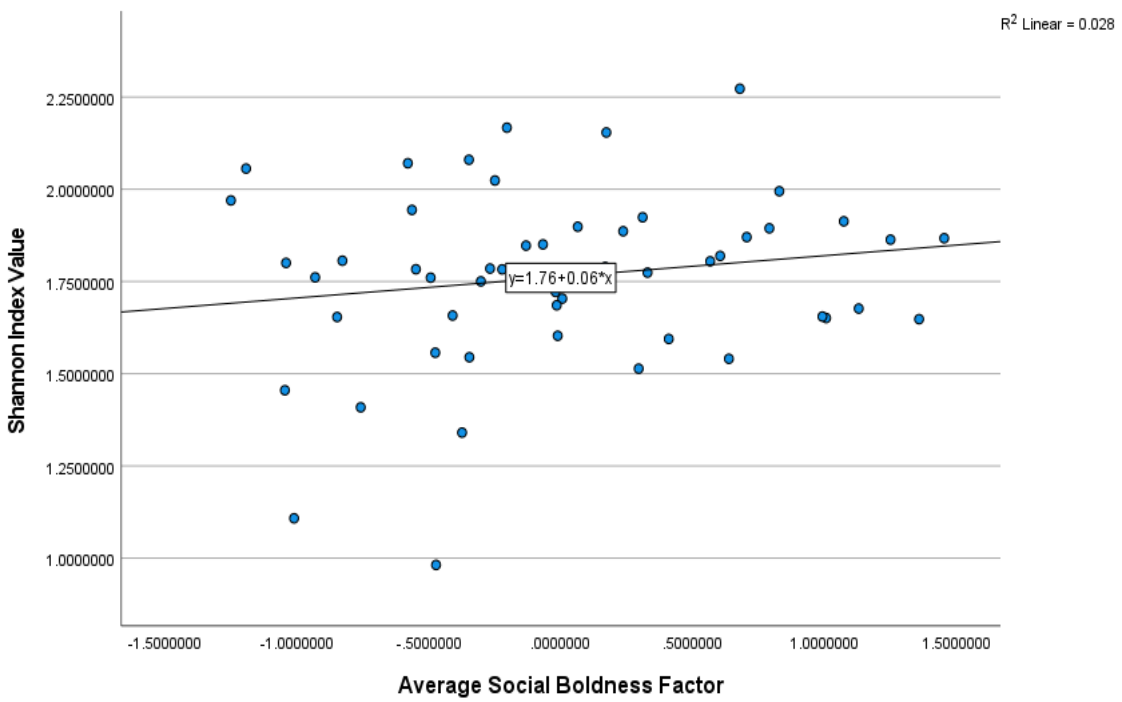


Figure 1

This scatterplot displays the relationship between the Shannon Index values and the average social boldness score for all rats. (R_{52} = 0.167; p-value = 0.236)

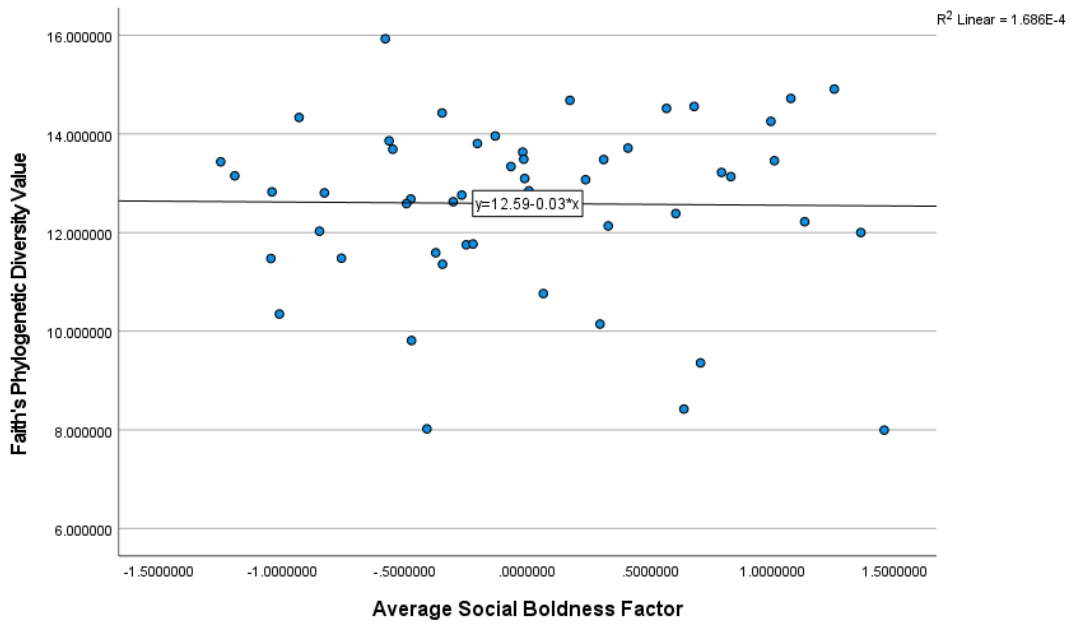


Figure 2

This scatterplot shows the association between Faith's Phylogeny values and the average social boldness score for all rats. (R_{52} = -0.013; p-value = 0.927)

Relationship Between Mean Shannon Index value and Social Boldness Categories

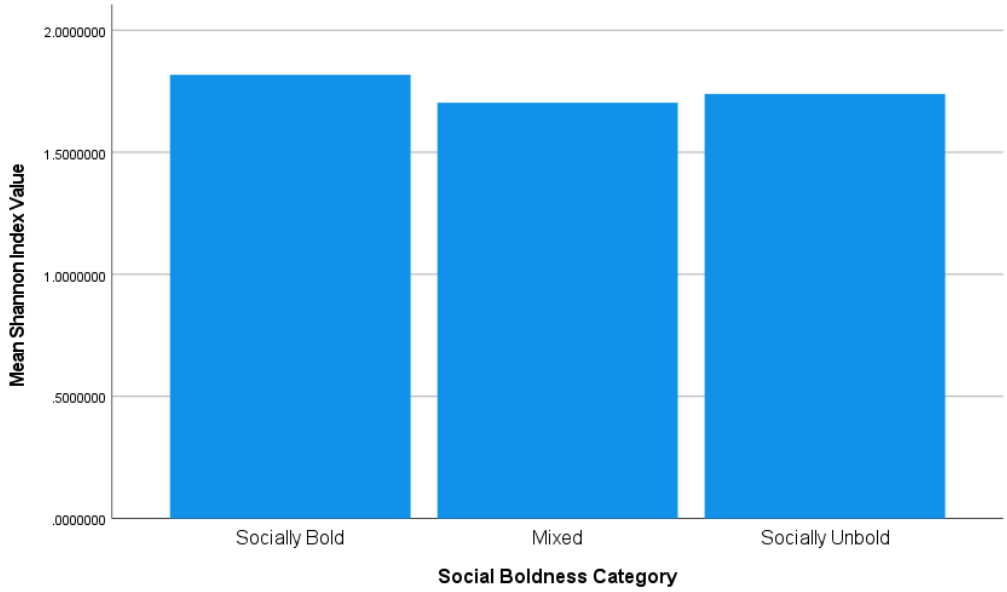


Figure 3

The mean Shannon Index values for each of the social boldness temperament categories. Socially bold rats have slightly greater mean Shannon Index values. ($F_{2,51}$ = 0.961; p-value = 0.390).

Relationship Between Mean Faith's Phylogenetic Values and Social Boldness Categories

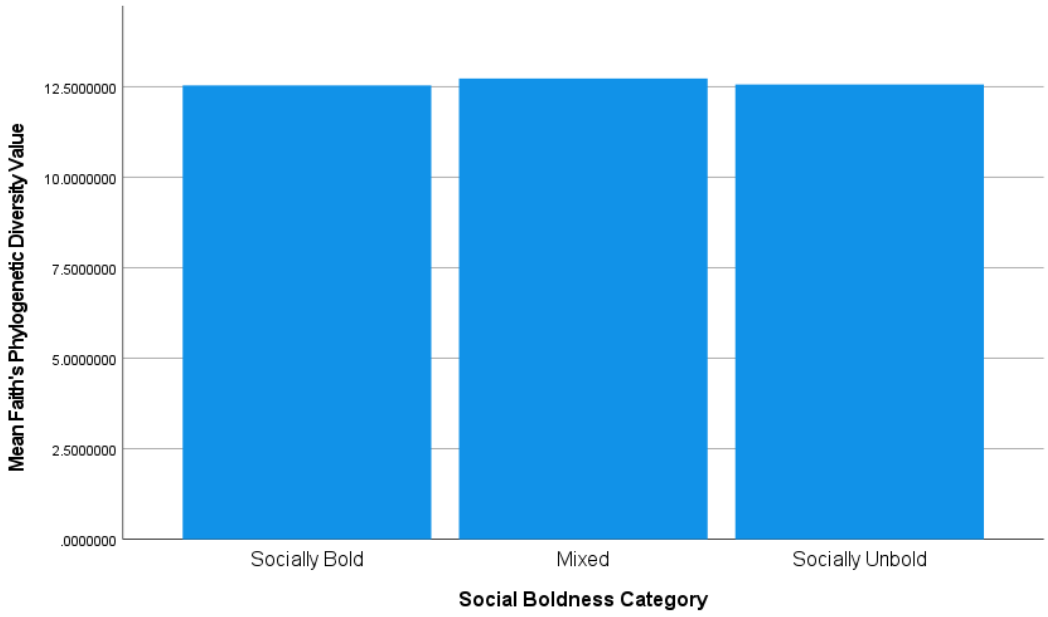


Figure 4

The mean Faith's Phylogenetic Diversity values for each of the social boldness temperament categories is shown in this graph. Little differentiation is observed between the groups. ($F_{2,51}$= 0.961; p-value = 0.956).

Behavioral Inhibition

For the second hypothesis on Behavioral Inhibition as it relates to measures of species richness, two correlations and two ANOVAs were once again conducted. Figure 5 displays the relationship between the natural log of the average latency scores from the novel physical and novel social tests and the Shannon Index values. There is a slight negative trend in the data with a slope of -0.06 for the line of best fit. However, this correlation was not statistically significant as demonstrated by the p-value of 0.125. This indicates that there is no association between Shannon Index and Behavioral Inhibition (R_{52} = -0.162; p-value = 0.125). For the association between Faith's Phylogenetic Diversity values and latency scores measuring Behavioral Inhibition, the results are shown in Figure 6. The scatterplot shows that there is a slight negative trend in the data, meaning that as species richness decreases, measures of Behavioral Inhibition increase. The slope of the line of best fit is -0.44, but the relationship was not statistically significant as shown by the p-value of 0.101 (R_{52} = -0.180; p-value = 0.101). Figure 7 shows very similar mean Shannon Index values between the behaviorally inhibited, not behaviorally inhibited, and mixed groups. As evidenced by the results of the ANOVA, there was no significant relationship between behaviorally inhibited temperament categories and Shannon Index values given that the p-value is 0.885 ($F_{2,51}$ = 0.122; p-value = 0.885). Figure 8 displays the relationship between Behavioral Inhibition category and the mean Faith's Phylogenetic Diversity values. The behaviorally inhibited rats had a slightly lower mean Faith's Phylogenetic Diversity value compared to the mixed and not behaviorally inhibited rats. However, the results of the ANOVA demonstrate that there is no significant relationship between the level of Behavioral Inhibition and mean Faith's Phylogenetic diversity values ($F_{2,51}$ = 1.729; p-value = 0.188).

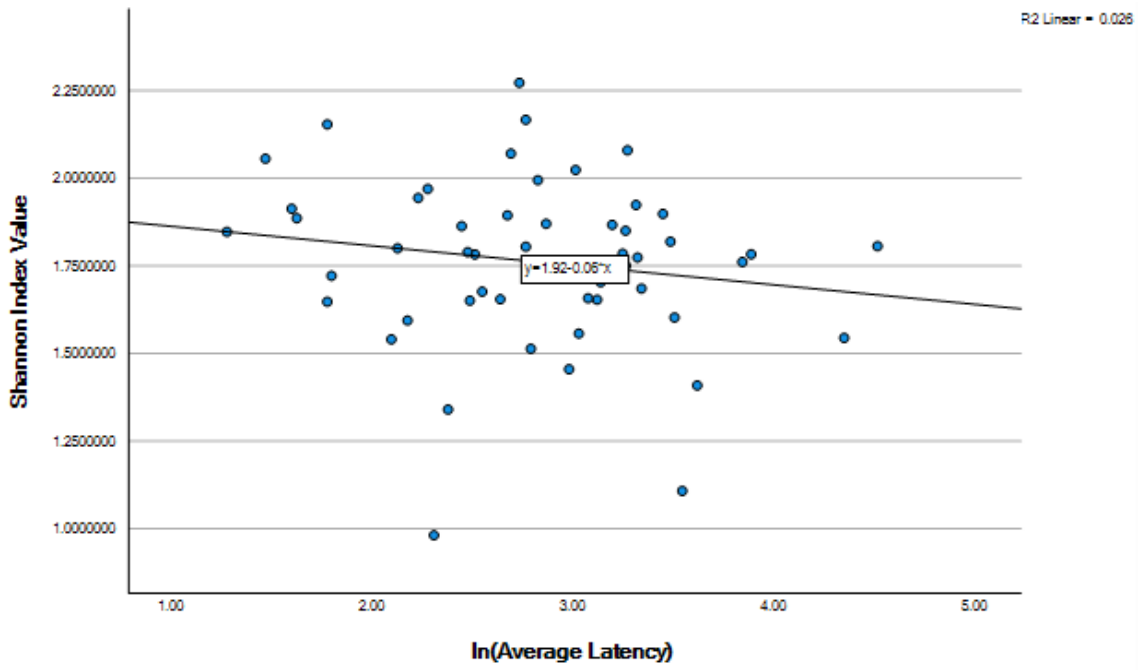


Figure 5

This scatterplot displays the correlation between Shannon Index values and the natural log of the average latency to approach a novel object or novel social partner. The average latency scores were compiled from the first two timepoints of the novel physical and novel social tests. This is meant to demonstrate the association between behavioral inhibition and species richness. (R_{52} = -0.162; p-value = 0.125)

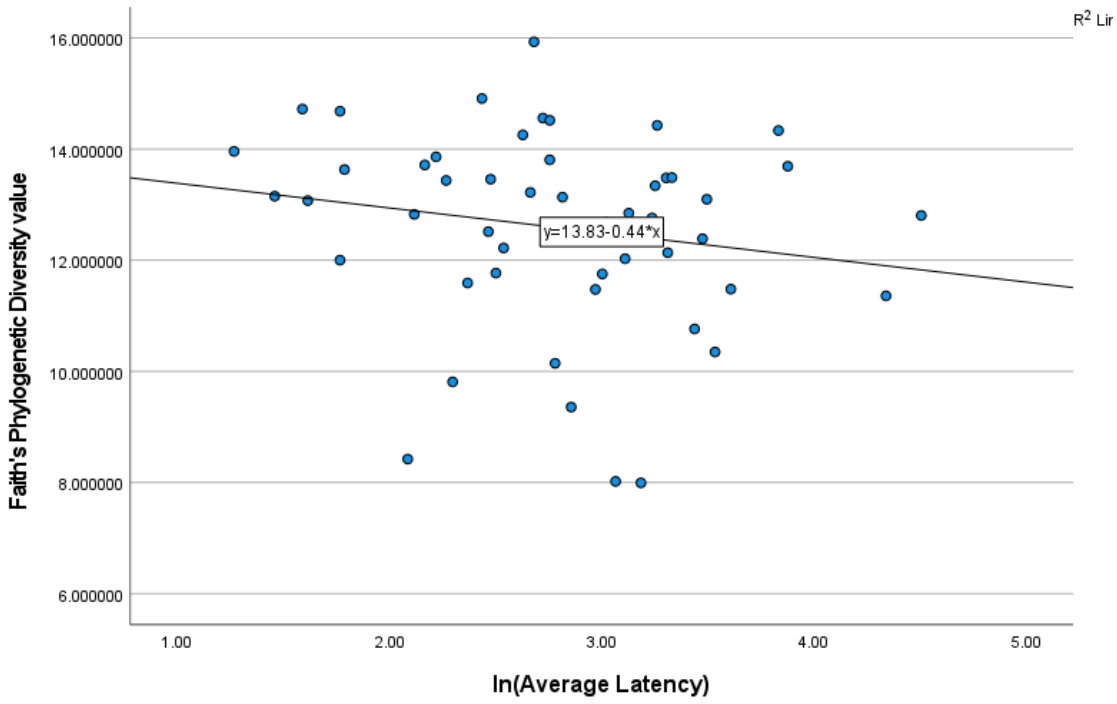


Figure 6

This scatterplot shows the relationship between the Faith's Phylogenetic Diversity values and the natural log of average latency to approach novel objects and novel social partners. Once again, this is meant to demonstrate the association between behavioral inhibition and species richness. (R_{52} = -0.180; p-value = 0.101)

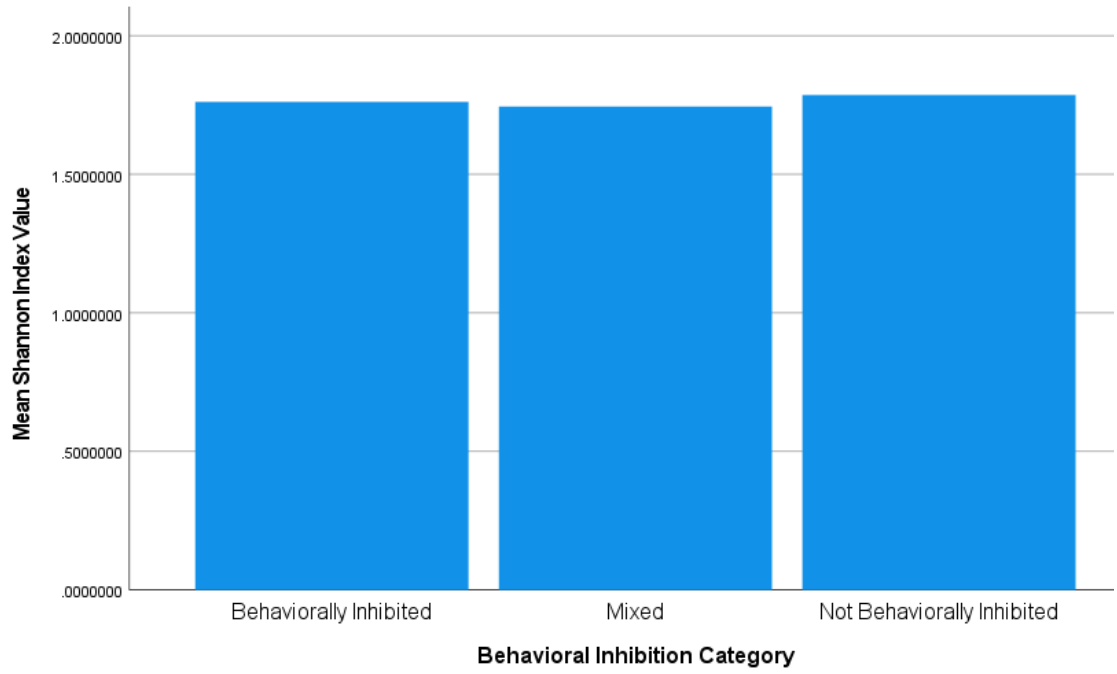


Figure 7

This graph shows little differentiation between the behaviorally inhibited, not behaviorally inhibited, and mixed rats. The rats that were not behaviorally inhibited had a slightly greater mean Shannon Index value. ($F_{2,51}$ = 0.122; p-value = 0.885)

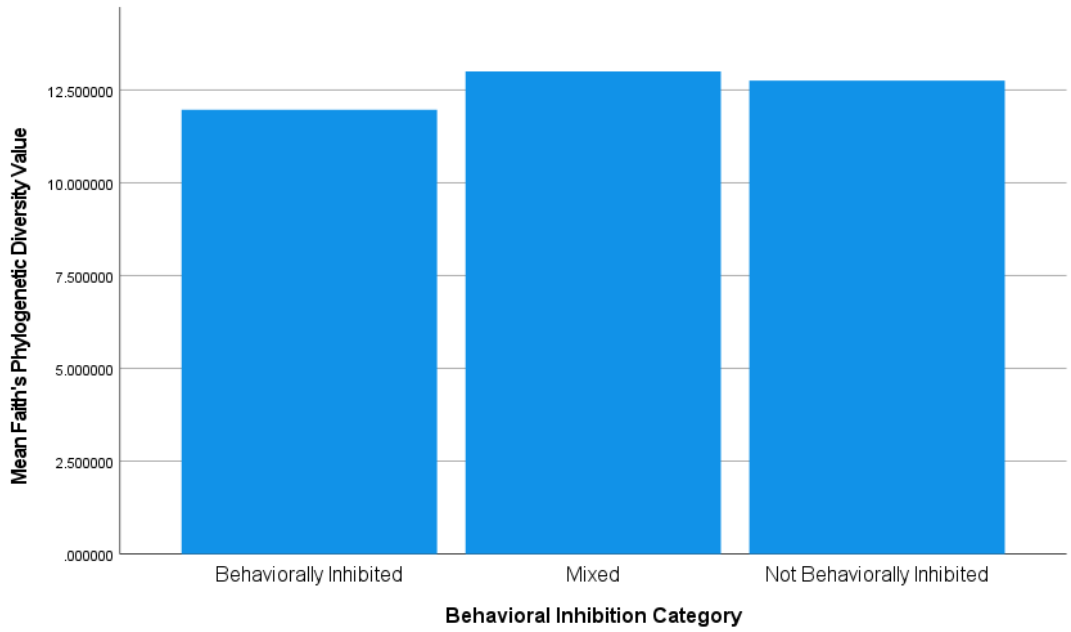


Figure 8

The mean Faith's Phylogenetic Diversity values for each of the behavioral inhibition temperament categories is shown in this graph. Behaviorally inhibited rats had a slightly lower level of species richness as indicated by the Faith's Phylogeny values. The rats that were mixed and not behaviorally inhibited had slightly higher mean Faith's phylogeny values. ($F_{2,51}$ = 1.729; p-value = 0.188)

The results of all correlations and ANOVAs refute my initial hypotheses, suggesting there is no relationship between gut microbial species richness and socially bold or behaviorally inhibited temperaments.

Chapter 4

Discussion

Interpretation and Comparisons

The current study determined if gut microbial diversity was related to temperament with the goal of ascertaining if gut microbial diversity could explain the relationship between temperament and mental/behavioral health outcomes. These studies were conducted in a naturalistic, observational manner. A series of behavior tests were used to classify the rats into temperament categories pertaining to social boldness and behavioral inhibition, two traits that have been associated with mental health outcomes in humans. The data from the tests and the temperament classifications were used to find a potential association with species richness, a measure of gut microbial alpha diversity.

Correlational analyses and ANOVAs used to test the associations between Social Boldness and gut microbial species richness demonstrated no significant relationship between the two variables. This refutes the first hypothesis that greater measures of Social Boldness predict greater levels of species richness in the gut. Evidence in the literature supports that behaviors indicative of Social Boldness are associated with the gut microbiome. Other studies do not explicitly use the term Social Boldness, often opting for terms such as “boldness” or “social behavior,” but do conduct similar tests and measure similar behaviors to the current study. For example, several mouse studies have found relationships between social behavior and gut microbial status. In a test similar to the Partner Preference test that was used in the current study, GF mice demonstrated decreased preference for a novel partner compared to controls and in the

three-chambered sociability test, germ-free mice displayed increased social avoidance (Desbonnet et al., 2014). Additionally, mice with lower gut microbial diversity have shown reduced preference for interacting with a novel social partner versus a familiar partner compared to controls (Buffington et al., 2016). These studies examined behaviors indicative of Social Boldness and demonstrated that these traits are associated with gut microbiota both in and outside of the germ-free model.

The second set of results of the current study indicated that measures of Behavioral Inhibition were not associated with species richness either, refuting the second hypothesis that increased Behavioral Inhibition is associated with reduced species richness. Unlike the current study, other research has found evidence of a relationship between the gut microbiome and traits related to Behavioral Inhibition. In the previously mentioned study from Crumeyrolle-Arias and colleagues (2014), GF rats displayed significantly less time sniffing a novel social partner in the first two minutes of the social interaction test. This increased latency to approach a novel partner is a trait indicative of Behavioral Inhibition. In the same study, GF rats displayed increased latencies in the Open Field Test (OFT), reflective of anxiety-like behavior (Crumeyrolle-Arias, 2014). The researchers in the Crumeyrolle-Arias study may have found different results due to their use of germ-free rats The interaction of gut microbes with other physiological systems is incredibly complex; therefore, the presence of any microbes may lead to behavioral differences compared to GF animals. In addition to evidence in rats, when Balb/c mice, a habitually anxious strain, are colonized with microbes from the bold and exploratory NIH Swiss strain, the Balb/c mice begin to display similar behaviors to the NIH Swiss mice (Bercik et al., 2011). Although the experimental design is quite different from the current study, the results from Bercik and colleagues show that temperament is altered by changes in microbial profile. Moreover,

temperaments that are associated with negative mental health outcomes can be changed through fecal microbiota transplant (Bercik et al., 2017).

The findings on temperament and the gut microbiome in human studies demonstrates that different analyses of the gut microbiome may provide evidence supporting a relationship. In line with the results of the present study, a study on human infants did not find any significant association between species richness indicated by Shannon Index and behavioral temperament (Kelsey et al., 2021). This contradicts the results of the previously mentioned study from Aatsinki and colleagues (2019) who found that increased alpha diversity was associated with reduced negative emotionality and fear reactivity. Notably, the researchers only found a significant association when adjusting for factors such as infant age, sex, mode of delivery, breastfeeding, and antibiotics intake suggesting that an extended level of experimental control may be necessary to observe an association between gut microbial diversity and temperament (Aatsinki et al., 2019). These inconsistencies illustrate that there is not a concrete consensus on whether gut microbial alpha diversity is associated with temperament. However, Kelsey and colleagues (2021) did find an increased abundance of certain bacterial genera in association with negative emotionality and the Aatsinki study (2019) also found associations between negative emotionality and the relative abundance of specific genera of bacteria. These findings suggest that measures of gut microbial diversity may not capture the nuance of microbial profiles, and basing conclusions solely off data on alpha diversity may not reveal the entirety of the gut-temperament connection. Species richness simply represents the number of different species present in the gut; therefore, in future studies, examining the relative abundance of specific taxa may point to associations between the gut microbiome and temperament.

The absence of a relationship between both Behavioral Inhibition and gut microbial species richness and Social Boldness and species richness in the current study does not support the initial speculation that gut microbial diversity is a mechanism by which these two temperaments affect the development of mental and behavioral health disorders. Some research has supported that the gut microbiome may link temperament and mental/neurodevelopmental disorders. Maternal immune activation (MIA) models autism in mice. MIA mice display unconventional behaviors characteristic of autism spectrum disorder such as altered social interaction. However, these behaviors are altered when MIA mice are colonized with B. *fragilis* specifically (Hsiao et al., 2014). Probiotic treatment, which increases gut microbial diversity, has been shown to reduce depressive-like behavior in rats that faced maternal separation (Desbonnet et al., 2010), and have reduced the anxiety-like behavior characteristic of Balb/c mice (Bravo et al., 2011). The animals in these three studies were either bred or manipulated in a way to create temperaments that are more socially inhibited or anxious, reminiscent of Behavioral Inhibition and a deficit of Social Boldness. Each of these studies demonstrated that supplementing the gut microbiome can ameliorate behaviors linked to mental illness and neurological conditions. These findings do not help determine if increasing alpha diversity of the gut will improve symptoms of these conditions given that the introduction of specific genera of bacteria may be necessary to observe these results as is the case with the Hsiao study. Once again, this demonstrates the need to further evaluate relative abundance of certain bacteria and their relationship to temperament.

Strengths, Limitations, and Future Directions

The current study is well designed to evaluate temperament because it uses multiple tests and the behaviors measured through those tests to classify temperament. Previous studies have conducted several tests to quantify behaviors such as latency (Cavigelli et al., 2007) and have detected temperaments such as boldness based on the presence of multiple behaviors (Dingemanse et al., 2007). The current study used two tests each to quantify Social Boldness and Behavioral Inhibition and collected data on numerous behaviors. Other studies only use one type of test to analyze behavioral traits which may not be fully indicative of temperament (Bercik et al., 2011) (Neufeld et al, 2011). Furthermore, using factor analyses addressed the multi behavioral and multi contextual aspects of Social Boldness by combining behaviors across tests into factors (McMahon et al., 2022). Previously mentioned animal studies that evaluate behavior and the gut microbiome have used multiple tests and behaviors to classify behavioral traits but did not document as many behaviors as we did (Crumeyrolle-Arias et al., 2014). Also, they did not use factor analyses to amalgamate behavioral data into latent constructs like the present study did. Therefore, it is possible that other studies did not assess temperament as thoroughly as the current study.

Another strength is the selection of rats to study the relationship between temperament and the gut microbiome. Many studies evaluating this relationship use mice instead of rats. Although mice are easier to manipulate genetically, they do not exhibit as many prosocial behaviors as rats do (Ellenbroek & Youn, 2016). Therefore, it can be more advantageous to study rats when evaluating Behavioral Inhibition and Social Boldness which are both at least partially based on social behaviors. Additionally, the current study did not use germ free rats which may have made the results more translational to humans.

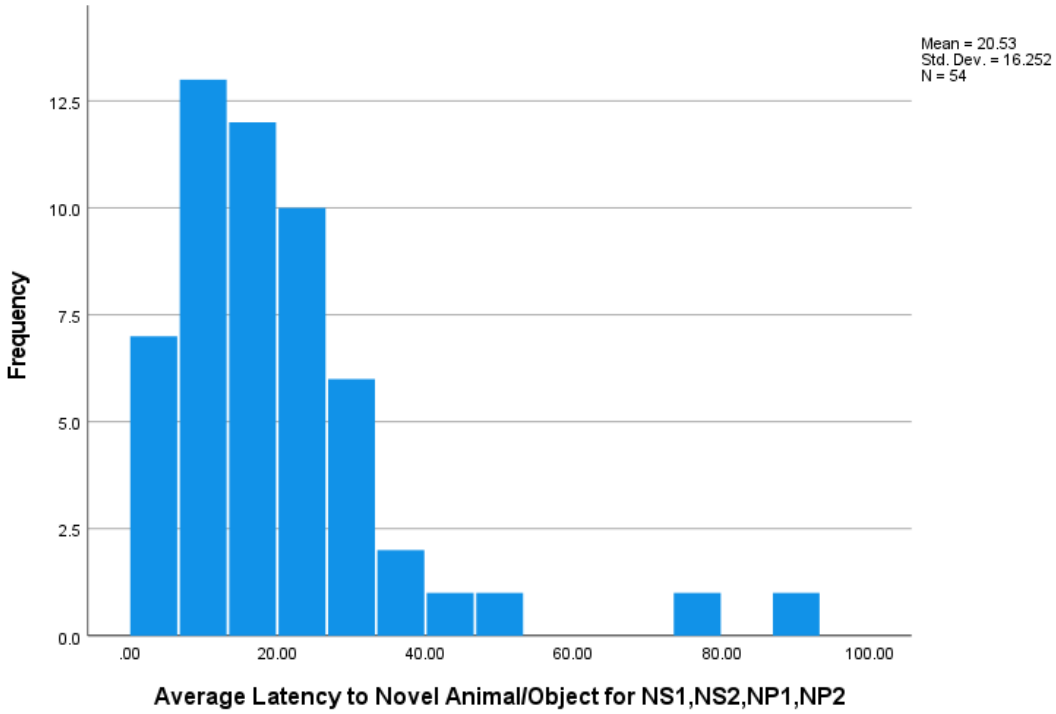
There are several limitations to the current study that could have impacted the results. The current study only evaluated species richness, a metric of alpha diversity, to analyze the gut microbiome. Analyzing alpha diversity alone may not have revealed the extent of the microbiome profile of each rat. Future studies should evaluate the abundance of genera that have been associated with stress, depression, and autism such as *Akkermansia, Prevotella, Bifidobacterium, Clostridium*, and *Lactobacillus* to name a few (Johnson, 2020). The current study is also limited by the lack of research on Behavioral Inhibition or Social Boldness related traits and their relation to gut microbiome diversity specifically in rats. Most of the research in this field has been conducted using murine models. Although it is easier to manipulate mice genetically, rats display more complex social behaviors that are more reflective of humans. This could be useful when studying temperaments and mental health conditions that impact social behavior. Another limitation in the experimental design is that rats were subjected to a series of physiological manipulations as part of a larger study conducted by the lab including LPS injections, blood collection, and corticosterone administration. These stressors could have interfered in typical temperament-microbial dynamics.

Several measures can be taken in the future to improve the current study. Firstly, female rats could be included in the study to increase generalizability of the results. More invasive measures to evaluate gut microbial composition and diversity can be conducted such as cecal analyses. Other potential experimental manipulations include dietary interventions and experimentation in different rat strains or rodent species. Moreover, gene expression of neurotransmitters and their transporters or receptors can be analyzed in the brain in conjunction with gut microbial analyses. Specifically, neurotransmitters implicated in psychiatric conditions

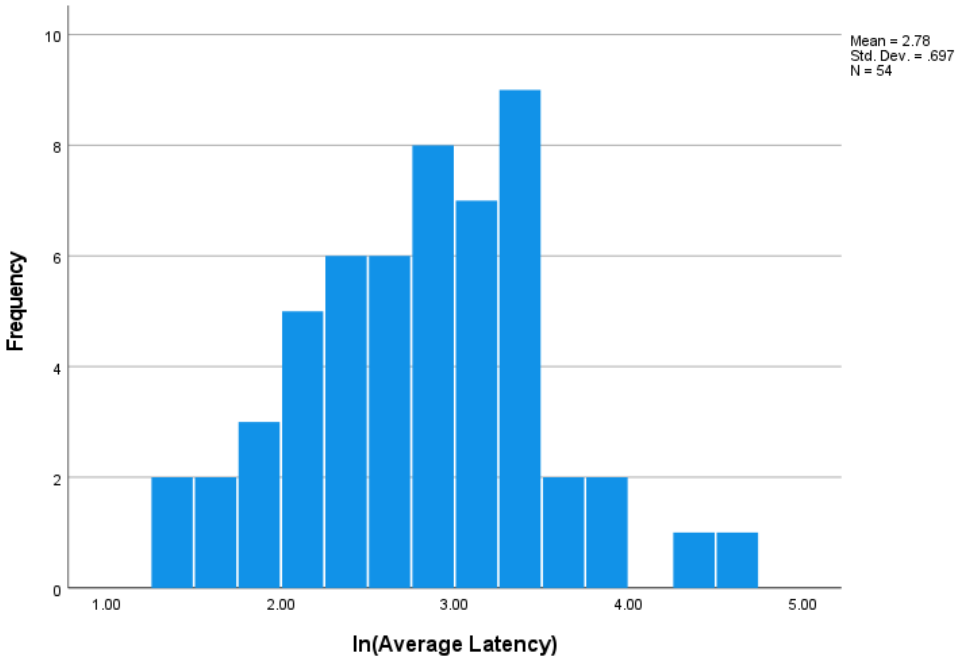
such as serotonin or dopamine. This could help to further understand the complexities of the gut-brain axis and its role in behavior.

The current study yielded no significant findings and did not support the proposed role of gut microbial diversity in the connection between socially bold or behaviorally inhibited temperaments and the development of psychiatric disorders. Yet, there are plenty of modifications to the study that can be made and a plethora of research demonstrating that this is a subject that should continue to be researched. We have shown that certain temperaments are risk factors for the development of neurodevelopmental and mental health disorders; therefore, it is of paramount importance to continue studying the microbiome's role in this as the literature has shown that it may affect a host of human health concerns.

Appendix



Histogram of the average latency values for all animals across the four tests, showing skewed distribution.



Histogram of the natural log of the average latency values for all animals across the four tests, showing a more normal distribution.

Correlations

		Shannon_Index_Value	Average_Social_Boldness_Factor
Shannon_Index_Value	Pearson Correlation	1	.167
	Sig. (2-tailed)		.236
	N	52	52
Average_Social_Boldness_Factor	Pearson Correlation	.167	1
	Sig. (2-tailed)	.236	
	N	52	54

Correlation between average social boldness score and Shannon Index

Correlations

		Faith_Phylogeny_Diversity_value	Average_Social_Boldness_Factor
Faith_Phylogeny_Diversity_value	Pearson Correlation	1	-.013
	Sig. (2-tailed)		.927
	N	52	52
Average_Social_Boldness_Factor	Pearson Correlation	-.013	1
	Sig. (2-tailed)	.927	
	N	52	54

Correlation between average social boldness score and Faith's Phylogenetic Diversity values

ANOVA

ShannonIndex

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.113	2	.056	.961	.390
Within Groups	2.872	49	.059		
Total	2.985	51			

Association between Shannon Index and Social Boldness categories

ANOVA

Faith_Phylogeny_Diversity_value

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.288	2	.144	.045	.956
Within Groups	155.089	49	3.165		
Total	155.377	51			

Association between Faith's Phylogenetic Diversity and Social Boldness categories

Correlations

		ln(Average Latency)	Faith_Phylogeny_Diversity_value
ln(Average Latency)	Pearson Correlation	1	-.180
	Sig. (1-tailed)		.101
	N	54	52
Faith_Phylogeny_Diversity_value	Pearson Correlation	-.180	1
	Sig. (1-tailed)	.101	
	N	52	52

Correlation between average latency and Faith's Phylogenetic Diversity values

Correlations

		ln(Average Latency)	Shannon_Index_Value
ln(Average Latency)	Pearson Correlation	1	-.162
	Sig. (1-tailed)		.125
	N	54	52
Shannon_Index_Value	Pearson Correlation	-.162	1
	Sig. (1-tailed)	.125	
	N	52	52

Correlation between average latency and Shannon Index values

ANOVA

Shannon_Index_Value

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.015	2	.007	.122	.885
Within Groups	2.970	49	.061		
Total	2.985	51			

Association between Shannon Index and Behavioral Inhibition categories

ANOVA

Faith_Phylogeny_Diversity_value

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.241	2	5.121	1.729	.188
Within Groups	145.136	49	2.962		
Total	155.377	51			

Association between Faith's Phylogenetic Diversity and Behavioral Inhibition categories

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ACADEMIC VITA

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EDUCATION

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Minor in French and Francophone Studies
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Full professional proficiency in French
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RESEARCH EXPERIENCE

Research Assistant — May 2022-present
Penn State International Health and Nutrition Equity Lab

- Write case studies on projects funded by the International Fund for Agricultural Development
- Interview teams implementing health interventions in Rwanda, Zimbabwe, Malawi, and Burundi
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Research Assistant
Penn State Behavioral Neuroendocrinology Lab — September 2020-present

- Conducting individual project on relationship between microbiome diversity and temperament
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PSU Global Health Case Competition Team — December 2022-present

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