Examining the interplay of social status and host gut microbiome composition in male zebrafish (*Danio rerio*)

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The gut and the brain, both vastly different in physiologic function, have been linked in a variety of different neurological and behavioral disorders. The bacteria that comprise the gut microbiome communicate with other systems within the body including neurological systems. Specifically, the gut is being associated with the development and onset of many behavioral disorders. Zebrafish (*Danio rerio*) form social relationships of dominants and subordinates, but the relationship between social status and host gut microbiome is unknown. The purpose of this study was to determine if social status impacts the composition of the host zebrafish by examining how the evolution of social status affects the composition and species diversity of the host gut. After initial isolation, male zebrafish were assigned to one of three experiment groups: paired (n=12), isolate (n=6), or communal (n=6). Over the course of fourteen days, the agonistic interactions of paired zebrafish were observed. To examine fish microbiomes, fecal samples were collected from each fish from each group at four different times: during isolation, on day 0, day 7, and day 14 of pairing. After fecal sample processing and 16S rRNA Illumina sequencing, we found that social status affected host gut microbiome composition. More specifically, bacterial composition differed between fish of different social status and time of experiment. These results are indicative that social status in zebrafish can impact the host gut microbiome with potentially similar effects on other social organisms. This supports the assumptions that social factors may be linked to components of the gut.

EXAMINING THE INTERPLAY OF SOCIAL STATUS AND HOST GUT MICROBIOME COMPOSITION IN MALE ZEBRAFISH (*DANIO RERIO*)

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Chapter I

INTRODUCTION

Social behaviors are an important part of the establishment and development of relationships in humans and animal models. These social behaviors vary depending on an individual's health and negative social behaviors can be associated with certain behavioral disorders. Zebrafish serve as an excellent model for behavioral research, as they exhibit certain social behaviors similarly to humans as they form hierarchal relationships. Host behavior in zebrafish involves the development of social hierarchies which is a key aspect in the development of relationships among many animals, including humans [14]. The development of social hierarchies has been studied in relation to neural circuit patterns in the brain associated with aggressive behaviors (dominance, submission) [14]. However, the gut microbiome has only recently been questioned as a contributing factor in neurological processes related to social behaviors [39].

Dominance and aggression in zebrafish have been studied to determine neural circuit patterns in the brain associated with specific behaviors [3]. With that being said, the mechanisms behind dominance and aggression revolve around hormone and neurotransmitter regulation and these behaviors play an important role in developmental success. Further, aggression animals typically establish dominance and have more success in finding food and access to mates. Conversely, animals displaying minimal aggression fall into the hierarchal rank of subordinance and may experience higher levels of stress compared to their counterparts. The effects of stress on an animal are detrimental and have been adversely linked to anxiety-like behavior relevant for survival in zebrafish and other social species [18]. Specifically, reduction of glucocorticoid signaling activity (caused by insufficient hormone availability) in chronic stress models has been

associated with stress-related disorders [19]. Interestingly, the gut microbiota has recently been thought to modulate downstream glucocorticoid receptors in the hippocampus resulting in behavioral abnormalities [20]. Altogether, this highlights the relationship between stress responses, the gut microbiome, and associated behavioral deficits.

While the establishment of dominance hierarchies dictate rank and organization within zebrafish populations, understanding the factors contributing to the formation of social dominance is critical. Although the effects of social dominance and anxiety on brain function have been studied to great extent [14], little effort has been invested in probing the effects of the social dominance on the brain-gut microbiome axes. Knowledge of how social behavior influences the gut microbiome health in zebrafish will provide important insight into the gut-brain relationship in many organisms. Understanding the correlation of neural pathways with specific behaviors in model species is critical to also understanding similar behaviors and social relationships in humans. In addition, understanding the gut microbiome and its relationship to social behaviors may lead to the development of treatment and preventative measures to control behavioral disorders and possibly neurodegeneration.

The gut microbiome is known to play key roles in biochemical functions of vertebrates. Gut bacterial metabolism has been linked to many systemic diseases in humans and plays an important role in immune system pathways [1]. Commensal bacteria within the gut microbiome serves as a line of defense to viral infections through interferon signaling [2]. Along with these known functions of the gut, it has recently been linked to the brain in many neurodevelopmental, behavioral, and neurodegenerative disorders. The gut microbiome produces certain biochemicals (i.e., dopamine and oxytocin) [3] that control certain neural circuit activity responsible for the regulation of social behaviors [4]. This recently discovered link is known as the “microbiota-gut-

brain axis" and dysfunction of this axis has been associated with many behavioral disorders such as autism spectrum disorder, depression, and anxiety [5]. These disorders can have long lasting and detrimental impacts on social behavior, thus, understanding the mechanisms of onset and progression related to the gut microbiome is critical.

The typical gut microbiome consists of both commensal and pathogenic bacteria. While it is known that pathogenic bacteria are more prevalent in gut microbiomes of diseased organisms, research in multiple animal models has shown that a lack of commensal bacteria can also contribute to the detrimental progression of various behaviors and behavioral disorders. In Autism Spectrum Disorder mouse models, the abundance of commensal bacteria species, *Lactobacillus reuteri* is significantly reduced in comparison to wild type mice [3]. Further, *Lactobacillus plantarum* has been used as a probiotic in zebrafish models to reduce stress-related behaviors and prevent stress-induced microbiome dysbiosis [9]. *L. plantarum* modulates anxiety related behavior through the GABAergic and serotonergic pathways, as levels of genes that encode for the GABA-A receptor alpha 1 and serotonin transporters were upregulated in *L. plantarum* treated fish [9]. Moreover, gut bacterial composition influences host behavior through the modulation of Brain-Derived Neurotrophic Factor (BDNF) levels and serotonin metabolism [8]. Probiotic treatment with *Lactobacillus rhamnosus* in zebrafish resulted in differences in shoaling behavior and brain expression levels of *bdnf* and other genes linked to serotonin pathways compared to untreated groups [8]. Serotonin is an important neurotransmitter involved in behavior, while changes in BDNF signaling has been linked to multiple psychiatric disorders and is relevant to behavioral systems regarding stress responses [10, 11]. This evidence supports the idea that certain bacteria can modulate certain neurotransmitter pathways responsible for social behaviors in zebrafish. In addition to the modulation of neurotransmitters, bacteria from the genera Lactobacillus can

activate afferent neurons in the intestine which modulate pain sensation and actions of defense behaviors in response to stress [12]. These relationships between host gut bacteria and neurotransmitter production, supports the idea of a link between the gut microbiome and associated host behavior.

Given the previously known relationships of the gut microbiome and behavior in both mice and zebrafish, this study aims to determine if and how social status in male zebrafish affects the abundance, diversity, and overall composition of the host gut microbiome. Additionally, we aim to analyze those compositional differences and characterize the male zebrafish gut microbiome at multiple time points during social dominance formation. It is predicted that there will be compositional changes between fish of dominance and subordinance and that these compositional changes will evolve as dominance relationships mature.

Thesis Outline

The objective of this thesis is to determine the interplay between social status and host gut microbiome abundance and diversity in male zebrafish. Further, I aim to examine the compositional differences between zebrafish of different social status to determine the role of certain social behaviors in shaping the gut microbiome bacterial communities. The second chapter of discusses the progression of social status in male zebrafish and associated changes in species richness, diversity, and evenness. The second chapter discusses the multivariate patterns of bacterial community composition. The results indicate that there are significant differences in community composition based on both social status and day of experiment.

The third and final chapter aims to discuss the community compositional differences and how they compare to similar zebrafish microbiome research. Altogether, I will summarize how

compositional differences in zebrafish based on social status may contribute to ongoing research surrounding the gut-brain axis related to behavior. While this study offers an insight into the relationship between the social behavior and the gut, more research is needed to characterize bacterial community composition based on social status and evolution of time.

Chapter II

Introduction

The gut and its 100 trillion bacterial inhabitants play a critical role in the overall health and survival of the host individual. The gut controls how our body breaks down food and in turn, how our bodies absorb specific nutrients to produce energy, maintain metabolism, and regulate immune responses. On the other end of the spectrum, the brain and the nervous system are responsible for thoughts, behavior, movement, and quite literally, every process that regulates the body. When two seemingly independent physiological systems are studied together, research is showing the very dependent link between the gut and the brain and how this link can regulate many neurological and behavior-related functions and associated microbial composition.

In zebrafish, social behaviors associated with the formation of dominance relationships have been studied. When paired, zebrafish quickly form dominance relationships that are stable and long lasting . The dominant fish exhibits increased aggression towards the subordinate fish and thus controls the area of the tank. This behavior allows the dominant fish to acquire more food and other resources necessary for survival [14]. The subordinate fish exhibits less aggression and is typically confined to a small area of tank which limits access to resources. The dominant/subordinate relationship and the neurological pathways involved have been previously researched; however, the potential association with the gut microbiome and these neurological pathways involved with social behaviors is less understood.

The adult zebrafish gut microbiome is composed of bacteria within the Proteobacteria, Firmicutes, and Fusobacteria phyla [6]. Pathogenic bacteria within the Proteobacteria phylum are found in significantly lower abundance of healthy human gut microbiomes and higher abundance of Proteobacterial members can be used as a diagnostic for disease and dysbiosis [7]. In zebrafish,

Proteobacteria including pathogenic genera *Vibrio* and *Plesiomonas,* were reduced in probiotic treated fish as opposed to commensal Firmicutes bacterial members [8]. Moreover, the presence of pathogenic bacteria in both zebrafish and humans can indicate microbial dysbiosis and potential health issues.

The development of zebrafish has been researched in multiple aspects, and it has recently been discovered that the gut microbiome composition of host zebrafish changes through the stages of growth. Based on developmental changes, bacterial abundance and diversity change between embryonic and adult stages. Specifically, diversity of gut bacteria decreased significantly over the course of development in terms of the number of Operational Taxonomic Units (OTUs), which are clusters of bacteria that exhibit high sequence similarity of the 16S gene [15, 38]. However, there were not significant differences between stages of adult fish which indicates that major gut microbial changes occur before and during major development changes such as sexual differentiation [15]. Further, bacterial phylum Proteobacteria and phyla's Firmicutes and Fusobacteria are present at all stages of the zebrafish developmental life cycle including the adult stage [6].

When the typical gut microbiome composition or host microbiome development is disrupted, the associated neurological consequences range from behavioral disorders like anxiety [14] to neurodegenerative disorders like Parkinson's Disease [16]. In mouse models, individuals with social deficits (e.g., Autism Spectrum Disorder, ASD) showed a reduction in the commensal bacteria, *Lactobacillus reuteri*, which is been known to modulate oxytocinergic brain circuits associated with social behavior [3]. Interestingly, treatments of *L. reuteri* rescued the social deficits in ASD models and it was discovered that *L. reuteri* works independently of other bacteria in the gut to modulate social behavior via the oxytocinergic system [3]. Oxytocin, a neurohormone is

implicated in regulating many aspects of behavior including social interactions, pair bonding, and sexual reproduction [3]. In addition to oxytocin, a study examining the relationship between the gut and onset of Parkinson's disease, discussed the idea of neurotransmitters associated with social behavior being expressed and regulated by certain bacteria in the gut [16]. In humans, bacterial species, *Bacteroides*, *Parabacteroids*, and *Eserichia*, express GABA (y-aminobutyric acid)-producing pathways which ultimately control the production and regulation of dopamine, a neurotransmitter responsible for emotional responses and behavior [17]. Relative abundance of fecal *Bacteroides* was significantly lower in human patients suffering from depression [17]. Understanding how and why the reduction or absence of *L. reuteri* and other commensal bacteria affect neurotransmitter production can aid in the understanding of the host microbiome's relationship to host behavior, and specifically the establishment of social hierarchy in the zebrafish, *Danio rerio*. Specifically in mice, social behaviors related to dominance and submission contribute to the composition of the gut microbiome [45]. Submissive mice, which spend less time interacting in the maze and has less access to food resources, have a reduction of certain commensal bacteria genera, *Paraprevotella* and *Prevotella* but an increased abundance of pathogenic bacteria belonging to the Rikenellaceae family [45]. Dominant mice, which spend more time interacting in the maze and has more access to food resources have higher abundances of the *Paraprevotella* and *Prevotella* genera and a decreased abundance of a pathogenic bacterial family, Rikenellaceae [45].

Because of these previous findings, not only do I expect to see compositional differences between fish of different social status, but a continuous reduction of commensal bacteria in isolate and subordinate fish. This hypothesis was supported in the fact that community composition differences were observed between fish of different social ranks, and the presence of commensal bacterial was reduced in both subordinate and isolate individuals.

Experimental Methods

Pairing and behavioral analysis:

Male zebrafish (n=24) were isolated in individual tanks for 7 days to minimize the effects of prior social experience. Then, 12 fish were randomly paired and placed into new tanks while 6 fish remained in isolation, and the remaining 6 fish were placed into one new tank to serve as communal controls. Over a period of 14 days, the 6 zebrafish pairs self-established rank and their behavior was monitored and recorded over a course of 14 days (2 weeks). Behavior will be observed visually, and written observations will be made regarding dominant and subordinate behavior among the paired and communal fish. Specifically, the number of attacks and retreats by each fish were recorded during a 5-minute observation period 3 times a week. Tank locations of each fish (top vs. bottom of tank) were monitored and recorded as stressed or subordinate fish tend to stay lower in the tank and more aggressive/dominant fish claim the top of the tank.

Fecal sample collection:

Initial fecal samples were collected from each fish on day 3 of isolation period. This fecal collection before pairing or establishment of behaviors serves as a method of comparison for later fecal collections. After pairing of fish after isolation period of 7 days, fecal samples were collected on day 0 (day of pairing), day 7 (one week after pairing), and day 14 (2 weeks after pairing). Collections were made throughout the progression of social hierarchal development to determine whether the gut microbiome composition of host fish evolves as the establishment of behavioral rank progresses.

On days of fecal collection, fish were removed from tanks and each fish was placed into individual containers to prevent any cross contamination of fecal matter (figure 1). Before pairing, each fish was carefully observed and distinct markings, size differences, and color differences

were noted to ensure easy recognition of each fish upon isolation and fecal sample collection. 2-4 hours after separation and feeding each fish, fecal samples were collected. Fecal samples from each fish were extracted using sterile micropipette tips and were placed into 500 microliters of DNAse free water. Gloves were always worn when handling fecal samples and fish, to minimize any cross contamination. After samples were collected, they were stored at -80 °C until DNA extractions and processing were performed.

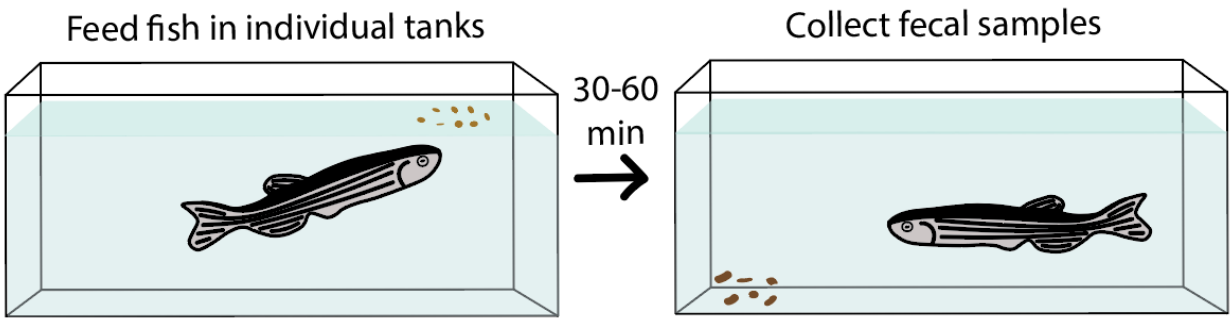

Figure 1. Fecal sample collection methodology. fecal samples were collected 30-60 minutes after fish were fed.

Water sample collection:

To ensure that the tank water bacterial composition was accounted for, two liters of water were collected at two different time points (before and after water system cleaning). Water biomass was collected after 200 mL of collected sample was concentrated on 0.22 μm sterile filters through a vacuum filtration system within 24 hours of collection. Filters containing biomass were stored at -80 °C until extractions and processing were performed.

Microbiome Analysis:

To determine differences in microbial composition in zebrafish based on social status, microbial communities in each fecal sample were characterized with Illumina sequencing of the highly conserved 16S rRNA gene [40]. Following fecal sample collection, DNA extractions were performed on each sample using the PowerLyzer PowerSoil DNA protocol. Approximately 0.02g of feces were collected from each fish for extractions. This extracted DNA was used for PCR reactions, where barcoded primers (515FB-806RB) were used to target the V4-V5 region of the 16S rRNA gene [40, 41]. To ensure the 515FB-806RB primers were working correctly, spot PCR checks using primers, P5 and P7 Illumina adapters were completed after initial PCR. Agarose gel electrophoresis (4%) to examine DNA concentration, and NanoDrop spectrophotometer quantification was completed for all PCR products to ensure amplification of DNA. PCR cleanup using AMPure xp Mg+ beads was performed to ensure any contaminants were removed from the pooled PCR product. After successful cleanup, DNA concentrations (ng/mL) from each sample were quantified using Qubit High Sensitivity (HS) setting. Recorded DNA concentrations (ng/mL) using HS measurements were converted to the final DNA concentration (ng/µl) and then all PCR products were diluted to the same concentration. PCR products were pooled in equimolar concentrations and sequenced using the paired-end Illumina MiSeq platform at the Center for Genomics and Bioinformatics at Indiana University.

Genome sequencing data was obtained and processed using the Mothur standard operating procedure (SOP) analysis pipeline [33]. The sequence contigs were organized and trimmed and sequences were aligned to the Silva Database [42] and the VSEARCH algorithm [43] was used to remove chimeric sequences. All sequences identified as anything other than bacteria were

removed. Using 97% sequence similarity threshold, sequences were clustered into operational taxonomic units (OTUs).

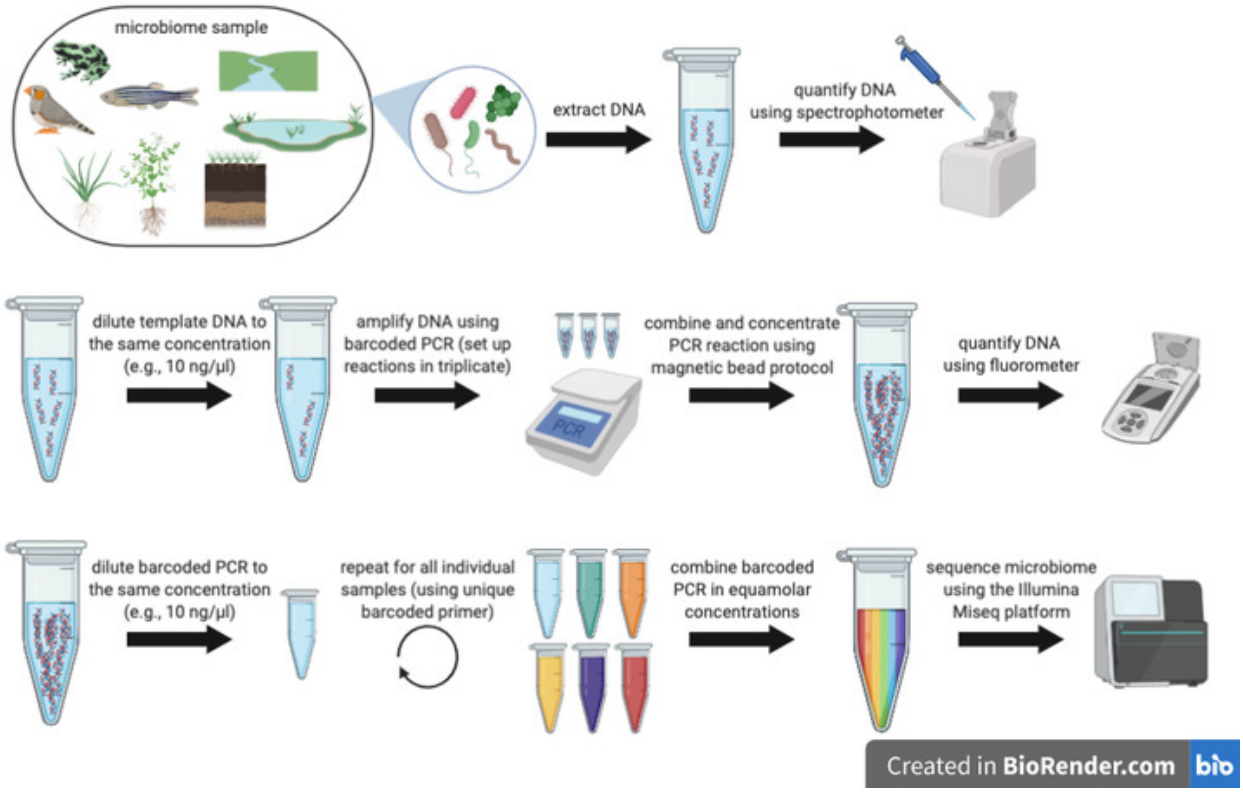

Figure 2. Experimental design of microbiome analysis protocol representing the individual steps necessary for preparing a microbiome fecal sample for microbiome sequencing via Illumina Miseq platform. After DNA extractions, the 16S rRNA gene from each sample was amplified using barcoded PCR and all products were diluted to equimolar concentrations for sample pooling and sequencing.

Compositional Analysis:

Taxonomic approaches were used to characterize diversity and composition of bacterial communities in each fecal sample. We first determined alpha diversity using Shannon's Diversity after we rarefied the OTU table to 6,000 observations. Bray-Curtis distance was calculated for taxonomy compositions were used to analyze bacterial community composition in each sample and at different time points. These differences in composition were observed with Principal Coordinate Analysis (PCoA).

Statistical Analysis:

All data analysis was completed using the R software (R v4.1.0, R Core Development Team 2021). Using the lmer() function from the lmerTest package (Kuznetsova et al., 2017), a linear mixed effects model with 'Day' and 'SocialStatus' as fixed effects was run to analyze bacterial diversity metrics. Species richness, Shannon diversity (H'), and Simpson's evenness were calculated and summarized based on social status and day of experiment. Following diversity metrics analysis, community composition analyses using permutational multivariate analysis of variance (PERMANOVA) and Principal Coordinates Analysis (PCoA) were completed. PCoA was performed based on the Bray-Curtis dissimilarity of bacterial community composition and the PERMANOVA analysis allowed us to determine significant differences in bacterial communities based on social status and time. We performed PERMANOVA using the adonis() function from the vegan package (Oksanen et al., 2017) and the inval() function in the indispecies package (Caceres & Jansen, 2016). For diversity metrics and PCoA analysis, statistical significance of $p < 0.05$ was used. We next found bacterial species that represented each treatment the most using indicator species analysis. Finally, specific phylum and genus level compositions with relative abundances greater than 0.05 were plotted.

Results:

Univariate Diversity Metrics:

To determine if there were notable differences between the gut microbiomes of zebrafish of different social statuses, microbiome analysis was performed. Linear mixed model analysis indicated that species richness, diversity and evenness were similar across social status and time ($p = 0.5481$, $F = 1.77$, $df = 15$). Type two Analysis of Variance (ANOVA) also indicated that species richness ($p = 0.2914$, $F = 1.268$, $df = 3$), diversity ($p = 0.89925$, $F = 0.195$, $df = 3$), and evenness ($p = 0.1627$, $F = 1.756$, $df = 3$) were similar overtime based on social status (Supplemental Table 3). However, species richness ($p = 0.439$, $F = 2.831$, $df = 3$) and evenness ($p = 0.0186$, $F = 3.533$, $df = 3$) were significantly different based on day of pairing (Supplemental Table 3). Additionally, interesting trends with these plots were observed. Subordinate data points plotted similarly to isolate data points during the isolation period (IP) before any pairing or rank establishment. Dominant data points trended similarly to those of communal status during the isolation period. These trends were observed for richness (figure 3), diversity (figure 4), and evenness (figure 5). Diversity was also visualized between social and antisocial individuals, where pairs and communals were social and isolates were considered nonsocial through a linear mixed model. Again, species richness and diversity were similar across social group and time ($p=0.6071$, $F=0.7785$, $df=7$), however it appears that social individuals have increased richness (figure 6) and diversity (figure 7) during the isolation period compared to nonsocial individuals. Type two Analysis of Variance (ANOVA) indicated as well that bacterial diversity remained the same based on social group and day (Supplemental Table 4)

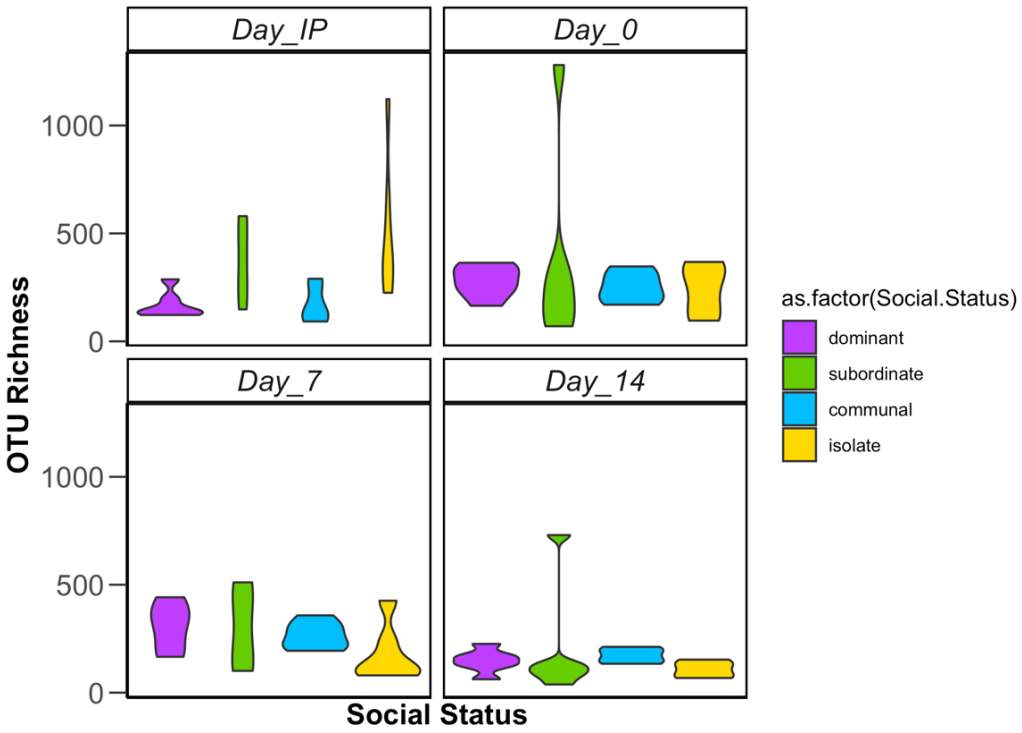

Figure 3. Violin plots representing OTU richness between individuals from dominant, subordinate, communal, and isolate social statuses throughout the pairing period with Day IP representing collection before pairing and Day 14 representing the end of socialization. The violin plots are a visual representation of summary statistics: the median, the 25% and 75% percentiles, and the feasible range of data ±1.5 x the interquartile range. Thicker violin sections indicate more data points. Summary of statistical output in Appendix table 1.

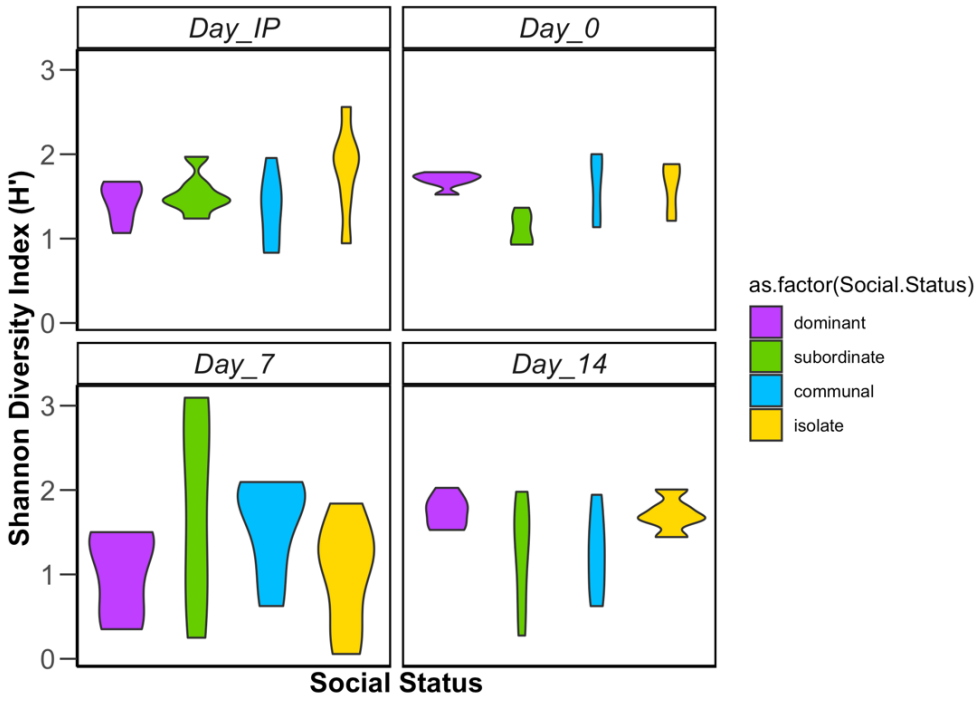

Figure 4. Violin plots representing species diversity between individuals from dominant, subordinate, communal, and isolate social statuses throughout the pairing period with Day IP representing collection before pairing and Day 14 representing the end of socialization. The violin plots are a visual representation of summary statistics: the median, the 25% and 75% percentiles, and the feasible range of data ±1.5 x the interquartile range. Thicker violin sections indicate more data points. Summary of statistical output in Appendix table 1.

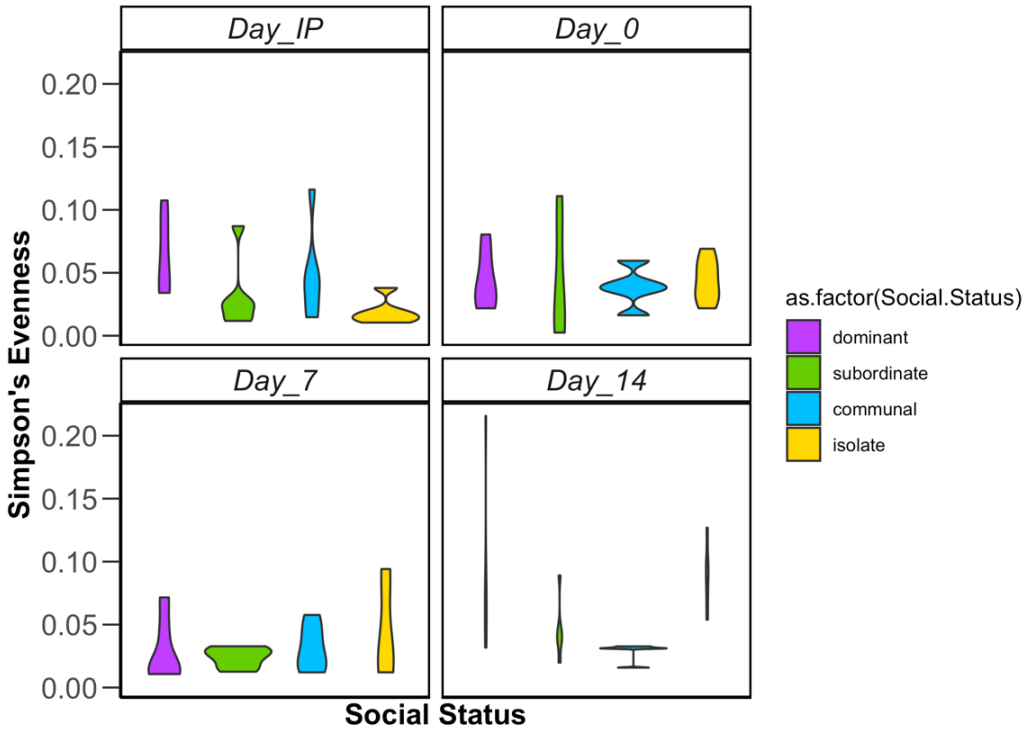

Figure 5. Violin plots representing species evenness between individuals from dominant, subordinate, communal, and isolate social statuses throughout the pairing period with Day IP representing collection before pairing and Day 14 representing the end of socialization. The violin plots are a visual representation of summary statistics: the median, the 25% and 75% percentiles, and the feasible range of data ±1.5 x the interquartile range. Thicker violin sections indicate more data points. Summary of statistical output in Appendix table 1.

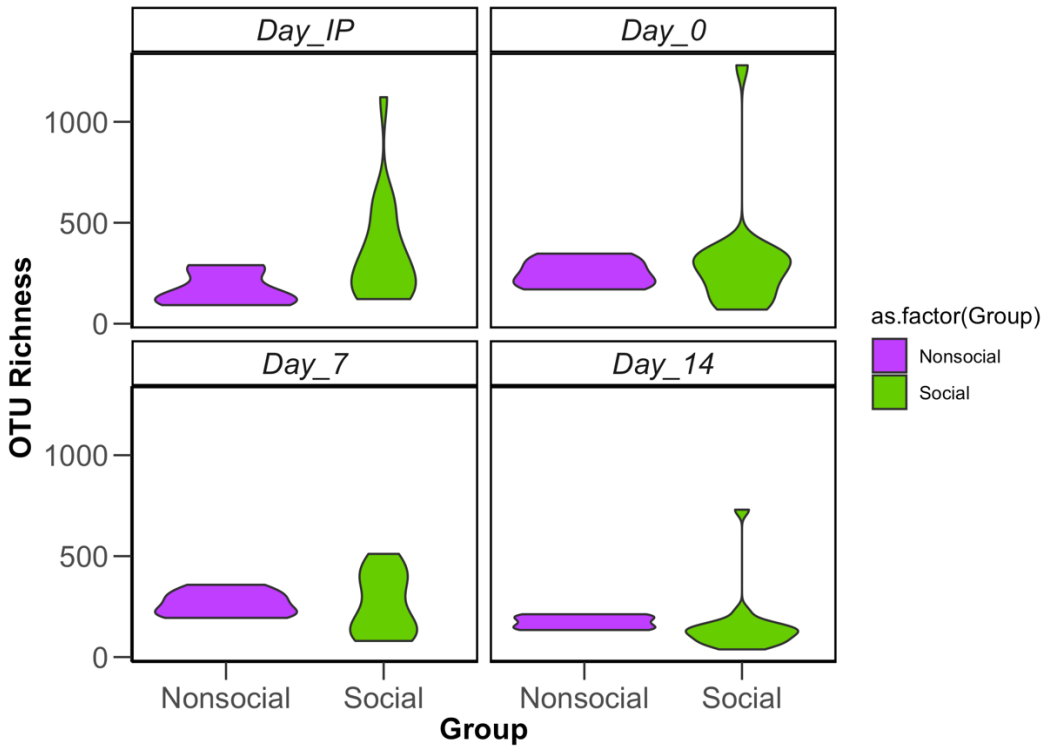

Figure 6. Violin plots representing OTU richness between individuals from social and nonsocial social status groups throughout the pairing period with Day IP representing collection before pairing and Day 14 representing the end of socialization. The violin plots are a visual representation of summary statistics: the median, the 25% and 75% percentiles, and the feasible range of data ±1.5 x the interquartile range. Thicker violin sections indicate more data points. Summary of statistical output in Appendix table 2.

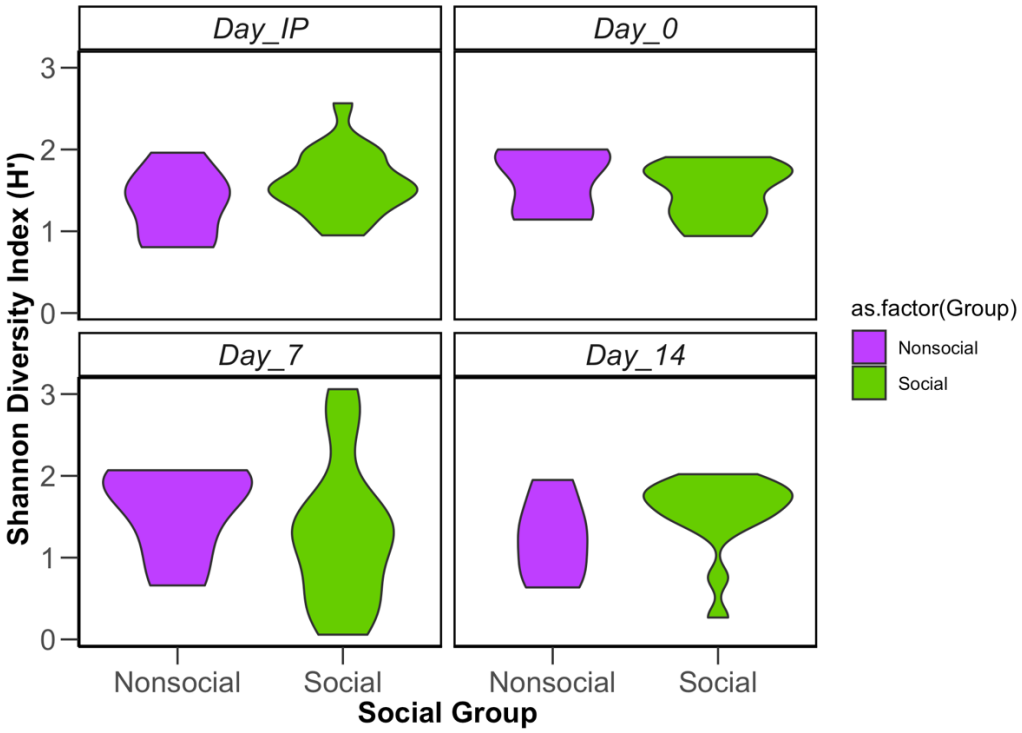

Figure 7. Violin plots representing species diversity between individuals from social and nonsocial social status groups throughout the pairing period with Day IP representing collection before pairing and Day 14 representing the end of socialization. The violin plots are a visual representation of summary statistics: the median, the 25% and 75% percentiles, and the feasible range of data ±1.5 x the interquartile range. Thicker violin sections indicate more data points. Summary of statistical output in Appendix table 2.

Community Composition Analysis:

Using multivariate ANOVA analysis with permutations, a PERMANOVA test was performed to determine if the centroids from the sample clusters based on social status and day of experiment were different. This analysis showed that bacterial composition differed significantly based on both social status and day ($p = 0.00099$, $R^2 = 0.1436$, $df = 9$). Using PCoA, we were able to visualize these differences in compositional clusters at different social statuses and time of experiment (figure 8). Interestingly, there is clustering of bacterial species specific to the dominant experimental group.

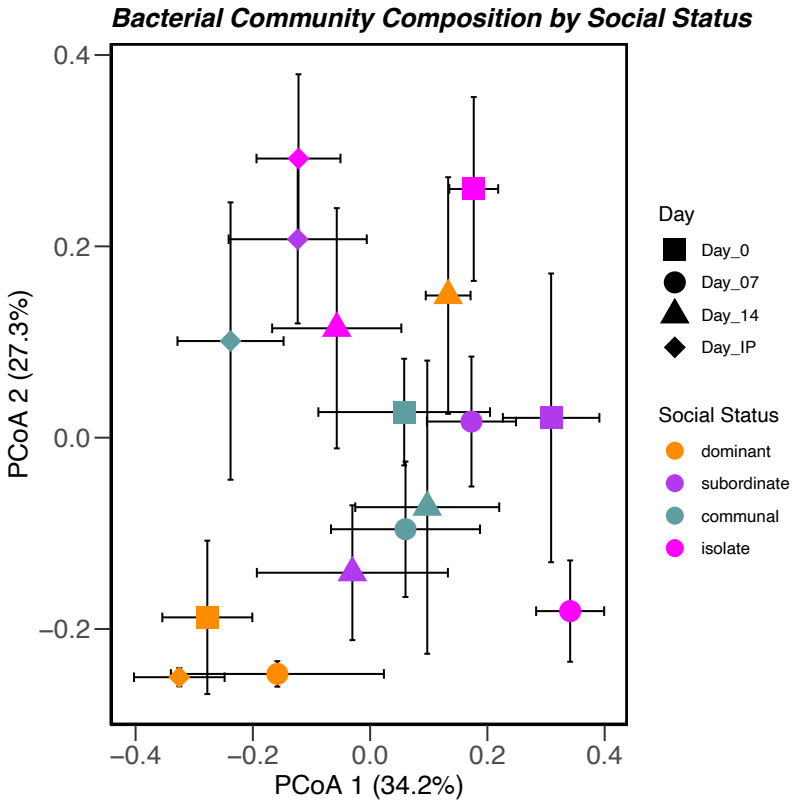

Figure 8. Ordination based on Principal Coordinate Analysis (PCoA) presenting bacterial community compositional based on 1) social status and 2) day of pairing. Symbols are colored according to social status (orange = dominant individuals, purple = subordinate individuals, teal = communal individuals, pink = isolate individuals) and day (diamond = isolation period (IP), square = first day of pairing (D0), circle = middle of pairing (D7), triangle = end of pairing (D14)). Summary of statistical output in Appendix table 5.

Once significant differences between fish of different social status and day of experiment were confirmed, taxonomic analysis was performed to see how phylum and genus level community composition varied between social status at different time points during the pairing period. Initial phylum level differences were observed based on social status (figure 9). The three main phyla: proteobacteria, firmicutes, and fusobacteria had different abundances dependent on social status. Further, Proteobacteria abundance was reduced in dominant individuals, whereas it was significantly higher in subordinate, communal, and isolate individuals. On the contrary, there was a higher abundance of firmicutes and fusobacteria phyla in dominant individuals compared to

the subordinate counterpart. To display variance of these taxonomic differences, box plots illustrating differences in all taxa present and high levels of variance were observed in many of the plots. While high levels of variance were seen within the taxonomic differences, notable trends were still observed. As seen initially, Actinobacteria, Fusobacteria, and Firmicute communities were higher in dominant individuals. These phyla are composed primarily of commensal bacteria with a few exceptions, whereas Proteobacteria is largely comprised of pathogenic bacteria. Interestingly, the abundance of Proteobacteria communities was reduced in dominant individuals compared to subordinate, isolate, and communal individuals (figure 10).

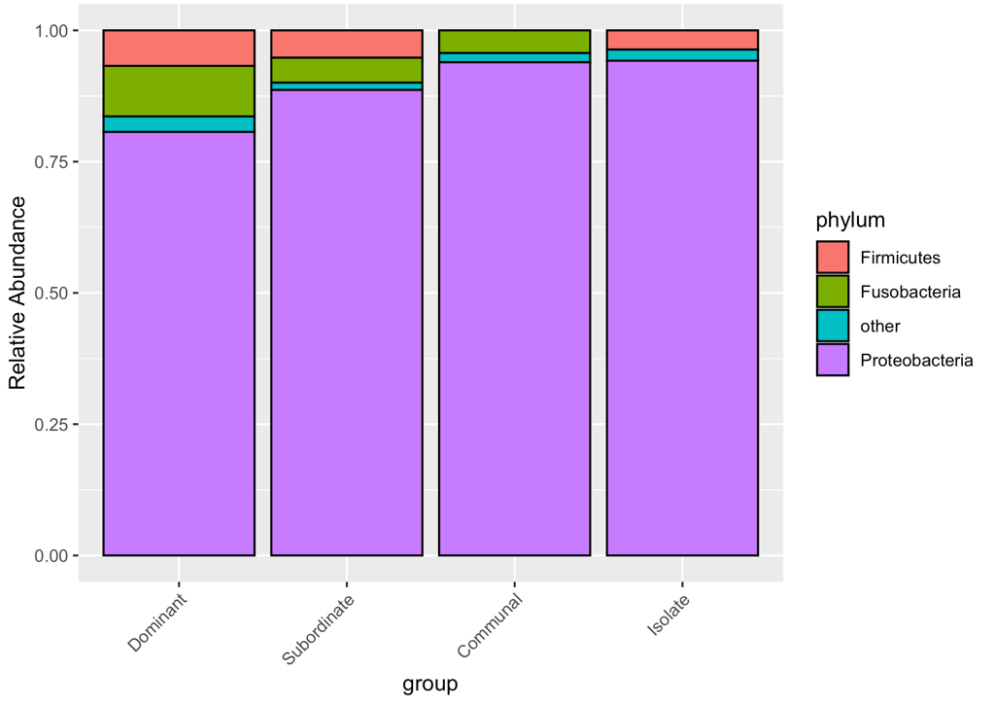

Figure 9. Stack bar plot analysis illustrating phylum level bacterial abundance in zebrafish based on social status. Colors represent individual phyla (pink = Firmicutes, green = Fusobacteria, purple = Proteobacteria, blue = other phyla). Thickness of each color represents relative abundance with Proteobacteria having the highest abundance in all individuals.

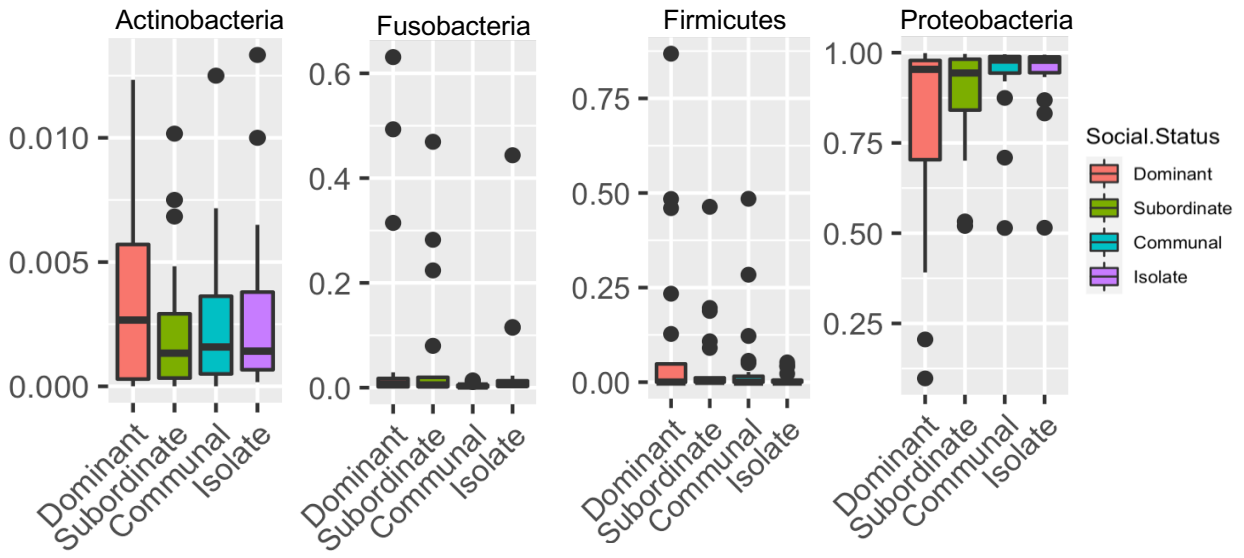

Figure 10. Taxonomic box plots highlighting abundance based on social status for the top phyla present in the zebrafish gut. Colors represent social status (pink = Dominant, green = Subordinate, blue = Communal, purple= Isolate). Actinobacteria, Fusobacteria, and Firmicutes phyla represent commensal bacteria primarily while the Proteobacteria phylum is representative of many pathogenic bacteria. Thicker box plot indicates less variation of data points.

Community compositional analysis showed significantly different clusters based on social status and day of experiment. These clusters of similar sequences represent taxonomies of bacteria at the genus level of classification. Bacterial communities within the Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Planctomycetes, and Chloroflexi phyla were observed. Interestingly, the Proteobacteria phylum made up most of the compositional analysis. This was expected; however, as many proteobacteria genera are waterborne [34]. After observing that the abundance levels of these specific phyla differed significantly based on social status, we next examined whether these differences were also present based on the day of pairing. By looking at phylum composition greater than 5%, we see that day of experiment had a significant impact on gut microbiome composition in addition to social status (figure 11). At initial glance, we don't many changes in gut microbiome composition, but on day 14 of pairing in dominant individuals, abundance levels of Proteobacteria, Firmicutes, and Fusobacteria are significantly

different. Specifically, Proteobacteria community abundance is reduced by almost half compared to dominant individuals at other time points and individuals of different social status. On day 14 in dominant individuals, Firmicute and Fusobacteria abundance increased to compensate for the reduction of Proteobacteria. Again, these changes were specifically seen at the end of pairing and the increase in commensal phyla was not observed in fish of subordinate, communal, or isolate groups.

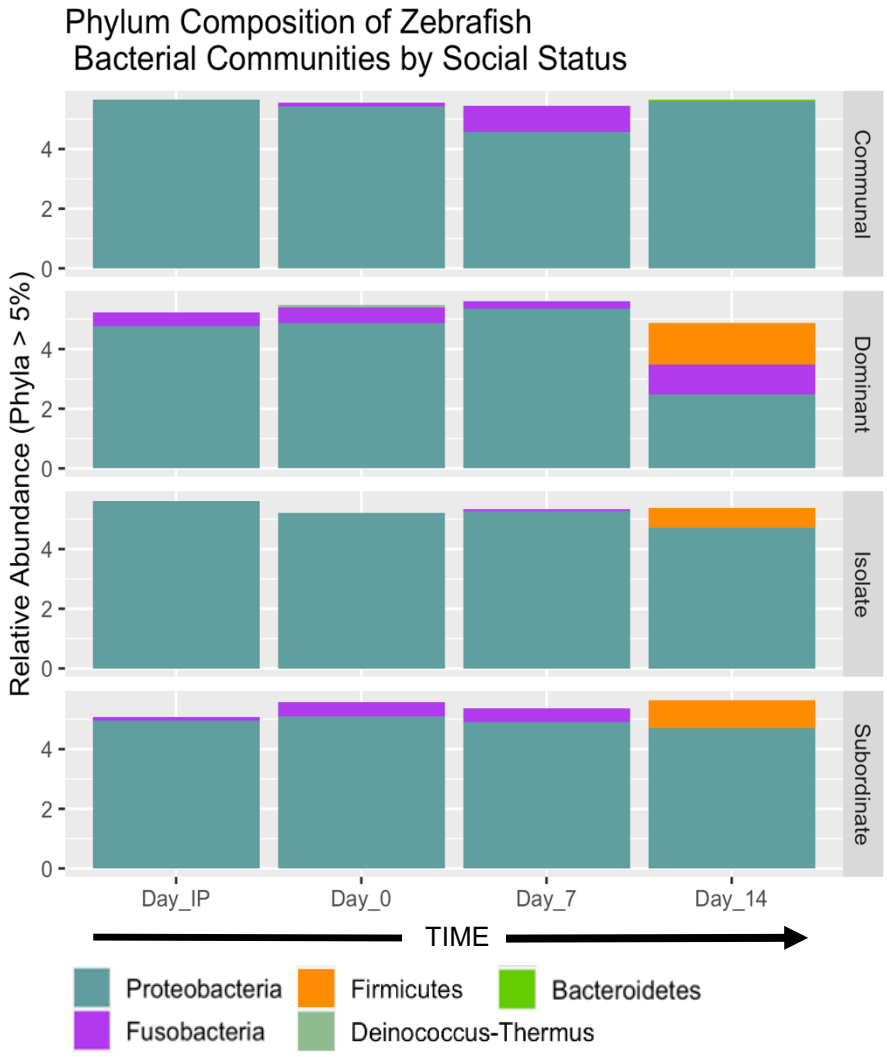

Figure 11. Phylum-level community composition in zebrafish by social status and day of pairing Colors represent individual phyla present within each social status at certain times of pairing and the thickness of color indicates relative abundance of certain phyla. Each stacked box plot represents relative abundance of phyla based on social status (rows) and day of pairing (columns).

We next wanted to see if similar trends were seen at the genus level composition. While community composition varied between different social statuses and day of pairing, we again noticed significant changes in dominant individuals on day 14 of pairing (figure 12). Like at the phylum level, we noticed a reduction in pathogenic genera that were consistently high in abundance in other social status groups and throughout the pairing period. Specifically, *Aeromonas* and *Enterobacteriaceae* community abundance was lower in dominant individuals on day 14, whereas abundance of these genera remained higher in other groups. We also saw an increased abundance of *Chitinobacteria, Vibrio*, and *Pseudomonas* in individuals of subordinate, isolate, and communal social status and the presence of these genera were maintained before and during pairing. Alternatively, certain commensal genera such as *Exiguobacterium* and *Cetobacterium* increased in abundance in dominant individuals on day 14 of pairing. Interestingly, these genera were present in dominant individuals throughout pairing, but on day 14, the abundance increased, especially for the *Cetobacterium* genus.

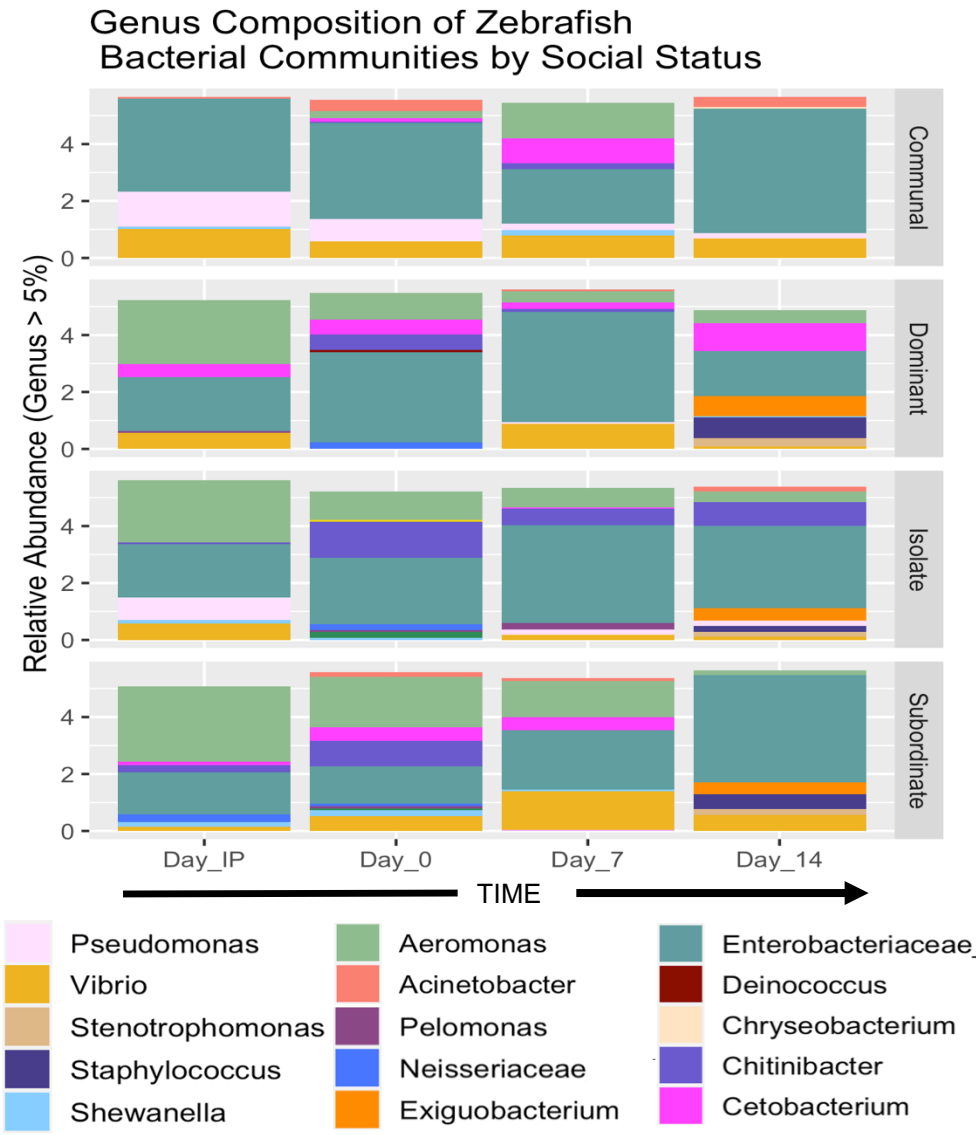

Figure 12. Genus-level bacterial community composition by social status and day of pairing. Colors represent individual genera present within each social status at certain times of pairing and the thickness of color indicates relative abundance of certain phyla. Each stacked box plot represents relative abundance of genera based on social status (rows) and day of pairing (columns).

Discussion

Determining the relationship between social status in zebrafish and host gut microbiome composition is a critical component to understanding the gut-brain axis related to social behaviors and disorders. Previous research highlights communication between gut microbes and neurological pathways but the results from this study demonstrate the presence of certain bacterial communities dependent on social status and day of socialization. As a general foundational conclusion, dominant zebrafish had decreased abundance of pathogenic bacterial phyla and genera and an increased abundance of commensal bacteria, specifically at the end of the socialization period. Conversely, subordinate individuals have a decreased presence of commensal bacterial phyla and genera and an increased abundance of pathogenic bacteria.

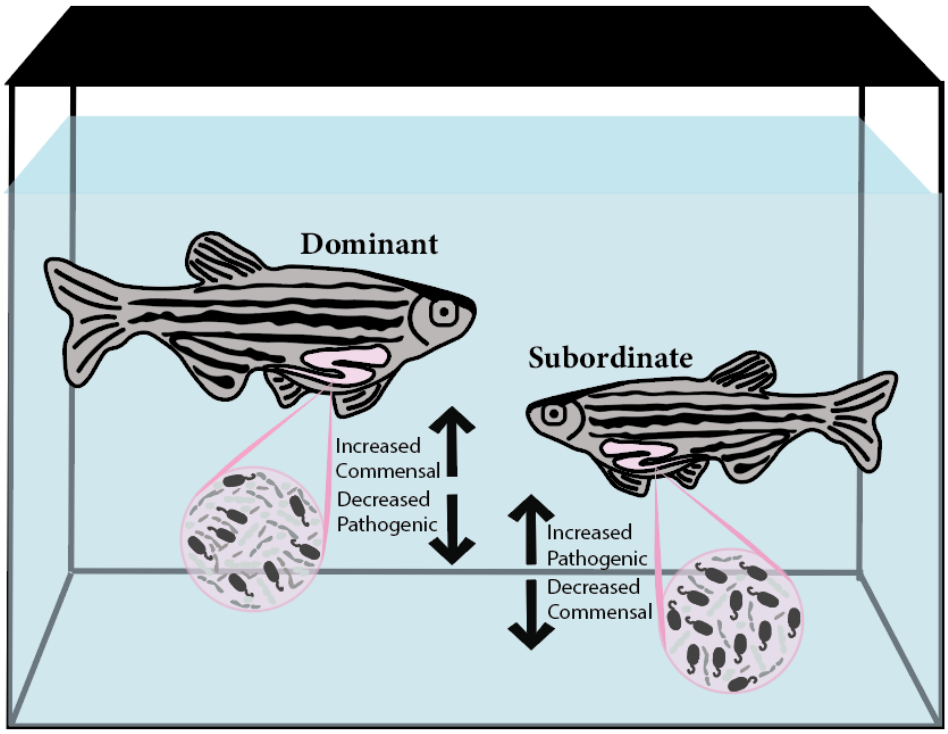

Figure 13. Proposed model of zebrafish gut microbiome composition based on social status.

While there were no significant differences in gut microbiome diversity or richness of zebrafish of different social status, the compositional differences provide a foundational insight into the gut microbiomes of organisms exhibiting different social behaviors. The typical zebrafish gut microbiome is comprised of the phyla firmicutes, fusobacteria, and proteobacteria [6]. These findings are consistent with our study, as Proteobacteria dominated the gut microbiota, but firmicutes and fusobacteria phyla were also present. Actinobacteria, Acidobacteria, Planctomycetes and Chloroflexi and a few other phyla made up the remaining community composition. While these are not dominant phyla in the zebrafish gut microbiome, analysis of the zebrafish core gut microbiota in other studies has included these two phyla [6].

Based on previous research in zebrafish and other animal models, the genus, *Lactobacillus,* from phylum Bacteroidetes, is a common protective bacterial species often found in the guts of many animal models [9]. Because of these qualities, the *Lactobacillus* genus is often used as a probiotic treatment in both zebrafish and mouse models to rescue stressed phenotypes [9, 3]. Interestingly, the *Lactobacillus* genus was only present in low abundances and only in the isolate social status group. Another bacterial genus from the Bacteroidetes phylum was *Chryseobacterium*. While this genus doesn't come from the same family as Lactobacillus, both genera seem to have similar protective qualities in zebrafish gut microbiota. In a study examining bacterial key players in pathogenic infection in zebrafish, *Chryseobacterium massillae* was determined to important in protecting both larvae and adult zebrafish from pathogenic infection [21]. This bacterial genus was interestingly only present in low abundances in both communal and dominant individuals (figure 8).

Within the firmicutes phylum, the bacterial genus *Exiguobacterium* was also determined as a component of compositional analysis of the zebrafish gut in this study. This genus of bacteria

has been shown to produce cyclic dipeptides, which have many antimicrobial, antifungal, antiviral, and anti-inflammatory properties in humans and other animals [23, 24]. Intriguingly, *Exiguobacterium acetylicum* has therapeutic properties in zebrafish colorectal cancer models [22]. This research highlights the protective properties of this genus of bacteria in the gut of both zebrafish and humans. This genus increased in abundance in dominant individuals at the end of pairing but was also present in both isolate and subordinate individuals at lower abundances (figure 8). This genus was not observed in communal individuals which is interesting considering their social behaviors may be like dominant fish. Another genus found in high abundance in dominant individuals was *Cetobacterium*. This bacterium has been used as a probiotic in zebrafish and serves to promote glucose homeostasis via activation of the parasympathetic nervous system [46]. In certain grouper species, bacteria within the *Psychrobacter* genus can be used as a probiotic to promote diversity in the intestines of the host fish [26]. Interestingly, the probiotic treatment of *Psychrobacter* in these fish also significantly increased abundance of other commensal bacteria and decreased the abundance of *Staphylococcus* and other pathogenic species [26]. This bacteria genus was present in dominant individuals, but at very low abundance levels (< 5%).

Given the high percent of proteobacteria communities in general zebrafish gut microbiomes, the presence of pathogenic bacteria isn't uncommon. Additionally, a large portion of the species in these pathogenic genera are waterborne. However, the varying abundance of certain pathogenic bacteria like *Aeromonas, Enterobacteriaceae, Staphylococcus, Vibrio,* and *Pseudomonas* in the community composition is interesting. *Aeromonas and Enterobacteriaceae* were the dominant pathogenic genera in all groups and at all time points during pairing, but several pathogenic genera were primarily seen in subordinate, isolate, and even communal individuals. Many of these genera have been used in zebrafish infection models. A model of *Staphylococcus*

infection in zebrafish has been studied, and the interactions between *Staphylococcus aureus* and other bacteria in the gut to create infection [29]. This study specifically examined the immune response of the fish in response to this infection, but it is important to note the pathogenesis associated with *Staphylococcus* infection. Interestingly, Staphylococcus abundance is increased in dominant individuals even though other pathogenic genera are decreased. This could contribute to the increase in Firmicute abundance seen in dominant individuals. While it is increased in dominant individuals, it is also present in subordinate individuals but has no presence in communal or isolates. This finding is inconsistent with other trends observed, but still provides insight into gut microbiota in all fish dependent upon different social statuses. A potential explanation to the increased abundance of *Staphylococcus* in dominant individuals could surround the hypothesis that Staphylococcus contributes to the production of Tyrosine, a precursor to the neurotransmitter dopamine [42]. Given that this genus is only found in socially paired individuals, it poses an interesting question as to whether the presence of *Staphylococcus* could contribute to increasing dopamine expression, which is a very relevant neurotransmitter in social behaviors and the establishment of dominance in zebrafish [43].

In another zebrafish pathogenesis model, bacteria within the *Pseudomonas* genus can lethally infect larval zebrafish [30]. Another similar study also saw this trend and found that *Pseudomonas aeruginosa* could cause lethal infection in zebrafish model whereas other pathogenic bacteria like *Escherichia coli* could not induce such pathogenic infection [31]. The *Vibrio* genus has also been researched in pathogenesis in zebrafish, but some *Vibrio* species are harmless. Many species of *Vibrio*, both non-pathogenic and pathogenic are present in water sources, but specific pathogenic species of *Vibrio* have been researched in zebrafish and humans [35]. Many species of

Vibrio are colonized in zebrafish models normally, so the presence of this genus isn't as indicative of pathogenesis compared to other genera (e.g., *Staphylococcus, Aeromonas*).

The Aeromonas genus, which has a high abundance in almost all zebrafish individuals regardless of social status, has been observed in several pathogenic models of zebrafish. Interestingly, specific species within the Aeromonas genus have been seen to induce severe infection of zebrafish models by influencing the release of reactive oxygen species (ROS) and nitrogen free radicals [36]. Consequently, immune responses were triggered in these fish models which ultimately resulted in a massive inflammatory response and eventual death of fish. While we do not know the specific species of *Aeromonas* present, higher abundances of this genus may result in pathogenesis, but further research surrounding this topic needs to be done in zebrafish social rank models.

Finally, the unclassified-Enterobacteriaceae genus had a very high abundance in many zebrafish. This family contains many pathogenic genera such as *Shigella, Salmonella, Edwardsiella,* and *Klebsiella.* Several of these genera have been observed in zebrafish pathogenesis models. Specifically, *Edwardsiella* infections in zebrafish led to an elevated cytokine response and Edwardsiellosis, which is one of the most studied bacterial diseases on fish [37]. More analysis needs to be done to examine specific genera of this Enterobacteriaceae family present in zebrafish bacterial composition, but research examining these specific genera are indicative of pathogenesis and disease in fish with high abundances of these bacteria.

Given the presence of certain bacterial genera, both pathogenic and commensal, we can foundationally characterize the zebrafish gut microbiome based on social status. Since dominant zebrafish have a higher abundance of commensal bacteria in addition to a decreased pathogenic bacterial abundance, we can speculate that certain social behaviors exhibited by these zebrafish

(i.e., aggression) may play a role in gut-brain communication. Alternatively, certain social behaviors exhibited by subordinate individuals (i.e., fear, anxiety, stress-related behaviors) may also play a role in gut-brain communication, but negatively.

Chapter III

GENERAL DISCUSSION

The link between the gut and the brain is a relatively recent discovery. With that said, the research surrounding the gut and its association with the brain is rapidly ongoing. Since there appears to be a relationship between the gut microbiome and social status in zebrafish, this points towards the assumptions that the gut microbiome may be associated with other social behaviors, or behavioral disorders. In mouse models, there seems to be a causative relationship between the gut and anxiety/depression phenotypes [3] in which individuals experiencing social deficits also have microbiome dysbiosis. These results are similar to animal model research examining how probiotic treatment changes behavior in stress-induced individuals. Specifically, zebrafish with prolonged, induced stress were treated with probiotics and the stress associated behaviors were rescued [9]. Further, the treatment of probiotics decreased blood cortisol levels in these stressed zebrafish which indicates that the gut may be communicating with hormonal pathways involved in stress regulation [9]. Given that subordinate zebrafish experience higher blood cortisol levels [44], subordinance may act as a stressor which could be a factor in associated gut microbiome composition. More research needs to be done surrounding this concept, however this information supports a microbiome mediated behavior hypothesis in which the gut microbiome and its bacterial inhabitants are associated with behaviors related to stress and anxiety. However, it is important to note that in many studies, the composition and diversity of the gut microbiome potentially affects behavior whereas we found that social status potentially shapes gut microbiome composition.

While we saw compositional differences between fish of different behaviors, these differences were also present based on the time of experiment which signifies that the physiological stress associated with certain social behaviors may directly impact bacterial

composition. Is also seems that certain social behaviors related to dominance and aggression promote an increased presence of commensal bacteria genera. Previous research in autism models show an inverse relationship between the gut and behavior, as individuals born with gut microbiota dysbiosis are predisposed to social deficits and autism-like behaviors. An initial question in this study was whether social status would be dependent on an individual's gut composition prior to pairing. Given that notable trends were not seen before pairing during the isolation period (Day IP), this hypothesis is not supported in this model. However, it would be interesting to see if probiotic or antibiotic treatment changed host zebrafish social status, which would indicate that the microbiome could directly affect behavior.

Future Experiments:

Given the community compositional differences between individuals of different social status, there is some connection between the gut and the neurological pathways associated with social behavior. It is also important to note that most notable changes in dominant individuals occurred on day 14 of pairing, it would be interesting to see if these changes would persist throughout longer pairing periods or post pairing in general. Further behavioral testing could be completed after initial experiments to examine if the gut microbiome changes effected by social status continue with new pairings. For example, if two established dominant fish were paired together, would the gut microbiome change further based on new social statuses?

Previous research has examined the relationship between gut microbiome composition and associated gene expression within the brain. Given that zebrafish experiencing induced stress exhibit changes in gut microbiome composition and brain gene expression [8] experiments testing this relationship in zebrafish of different social status could be informative. More specifically, it would be interesting to see if there are any transcriptional changes in the brains of dominant and

subordinate fish that could be linked to changes in bacterial abundance in the host gut. This may allow us to see if certain neurotransmitters (i.e., serotonin, GABA, dopamine) and their pathways are influencing the gut in a way that changes the overall community composition.

Experiments surrounding antibiotic treatment targeting certain bacterial communities would also be significant in examining the gut microbiome-social status link. Given that certain bacteria genera are present in fish of different social status, removing those specific communities could impact social behavior. Alternatively, probiotic treatment for fish lacking certain bacterial communities may also impact social behaviors in the host zebrafish. Probiotic treatment in zebrafish under induced stress has rescued both behavioral phenotypes and associated gene expression in the brain [8]. A similar experiment in fish based on social status could highlight the importance of certain bacteria in regulating social behaviors.

Conclusions:

While it was initially predicted that social status would affect both gut microbiome composition and species diversity, we did not see any significant diversity differences between fish of different social status. However, the differences in gut microbiome composition by social status and time of pairing tell an interesting story about the composition of zebrafish gut microbiota and the changes in composition depending on certain social behaviors exhibited by the host. These differences in bacteria communities between fish of different social status support the hypotheses surrounding the gut-brain axis and link between the gut and behavior. Interestingly, it appears that behaviors like aggression associated with social dominance may alter the gut microbiome composition in which commensal genera become more abundant and pathogenic genera decrease in abundance. Alternatively, social behaviors related to fear, stress, or anxiety in subordinate fish may contribute to the increased presence of pathogenic communities throughout pairing. In

conclusion, the findings from this study contribute to our knowledge about social behaviors and their impacts on the host gut microbiome. Furthermore, better understanding of this behavior-gut link provides foundational research that may help us better understand the relationship between the gut and the brain and how the onset of certain behavioral, neurodevelopmental, and neurodegenerative diseases and disorders may be associated with gut dysbiosis.

References

1. Kinross, James M., Ara W. Darzi, and Jeremy K. Nicholson. "Gut microbiome-host interactions in health and disease." *Genome medicine* 3, no. 3 (2011): 1-12.

2. Stefan, Kailyn L., Myoungjoo V. Kim, Akiko Iwasaki, and Dennis L. Kasper. "Commensal microbiota modulation of natural resistance to virus infection." *Cell* 183, no. 5 (2020): 1312-1324.

3. Sgritta, Martina, Sean W. Dooling, Shelly A. Buffington, Eric N. Momin, Michael B. Francis, Robert A. Britton, and Mauro Costa-Mattioli. "Mechanisms underlying microbial-mediated changes in social behavior in mouse models of autism spectrum disorder." *Neuron* 101, no. 2 (2019): 246-259.

4. Lynch, J. B., and E. Y. Hsiao. "Microbiomes as sources of emergent host phenotypes." *Science* 365, no. 6460 (2019): 1405-1409.

5. Vuong, Helen E., Jessica M. Yano, Thomas C. Fung, and Elaine Y. Hsiao. "The microbiome and host behavior." *Annual review of neuroscience* 40 (2017): 21-49.

6. Roeselers, Guus, Erika K. Mittge, W. Zac Stephens, David M. Parichy, Colleen M. Cavanaugh, Karen Guillemin, and John F. Rawls. "Evidence for a core gut microbiota in the zebrafish." *The ISME journal* 5, no. 10 (2011): 1595-1608.

7. Shin, Na-Ri, Tae Woong Whon, and Jin-Woo Bae. "Proteobacteria: microbial signature of dysbiosis in gut microbiota." *Trends in biotechnology* 33, no. 9 (2015): 496-503.

8. Borrelli, Luca, Serena Aceto, Claudio Agnisola, Sofia De Paolo, Ludovico Dipineto, Roman M. Stilling, Timothy G. Dinan, John F. Cryan, Lucia F. Menna, and Alessandro Fioretti. "Probiotic modulation of the microbiota-gut-brain axis and behaviour in zebrafish." *Scientific reports* 6, no. 1 (2016): 1-9.

9. Davis, Daniel J., Holly M. Doerr, Agata K. Grzelak, Susheel B. Busi, Eldin Jasarevic, Aaron C. Ericsson, and Elizabeth C. Bryda. "Lactobacillus plantarum attenuates anxiety-related behavior and protects against stress-induced dysbiosis in adult zebrafish." *Scientific reports* 6, no. 1 (2016): 1-11.

10. Ventriglia, Mariacarla, Roberta Zanardini, Cristina Bonomini, Orazio Zanetti, Daniele Volpe, Patrizio Pasqualetti, Massimo Gennarelli, and Luisella Bocchio-Chiavetto. "Serum brain-derived neurotrophic factor levels in different neurological diseases." *BioMed research international* 2013 (2013).

11. Mondelli, Valeria, Annamaria Cattaneo, Martino Belvederi Murri, Marta Di Forti, Rowena Handley, Nilay Hepgul, Ana Miorelli et al. "Stress and inflammation reduce brain-derived neurotrophic factor expression in first-episode psychosis: a pathway to smaller hippocampal volume." *The Journal of clinical psychiatry* 72, no. 12 (2011): 0-0.

12. Chiu, Isaac M., Balthasar A. Heesters, Nader Ghasemlou, Christian A. Von Hehn, Fan Zhao, Johnathan Tran, Brian Wainger et al. "Bacteria activate sensory neurons that modulate pain and inflammation." *Nature* 501, no. 7465 (2013): 52-57.

13. O'Donnell, Michael P., Bennett W. Fox, Pin-Hao Chao, Frank C. Schroeder, and Piali Sengupta. "A neurotransmitter produced by gut bacteria modulates host sensory behaviour." *Nature* 583, no. 7816 (2020): 415-420.

14. Miller, Thomas H., Katie Clements, Sungwoo Ahn, Choongseok Park, Eoon Hye Ji, and Fadi A. Issa. "Social status–dependent shift in neural circuit activation affects decision making." *Journal of Neuroscience* 37, no. 8 (2017): 2137-2148.

15. Stephens, W. Zac, Adam R. Burns, Keaton Stagaman, Sandi Wong, John F. Rawls, Karen Guillemin, and Brendan JM Bohannan. "The composition of the zebrafish intestinal microbial community varies across development." *The ISME journal* 10, no. 3 (2016): 644-654.

16. Sampson, Timothy R., Justine W. Debelius, Taren Thron, Stefan Janssen, Gauri G. Shastri, Zehra Esra Ilhan, Collin Challis et al. "Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease." *Cell*167, no. 6 (2016): 1469-1480.

17. Strandwitz, Philip, Ki Hyun Kim, Darya Terekhova, Joanne K. Liu, Anukriti Sharma, Jennifer Levering, Daniel McDonald et al. "GABA-modulating bacteria of the human gut microbiota." *Nature microbiology* 4, no. 3 (2019): 396-403.

18. Chrousos, George P., and Philip W. Gold. "The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis." *Jama* 267, no. 9 (1992): 1244-1252.

19. Raison, Charles L., and Andrew H. Miller. "When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders." *American Journal of Psychiatry* 160, no. 9 (2003): 1554-1565.

20. Luo, Yuanyuan, Benhua Zeng, L. I. Zeng, Xiangyu Du, B. O. Li, Ran Huo, Lanxiang Liu et al. "Gut microbiota regulates mouse behaviors through glucocorticoid receptor pathway genes in the hippocampus." *Translational psychiatry* 8, no. 1 (2018): 1-10.

21. Stressmann, Franziska A., Joaquin Bernal-Bayard, David Perez-Pascual, Bianca Audrain, Olaya Rendueles, Valérie Briolat, Sebastian Bruchmann et al. "Mining zebrafish microbiota reveals key community-level resistance against fish pathogen infection." *The ISME Journal* 15, no. 3 (2021): 702-719.

22. Ström, Katrin, Jörgen Sjögren, Anders Broberg, and Johan Schnürer. "Lactobacillus plantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and

cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid." *Applied and environmental microbiology* 68, no. 9 (2002): 4322-4327.

23. Graz, C. J. M., G. D. Grant, S. C. Brauns, A. Hunt, H. Jamie, and P. J. Milne. "Cyclic dipeptides in the induction of maturation for cancer therapy." *Journal of pharmacy and pharmacology* 52, no. 1 (2000): 75-82.

24. Jinendiran, Sekar, Weilin Teng, Hans-Uwe Dahms, Wangta Liu, Vinoth Kumar Ponnusamy, Charles Chien-Chih Chiu, BS Dileep Kumar, and Natesan Sivakumar. "Induction of mitochondria-mediated apoptosis and suppression of tumor growth in zebrafish xenograft model by cyclic dipeptides identified from Exiguobacterium acetylicum." *Scientific reports*10, no. 1 (2020): 1-17.

25. Stagaman, Keaton, Thomas J. Sharpton, and Karen Guillemin. "Zebrafish microbiome studies make waves." *Lab Animal* 49, no. 7 (2020): 201-207.

26. Yang, H. L., Y. Z. Sun, R. L. Ma, J. S. Li, and K. P. Huang. "Probiotic Psychrobacter sp. improved the autochthonous microbial diversity along the gastrointestinal tract of grouper Epinephelus coioides." *J Aquac Res Development S* 1 (2011): 001.

27. Loftie-Eaton, Wesley, Angela Crabtree, David Perry, Jack Millstein, Justin Baytosh, Thibault Stalder, Barrie D. Robison, Larry J. Forney, and Eva M. Top. "Contagious Antibiotic Resistance: Plasmid Transfer among Bacterial Residents of the Zebrafish Gut." *Applied and Environmental Microbiology*87, no. 9 (2021): e02735-20.

28. Prajsnar, Tomasz K., Vincent T. Cunliffe, Simon J. Foster, and Stephen A. Renshaw. "A novel vertebrate model of Staphylococcus aureus infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens." *Cellular microbiology* 10, no. 11 (2008): 2312-2325.

29. Clatworthy, Anne E., Jenny See-Wai Lee, Mark Leibman, Zachary Kostun, Alan J. Davidson, and Deborah T. Hung. "Pseudomonas aeruginosa infection of zebrafish involves both host and pathogen determinants." *Infection and immunity* 77, no. 4 (2009): 1293-1303.

30. Llamas, María A., and Astrid M. van der Sar. "Assessing Pseudomonas virulence with nonmammalian host: zebrafish." In *Pseudomonas Methods and Protocols*, pp. 709-721. Humana Press, New York, NY, 2014.

31. Kozich, James J., Sarah L. Westcott, Nielson T. Baxter, Sarah K. Highlander, and Patrick D. Schloss. "Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform." *Applied and environmental microbiology*79, no. 17 (2013): 5112-5120.

32. Jin, Decai, Xiao Kong, Bingjian Cui, Shulan Jin, Yunfeng Xie, Xingrun Wang, and Ye Deng. "Bacterial communities and potential waterborne pathogens within the typical urban surface waters." *Scientific reports* 8, no. 1 (2018): 1-9.

33. Runft, Donna L., Kristie C. Mitchell, Basel H. Abuaita, Jonathan P. Allen, Sarah Bajer, Kevin Ginsburg, Melody N. Neely, and Jeffrey H. Withey. "Zebrafish as a natural host model for Vibrio cholerae colonization and transmission." *Applied and environmental microbiology* 80, no. 5 (2014): 1710-1717.

34. Rodríguez, Iván, Beatriz Novoa, and A. Figueras. "Immune response of zebrafish (Danio rerio) against a newly isolated bacterial pathogen Aeromonas hydrophila." *Fish & shellfish immunology* 25, no. 3 (2008): 239-249.

35. Pressley, Meagan E., Peter E. Phelan III, P. Eckhard Witten, Mark T. Mellon, and Carol H. Kim. "Pathogenesis and inflammatory response to Edwardsiella tarda infection in the zebrafish." *Developmental & Comparative Immunology* 29, no. 6 (2005): 501-513.

36. Chen, Wei, Clarence K. Zhang, Yongmei Cheng, Shaowu Zhang, and Hongyu Zhao. "A comparison of methods for clustering 16S rRNA sequences into OTUs." *PloS one* 8, no. 8 (2013): e70837.
37. Sylvia, Kristyn E., and Gregory E. Demas. "A gut feeling: microbiome-brain-immune interactions modulate social and affective behaviors." *Hormones and behavior* 99 (2018): 41-49.

38. Caporaso, J. Gregory, Christian L. Lauber, William A. Walters, Donna Berg-Lyons, James Huntley, Noah Fierer, Sarah M. Owens et al. "Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms." *The ISME journal* 6, no. 8 (2012): 1621-1624.

39. Apprill, Amy, Sean McNally, Rachel Parsons, and Laura Weber. "Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton." *Aquatic Microbial Ecology* 75, no. 2 (2015): 129-137.

40. Yilmaz, Pelin, Laura Wegener Parfrey, Pablo Yarza, Jan Gerken, Elmar Pruesse, Christian Quast, Timmy Schweer, Jörg Peplies, Wolfgang Ludwig, and Frank Oliver Glöckner. "The SILVA and "all-species living tree project (LTP)" taxonomic frameworks." *Nucleic acids research* 42, no. D1 (2014): D643-D648.

41. Rognes, Torbjørn, Tomáš Flouri, Ben Nichols, Christopher Quince, and Frédéric Mahé. "VSEARCH: a versatile open source tool for metagenomics." *PeerJ* 4 (2016): e2584.

42. Luqman, Arif, Mulugeta Nega, Minh-Thu Nguyen, Patrick Ebner, and Friedrich Götz. "SadA-expressing staphylococci in the human gut show increased cell adherence and internalization." *Cell reports* 22, no. 2 (2018): 535-545.

43. Filby, Amy L., Gregory C. Paull, Tamsin FA Hickmore, and Charles R. Tyler. "Unravelling the neurophysiological basis of aggression in a fish model." *BMC genomics* 11, no. 1 (2010): 1-17.

44. Tea, Jonathan, Sarah L. Alderman, and Kathleen M. Gilmour. "Social stress increases plasma cortisol and reduces forebrain cell proliferation in subordinate male zebrafish (Danio rerio)." *Journal of Experimental Biology* 222, no. 2 (2019): jeb194894.

45. Agranyoni, Oryan, Sapir Meninger-Mordechay, Atara Uzan, Oren Ziv, Mali Salmon-Divon, Dmitry Rodin, Olga Raz et al. "Gut microbiota determines the social behavior of mice and induces metabolic and inflammatory changes in their adipose tissue." *NPJ biofilms and microbiomes* 7, no. 1 (2021): 1-14.

46. Wang, Anran, Zhen Zhang, Qianwen Ding, Yalin Yang, Jérôme Bindelle, Chao Ran, and Zhigang Zhou. "Intestinal Cetobacterium and acetate modify glucose homeostasis via parasympathetic activation in zebrafish." *Gut microbes* 13, no. 1 (2021): 1-15.

Appendix

Supplemental Table 1. Statistical output of linear mixed model with social status (dominant, subordinate, isolate, communal) and day (Day IP, Day 0, Day 7, Day 14) as mixed effects.

	Minimum	**1Q**	**Median**	**3Q**	**Maximum**
Richness	-298.00	-84.83	-29.33	62.17	912.00
Diversity	-1.5392	-0.2825	0.0581	0.2526	1.2440
Evenness	-0.0470	-0.0145	-0.0048	0.0074	0.1031

Supplemental Table 2. Statistical output of linear mixed model with social group (social, nonsocial) and day (Day IP, Day 0, Day 7, Day 14) as mixed effects.

	Minimum	**1Q**	**Median**	**3Q**	**Maximum**
Richness	-227.89	-86.12	-31.20	44.67	983.67
Diversity	-1.2960	-0.3584	0.0487	0.2842	1.7729
Evenness	-0.0441	-0.0204	-0.0046	0.0081	0.1107

Supplemental Table 3. Summary of two-way Analysis of Variance Table comparing bacterial diversity metrics (OUT richness, Shannon Diversity Index H', and Simpson's Evenness associated with Social Status (dominant, subordinate, isolate, communal) and Day of socialization (Day IP, Day 0, Day 7, Day 14).

(A) OTU Richness

Fixed Effect	**SumSq**	**MeanSq**	**NumDF**	**F-value**	**Pr(>F)**
SocialStatus	131644	43881	3	1.268	0.2914
Day	293917	97972	3	2.831	**0.0439**
SocialStatus:Day	497947	55327	9	1.599	0.1306

(B) Shannon's Diversity (H')

Fixed Effect	**SumSq**	**MeanSq**	**NumDF**	**F-value**	**Pr(>F)**
SocialStatus	0.150	0.0499	3	0.195	0.8993
Day	0.1378	0.1378	3	0.539	0.6570
SocialStatus:Day	6.148	0.6831	9	2.672	**0.0094**

(C) Simpson's Evenness

Fixed Effect	**SumSq**	**MeanSq**	**NumDF**	**F-value**	**Pr(>F)**
SocialStatus	0.0045	0.0015	3	1.756	0.1627
Day	0.0090	0.0030	3	3.533	**0.0186**
SocialStatus:Day	0.0148	0.0016	9	1.924	0.067

Supplemental Table 4. Summary of two-way Analysis of Variance Table comparing bacterial diversity metrics (OTU richness, Shannon Diversity Index H', and Simpson's Evenness associated with Social group (social, nonsocial) and Day of socialization (Day IP, Day 0, Day 7, Day 14).

(A) OTU Richness

Fixed Effect	**SumSq**	**MeanSq**	**NumDF**	**F-value**	**Pr(>F)**
Group	44379	44379	1	1.199	0.2766
Day	293265	97755	3	2.641	0.0546
Group:Day	104921	37974	3	0.945	0.4227

(B) Shannon's Diversity (H')

Fixed Effect	**SumSq**	**MeanSq**	**NumDF**	**F-value**	**Pr(>F)**
Group	0.011	0.0115	1	0.039	0.843
Day	0.411	0.1371	3	0.470	0.704
Group:Day	1.160	0.3868	3	1.325	0.272

(C) Simpson's Evenness

Fixed Effect	**SumSq**	**MeanSq**	**NumDF**	**F-value**	**Pr(>F)**
Group	0.00137	0.0014	1	1.480	0.2271
Day	0.00895	0.0030	3	3.324	**0.0263**
Group:Day	0.00512	0.0017	3	1.852	0.1440

Supplemental Table 5. Summary of Permutational Analysis of Variance (PERMANOVA) comparing bacterial compositional differences between social status (dominant, subordinate, isolate, communal) and time (Day IP, Day 0, Day 7, Day 14). Number of permutations was 1000 and PERMANOVA was performed using Bray-Curtis dissimilarities in bacterial community composition.

	SumSq	**MeanSq**	**NumDF**	**F-value**	**Pr(>F)**
SocialStatus	2.301	0.767	3	3.671	**0.00099**
Day	3.156	1.052	3	5.035	**0.00099**
SocialStatus:Day	3.613	0.402	9	1.920	**0.00099**