THE EFFECTS OF SOCIAL HOUSING CHANGES, TEMPERAMENT AND SOCIAL RANK ON THE MICROBIOME COMPOSITION AND DIARRHEA RATES IN A COLONY OF MAURITIUS-ORIGIN MACACA FASCICULARIS

by
Keely McGrew

A thesis submitted to the Psychology Department,
College of Liberal Arts
in partial fulfillment of the requirements for the degree of

MASTER OF ARTS

in Psychology

Chair of Committee: Terri Kosten, PhD

Committee Member: Beau Alward, PhD

Committee Member: Julia Benoit, PhD

Committee Member: Nicole Monts de Oca, DVM ACLAM

University of Houston
May 2021

This thesis is dedicated to my husband, Jason McGrew, who supports my efforts by managing the house and children when I am at work and school. You are my rock, and I couldn't do all that I do without you.

ACKNOWLEDGMENTS

I could not have done this project without the support of Dr Nicole Monts de Oca and the team at Charles River. The processing team, led by Katelyn Ballard during the time of this project, worked with me on coordinating fecal sample collection. The processing team, particularly Ricardo Guerra, helped me collect samples when processing was occurring during times I needed to be in class. The vet technicians, particularly Roberto Ramirez, were very helpful in informing me when new diarrhea cases were noted in the pens, and letting me know if the animals were going to be relocated for treatment. My team of behaviorists helped support this project by picking up the slack in the program when I was busy working on collecting fecal samples and data.

Kourtney Nickerson and Danielle White from the Charles River RADS department were instrumental in walking me through the process of getting my fecal samples ready to send, processed, and then getting the fastq files and OneCodex report to me. Ken Henderson at CRL got me in touch with Kourtney Nickerson in the first place, and was instrumental in obtaining internal support for this project.

I would like to thank my lab mate Shadab Fourazan, who offered me so much support and advice over the past couple of years. Colin Hale offered me much guidance and support during the cortisol ELISA process and subsequent analysis. Kristi Hoffman at Baylor Metagenomics lab helped me get the fastq files provided by Charles River into the ATIMA program, and gave me a tutorial on how to use ATIMA to produce my microbiome graphs. Dr Julia Benoit met with me virtually to give assistance on statistical analysis. I would also like to thank Dr Kosten for her mentorship and support.

ABSTRACT

The gut-brain axis has been implicated in health outcomes related to physical and mental health in humans and animals. Social housing changes can be a source of stress in laboratory animals, and this stress may cause a negative shift in the gut flora ("gut dysbiosis") that can play a role in diarrhea. Diarrhea is the most common health problem noted in captive macaque populations, and it can have significant consequences. This study characterized changes in the microbiome of primates (*Macaca fascicularis*) that experienced a change in social housing and were exhibiting diarrhea. As an adjunct, behavior aspects (temperament and social status) and fecal cortisol (a measure of stress) levels were evaluated to see if there is correlation with presence and severity of diarrhea. Fecal samples of recently-imported animals were collected during specific routine sedation events. As the animals experienced a housing change, the entire cohort was monitored for diarrhea. Matched-case samples (one sick, one healthy animal) were collected when the diarrhea outbreaks began. Samples from each time point per animal were evaluated via NexGen 16S microbiome analysis and cortisol levels. No significant correlation was determined between temperament test results/social rank, HPA axis activity and diarrhea. Significant changes in alpha and beta diversity and in abundance levels of several taxa were noted on the microbiome analysis between the two time points and animals with and without diarrhea. Characterization of these changes will direct future interventions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1 Specific Aims

Idiopathic diarrhea is a common health condition in macaques, and methods to treat and help prevent diarrhea are always being investigated (Ferrecchia & Hobbs, 2013). Incidence rate can vary within facilities, but has been reported to affect as much as 15-39% of the population (Prongay *et al.*, 2013). It can result in mortality if it becomes acute or chronic (Howell *et al.*, 2012).

In the import/export colony of cynomolgus macaques (*Macaca fascicularis*) housed at the Charles River facility in Houston, there is a dramatic increase in diarrhea rates in the animals that originate from the island of Mauritius after they are moved from the quarantine building into a building that holds conditioned animals. The diarrhea rates tend to peak 4-6 weeks after the animals are moved from the quarantine building, where they are pair-housed during the 31-35 days of quarantine, to the group-housing units in a building that houses other conditioned animals. During this relocation, the animals are placed in groups of 8-10 with other members of their cohort (same shipment).

Approximately a dozen animals from each newly-arriving cohort (112-120 animals) into this building will display diarrhea. There are occasional cases that either become chronic and unresponsive to treatment, or that are severely acute and the animal reaches a state where therapeutic resolution is not possible. These acute or chronic cases are typically euthanized at the point of no resolution, and this is the primary cause of mortality in this colony. This mortality, although a small rate

compared to the number of animals that come through the facility, has a high cost to personnel in terms of time spent caring for these animals, and also represents a financial loss to the company. Therefore, it would be a refinement to existing practices to develop a method of determining animals that were most susceptible, or work towards a preventative or therapeutic intervention.

This project proposes to establish connections between biological, behavioral and physical outcomes to point towards the optimal direction for enhanced behavioral management as well as specific treatment interventions. The goals of the study are to determine if there are changes in the macaque microbiome related to diarrhea and social housing changes, and to determine if temperament or social rank is associated with diarrhea. A secondary aim of this study is to determine if the changes in the microbiome are associated with stress.

Specific Aim 1: Identify if nonhuman primates (*Macaca fascicularis*) displaying diarrhea after a social housing change have differences in their microbiome composition when compared to age/weight/sex matched healthy controls.

Specific Aim 2: Determine if presence of diarrhea and/or microbiome shifts are predicted by temperament, social rank, or stress reactivity (cortisol levels).

1.2 Background

The gut-brain axis has emerged as a focus of many studies looking at its role in emotional well-being. The gut-brain axis refers to the communication between the gut and the brain, which occurs via three routes: the endocrine system via hormones and their metabolites, the immune system, and the enteric system via the vagus nerve and

spinal column (Farzi *et al.*, 2018). Neurochemicals produced in the gut travel up to the brain and initialize changes in the brain that then affect behavior. Gut composition is influenced by birth and early life experiences, diet, social networks, and environment (Dettmer *et al.*, 2019).

The microbiota of the gut plays an important role in protecting the organism from enteric pathogens (Seekatz *et al.*, 2013). Studies suggest that in certain cases, diarrhea may associate with a stress-related "gut dysbiosis" or negative shift in gut flora (Mayer, Savidge, & Shulman, 2014). Dysbiosis also has effects on immune function, potentially allowing an organism to be more susceptible to pathogens. Additionally, dysbiosis contributes to "leakiness" of the gut, malabsorption of nutrients, and idiopathic diarrhea (Laing *et al.* 2018).

Gut dysbiosis is associated with anxiety, depression, and elevations of the HPA axis. Emotional stress can also affect microbiome composition (Dinan & Cryan, 2012). Macaques are social animals with a linear hierarchy and a range of temperaments. Stress can be induced by social changes within individual group hierarchies (Gottlieb *et al.* 2018). Social transitions can cause alterations in the stress pathways that can last for several days followed by a period of recovery (Capitanio *et al.* 2011).

Due to the nature of the work conducted at Charles River's import-export NHP quarantine site, social dynamics within the colony are in a constant state of flux. Therefore, this social flux makes it difficult to minimize stress that a specific individual may experience. This complex social behavior may have an impact on the

stress circuits (e.i. HPA axis activity) and provide insight into predictors of disease susceptibility (Sapolsky 2005). Nervous temperaments in primates are a risk factor in developing diarrhea (Elfenbein *et al.,* 2016). Temperament is used interchangeably with "personality" in NHPs, and is typically measured by behavioral responses within a context. In addition, personality has been noted to affect the immune response of NHPs in response to a housing relocation (social stressor) by measurement of antibody response to a vaccine (Maninger *et al.,* 2003), in which it was determined that individuals may be more or less likely to experience a negative health outcome based on aspects of personality. Association between a host organism's behavior and the gut microbiota is demonstrated in several sources (review, Parashar & Udayabanu, 2016). However, the relationship between social aspects of primates such as temperament and social status and microbiome composition is an area where there is a gap in the current literature (Dettmer *et al.,* 2019).

Social networks could alter the microbiome of individuals. Combining primates into new social groups results in microbial convergence by two weeks (Amaral *et al.,* 2017). Also, shared space and same diet could cause microbiome shifts towards a more similar composition (Archie & Tung, 2015). These are two examples of microbial convergence, but examining how gut microbiota differs can also be meaningful to understanding how the microbiome changes as a result of stress. Bailey and Coe (1999) examined the effect of stress (maternal separation) on the microbiome of infant rhesus and found temporary changes in *Lactobacilli* that correlated with stress-indicative behaviors and susceptibility to infection, but not with cortisol

secretion (an indicator of HPA axis dysregulation). A change in the populations of bacteria in the cecum in mice were noted in response to social disruption (decrease in *Bacterioides* and increase in *Firmicutes*, *Clostridium*), as well as a corresponding decrease in microbial diversity and richness (Bailey *et al.*, 2011).

2. MICROBIOME

2.1 Characterization of the Primate Microbiome

In order to understand the changes associated with microbiota in response to changes, some understanding of the composition of the primate microbiome is essential. Relevant taxonomic levels associated with microbiota include kingdom, phylum, class, order, family, genus, and species. Gut bacteria in mammals are similar across species at the phylum level, but differ in terms of specific genus and species (Bleich & Fox, 2015). Comparison of the microbiome of wild vs captive primates demonstrates that the state of captivity results in microbiome changes that shift towards a more human-like composition (Clayton *et al.*, 2016). In captivity, the primate microbiome shifts to higher relative abundance of *Prevotella* (genus level) and *Bacteriodes* (phylum level). In the human microbiome, *Prevotella* has a strong connection to high fiber diet (Yatsunenko, 2012), shows correlation with differences between lean and obese people, and is a feature of dysbiosis seen in autoimmune conditions (O'Keefe *et al.,* 2015). In a colony of young rhesus, *Prevotella* was the dominant taxon at the genus level (Amaral *et al.,* 2017).

Factors that promote gut health include richness and diversity of microbial species, which helps protect against pathogenic invasion. Microbial diversity was

lower in animals with colitis (as measured by the Shannon Index) compared to healthy macaques (McKenna *et al.,* 2008). *Campylobacteria* was more common in sick animals (5 out of 10 of the sick compared to 0 of the healthy animals). Richness and diversity measures may differ across populations from different geographic origins (Seekatz *et al.,* 2013). Sex differences are also noted, such as more abundant *Lachnospiracea*, *Bacteriodes*, and *Treponema* in males (McKenna *et al.,* 2008).

Analysis of microbiome composition in pilot samples sent from Charles River Houston to Charles River's Diagnostic Laboratory showed that the primary microbiota at the genus level of the Mauritius-origin cynomolgus macaques (n = 32) were *Prevotella, Lactobacillus, Treponema, Streptococcus, Ruminococcus, Eubacterium, Blautia, Lachnoclostridium, Oscillibacter, Prevotellamassilia, and Faecalibacterium.* Comparison of affected and healthy animals demonstrated a significant increase in *Faecalibacterium* (p = .02) and decrease in *Streptococcus* (p = .05) and *Ruminococcus* (p = .04) in diarrhea animals compared to controls (n = 16). There was a 10% decrease in abundance of Firmicutes at the phylum level in diarrhea animals versus controls. This provided preliminary evidence of a microbiota shift associated with diarrhea.

2.2 Diarrhea and Microbiome

Gut dysbiosis associates with diarrhea in many species of animals, such as dogs (Guard *et al.* 2015*),* cats (Suchodolski *et al.* 2015), pigs (Fouhse, Zijlstra & Willing, 2016) and cattle (Zeineldin, Aldridge & Lowe, 2018). Clinical diarrhea score also associates with many genera, including the genera *Flavobacterium, Pedobacter,*

Yersinia, and *Enterococcus*, which are rare genera, following a Shigella challenge in a 2013 study in macaques (Seekatz *et al.*).

A comparison of microbiome composition between colobines housed at the San Diego zoo showed a distinctly different composition in animals with poor GI health compared to healthy ones (Amato *et al.*, 2016). This study also demonstrated that GI flora does not vary along all sections of the GI tract, validating fecal collection as a reliable method of sample collection.

3. STRESS

3.1 Measurement of Stress

Measurements of glucocorticoids through blood, urine, feces and hair are frequently evaluated markers of biological stress in primates (Field *et al.*, 2015). In addition, an increase in circulating cortisol levels in response to stress is long been established in the literature (Rosso *et al.*, 2016). Exposure to a stressor leads to elevation of the hypothalamic-pituitary-adrenal axis, which releases glucocorticoids in response. Measurement of the level of these glucocorticoids in a fecal sample can be representative of the magnitude of a perceived stressor in the past several hours to several days (Field *et al.*, 2015). One study demonstrated that fecal glucocorticoid levels showed elevation 26 hours after a stressor, peaked at 38 hours, and returned to baseline at 48 hours (Lee *et al.*, 2010). The social stress that the primates in this study may be experiencing are expected to be related to the social housing change and subsequent social reconfiguration, which occurred presumably within days to a few weeks of diarrhea outbreak.

4. METHODS

4.1 Ethics Statement

All procedures were approved by the Animal Care and Use Committee at Charles River's Houston location.

4.2 Subjects and housing

Cynomolgus macaques (*Macaca fascicularis*) imported from the island of Mauritius to Charles River's import-export site during a time period from February to June 2020 were included in this study. The animals came from two different vendors (breeding farms) in the island of Mauritius, and seven different shipments (five that arrived close to the same time frame). Data were collected during quarantine for 776 primates (413 females/363 males). However, only data from 528 animals were included in the final analysis, as the remaining animals did not move out to the social housing unit, and therefore information on diarrhea and social status was unavailable for analysis. All animals were juveniles, in the age range of 1.61-3.81 years (mean 2.27, sd 0.52). The subjects ranged in weight from 1.9-5.9 kg (mean 2.62 kg, sd 1.33). The portion of animals included in the microbiome analysis portion of the study were all from the same farm and arrived in the facility close to the same time.

All the animals were housed indoors. The animals were initially housed in a CDC-approved quarantine building in quad rack cages. The quarantine rooms consist of quad-rack style caging with 4.69 sq ft of floor space per animal. The dividers between the two cages in both upper and lower portions of the quad rack caging were removed,

allowing two animals of the same sex to share two cage spaces in a pair-housing relationship.

Following quarantine, most of the animals were relocated to another building, where they were housed in groups. The group housing area is composed of 120 (6 X 8 X 6.5 ft) stainless steel social housing units. Each unit can house up to 11 monkeys weighing less than 10 kg per the USDA and *Guide* NHP housing recommendations. Typically, the animals are housed at a density of 8 individuals per group. Each unit may include structures such as perches at varying levels, swings, toys, or other devices to promote exercise and provide visual barriers, as outlined by our institute's husbandry and care program.

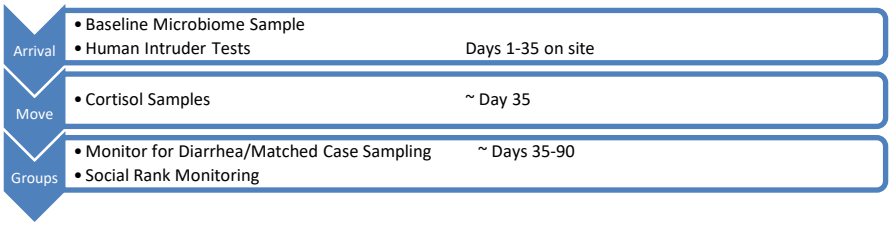
The housing environment was maintained at 69 to 75 °F (22.6 to 23.9 °C), with relative humidity of 30% to 70%, and on a 12:12-h light:dark cycle. Chlorinated and filtered municipal water was provided *ad libitum* through an automated watering system. All animals were examined by a veterinarian at import, dosed with ivermectin and praziquantel (Droncit), and tested for tuberculosis. All the animals were fed a commercial monkey chow (Lab Fiber-Plus Monkey Diet, PMI International, Brentwood MO) and given supplemental treats and fresh produce on a regular rotating basis.

4.3 Groups and Design

The study design chosen was a matched case-control study, which is a method for studying diseases with low incidence in the population in which cases of the affected population are matched to a healthy control for data analysis (Niven *et al.*

2012). Effect size is unknown, as similar studies have not been conducted, but a sample size of 60 per matched case (30 diarrhea, 30 control) with two time points was believed to be effective at determining a large or medium effect size. Typically, animals from incoming shipments have been breaking with diarrhea at about a 10% incidence rate around the 4-6 weeks after the housing change. When an animal is noted to have diarrhea, fecal samples were collected from the affected animal and a healthy animal in its same shipment cohort that was not showing signs of diarrhea. This healthy animal's sample served as the matched control. In order to determine whether there may be a difference in the way that stress is processed by sex, we attempted to gather samples from equal numbers of groups of males and females. The samples included in microbiome and cortisol analysis were derived from 28 females and 29 males. The experiment ended 8 weeks after the animals moved to the group housing unit.

Timeline of events:



4.4 Specimen Collection

Fecal samples of all animals of Mauritius imports (112-120 animals) were collected during specific routine sedation events for a total of 7 imports. The first fecal samples were collected at the first TB test, approximately 6 days after arrival into

the facility from Mauritius. This sample served as a baseline measure of the microbiome composition at the time of arrival. Samples were collected by inserting a fecal loop into the rectum of the animal, and then relocating the feces into a cryotube using a cotton tipped applicator. Samples were stored in the -80 freezer until lab analysis is warranted. Samples from all incoming animals were collected and stored, as it was not known at the time of arrival which animals would break with diarrhea.

A second fecal sample was collected at the 3rd TB test, four weeks after the first test. This sample was the one used for the cortisol assay, to measure the amount of HPA activation experienced by the individual animal within the 1-2 days prior to sample collection. This sample timepoint served as a baseline cortisol sampling per animal and the resulting value was compared to cortisol levels from another sample from the same animals taken at the time the diarrhea outbreak starts in the group housing units. The cortisol assay was conducted at University of Houston using Cayman Cortisol ELISA kits.

4.5 Behavioral observations in quarantine. Behavior observations were conducted to determine if there is a behavioral profile that correlates with health outcomes that predicts the susceptibility of primates to diarrhea. The temperament of the animals was assessed during the quarantine period using a modified version of the Human-Intruder test. This specific modified version has been used by the author for over 8 years at the facility and has been used reliably as a temperament assessment tool for adult male primates for pair housing purposes (McGrew 2017).

This test consists of recording behaviors offered by the animal during an "intruder threat". The test process involves separating the animals from their social partner. Each set of separated animals is given five minutes following separation to habituate to being separated before the test began. Following that time, an unfamiliar human stands one foot away from the front of the animal's cage. The human offers first their side profile (profile phase) by looking at the opposite wall, and then a direct stare (stare phase) by turning and facing the animal. The profile and stare phase consisted of 30 seconds each, and all behaviors observed were recorded. Definitions of recorded behaviors can be found in Table 1.

4.6 Behavioral observations in group housing. Social housing data was collected on 530 of the 776 total animals on the study. The remaining 246 animals were not moved to the group housing building, either due to leaving the facility directly from the quarantine building, or being retained in a holding room in the quarantine facility due to a lack of available space in the group housing unit. The animals included in this group housing population (530) included 317 females and 213 males in an age range of 1.63-3.81 years (mean 2.35 years, sd .54) and a weight range of 1.9 – 5.0 kg (mean 2.62, sd 1.31). Table 2 provides a summary of the characteristics of animals included in this population. Assessment of the behaviors of the animals within the group was conducted using a standardized ethogram for five minutes once a week after moving out to the social housing unit. The number of weekly observations performed ranged from 1 -7 observations (mean 3.72, sd 1.52). A rank certainty ratio was calculated by

dividing the number of observations of the most common rank assignment divided by the total number of observations. The average rank certainty equaled 0.75.

The social rank observations consisted of focal-animal sampling of the entire group for five minutes in which all behaviors of all individuals are noted, with interactions between individuals noted. Use of this technique for group observation is possible due to the design of the enclosure, which allows for continuous visibility for all members of the group for the extent of the sampling period (Altmann 1974). Following this, an identification of social rank status (high, mid or low rank) was noted for each individual. This observation was determined by behaviors observed within the five-minute focal sampling, and by body language and facial expressions directed towards or away from other individuals within the group.

Video-taping of group housing assessments was considered, but ultimately rejected due to the following reasons: institutional and management concern about security and storage of recorded videos and consultation with expert primate behaviorists (personal communication with Paul Honess, Carol Shively and Susan Lambeth-Pavonetti) who all recommended human visual observation only. Other reasons provided included that the animals were already habituated to human presence, that video would remove context from behaviors, and also that it would be expensive to store and code.

4.7 Diarrhea observations. After each import cohort was moved to group housing, the cohort was monitored for diarrhea during weeks 2-8 post-relocation. Every effort was made to minimize social movements in and around those pens in order to minimize social disruption. Monitoring for diarrhea consisted of reading daily health

reports filled out by trained animal care technicians. If diarrhea was observed inside the social housing unit, the technicians would record a score (0-6, with a 4 being loose stool, and 5 and 6 indicating progressively more liquid stool). Following a report of a fecal score of 5 or 6, an attempt was made to determine the individual animal with diarrhea by standing inside the group housing unit with the animals and watching them defecate. If the animals seemed shy to defecate and were nervous with the observer inside the unit, then they were watched from outside the unit.

Once an animal was identified with diarrhea, a fecal sample was collected by one of two methods. If a fresh sample was immediately provided by the animal, then a sterile cotton tipped applicator was used to collect a small amount (approximately the grain of rice) of the top layer of the fresh feces. If the animal did not defecate within a five-minute observation period, then an animal care technician would hand-catch the animal for a sample to be obtained either by direct collection (if the animal defecated during capture), or by insertion of a fecal collection loop into the anus. A sterile cotton tipped swab was used to collect the sample from the outside of the anus or the fecal collection loop. A second fecal sample was collected using the same method from a healthy animal from the same social housing unit. Animals in the same housing unit were matched by age, weight and sex, and were exposed to the same food, environment, care staff. This sample served as the control sample. This control animal was chosen randomly by selecting the first animal observed to defecate a normal stool during the observation period. The feces were placed in two separate 2 ml cryotubes, labeled, and placed in the -80 freezer.

Upon completion of collection of all desired samples, the samples stored in the freezer were removed, sorted, and those final samples plus their corresponding frozen baseline samples were shipped off to the diagnostic lab for microbiome analysis, to determine if there is a significant shift in microbiome composition in affected animal compared to controls.

4.8 Microbiome Analysis Microbiome analysis was conducted by the RADS department of Charles River's diagnostic lab. DNA was isolated from submitted samples using optimal sample-dependent column or magnetic purification kit per the manufacturer's protocol. Recovery yield and DNA quality was determined by fluorometric analysis. DNA concentration was adjusted to specifications and amplified using broadly reactive 16s rRNA primers spanning the V3 and V4 regions. Resulting amplified PCR products were analyzed for quantity and correct product size then purified and amplified with primers containing unique sample nucleotide barcodes. PCR products quality and quantity were further analyzed by SYBR green qPCR. All samples were pooled and adjusted to a normalized concentration. The DNA library pool was denatured with sodium hydroxide, normalized to optimal loading concentration and combined with PhiX control. Extended read lengths up to 2 X 300 bp was used for cluster generation and sequencing. Following the sequencing run, the sequence data was de-multiplexed based on the nucleotide barcode and compared to the One Codex Targeted Loci Database for taxonomy identification and subsequent alpha and beta diversity analysis.

All raw sequences were analyzed using the One Codex Targeted Loci Database (TLDB)1. The One Codex Targeted Loci Database is specifically designed for marker gene sequencing and built using the most commonly used genes for microbial surveys, including 16S, ITS, 18S, and others. The Targeted Loci Database contains 250,000 curated gene records spanning the known microbial world bacteria, archaea, fungi, protists, algae, etc. To analyze samples against the Targeted Loci Database, every read is aligned with high sensitivity (using SNAP) and the best alignments to the database are identified. Each read is assigned to the most specific taxonomic grouping that the data support, down to the species-level where appropriate. Abundance-based filtering is performed next to minimize the number of false positive assignments that may be introduced by sequencing error. In this filtering step, any organism whose readcount (with children) makes up less than 0.00005x of the total are reassigned to its parent. In addition, any organism at the genus level or below whose readcount (with children) makes up less than 0.01x of its immediate parent is also reassigned to its parent.

Following the processing of microbiome samples by Charles River, the fastq files were sent to the Baylor Metagenomics Lab for bioinformatics. The fastq files were uploaded into the *Agile Toolkit for Incisive Microbial Analyses* (ATIMA) platform, an in-house R-based platform created by the Baylor Metagenomics lab, for sample comparison. ATIMA is a stand-alone tool for analyzing and visualizing microbiome data sets that combines publicly available packages (Paradis, Claude & Strimmer, 2004, Oksanen *et al.* 2017) with purpose-written code to import sample data and identify trends in taxa abundance, alpha-diversity, and beta-diversity as they relate to

sample metadata. ATIMA enables analysis of rarefied count or non-rarefied relative abundance data. Categorical variables are evaluated using the non-parametric Mann-Whitney U (Mann & Whitney, 1947) and Kruskal-Wallis (Kruskal & Wallis, 1952) tests for variables with two groups or three or more groups, respectively. Relationships with continuous variables are tested using R's base function for linear regression models. Differences in community composition use PERMANOVA (Dwass 1957, Anderson 2001) to estimate p-values. ATIMA heatmaps cluster by complete linkage and are generated via the R package 'pheatmap' (Kolde 2015), using default parameters. All p-values are adjusted for multiple comparisons with the Benjamini-Hochberg false discovery rate (q=0.05) (Benjamini & Hochberg, 1995).

Comparison between groups (diarrhea vs control) in the matched sample set in microbiome composition and statistically significant changes in the abundance of bacteria at the genus and phylum level were initially analyzed using multiple ANOVA in R, an open-source statistical analysis platform. The significance level was set at α=0.05. The ATIMA platform allowed for comparisons via Mann Whitney and Kruskal-Wallis tests, with FDR correction for multiple comparisons for the relative abundance level comparisons. We expected to see that GI-unhealthy macaques have a microbiome composition that is distinct from GI-healthy macaques, and to see a shift in composition in within-group samples.

Multiple regression was used to determine correlation between results on the human intruder test, social rank assessment, and diarrhea (present or absent) in the larger (full, not matched) sample set. We hoped to identify behavioral profiles that

predict diarrhea and identify relationships that can be utilized to target specific groups of animals for intervention strategies.

4.9 Cortisol Measures Cortisol was measured in the feces utilizing the Cayman Chemical Cortisol ELISA kit #500360. This is a competitive assay that has a range from 6.6-4000 pg/ml and a sensitivity of approximately 35 pg/ml. The assay is based on a competition between cortisol and cortisol-acetylcholinesterase (AChE) for a limited number of cortisol-specific mouse monoclonal antibody binding sites. For an extraction of cortisol, wet fecal samples were weighed to 0.1g. The feces were added to 1ml of 100% methanol in a plastic centrifuge tube and homogenized using a metal stirring rod. Each sample was vortexed for 30 seconds and then centrifuged for 10 minutes at 2500rpm. The supernatant was removed and put into an Eppendorf tube using a micropipette. The sample was diluted (in MeOH) 1:20 using the Cayman EIA buffer (20ul + 380ul buffer) and then standard protocol completed the assay (Cayman). Microplates were placed on a Biotek absorption reader and analyzed to assess the amount of light absorption per sample using the Gen5 software. Readings were recorded at 405, 410 and 420nm. The blank values were too high at 405 and 410 nm and the most reliable readings were determined to be the ones read at 420nm. Each sample was processed in duplicates and final concentrations for the duplicates were averaged for a baseline and post stress measurements for each animal.

5. Results

5.1 Behavioral observations in quarantine. The total sample population of 776 primates were tested exactly once on this test during the 31 days of their quarantine

period. Behavioral responses were used to categorize the animals into temperament types by assessing behavioral clusters by use of Principal Component Analysis. Behavior observations conducted in quarantine on 528 of the primates who were eventually moved to the social housing unit were analyzed through *Statistica* software using a Principal Components Analysis with varimax normalized factor rotation. Two individuals were removed from the analysis due to incomplete data. The two phases of the test were run separately. Table 3 shows the results from the Profile phase, and Table 4 shows results from the Stare phase.

Variables showing low numbers of observations (defecate, tooth grind, grimace and Other) were eliminated, and the factor analysis was rerun. In this second analysis, two dimensions showed higher eigenvalues than the third. Based on the behaviors represented in the plot (Figure 1), it was determined that the two dimensions revealed from the data were a spectrum of Boldness vs Fear (Factor 1), and Friendliness vs Aggression (Factor 2). These factors explain approximately 24% of the variability in the data.

The singular behaviors were compared to diarrhea incidence via a correlation matrix using R software, to determine if expressed behaviors related to an increased likelihood of an animal exhibiting diarrhea. Figure 2 shows the univariable regression analysis for behaviors exhibiting during the Profile phase, and Figure 3 shows univariable regression for the behaviors exhibited during the Stare phase. There was no strong correlation between diarrhea and behaviors observed in quarantine.

For the purposes of further analysis, the behaviors expressed in each cluster per animal were summed up, and a fifth aspect to score called “Anxious” was scored, based on behaviors seen in the middle of the PCA axis. This is a summary of what behaviors were summed up to compose each “temperament” category:

Aggressive = Open mouth threat + lunge + coo

Friendly = Peering + Girney + Lip Smack + Divider Pressing

Fearful = Fear vocalization + freeze

Bold = Bark + Crouch + Approach

Anxious = Gaze Avert + Movement Stereotypy

A regression matrix was created in R using the cor.plot function in the *psych* package to examine correlation between these temperament categories, diarrhea observation, and the results of the behavioral observations in group housing (Figure 4). No significant correlations were observed.

5.3 Behavioral observations in group housing. The groups of animals were observed using the ethogram on a weekly basis, and then based on observed behaviors and body language, the animals were assigned to either “high”, “mid” or “low” rank. A proportion of animals were given the same social rank each time, but some had differing ranks. This may have to do with the fact that these were juvenile animals in newly formed groups. Hierarchy stability was not expected to be completely stable for this reason. In addition, rank relationships for macaques in general are not believed to be completely stable until they reach 4-5 years of age (Paul Honess,

personal communication). A ratio of the dominant social status assignment compared to the number of assessments was used to calculate “rank certainty”.

For analysis, the social rank was given a numerical score on a range, with animals always scoring “low” assigned a 1, Mid” only was a 4, and those always assigned to “high” a 6. If the animal was assigned at an equal ratio to “Low” and “Mid”, it was a 2, “, equal “Low”, “Mid” and High” a 3, and “Mid” and “High” a 5. This number was utilized to examine correlation between diarrhea and other variables. The correlation between social status as well as rank certainty to diarrhea showed little relationship. Weight and age contributed a significant effect on diarrhea presence over and above social rank and rank certainty alone, F (1, 524) = 9.49, $p < 0.00$.

5.4 Diarrhea Observations

A total of 527 animals was observed for diarrhea during the group housing phase. Of those, 60% were female (316) and 40% were male (211). The overall proportion of males who exhibited diarrhea (n = 29, 13.7%) was significantly higher than the proportion of females (n = 28, 8.8%). A total of 33 samples from diarrhea animals was obtained to submit for microbiome and cortisol analysis. The study protocol called for collection of fecal material within the first two days of diarrhea being noted on health observations, but veterinary intervention was typically not given until the third day of diarrhea persistence. Fourteen of the animals whose sample was collected for analysis had their diarrhea resolve before the third day, so treatment was not initiated and their diarrhea was noted as “transient”. Eight animals were treated with the standard diarrhea protocol (Bismuth Subsalicylate, L’il Critters Brand Gummy

Fiber & Gummy Probiotic) and resolved. These animals were categorized as "mild" on comparison measures for microbiome composition. The rest of the animals had diarrhea that persisted or reoccurred after initial treatment (moderate severity), with one animal being euthanized during the observation period for chronic, unresolving diarrhea (severe). A relationship between overall temperament of animals and diarrhea incidence was explored (Table 5), but a chi-square test of independence showed there was no significant relationship.

5.5 Cortisol

Cortisol results were evaluated using R. These results are illustrated in Figure 6. A two-way ANOVA was performed in R to compare the two levels of Group (control, diarrhea,) and two levels of Time (Time 1 and Time 2). There was no significant effect of group or of time. Figure 7 shows a plot representing sex differences at the two time points. There was a trend noted of lower cortisol in diarrhea females compared to males when examining the figure. However, the effect of sex was also explored by one way ANOVA, and this result was also not significant, nor was there a significant finding of Group x Time x Sex. The interactions of Sex x Time and Sex x Group were probed as well, and no significance was found.

5.6 Microbiome Analysis

Alpha diversity measures the ecological diversity within each sample, within-sample variance (Finotello, Mastrorilli & Di Camillo, 2018). It is usually measured as a single number from 0 (no diversity) to infinity. The methods of calculation used to examine this in these samples included the Observed OTUs and the Shannon index.

Observed OTUs compares number of reads obtained per sample. The Shannon Index measures how much unique information is contained in a given sample and is an estimator of the richness and evenness of the abundances of all taxa in the sample (Hagerty *et al.* 2020). Figure 8 shows a comparison of alpha diversity between the four microbiome sample groups: baseline of the control group, baseline of the group that later got diarrhea, post-relocation of the control group and post-relocation of the group that had diarrhea at this time point. The results showed a significant difference between the baseline samples of both groups and the relocation samples of both groups (Kruskal-Wallis, FDR-adjusted $p < 0.00$) for both Observed OTUs and the Shannon Index.

Beta Diversity is the relative distance or dissimilarity between two communities or samples (Finotello, Mastrorilli & Di Camillo, 2018). This is calculated by performing pairwise comparisons between all pairs of samples, and this data is used to identify similar clusters of samples using tools such as Principal Coordinates Analysis. The Principal Coordinates Analysis demonstrated in Figure 9 shows some separation of samples in beta diversity by group, with PC1 explaining 47% of the variance in samples and PC2 explaining 17%. Figure 10 illustrate the Beta Diversity between the four groups of animals by showing spread away from a centroid. Figure 11 examines the beta diversity between animals with differing levels of diarrhea severity. The sex trend in the diarrhea incidence rate and the cortisol results was explored further by comparing beta diversity by Group and Time and separated by Sex in Figure 12. In this figure, the more complete separation of colored samples in the male column

compared to females indicates that male animals that presented with diarrhea had a greater diversity from those who did not, compared to females. Figure 13 provides a 3D ordination plot view of the beta diversity spread between the four comparison groups.

Characterizing and comparing the relative abundances of predominant taxa is another method of making comparisons between groups of microbiome samples. To explore differences in relative abundance between significant taxa and summarize what the changes meant, the direction of abundance changes was explored in association with the significant findings. All abundances are calculated from the filtered results of the One Codex Targeted Loci Database classification. Table 6 summarizes the changes, before correction for multiple comparison was applied. Differences in time points quantify the changes between the baseline sample collected during the first week of quarantine and the second sample collected following relocation, at the beginning of diarrhea outbreaks. After relocation and during the diarrhea outbreaks, the following changes were noted: Phylum level increase in *Proteobacteria, Actinobacteria*; Genus level decrease in *Kineothrix, Streptococcus* and increase in *Ruminococcus, Lactobacillus, Clostridium*; Species level decrease in *Prevotella copri*, and increase in *Collinsella aerofaciens*.

Differences between the Diarrhea group and control group were quantified. The animals that were noted to get diarrhea showed the following differences from animals who did not get diarrhea: Phylum level increase in *Proteobacteria*, *Spirochaetes*, decrease in *Bacteroides*; Genus Level: Increase in *Treponema* and *Heliobacteria*;

Species Level: decrease in *Prevotella copri*, and an increase in *Collinsella aerofaciens*.

This analysis had looked at the differences as a whole between baseline and relocation, and between control and diarrhea animals. Following this, the data were further stratified to compare differences between animals who later got diarrhea and those who did not (control) at baseline and then at relocation. These data are represented by the bar graphs in Figure 16 (Phylum level), 19 (Genus level) and 22 (Species level). Final comparisons in relative abundance at the phylum level were determined by use of the Mann-Whitney test, with FDR correction. Table 7 provides details on the results of the test. In summary, between baseline and relocation, there were significant increases in Actinobacteria ($p < 0.00$) and Proteobacteria ($p = .039$). The control group showed increases after relocation in three phyla: Desulfobacterota ($p = .005$), Fibrobacterota ($p = .027$), and Spirochaetota ($p = .038$).

To categorize differences between groups and time points in abundance levels at the genus and species level, multiple ANOVAs were carried out in R using the ANOVA function on the read counts included in the raw data, with Bonferroni's correction applied for multiple comparisons ($p = .006$). Specific targets were chosen based on visualization of abundances based on Figures 18 & 19 for the genus level and 21 & 22 for species level. Genus level results showed Significant effects of Time/Not Group on *Lactobacillus* ($p = 0.001$), *Kineothrix* ($p < 0.000$), *Streptococcus* ($p < 0.000$), and *Clostridium* ($p = 0.001$), Time/not Sex or Group on *Sutterella* ($p = 0.001$), Group but not Time on *Treponema* ($p < 0.002$). The diarrhea group was more enriched with

Heliobacteria at baseline ($p = 0.02$) than at relocation, and enriched with *Kineothrix* following relocation (($p < 0.000$).

At the species level, the dominant taxa included *Collinsella aerofaciens, Prevotella copri, Kineothrix alysoides, Lactobacillus salivarius, Clostridium botulinum,* and *Lactobacillis reuteri*. Two species had significant differences between groups after relocation. *Prevotella copri* was reduced in diarrhea animals compared to controls ($p < 0.000$), and *Kineothrix alysoides* was enriched ($p < 0.000$). There were significant effects of Group and Time on *Prevotella copri* ($p < 0.000$ Time, P = .008 Group), and Time only on *Lactobacillus salivarius* ($p = 0.002$). No significant difference was noted in *Lactobacillus reuteri*. This particular species was explored because it is the main probiotic utilized for supplementation to improve digestive health.

6. DISCUSSION

The results of the present study demonstrate microbiome differences before and after relocation to group housing, and between animals with and without diarrhea. The changes explored in the microbiome analysis may indicate the role that particular bacteria play in the digestive health of cynomolgus macaques due to the presumed stress of relocation. No relationship was noted between temperament, social rank, rank certainty and diarrhea. Nor was there an association between cortisol levels and diarrhea presence or severity. However, it was noted that aggressive animals (4:1) and males (14:9) had higher incidence of diarrhea. A trend was noted in terms of sex effect in cortisol levels in that females who developed diarrhea tended to have lower cortisol

levels compared to baseline, but the difference between time points and interaction effects were not significant. Sex differences were also noted in beta diversity comparisons between animals who did and did not get diarrhea after relocation, indicating that male animals have more distinct changes in microbial composition after relocation than females do. These differences indicate the possibility of a sex-dependent difference in health outcomes in juvenile macaques.

Personality dimensions of Fearfulness vs Boldness and Aggression vs Friendliness, as determined by the Human Intruder tests were not informative in determining diarrhea susceptibility. However, the results are consistent with previous studies on animal personality, particularly in primates, that noted similar dimensions of Boldness vs Shyness, Aggression and Sociability (Koski 2014). Identifying clusters of behaviors was informative in characterizing how juvenile cynomolgus macaques behave typically in a home-cage version of the Human Intruder test. However, the two factors identified via PCA explained just 24% of the variance, which suggests further exploration of latent variables regarding behavioral expressions may be warranted.

Characterization of the macaque microbiome overall in the study was informative. *Prevotella* was noted to be a dominant organism. *Prevotella* species are normally considered to be beneficial symbionts that assist the host in digestion of plant material (David et al; 2014) but have also been associated with inflammatory disease and gut dysbiosis (Larsen 2017). At the species level, the dominant species identified was *Kineothrix alysoides*, a species identified in 2017 and described as a butyrate producing anaerobe (Haas & Blanchard, 2017).

Overall fecal microbiota composition of the diarrhea group differed from that of the control group. At the phylum level, *Proteobacteria* was noted to be increased both after the relocation event, and in animals with diarrhea. A high abundance of *Proteobacteria* has associated with dysbiosis and inflammation in hosts (Moon et al 2018) and are linked to stress perception and depression in humans (Peter et al 2017). This particular phylum is commonly found and fairly diverse, but it does include pathogenic bacteria such as campylobacter, salmonella and e. coli. However, there were no significant findings in these three pathogens. Campylobacter was noted in the overall read counts but there were no significant differences between time points or groups in this class. Salmonella and e.coli was not noted at a level high enough to compare between groups.

Differences noted after relocation included an increase in the phylum *Actinobacteria,* which has been associated with gut homeostasis. This phylum includes the class *Bifidobacterium*, commonly used as a probiotic to improve health (Binda *et al.*, 2018). A decrease in genus *Lactobacillus* at the genus level, as observed after relocation to group housing, could be indicative of stress (Lutgendorff *et al.*, 2008). A decrease in *Kineothrix* was also noted after relocation. In mice, increased *Kineothrix* associated in decreased anxiety-like behavior (Liddicoat *et al.,* 2020). Both relocation and diarrhea were associated with a decrease in *Prevotella copri* and an increase in *Collinsella aerofaciens*. Higher *P. copri* has been associated with health and growth of livestock (Amat *et al.*, 2020). *Treponema* was enriched in samples from animals with diarrhea. This genus is in phylum *Spirochaetes*, and is a known fiber

degrader, typically associated with high fiber diet (Rhoades *et al.*, 2019). Interestingly, in another study, *Treponema* abundance was decreased in juvenile macaques with idiopathic diarrhea compared to control animals (Westreich et al 2019). The role of *Treponema* in diarrhea in macaques warrants further explanation

A hypothesis tested in this study was that the diarrhea occurring after relocation was due to s stress-induced gut dysbiosis. However, there was little evidence of stress-related behavior on the behavioral observations of group housed animals. In addition, cortisol levels did not show any significant differences between groups (healthy vs diarrhea affected animals), time points, or sex. An interesting trend in the data in which female monkeys with diarrhea tended to have lower cortisol levels than controls was noted, but not significant. Lower cortisol in diarrhea animals versus healthy animals was noted in a previous study looking at chronic idiopathic diarrhea (Howell *et al*., 2012). A theory of low confidence or behavioral inhibition coupled with low cortisol levels may need further exploration. In addition, there may be differences in the way males and females process stressful events such as social changes, akin to the "tend and befriend" model proposed for females rather than the "fight or flight" model (Taylor *et al*., 2000).

Another possible explanation for the lower cortisol in females with diarrhea may be related to how female macaques interact with each other in a social housing setting compared to males. In our study, there was a higher number of males than females that presented with diarrhea, and also a higher number of animals who scored in the "Aggressive" category that developed diarrhea compared to those who did not.

It may be possible that once the females moved to the social housing group, they spent more time huddling and grooming with each other than the males did. One study examining *Shigella* infection rates compared to social interactions in macaques noted that in networks where more huddling and grooming was noted, there was less transmission of *Shigella*, whereas in social groups where there was more aggression noted, there were higher rates of transmission (Balasubramaniam *et al*., 2016). Performing a network analysis in future studies to examine rates of grooming, huddling, aggressive interactions in male social housing groups compared to female social housing groups may shed some light on to the difference between the diarrhea outcomes and cortisol level differences between the sexes.

Additional or different measurement of stress may provide more meaningful insight in future studies. Cortisol levels were consistently higher than what has been reported in zoo primates undergoing relocation stress (Cinque *et al*., 2017) in both time points throughout this study, possibly indicating long term rather than acute stress. Using hair cortisol (a measurement of chronic stress) may have been more insightful than using fecal cortisol as in this study, which measures HPA axis from the past 1-2 days.

Overall, the data demonstrated a microbiome composition shift associated with both the relocation itself and diarrhea status compared to healthy animals. Animals that later got diarrhea differed from animals who did not get diarrhea at baseline in only two abundance measures: higher levels of *Proteobacteria* and *Heliobacteria*. Relocation resulted in a higher number of changes in abundance levels between

diarrhea and control animals. The diarrhea animals continued to have higher enrichment of *Proteobacteria* over control animals, and also showed enrichment of *Kineothrix* at the Genus level and *Kineothrix alysoides* at the species level, a decrease in genus *Prevotella* and species *Prevotella copri*, and a decrease in genus *Streptococcus.* No significant behavioral or social predictors of diarrhea emerged in the data, and the diarrhea incidence did not appear to be associated with an increase in cortisol levels. An interesting effect of sex was noted in the cortisol level data and in diarrhea rates that may indicate a differing health response to stressors based on sex that warrants further exploration. The data suggest that behavioral testing, social status observations or measurement of HPA axis activity may not be beneficial in managing the colony to reduce diarrhea rates. However, the changes in microbiome composition imply that it would be beneficial to focus efforts on the microbiome changes for future intervention strategies.

7. REFERENCES

Altmann, J. (1974). Observational study of behavior: sampling methods. *Behaviour*, *49*(3-4), 227-266.

Amaral, W. Z., Lubach, G. R., Proctor, A., Lyte, M., Phillips, G. J., & Coe, C. L. (2017). Social influences on prevotella and the gut microbiome of young monkeys. *Psychosomatic medicine*, *79*(8), 888.

Amat, S., Lantz, H., Munyaka, P. M., & Willing, B. P. (2020). Prevotella in Pigs: The Positive and Negative Associations with Production and Health. *Microorganisms*, *8*(10), 1584.

Amato, K. R., Metcalf, J. L., Song, S. J., Hale, V. L., Clayton, J., Ackermann, G., ... & Schrenzel, M. D. (2016). Using the gut microbiota as a novel tool for examining colobine primate GI health. *Global Ecology and Conservation*, *7*, 225-237.

Anderson MJ (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26 (1): 32-46.

Archie, E. A., & Tung, J. (2015). Social behavior and the microbiome. *Current opinion in behavioral sciences*, *6*, 28-34.

Bailey, M. T., & Coe, C. L. (1999). Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. *Developmental Psychobiology: The Journal of the International Society for Developmental Psychobiology*, *35*(2), 146-155.

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G., & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain, behavior, and immunity*, *25*(3), 397-407.

Balasubramaniam, K., Beisner, B., Vandeleest, J., Atwill, E., & McCowan, B. (2016). Social buffering and contact transmission: network connections have beneficial and detrimental effects on Shigella infection risk among captive rhesus macaques. *PeerJ*, *4*, e2630.

Benjamini and Hochberg (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* Series B 57 (1): 289-300.

Binda, C., Lopetuso, L. R., Rizzatti, G., Gibiino, G., Cennamo, V., & Gasbarrini, A. (2018). Actinobacteria: a relevant minority for the maintenance of gut homeostasis. *Digestive and Liver Disease*, *50*(5), 421-428.

Bleich, A., & Fox, J. G. (2015). The mammalian microbiome and its importance in laboratory animal research. *ILAR journal*, *56*(2), 153-158.

Capitanio, J. P., Mendoza, S. P., & Cole, S. W. (2011). Nervous temperament in infant monkeys is associated with reduced sensitivity of leukocytes to cortisol's influence on trafficking. *Brain, Behavior, and Immunity*, *25*(1), 151-159.

Clayton, J. B., Vangay, P., Huang, H., Ward, T., Hillmann, B. M., Al-Ghalith, G. A., ... & Cabana, F. (2016). Captivity humanizes the primate microbiome. *Proceedings of the National Academy of Sciences*, *113*(37), 10376-10381.

Cinque, C., De Marco, A., Mairesse, J., Giuli, C., Sanna, A., De Marco, L., ... & Cozzolino, R. (2017). Relocation stress induces short-term fecal cortisol increase in Tonkean macaques (Macaca tonkeana). *Primates*, *58*(2), 315-321.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., ... & Biddinger, S. B. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559-563.

Dettmer, A. M., Allen, J. M., Jaggers, R. M., & Bailey, M. T. (2019). A descriptive analysis of gut microbiota composition in differentially reared infant rhesus monkeys (Macaca mulatta) across the first 6 months of life. *American journal of primatology*, *81*(10-11), e22969.

Dinan, T. G., & Cryan, J. F. (2012). Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. *Psychoneuroendocrinology*, *37*(9), 1369-1378.

Dwass M (1957). Modified randomization tests for nonparametric Hypotheses. *The Annals of Mathematical Statistics* 28 (1): 181-187.

Elfenbein, H. A., Rosso, L. D., McCowan, B., & Capitanio, J. P. (2016). Effect of indoor compared with outdoor location during gestation on the incidence of diarrhea in indoor-reared rhesus macaques (Macaca mulatta). *Journal of the American Association for Laboratory Animal Science*, *55*(3), 277-290.

Farzi, A., Fröhlich, E. E., & Holzer, P. (2018). Gut microbiota and the neuroendocrine system. *Neurotherapeutics*, *15*(1), 5-22.

Ferrecchia, C. E., & Hobbs, T. R. (2013). Efficacy of oral fecal bacteriotherapy in rhesus macaques (Macaca mulatta) with chronic diarrhea. *Comparative medicine*, *63*(1), 71-75.

Field, A. E., Jones, C. L., Kelly, R., Marko, S. T., Kern, S. J., & Rico, P. J. (2015). Measurement of fecal corticosterone metabolites as a predictor of the habituation of rhesus macaques (Macaca mulatta) to jacketing. *Journal of the American Association for Laboratory Animal Science*, *54*(1), 59-65.

Finotello, F., Mastrorilli, E., & Di Camillo, B. (2018). Measuring the diversity of the human microbiota with targeted next-generation sequencing. *Briefings in bioinformatics*, *19*(4), 679-692.

Fouhse, J. M., Zijlstra, R. T., & Willing, B. P. (2016). The role of gut microbiota in the health and disease of pigs. *Animal Frontiers*, *6*(3), 30-36.

Guard, B. C., Barr, J. W., Reddivari, L., Klemashevich, C., Jayaraman, A., Steiner, J. M., ... & Suchodolski, J. S. (2015). Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea. *PloS one*, *10*(5).

Gottlieb, D. H., Del Rosso, L., Sheikhi, F., Gottlieb, A., McCowan, B., & Capitanio, J. P. (2018). Personality, environmental stressors, and diarrhea in Rhesus macaques: An interactionist perspective. *American journal of primatology*, *80*(12), e22908.

Haas, K. N., & Blanchard, J. L. (2017). Kineothrix alysoides, gen. nov., sp. nov., a saccharolytic butyrate-producer within the family Lachnospiraceae. *International Journal of Systematic and Evolutionary Microbiology*, *67*(2), 402-410.

Hagerty, S. L., Hutchison, K. E., Lowry, C. A., & Bryan, A. D. (2020). An empirically derived method for measuring human gut microbiome alpha diversity: Demonstrated utility in predicting health-related outcomes among a human clinical sample. *Plos one*, *15*(3), e0229204.

Howell, S., White, D., Ingram, S., Jackson, R., Larin, J., Morales, P., ... & Wagner, J. (2012). A bio-behavioral study of chronic idiopathic colitis in the rhesus macaque (Macaca mulatta). *Applied animal behaviour science*, *137*(3-4), 208-220.

Kolde R (2015). pheatmap: Pretty heatmaps. R package version 1.0.12. Https://cran.r-project.org/web/packages/pheatmap/index.html

Koski, S. E. (2014). Broader horizons for animal personality research. *Frontiers in Ecology and Evolution*, *2*, 70.

Kruskal WH and Wallis WA (1952). Use of ranks in one-criterion variance analysis. *Journal of the American Statistical Association* 47 (260): 583-621.

Laing, S. T., Merriam, D., Shock, B. C., Mills, S., Spinner, A., Reader, R., & Hartigan-O'Connor, D. J. (2018). Idiopathic colitis in Rhesus macaques is associated with dysbiosis, abundant enterochromaffin cells and altered T-cell cytokine expression. *Veterinary pathology*, *55*(5), 741-752.

Larsen JM. The immune response to Prevotella bacteria in chronic inflammatory disease. Immunology. 2017;151:363–74.

Lee, V. K., Flynt, K. S., Haag, L. M., & Taylor, D. K. (2010). Comparison of the effects of ketamine, ketamine–medetomidine, and ketamine–midazolam on physiologic parameters and anesthesia-induced stress in rhesus (Macaca mulatta) and cynomolgus (Macaca fascicularis) macaques. *Journal of the American Association for Laboratory Animal Science*, *49*(1), 57-63.

Liddicoat, C., Sydnor, H., Cando-Dumancela, C., Dresken, R., Liu, J., Gellie, N. J., ... & Breed, M. F. (2020). Naturally-diverse airborne environmental microbial exposures modulate the gut microbiome and may provide anxiolytic benefits in mice. *Science of the Total Environment*, *701*, 134684.

Lutgendorff, F., Akkermans, L., & Soderholm, J. D. (2008). The role of microbiota and probiotics in stress-induced gastrointestinal damage. *Current molecular medicine*, *8*(4), 282-298.

Maninger, N., Capitanio, J. P., Mendoza, S. P., & Mason, W. A. (2003). Personality influences tetanus-specific antibody response in adult male rhesus macaques after removal from natal group and housing relocation. *American Journal of Primatology: Official Journal of the American Society of Primatologists*, *61*(2), 73-83.

Mann HB and Whitney DR (1947). On a test of whether one of two random variables is stochastically larger than the other. *The Annals of Mathematical Statistics* 18 (1): 50-60.

Mayer, E. A., Savidge, T., & Shulman, R. J. (2014). Brain–gut microbiome interactions and functional bowel disorders. *Gastroenterology*, *146*(6), 1500-1512.

McGrew, K. (2017). Pairing strategies for cynomologous macaques. *Handbook of primate behavioral management*, 255-264.

McKenna, P., Hoffmann, C., Minkah, N., Aye, P. P., Lackner, A., Liu, Z., ... & Bushman, F. D. (2008). The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. *PLoS pathogens*, *4*(2), e20.

Moon, C. D., Young, W., Maclean, P. H., Cookson, A. L., & Bermingham, E. N. (2018). Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *Microbiologyopen*, *7*(5), e00677.

Niven, D. J., Berthiaume, L. R., Fick, G. H., & Laupland, K. B. (2012). Matched case-control studies: a review of reported statistical methodology. *Clinical epidemiology*, *4*, 99.

O'Keefe, S. J., Li, J. V., Lahti, L., Ou, J., Carbonero, F., Mohammed, K., ... & Vipperla, K. (2015). Fat, fibre and cancer risk in African Americans and rural Africans. *Nature communications*, *6*, 6342.

Oksanen J, Kindt R, Legendre P, O'Hara B, Simpson G, Solymos P, Stevens MHH, and Wagner H (2017). Vegan: community ecology package. R package version 2.4-2. Https://cran.r-project.org/web/packages/vegan/index.html.

Paradis E, Claude J and Strimmer K (2004). APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* 20 (2): 289-290.

Parashar, A., & Udayabanu, M. (2016). Gut microbiota regulates key modulators of social behavior. *European Neuropsychopharmacology*, *26*(1), 78-91.

Peter, J., Fournier, C., Keip, B., Knoblich, L., & Moser, G. (2017). Psychological stress, intestinal microbiome and Irritable Bowel Syndrome. *Zeitschrift für Gastroenterologie*, *55*(05), A31.

Prongay, K., Park, B., & Murphy, S. J. (2013). Risk factor analysis may provide clues to diarrhea prevention in outdoor-housed rhesus macaques (Macaca mulatta). *American Journal of Primatology*, *75*(8), 872-882.

Rhoades, N., Barr, T., Hendrickson, S., Prongay, K., Haertel, A., Gill, L., ... & Messaoudi, I. (2019). Maturation of the infant rhesus macaque gut microbiome and its role in the development of diarrheal disease. *Genome biology*, *20*(1), 1-16.

Rolhion, N., Chassaing, B., Nahori, M. A., De Bodt, J., Moura, A., Lecuit, M., ... & Littman, D. R. (2019). A Listeria monocytogenes bacteriocin can target the commensal Prevotella copri and modulate intestinal infection. *Cell host & microbe*, *26*(5), 691-701.

Rosso, M. C., Badino, P., Ferrero, G., Costa, R., Cordero, F., & Steidler, S. (2016). Biologic data of cynomolgus monkeys maintained under laboratory conditions. *PloS one*, *11*(6), e0157003.

Sapolsky, R. M. (2005). The influence of social hierarchy on primate health. *Science*, *308*(5722), 648-652.

Seekatz, A. M., Panda, A., Rasko, D. A., Toapanta, F. R., Eloe-Fadrosh, E. A., Khan, A. Q., ... & Fraser, C. M. (2013). Differential response of the cynomolgus macaque gut microbiota to Shigella infection. *PloS one*, *8*(6).

Suchodolski, J. S., Foster, M. L., Sohail, M. U., Leutenegger, C., Queen, E. V., Steiner, J. M., & Marks, S. L. (2015). The fecal microbiome in cats with diarrhea. *PloS one*, *10*(5).

Taylor, S. E., Klein, L. C., Lewis, B. P., Gruenewald, T. L., Gurung, R. A., & Updegraff, J. A. (2000). Biobehavioral responses to stress in females: tend-and-befriend, not fight-or-flight. *Psychological review*, *107*(3), 411.

Westreich, S. T., Ardeshir, A., Alkan, Z., Kable, M. E., Korf, I., & Lemay, D. G. (2019). Fecal metatranscriptomics of macaques with idiopathic chronic diarrhea reveals altered mucin degradation and fucose utilization. *Microbiome*, *7*(1), 1-17.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., ... & Heath, A. C. (2012). Human gut microbiome viewed across age and geography. *nature*, *486*(7402), 222-227.

Zeineldin, M., Aldridge, B., & Lowe, J. (2018). Dysbiosis of the fecal microbiota in feedlot cattle with hemorrhagic diarrhea. *Microbial pathogenesis*, *115*, 123-130.

Table 1

Behavior Definitions for Human-Intruder Test

Open Mouth Threat—Aggressive gesture where jaw is open and teeth bared.
Lunge - Rushing forward in an assertive manner which may end with an abrupt stop
Tooth Grind- rubbing of molars against each other in manner that makes noise
Fear Grimace—Submissive facial expression with the corners of lips drawn back, exposing the lower & upper teeth
Freeze—Fixed or motionless stiff body posture, usually accompanied by averted gaze
Crouch—A crouched position with legs and arms held beneath the body, head lowered; often in back corner of cage
Fear Vocalization—Loud, high-pitched vocalization
Gaze Avert – Directing eyes away from observer, turning towards the back or sides of cage in effort to avoid eye contact
Locomotor Stereotypy: any nonfunctional repetitive movement
Divider Press – pressing any part of the body against the divider between cages
Bark – guttural, grunting vocalization, deep pitch, short duration
Lip Smack—Mouth slightly opened & closed rhythmically. As the mouth is opened, there is a smacking sound when the tongue is drawn across the palate
Coo call—vocalization of medium pitch and intensity, mouth opened in circle or diamond shape
Girney – vocalization of medium pitch but high intensity
Peering – turning of head to the side while maintaining eye contact, may turn body
Approach – coming to the front of the cage and looking at observer

Less Common:
Defecation, Urination, Rub nose, Tail curl, Scratch, Yawn, Head Toss

Table 2

Social Housing Data Characteristics

	F (n=316)	M (n=211)	Overall (n=528)
Weight			
Mean (SD)	2.60 (0.463)	3.01 (0.576)	2.77 (0.548)
Median [Min, Max]	2.50 [1.90, 4.20]	2.80 [2.10, 5.00]	2.65 [1.90, 5.00]
Age			
Mean (SD)	2.37 (0.577)	2.31 (0.491)	2.35 (0.544)
Median [Min, Max]	2.12 [1.65, 3.81]	2.13 [1.63, 3.69]	2.13 [1.63, 3.81]
Sex			
F	316 (100%)	0 (0%)	316 (59.8%)
M	0 (0%)	211 (100%)	211 (40.0%)
Missing	0 (0%)	0 (0%)	1 (0.2%)
Social Rank in Group Housing			
High	60 (19.0%)	52 (24.6%)	113 (21.4%)
Low	103 (32.6%)	64 (30.3%)	167 (31.6%)
Low/High	7 (2.2%)	3 (1.4%)	10 (1.9%)
Low/Mid	20 (6.3%)	18 (8.5%)	38 (7.2%)
Low/Mid/High	5 (1.6%)	4 (1.9%)	9 (1.7%)
Mid	102 (32.3%)	61 (28.9%)	163 (30.9%)
Mid/High	19 (6.0%)	9 (4.3%)	28 (5.3%)
Rank Certainty			
Mean (SD)	0.746 (0.213)	0.759 (0.205)	0.751 (0.210)
Median [Min, Max]	0.670 [0.330, 1.00]	0.750 [0.300, 1.00]	0.750 [0.300, 1.00]

Table 3

Principal Component Analysis HIT -Profile Phase

Variable	Factor Loadings (Varimax normalized) (masters behavioral data) Extraction: Principal components (Marked loadings are >.700000) Factor 1	Factor 2	Factor 3
lip smack 1	-0.656966	0.093812	-0.111682
divider 1	-0.352943	0.000273	0.408058
bark 1	0.148348	0.555909	0.288778
fear vocal 1	0.067258	-0.484657	0.142654
gaze avert 1	0.024906	-0.170348	0.579367
open mouth 1	0.676842	0.285913	-0.030750
peering 1	-0.346931	0.153704	0.053220
crouch 1	0.057227	0.436402	-0.285193
stereo 1	-0.114564	0.000256	-0.665203
freeze 1	-0.047203	-0.508076	0.103340
approach 1	0.030403	0.547485	0.073676
lunge 1	0.606615	0.055916	-0.223399
coo 1	0.424637	0.132356	0.178276
girney 1	-0.265804	-0.091374	-0.169458
Expl.Var	1.800303	1.464440	1.272513
Prp.Totl	0.128593	0.104603	0.090894

Table 4

Principal Component Analysis HIT - Stare Phase

Variable	Factor Loadings (Varimax normalized) (masters behavioral data) Extraction: Principal components (Marked loadings are >.700000) Factor 1	Factor 2	Factor 3
Lip smack 2	*0.738929*	0.198691	0.027445
divider 2	0.093288	0.006449	*-0.766103*
bark 2	-0.198346	0.551976	-0.175933
fear vocal 2	0.103434	-0.576087	0.007439
gaze avert 2	-0.043312	-0.500751	-0.184500
open mouth 2	*-0.712666*	0.019105	0.153459
peering 2	0.262725	0.024206	0.420579
crouch 2	-0.004760	0.427322	-0.080022
stereo 2	0.071209	-0.139233	0.424944
freeze 2	0.001910	-0.406261	0.095826
approach 2	0.110380	0.373155	0.292999
lunge 2	-0.587135	0.214507	0.041209
coo 2	-0.313052	0.077278	-0.159446
girney 2	0.170088	-0.008722	-0.342189
Expl.Var	1.672492	1.486120	1.279378
Prp.Totl	0.119464	0.106151	0.091384

Table 5

Temperament Categories and Diarrhea Incidence

Temperament	**Control Animals**	**Diarrhea Animals**
Fearful	3	4
Anxious	13	14
Friendly	9	7
Aggressive	2	8

Note: Date presented in this table is from the matched pairs set that was sent off for microbiome composition analysis and utilized in the cortisol ELISA tests.

Table 6

Summary of Microbiota Composition Differences Between Times/Groups

Taxa	Baseline	Relocation	Difference	p-value	Control	Diarrhea	Difference	p-value
Phylum:				=				
Bacteriodes	0.218669	0.189952			0.224142	0.186259	↓	=.04
Firmicutes	0.739106	0.735318			0.73281	0.740734		
Proteobacteria	0.001497	0.008753	↑	< .00	0.002044	0.008173	↑	=.01
Actinobacteria	0.02049	0.031479	↑	=.02	0.022217	0.029678		
Spirochaetes	0.014746	0.025588			0.011369	0.0283	↑	< .00
Genus:								
Prevotella	0.086568	0.045414	↓	< .00	0.073715	0.052217	↓	< .00
Kineothrix	0.036288	0.009189	↓	< .00	0.02421	0.021011		
Streptococcus	0.025736	0.012165	↓	< .00	0.020509	0.016085		
Treponema	0.005576	0.008361			0.004393	0.009521	↑	< .00
Ruminococcus	0.006236	0.007938	↑	< .00	0.007159	0.007085		
Clostridium	0.003579	0.005724	↑	< .00	0.00411	0.005256		
Heliobacteria	0.000337	0.000316			0.000281	0.000366	↑	=.02
Lactobacillus	0.002892	0.004651	↑	< .00	0.004332	0.003996		
Species:								
C. aerofaciens	0.011502	0.023357	↓	=.02	0.01358	0.020627	↓	< .00
P. copri	0.139987	0.08691	↓	< .00	0.13085	0.096281	↓	=.05
K. alysoides	0.036288	0.009189	↓	< .00	0.02421	0.021011		
L. salivarius	0.003367	0.009115	↑	< .00	0.008138	0.004919		
C. botulinum	0.004951	0.006656	↑	=.04	0.005629	0.006031		
L. reuteri	0.004395	0.005543			0.005524	0.004574		

Note: Test statistic used for comparisons: ANOVA

Table 7

Phylum Relative Abundance Comparisons

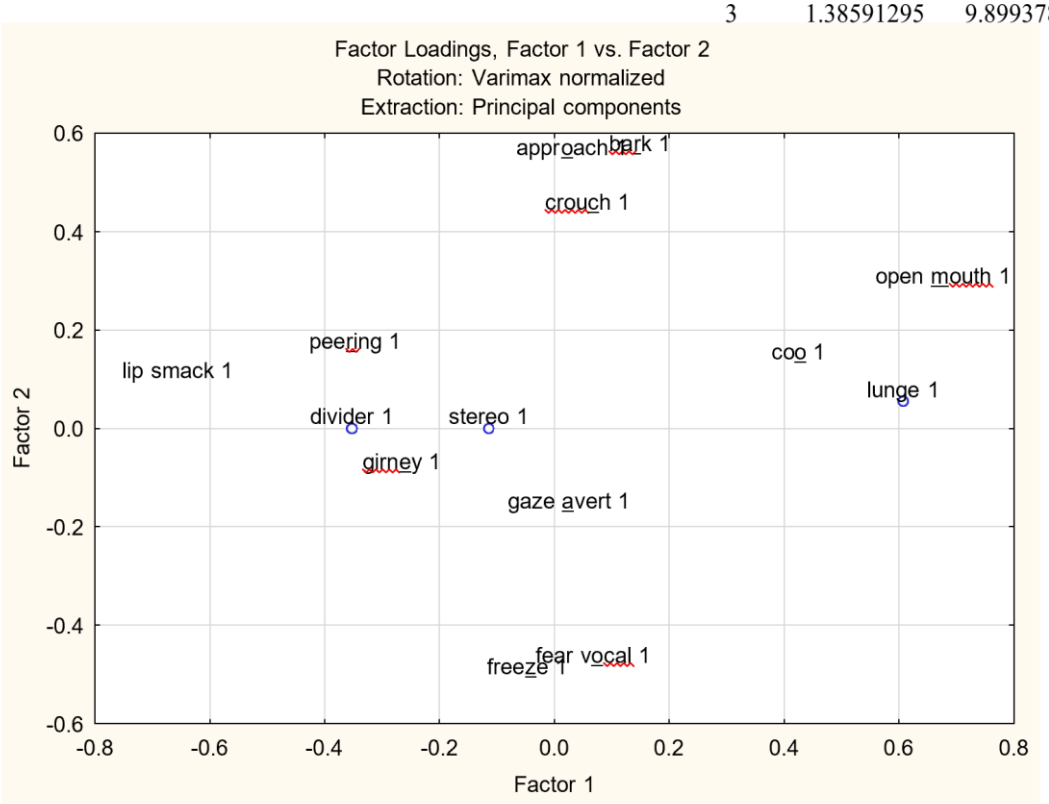
Taxa	Group	P-Value	FDR-Adj. P	Overall Mean	Mean of Baseline	Mean of Relocation
Bacteroidota	Control	0.067512	0.108019	0.526944	0.557240153	0.500976054
Desulfobacterota	Control	0.00067	**0.005361**	0.001457	0.000769998	0.002045066
Fibrobacterota	Control	0.010049	**0.027098**	0.000611	0.000260281	0.000910984
Firmicutes	Control	0.538604	0.538604	0.385461	0.378990977	0.391007288
Actinobacteria	Control	0.010162	**0.027098**	0.062908	0.050093267	0.073891946
Proteobacteria	Control	0.410611	0.46927	0.000981	0.000976054	0.00098535
Spirochaetota	Control	0.019225	**0.03845**	0.020552	0.010454624	0.029207258
Verrucomicrobiota	Control	0.330032	0.440042	0.000876	0.000976054	0.000790139
Bacteroidota	Diarrhea	0.256307	0.261109	0.45003	0.476843378	0.42484186
Desulfobacterota	Diarrhea	0.197937	0.261109	0.001651	0.001603667	0.001695771
Fibrobacterota	Diarrhea	0.219909	0.261109	0.00059	0.000747259	0.000441689
Firmicutes	Diarrhea	0.261109	0.261109	0.406384	0.424056691	0.389782784
Actinobacteria	Diarrhea	2.08E-07	**1.45E-06**	0.07599	0.044138637	0.105910747
Proteobacteria	Diarrhea	0.011188	**0.039159**	0.002176	0.000797636	0.003470415
Spirochaetota	Diarrhea	0.128968	0.261109	0.062524	0.051384528	0.072989131

Note: Test statistic used for comparisons: Mann-Whitney test. Significant findings after FDR correction indicated in bold.

Figure 1

Principal Component Analysis of Human Intruder Tests

Factor	Eigenvalue	% variance
1	1.985957	14.185413
2	1.385913	9.89938
3	1.38591295	9.8993782

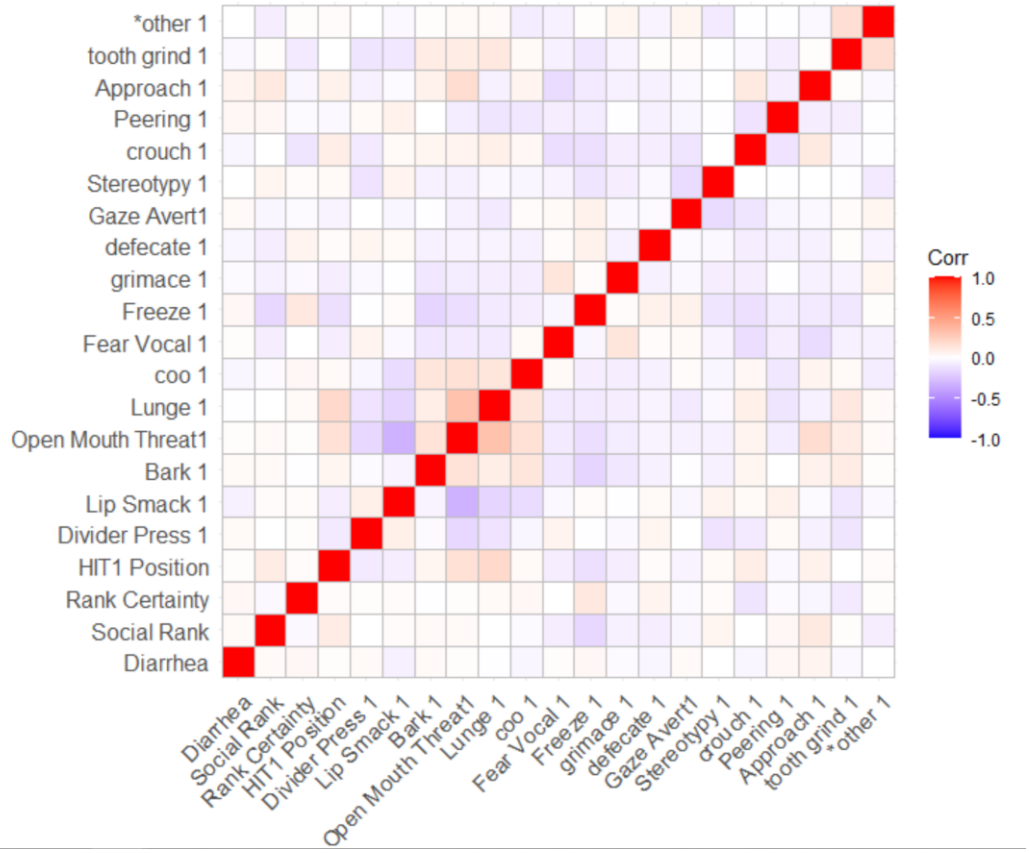


Note: This figure represents a principal components analysis performed on the first phase of the Human Intruder Test (Profile phase). This is why each behavior has a 1 following it in the figure. This figure was interpreted as a reflection of two dimensions of temperament indicated by behavioral clusters at specific areas of the graph. Behaviors along the horizontal plane in the middle of the graph are interpreted as representing a dimension of Friendliness vs Aggression, and the behaviors along the

vertical plane in the middle of the graph are interpreted as representing a dimension of Boldness vs Fearfulness.

Figure 2

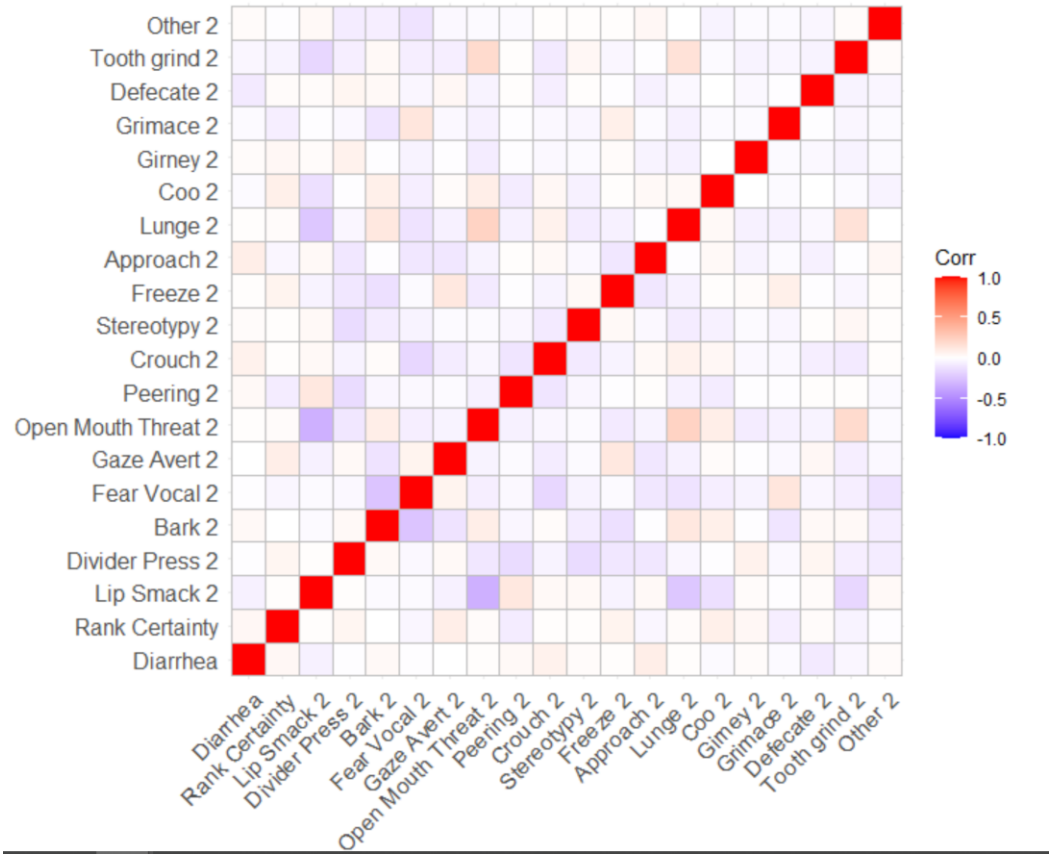
Correlation Matrix: HIT Profile Phase Behaviors and Diarrhea



Note: This figure depicts univariate regression between diarrhea (present or absent) and social/behavioral variables. Social ranks were given a numerical value between 1-6 (value assignment described in section 5.3). Rank certainty was calculated as a ratio of the most common social rank assessment divided by the number of observations. HIT 1 Position indicates the position of the primate in the cage during the observations and were coded as follows: back of cage (1), front of cage (2), and middle of cage (3). All other variables from Divider Press 1 to *other 1 represent individual behaviors, scored either as a 0 (not performed) or 1 (performed) during the profile phase of the test.

Figure 3

Correlation Matrix: HIT Stare Phase Behaviors and Diarrhea



Note: This figure depicts univariate regression between diarrhea (present or absent) and social/behavioral variables. Rank certainty was calculated as a ratio of the most common social rank assessment divided by the number of observations. All other variables from Lip Smack 2 to Other 2 represent individual behaviors, scored either as a 0 (not performed) or 1 (performed) during the stare phase of the test.

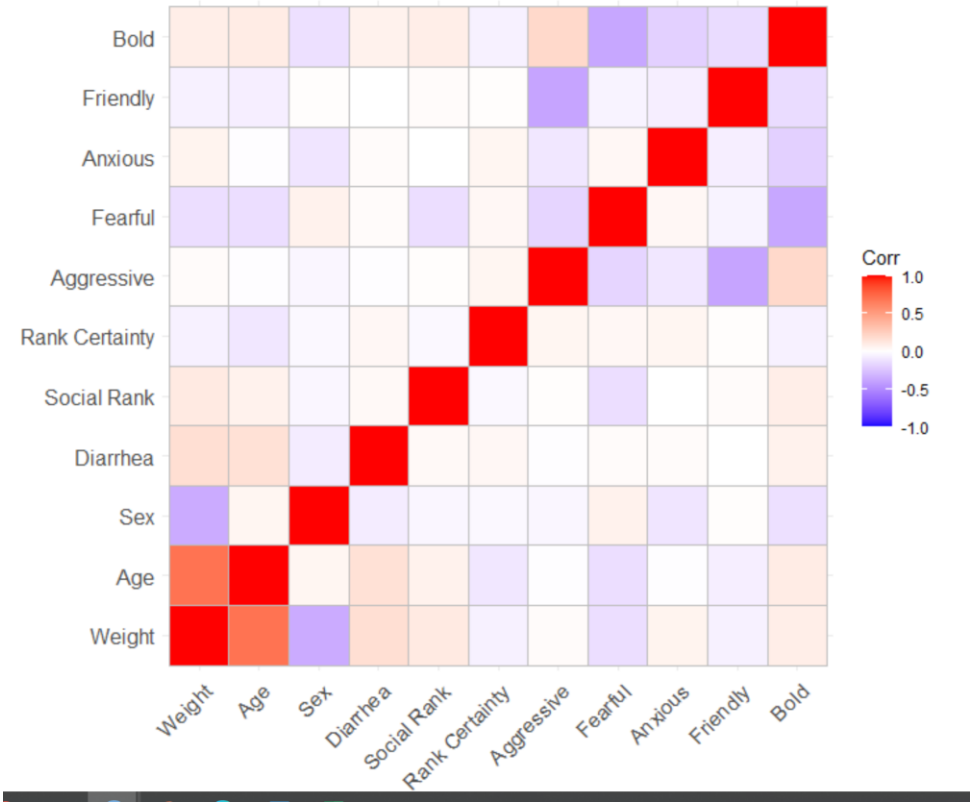
Figure 4

Correlation Matrix Between Diarrhea, Social and Behavioral Variables

Note: This figure offers correlation coefficients between Diarrhea (present = 1, absent = 0) and Social Rank (numerical value as described in Section 5.2), Rank Certainty (ratio of predominant rank/number of observations) and a sum of individual behaviors performed that were included in each of the temperament categories determined as outlined in Section 5.1 during both phases of the Human-Intruder Test total.

Figure 5

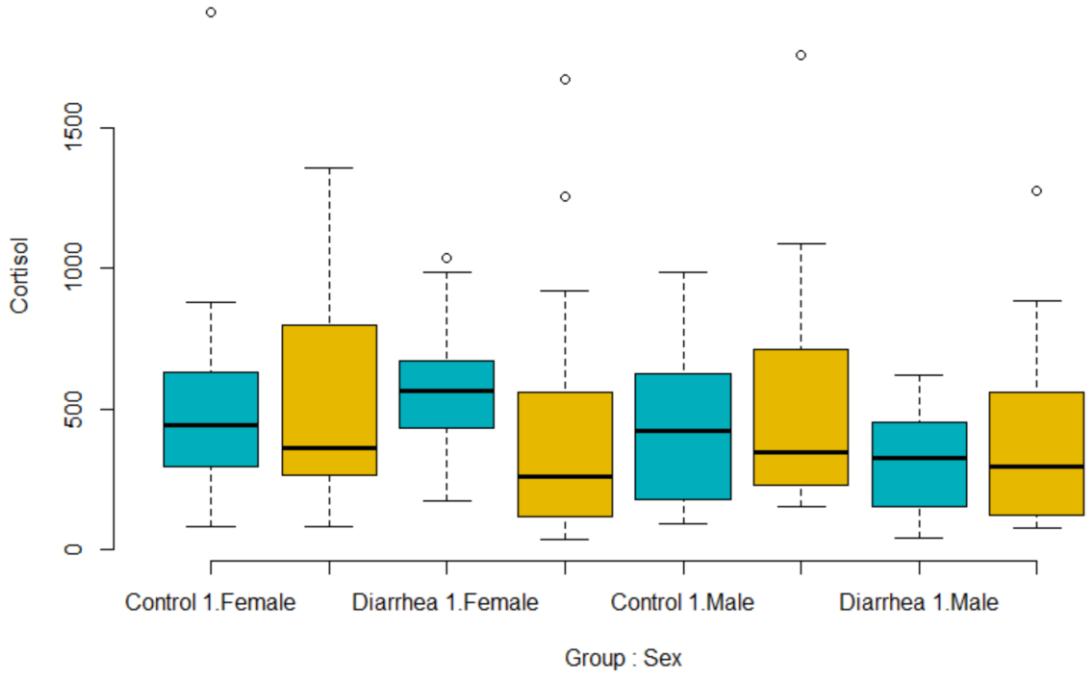
Correlation Heat Map Between Diarrhea and Independent Variables



Note: This figure illustrates the relationship between independent variables (weight, age, sex), the dependent variable of diarrhea (present = 1, absent = 0) and social/behavioral independent variables: Social Rank (numerical value as described in Section 5.2), Rank Certainty (ratio of predominant rank/number of observations) and a sum of individual behaviors performed that were included in each of the temperament categories determined as outlined in Section 5.1 during both phases of the Human-Intruder Test total.

Figure 6

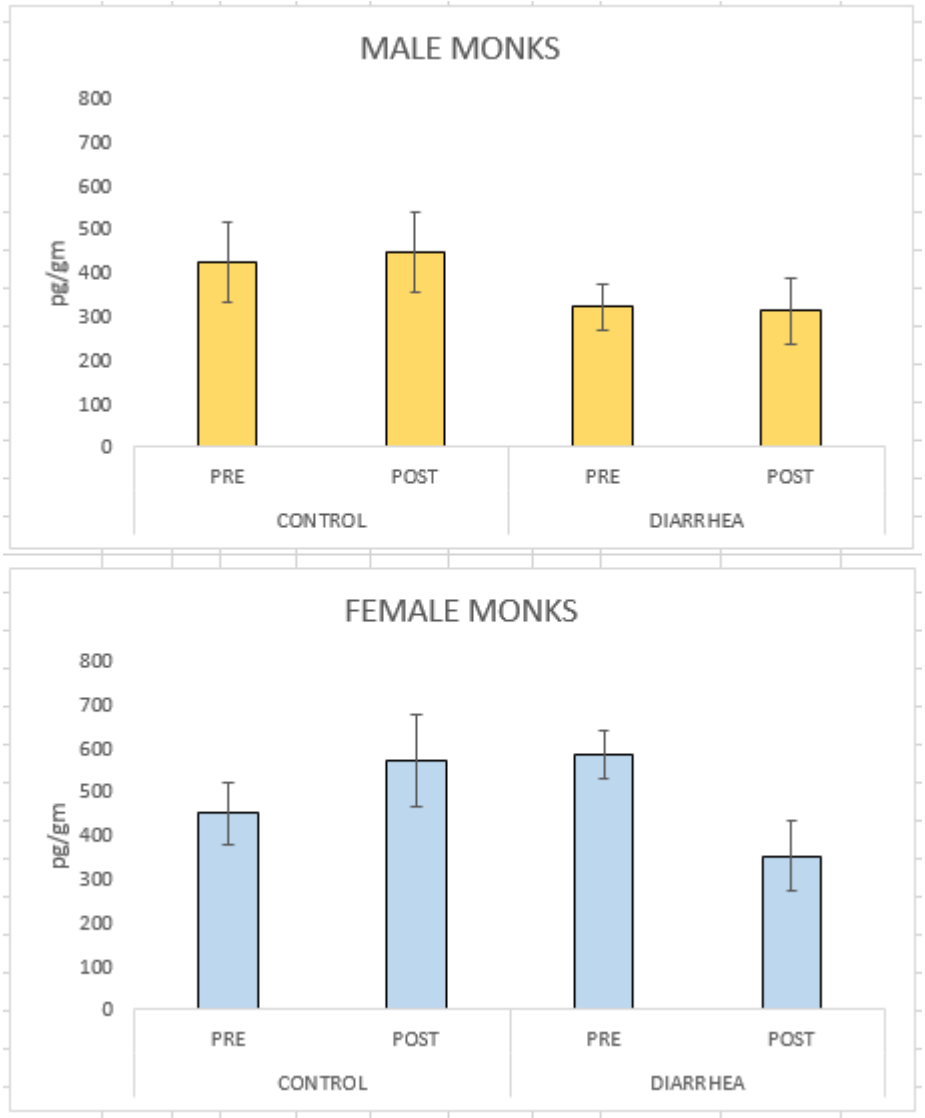
Cortisol Results by Group and Sex



Note: Cortisol values measured as pg/gm are represented in a box plot in this figure, with the blue boxes representing baseline time point values for each group, and the gold boxes representing the relocation time point values. The first set of blue and gold boxes represent the cortisol values of the control group females, the second set are the females who developed diarrhea, the third set are the control group males, and the fourth set of boxes are values from the males who developed diarrhea.

Figure 7

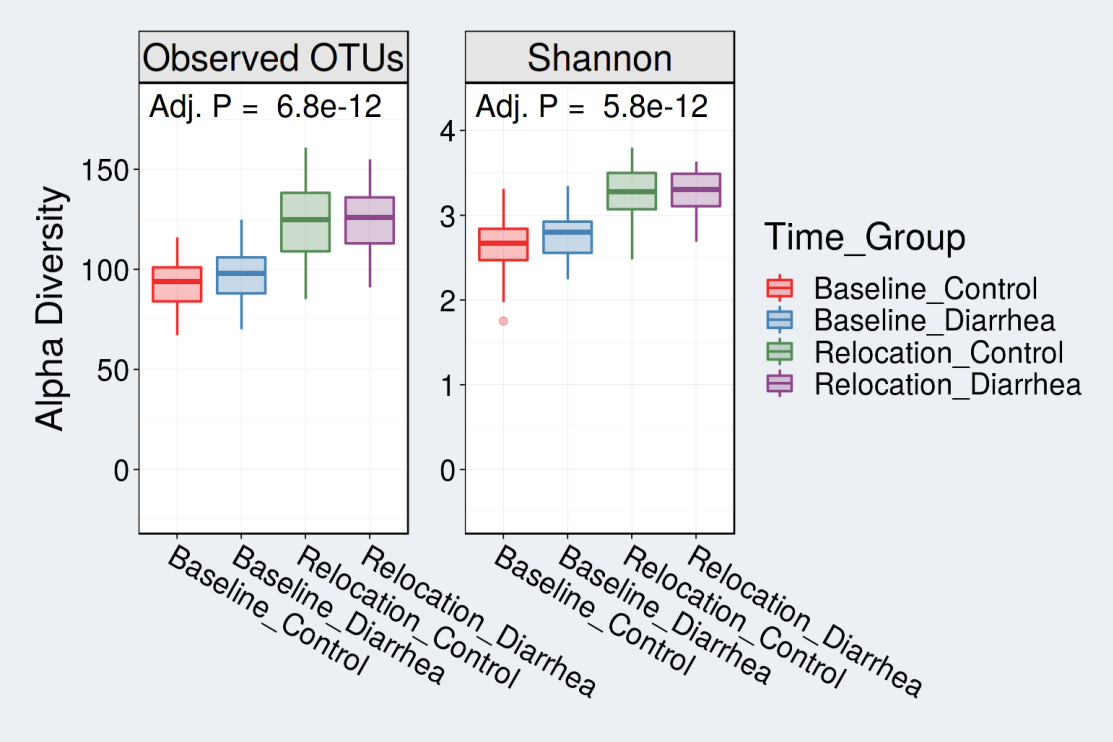
Cortisol Results by Group (Pre/Post) and Sex



Note: Cortisol values per sex are represented with a comparison between baseline (PRE) and relocation (POST) for each set of group and sex: control males vs diarrhea males (gold bars) and control females vs diarrhea females (blue bars).

Figure 8

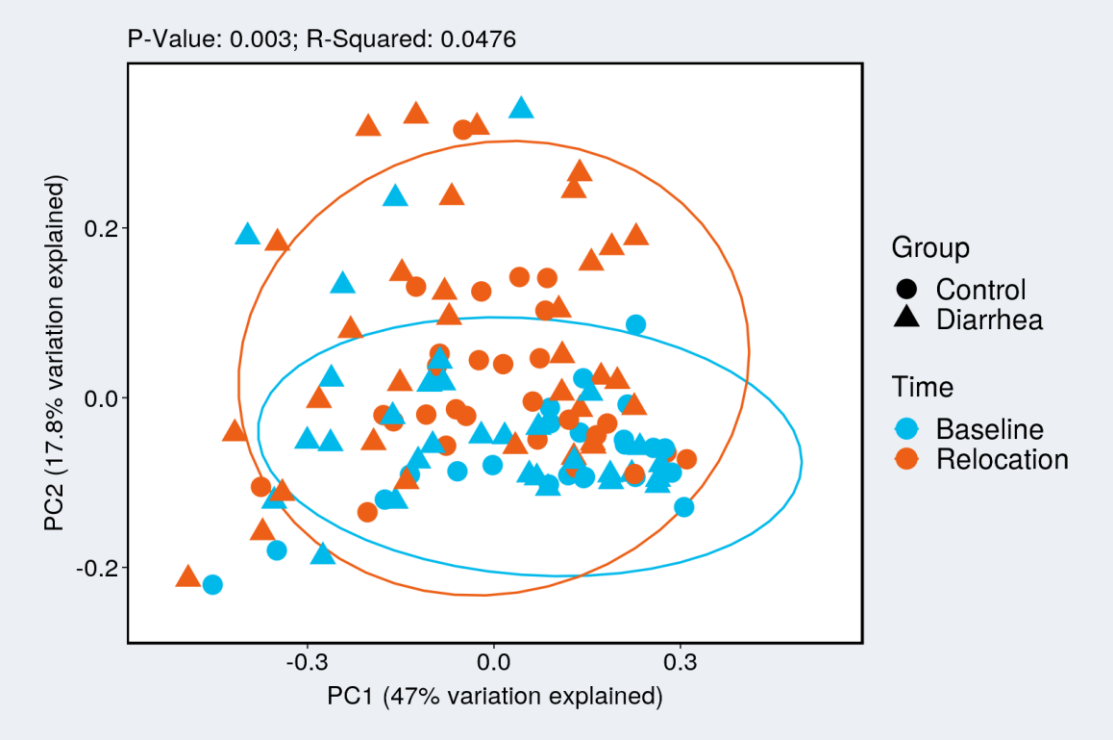
Alpha Diversity Comparison Between Groups Divided by Time



Note: Alpha Diversity comparisons via two different methods (Observed OTUs and the Shannon Index) between each of the two groups at the two time points are represented by these box plots. The Adj. P on each plot represented the *p* value associated with the Mann-Whitney test used to compare groups, adjusted for False Discovery Rate.

Figure 9

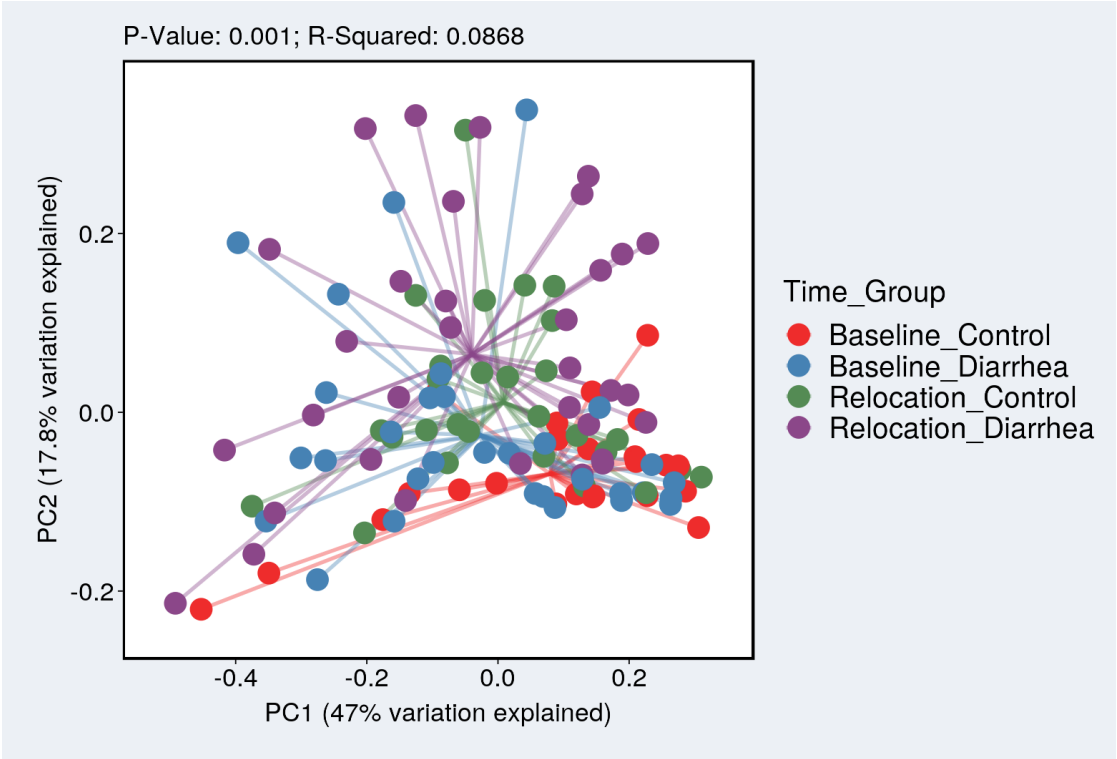
PCoA Measuring Beta Diversity by Time Points, Shaped by Group



Note: the portion of samples included within each colored ellipse are more similar to each other in microbial composition. The blue (baseline) and red (relocation) samples have some samples with similar compostion and some separation, indicated by the fact that the two ellipses share space with each other. Samples included outside the ellipses have microbial compositions that differ from those within the ellipses. The number of blue samples, representing samples collected at baseline, within the blue ellipses are higher within the ellipse than the number outside the ellipse, representing a tendency to be more similar to each other in microbial composition. The relocation samples, noted in red, are included in roughly similar amounts in both red and blue ellipses, showing some shared microbial compositions between each group.

Figure 10

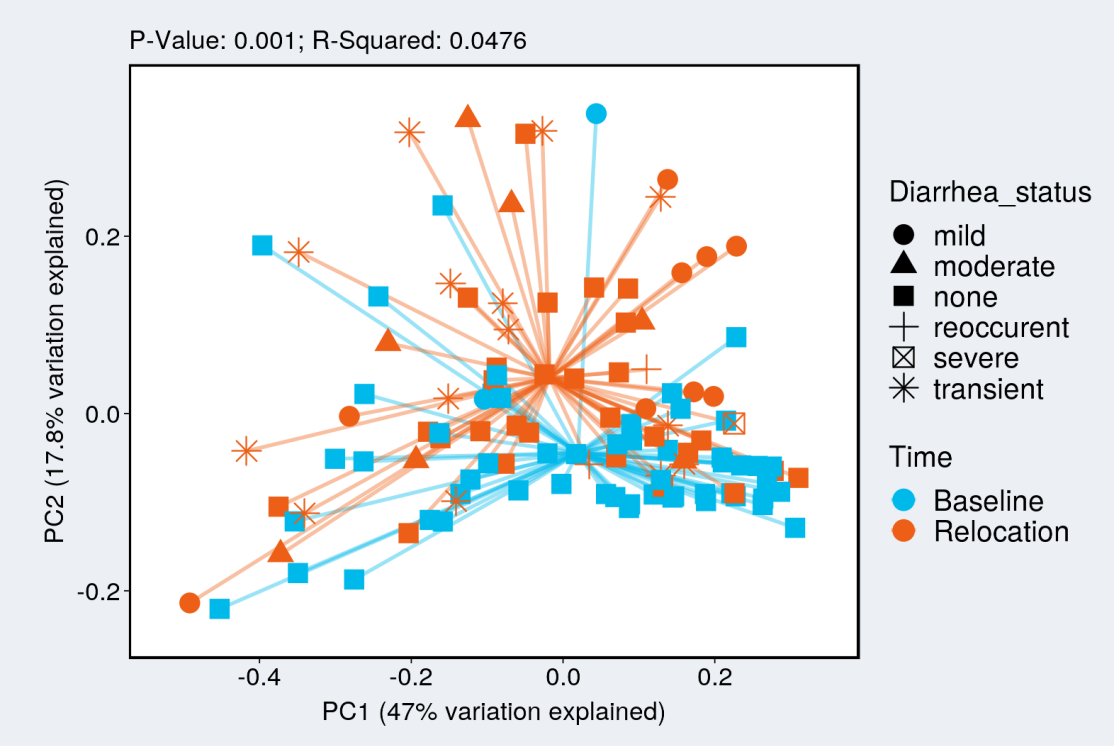
Centroid Cluster Measuring Beta Diversity By Time x Group



Note: Beta Diversity between the four groups of animals illustrated by showing spread away from a centroid cluster. Red (Baseline Control samples) and blue (Baseline Diarrhea) samples are clustered together near the bottom right, indicating a similar microbiome composition. Purple samples (Relocation Diarrhea) samples appear further away from this cluster of red and blue samples, indicating a microbiome composition that differs from baseline samples.

Figure 11

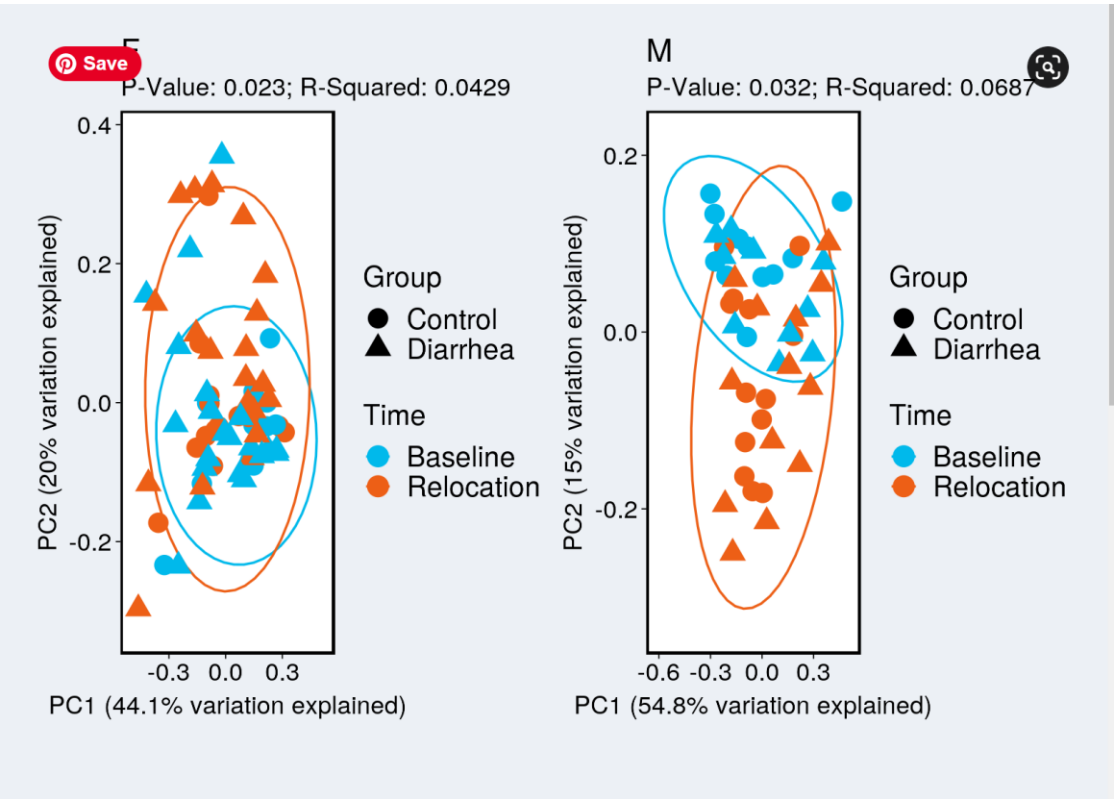
Beta Diversity by Diarrhea Status, Colored by Time Point



Note: Beta Diversity between the four groups of animals illustrated by different colors for time points (blue for baseline samples and red for relocation samples), and different diarrhea statuses indicated by shape. Samples from animals that did not develop diarrhea are indicated with boxes. These samples tend to cluster together at the bottom right of the centroid, indicating similar microbial composition. Differing levels of diarrhea severity show a longer line away from the center cluster of baseline/no diarrhea samples, indicating differing microbial composition in animals with diarrhea than those with no diarrhea. The difference between levels of diarrhea severity is outlined in Section 5.4

Figure 12

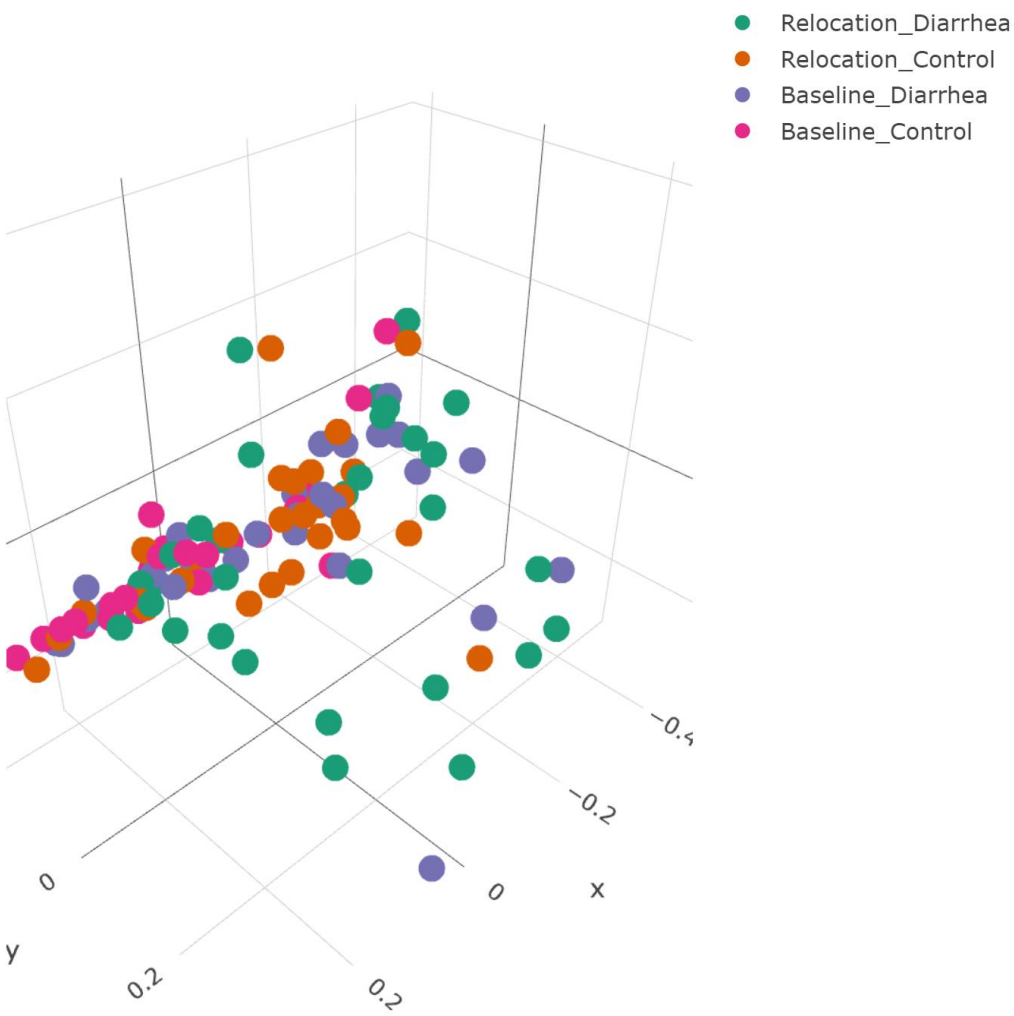
Beta Diversity Comparisons by Group and Time Faceted by Sex



Note: Two boxes separate the beta diversity measurements of females and males. The portion of samples included within each colored ellipse are more similar to each other in microbial composition. The blue (baseline) and red (relocation) samples have some samples with similar compostion and some separation, indicated by the fact that the two ellipses share space with each other. There is more clear separation between the ellipses in males than there are in females, indicating that male animals have more distinct changes in microbial composition after relocation than females do.

Figure 13

3D Ordination Plot by Time x Group



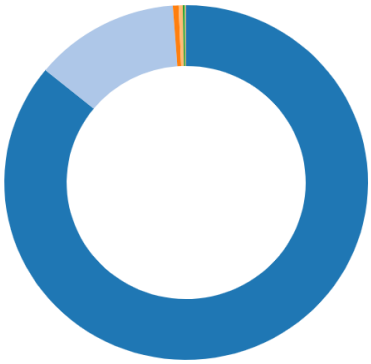
Note: differences within an ordination plane is another way to represent beta diversity. In this figure, the pink (control) and purple (diarrhea) points represent samples taken at baseline, and tend to cluster together. The green points represent samples from animals taken after relocation that developed diarrhea, and are further away on the

ordination plane from the baseline points. This indicates that diarrhea samples had a differing microbial composition than both sets (control and diarrhea) of samples taken at baseline.

Figure 14

Phylum Overall Read Counts

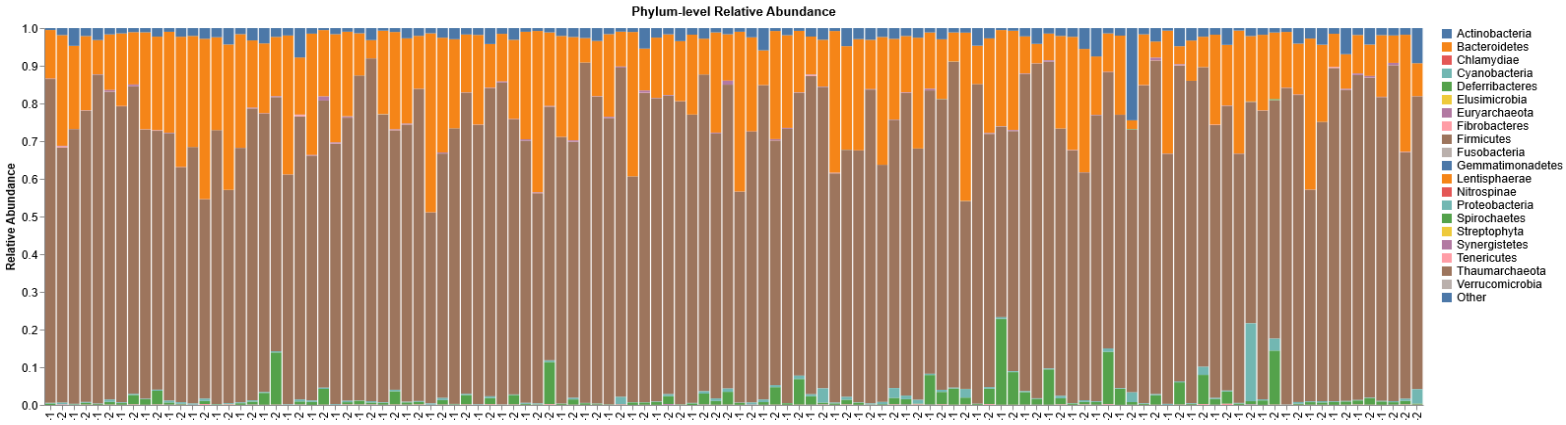
Name	Readcount (% of classified reads)
Firmicutes	163023 (85.69%)
Bacteroidetes	24481 (12.87%)
Actinobacteria	962 (0.51%)
Spirochaetes	696 (0.37%)
Proteobacteria	340 (0.18%)
Euryarchaeota	168 (0.09%)
Synergistetes	24 (0.01%)
Fibrobacteres	20 (0.01%)
Lentisphaerae	10 (0.01%)



Note: Characterization of the microbiome relative abundance at the phylum level for all samples is illustrated in this figure. Dominant phyla of all the samples overall are ranked in order of percent of classified reads.

Figure 15

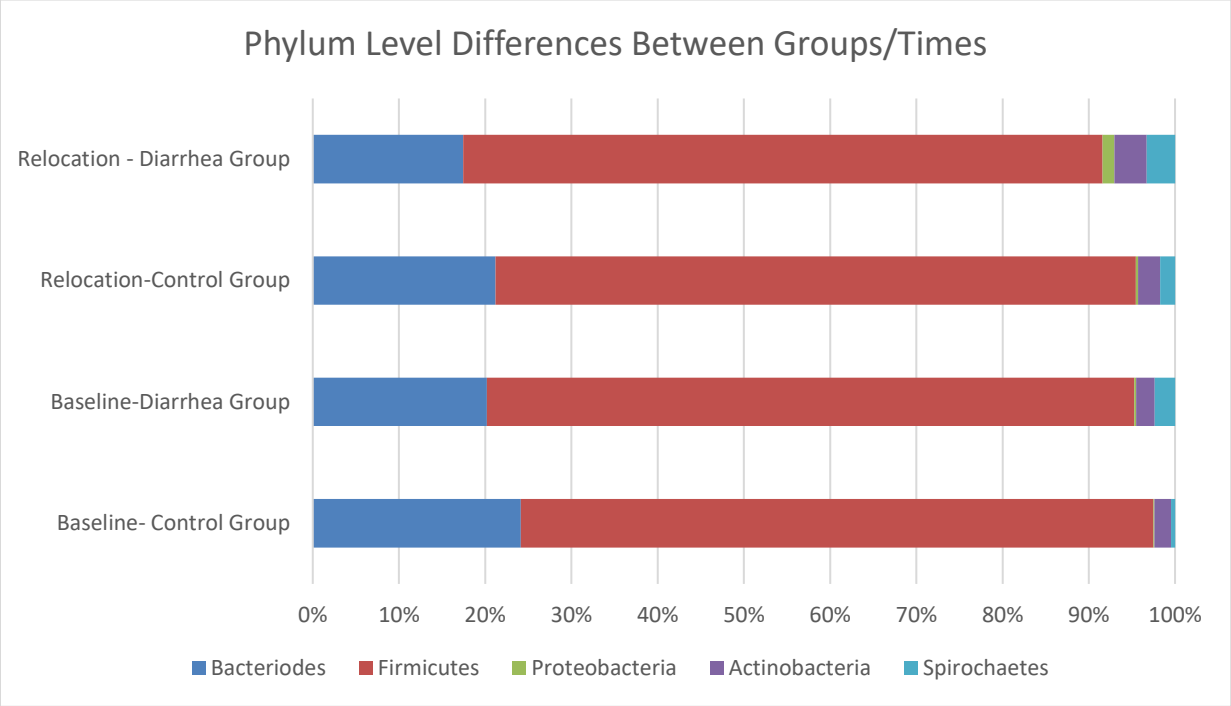
Phylum Level Abundance



Note: Relative abundance chart for all samples at the phylum taxonomic level. Samples are sorted from left to right by sample ID which corresponds to control samples on the left and diarrhea samples on the right. As part of the post-hoc filtering process, abundances are proportionalized such that the above plots are adjusted for variable sequencing depth.

Figure 16

Phylum Level Abundance Per Time/Group

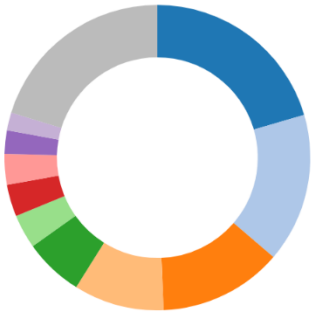


Note: Illustration of the differences in relative abundance per sample between the five most dominant phyla, differentiated by group and time point. This bar graph helps visually indicate which phyla differ the most between groups.

Figure 17

Genus Level Read Counts

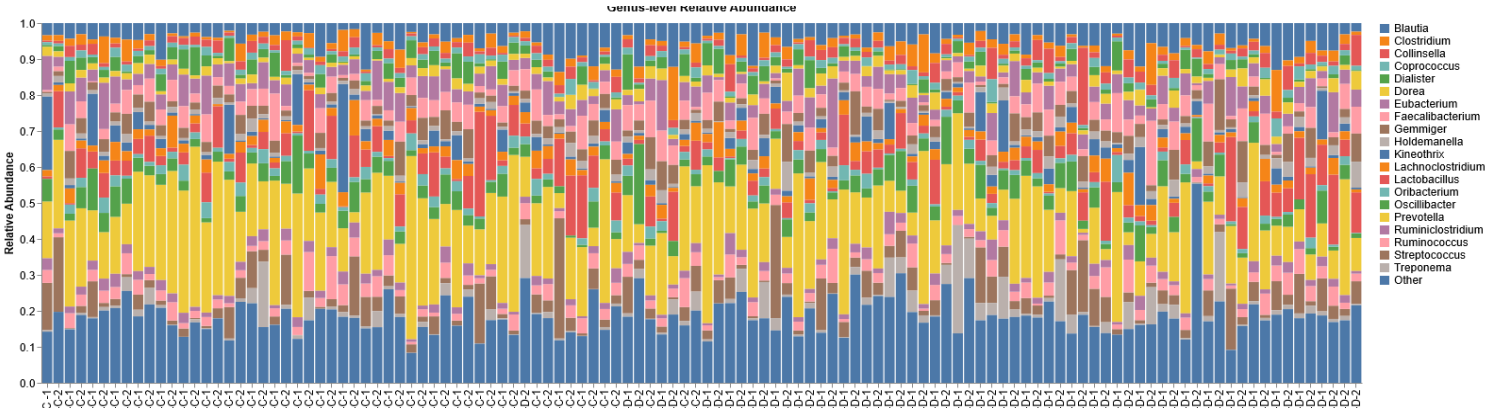
Name	Readcount (% of classified reads)
Kineothrix	29310 (15.41%)
Prevotella	22605 (11.88%)
Streptococcus	18704 (9.83%)
Eubacterium	13666 (7.18%)
Oscillibacter	8955 (4.71%)
Ruminococcus	5144 (2.7%)
Blautia	4838 (2.54%)
Ruminiclostridium	4664 (2.45%)
Dorea	3540 (1.86%)
Murimonas	2732 (1.44%)
(Remaining)	28936 (15.21%)



Note: Characterization of the microbiome relative abundance at the genus level for all samples is illustrated in this figure. Dominant genera of all the samples overall are ranked in order of percent of classified reads.

Figure 18

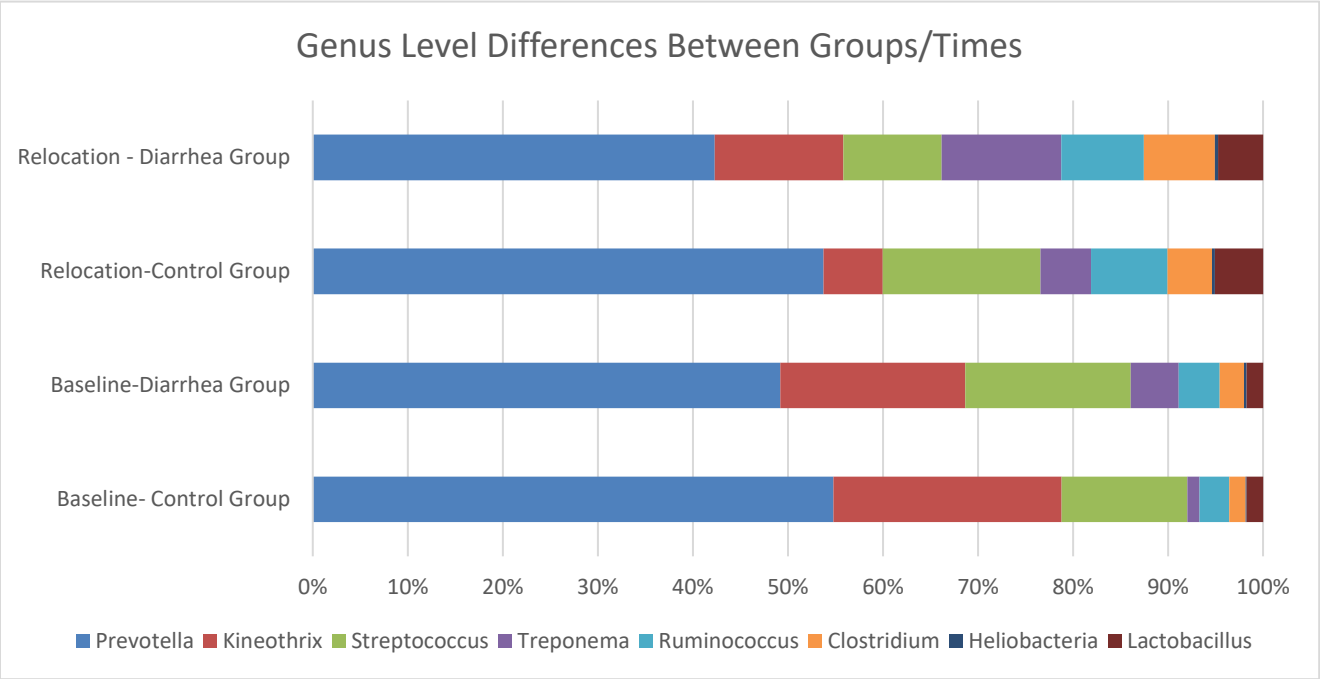
Relative abundance chart for all samples at the genus taxonomic level



Note: Relative abundance chart for all samples at the genus taxonomic level. Samples are sorted from left to right by sample ID which corresponds to control samples on the left and diarrhea samples on the right. As part of the post-hoc filtering process, abundances are proportionalized such that the above plots are adjusted for variable sequencing depth.

Figure 19

Genus Abundance Differences Between Group/Time

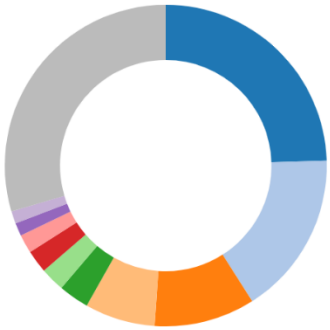


Note: Illustration of the differences in relative abundance per sample between the eight most dominant genera differentiated by group and time point. This bar graph helps visually indicate which genera differ the most between groups.

Figure 20

Species Level Read Counts

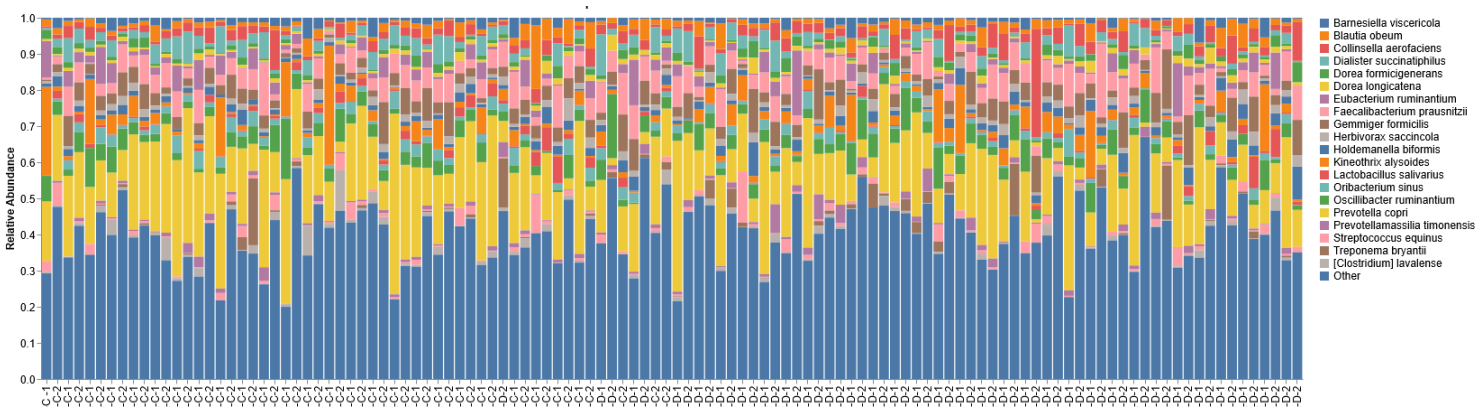
Name	Readcount (% of classified reads)
Kineothrix alysoides	29310 (15.41%)
Prevotella copri	19694 (10.35%)
Eubacterium ruminantium	12110 (6.37%)
Oscillibacter ruminantium	8453 (4.44%)
Streptococcus equinus	3654 (1.92%)
Dorea formicigenerans	2930 (1.54%)
Murimonas intestini	2732 (1.44%)
Blautia obeum	2252 (1.18%)
Anaerotignum propionicum	1556 (0.82%)
Clostridium botulinum	1501 (0.79%)
(Remaining)	35401 (18.61%)



Note: Characterization of the microbiome relative abundance at the species level for all samples is illustrated in this figure. Dominant species of all the samples overall are ranked in order of percent of classified reads.

Figure 21

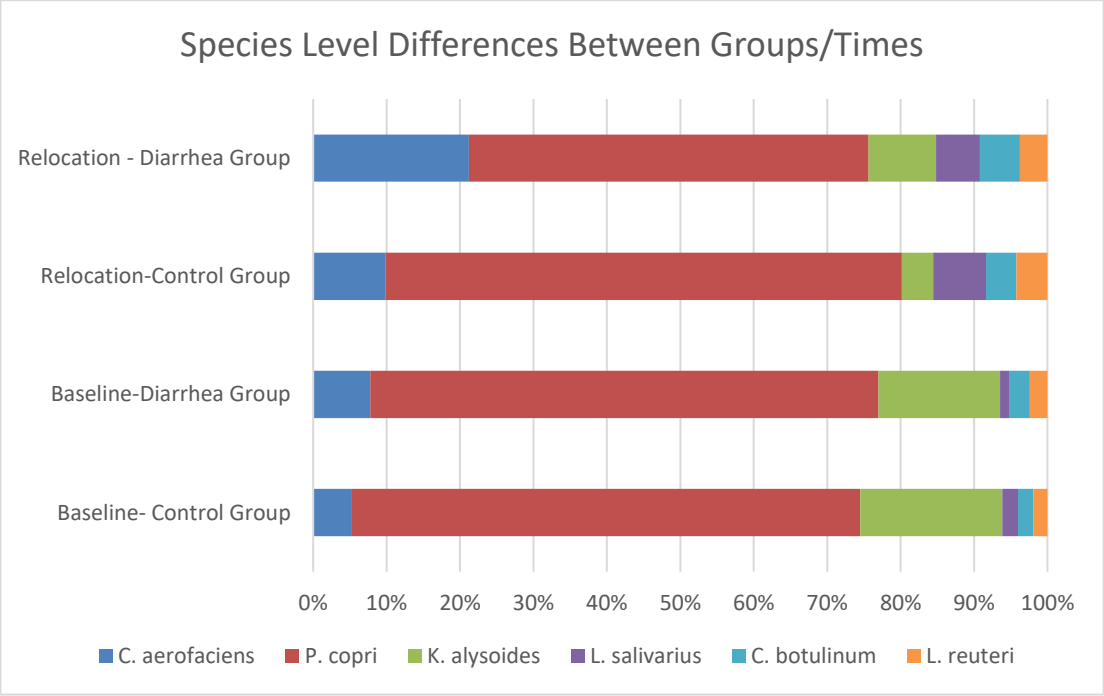
Species Level Abundance



Note: Relative abundance chart for all samples at the species taxonomic level. Samples are sorted from left to right by sample ID which corresponds to control samples on the left and diarrhea samples on the right. Abundances are proportionalized such that the above plots are adjusted for variable sequencing depth.

Figure 22

Species Abundance by Time/Group



Note: Illustration of the differences in relative abundance per sample between the six most dominant species differentiated by group and time point.