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Exploring the Utility of the Gut Microbiome as a Longitudinal Health Monitoring Tool in Sanctuary Chimpanzees (*Pan troglodytes*)

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ABSTRACT

The primary goal of captive primate management is to ensure optimal health and welfare of the animals in our care. Given that the gut microbiome interacts closely with host metabolism, immunity, and even cognition, it represents a potentially powerful tool for identifying subtle changes in health status across a range of body systems simultaneously. However, thus far, it has not been widely tested or implemented as a monitoring tool. In this study, we used longitudinal microbiome sampling of newly arrived chimpanzees at Chimp Haven to explore the feasibility of using the gut microbiome as a health and welfare biomarker in a sanctuary environment. We also tested the hypothesis that a transition to a new living environment, and integration into new social groupings, would result in temporal changes in chimpanzee gut microbiome composition. The collection of longitudinal microbiome data at Chimp Haven was feasible, and it revealed temporal shifts that were unique to each individual and, in some cases, correlated to other known impacts on health and behavior. We found limited evidence for microbial change over time after arrival at Chimp Haven that was consistent across individuals. In contrast, social group and enclosure, and to a lesser extent, age and sex, were associated with differences in gut microbiome composition. Microbiome composition was also associated with overall health status categories. However, many of the effects we detected were most apparent when using longitudinal data, as opposed to single time point samples. Additionally, we found important effects of technical factors, specifically outdoor temperature and time to collection, on our data. Overall, we demonstrate that the gut microbiome has the potential to be effectively deployed as a tool for health and environmental monitoring in a population of sanctuary chimpanzees, but the design must be carefully considered. We encourage other institutions to apply these approaches and integrate health and physiology data to build on the utility of gut microbiome analysis for ensuring the welfare of captive primates in a range of contexts.

Summary

- Longitudinal sampling of newly arrived chimpanzees at Chimp Haven showed that gut microbiome composition was stable over time but appeared to change in response to events such as injury.
- The effects of factors such as social group and general health status were most apparent with longitudinal samples as opposed to single time point samples.
- Technical factors such as outdoor temperature and time to collection influenced data and should be controlled or recorded in future studies.
- The gut microbiome represents a potentially powerful tool for identifying changes in captive primate health, but implementation must be carefully designed.

1 | Introduction

Central to the provision of optimal care for captive primates is the ability to monitor the welfare of individuals and groups and to quickly assess changes in primates' health. Repeated and ongoing health and behavioral monitoring can allow for the establishment of animals' baseline well-being, track changes over time, and assess recovery or response to various therapeutic interventions (Fultz et al. 2023). Such baseline, longitudinal monitoring may be especially valuable for long-lived primate species (Hopper et al. 2022). While various behavioral measures have been used for the long-term monitoring of captive primates' behavior, health, and welfare (Watters et al. 2021), in recent years, noninvasive approaches to measuring physiological markers of well-being have been developed. For example, endocrine correlates of reproductive health, stress, nutritional status, and even social compatibility (reviewed in Behringer and Deschner 2017) and biomarkers of immune function (Behringer et al. 2017; Lucore et al. 2022) have been developed, while disease burdens can be measured directly from biological samples (Gilardi et al. 2015) and indirectly from wastewater (Mathavarajah et al. 2022). As our understanding of the gut microbiota and its interactions with host biology and health has improved over the past two decades, there has been growing interest in using gut microbiome analysis as another noninvasive tool for long-term monitoring of animal health and welfare (Chen et al. 2021)

Variation in the gut microbiome is associated with a range of chronic diseases in humans, including diabetes, colitis, and Alzheimer's (Arora et al. 2020; Cani et al. 2012; Halfvarson et al. 2017), as well as with self-reported experiences of stress and anxiety (Foster and McVey Neufeld 2013). Many of these relationships have also been reported in nonhuman primates across a range of environments (Anzà et al. 2023; Sheh et al. 2022; Vlčková et al. 2018; Wu et al. 2020; Yang et al. 2022). For example, rhesus macaques (*Macaca mulatta*) at a primate research center with chronic diarrhea had a distinct microbiome composition with lower relative abundances of *Lactobacillus* compared to healthy rhesus macaques (McKenna et al. 2008). Although there is some debate regarding whether microbial shifts are a cause or a symptom of these health conditions, data describing changes or differences in the microbiome can be used to identify changes or differences in health. Because the microbiome interacts with multiple body systems and is sensitive enough to detect asymptomatic physiological shifts (Diaz and Reese 2021), it is particularly useful as a first line of evaluation both at the individual and group levels. Once microbial changes are reported, health and behavioral records can be used in conjunction with more specific physiological testing to pinpoint the cause.

Microbiome analyses can also be used to assess the health impact of environmental change. For example, changes in diet, social interactions, and even outdoor exposure are known to affect the gut microbiome in humans and other primates (Amato et al. 2015; Bisanz et al. 2019; David et al. 2014; Hicks et al. 2018; Meehan et al. 2018; Perofsky et al. 2017; Roslund et al. 2020; Tung et al. 2015). In turn, the gut microbiota can affect host metabolism, immunity, and even behavior (Belkaid and Hand 2014; Sylvia and Demas 2018; Thaiss et al. 2016; Visconti et al. 2019). Therefore, knowledge of how different environmental factors affect the gut microbiota could be important for predicting and shaping long-term health outcomes for captive primates.

Despite its potential utility, however, gut microbiome analysis is not commonly integrated into long-term monitoring of captive primates' health and welfare (Diaz and Reese 2021). Typically, measures of gut microbiome health are taken via "snapshot" measures, giving insights into specific time points or interventions or comparing the well-being of individuals or species (Frankel et al. 2019). A major obstacle to the integration of microbiome analysis with long-term welfare monitoring is the lack of clear links between primate species-specific environmental or health conditions and distinct microbial signatures. Generating long-term microbiome data for captive populations requires substantial resources in terms of sample collection and storage, as well as lab analyses and data processing. Many institutions are rightfully hesitant to dedicate these resources to a biomarker that has not been rigorously tested in their specific context. Ironically, though, without committing to long-term microbiome data generation, it is difficult to establish microbial baselines for captive populations. These baselines, and longitudinal deviations from them, are necessary to establish the required links between environment, health, and the microbiome.

In this context, an important first step toward facilitating the use of gut microbiome analysis as a noninvasive tool for monitoring welfare is to assess the feasibility of generating longitudinal gut microbiome data from captive nonhuman primate populations. Knowledge of the time and effort it takes to collect and store samples, as well as the technical variables that can affect data quality, will help institutions evaluate their ability to incorporate microbial biomarkers into their existing tool kits. Additionally, data describing baseline community composition in different captive nonhuman primate populations, the extent to which they change over time, and the biological variables that can affect these patterns will provide an important foundation for teams to build from as they consider implementing this approach.

Here, we aim to demonstrate the feasibility of long-term microbiome monitoring and create a foundation for its future use in captive chimpanzee (*Pan troglodytes*) populations. We

selected chimpanzees given their long lifespans and the variety of environments in which they are cared for and studied, including zoos, sanctuaries, research facilities, and in the wild. We collected longitudinal samples for gut microbiome analysis from a group of chimpanzees that were retired from research in 2015 and moved to Chimp Haven sanctuary, the United States, in 2019. Sampling lasted for one year, beginning when the individuals arrived at Chimp Haven, with the goal of tracking the health and welfare of the animals during their first year at the sanctuary. We hypothesized that the chimpanzees would exhibit changes in their gut microbiome composition over time as they underwent this transition in their living environment. We also opportunistically studied the introduction of one of our study groups to another resident social group already living at the sanctuary. Given the reported importance of social interactions on gut microbiome populations for certain primate species (e.g., Perofsky et al. 2017; Tung et al. 2015), we predicted that we would see initial differences between these two groups that would decrease with time following the introduction.

2 | Methods

2.1 | Subjects and Housing

This study was conducted at Chimp Haven. Chimp Haven is a sanctuary for chimpanzees retired from biomedical research in the United States. Chimpanzees have been housed at Chimp Haven since 2005, but in November, 2015, the National Institutes of Health (NIH) announced that they would no longer support biomedical research on chimpanzees. At this time, a plan was created for the remaining NIH-owned and -supported chimpanzees to be relocated to Chimp Haven (NIH 2016). At the time of publication, over 300 chimpanzees reside at Chimp Haven.

Two social groups of chimpanzees (*P. troglodytes*), comprised of 13 adult individuals in total, were the primary subjects of this research (see Table 1 for the demographic information of all subjects). The chimpanzees were studied starting from their arrival at the sanctuary and over the course of the following year (see Section 2.3). Due to the logistics surrounding the transfer of the chimpanzees, the researchers of this study could not directly evaluate the prior environments of the chimpanzees and did not have access to existing data. Therefore, we focused on understanding potential patterns of convergence among individuals that had previously occupied distinct environments and now occupied the same environment. To evaluate similar dynamics on a more local scale, during the last month of the study, we also collected samples from eight additional chimpanzees in a third social group after they were introduced with one of our primary social groups. No manipulations to the chimpanzees' care, diet, housing, or social groupings were made for the purpose of this opportunistic study.

When chimpanzees are relocated to Chimp Haven, they are initially housed in the McGrath Welcoming Center, which serves as the chimpanzees' housing while they are quarantined after arrival. This practice protects Chimp Haven's existing population from the introduction of infectious diseases and optimizes the health, behavior, and welfare of newly relocated chimpanzees. It also provides the chimpanzees with time to acclimate to the sanctuary and allows staff to get to know them. To ensure socialization and continuity of familiar bonds, the chimpanzees are housed in the groups they arrived in. The chimpanzees are typically quarantined from Chimp Haven's existing population for approximately 30 days at the discretion of the Attending Veterinarian. The McGrath Welcoming Center and its veterinary suite are separate from the rest of the Chimp Haven facility and provide dedicated staff care for the chimpanzees during quarantine. Housing consists of indoor/outdoor enclosures with either concrete or grass substrate. The groups in this study were housed in one of three indoor/outdoor enclosures in the McGrath Welcoming Center with concrete substrates (QC1, QC2, and QC3).

After the quarantine period, veterinary staff sedate the chimpanzees, conduct physical exams, clear the chimpanzees as safe to exit quarantine, and relocate them to enclosures throughout the facility. All of the chimpanzees in this study were relocated to one building, denoted as Building B, which is 0.45 km from the McGrath Welcoming Center. The housing in Building B consists of areas referred to as Play Yards (PY) which are large indoor/outdoor mesh enclosures with brachiation bars overhead. Outdoor areas are 6000 sq. ft., and indoor locations range from 395 to 4138 sq. ft. Outdoor areas include concrete, soil, or grass supplemented by pine straw, wood-wool, or hay as substrates (see Fultz et al. 2022 for more details). Animals in these PY have access to grass, soil, and surface water after it rains. The concrete edges are covered by pine straw or hay, and there is occasional spontaneous growth in the enclosures due to the humid subtropical climate. Chimpanzees at the sanctuary are often rotated into different areas as a form of enrichment and to provide novelty, when maintenance procedures are necessary, or to prepare for social introductions. Group 1 moved to PY33 initially, and in September 2019 they were moved to PY43. Group 2 moved to PYs 41 and 42. Group 2 and another group already resident at the sanctuary, Group 3, were combined in January 2020. Before this, Group 2 was housed in PY32. After the introduction, combined Groups 2 and 3 were housed in PY31.

During the course of this study, all the chimpanzees consumed the typical diet provided at Chimp Haven. This diet consists of Mazuri Primate Basix Biscuits ad libitum (Mazuri 5NAA, Brentwood, Missouri), which are provided twice daily. Animals are also given a morning produce meal and an afternoon vegetable. Produce varies seasonally and daily, but all animals typically receive fruits, vegetables, leafy greens, and starch daily. They also receive forage twice weekly, which includes nuts in the shells, various grains, and popcorn. A variety of food-based enrichment is provided ad libitum.

2.2 | Ethics

Chimp Haven is accredited by the Global Federation of Animal Sanctuaries (GFAS) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and is a member of the North American Primate Sanctuary Alliance (NAPSA). This Research adhered to the

TABLE 1 | Demographic, sampling, and analysis details for chimpanzees included in the study.

Subject	Group	Age	Sex	Status	Dates sampled	No. of samples	Variables
AM1A	1	Adult	Male	Incoming	2/12/19–2/12/20	13	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AM1B	1	Adult	Male	Incoming	2/14/19–2/12/20	12	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AF1C	1	Adult	Female	Incoming	2/14/19–3/18/20	14	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AM1D	1	Adult	Male	Incoming	2/14/19–2/12/20	17	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AF1E	1	Adult	Female	Incoming	2/15/19–2/10/20	15	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AF1F	1	Adult	Female	Incoming	2/22/19–2/12/20	14	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AM1G	1	Adult	Male	Incoming	2/22/19–2/12/20	15	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AF1H	1	Adult	Female	Incoming	2/22/19–3/18/20	13	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline
AM2I	2	Adult	Male	Incoming	3/26/19–3/20/20	15	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline, group merge
AF2J	2	Adult	Female	Incoming	3/26/19–3/9/20	13	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline, group merge
AF2K	2	Adult	Female	Incoming	3/28/19–3/20/20	16	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline, group merge
AF2L	2	Adult	Female	Incoming	4/1/19–3/4/20	12	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline, group merge
AF2M	2	Adult	Female	Incoming	4/2/19–3/26/20	12	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, age, sex, social group, health status, time since baseline, group merge
AF3N	3	Adult	Female	Resident	2/4/2020–3/19/20	2	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge
AF3O	3	Adult	Female	Resident	2/5/20–3/19/20	4	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge
AM3P	3	Adult	Male	Resident	2/5/20–3/20/20	3	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge

(Continues)

TABLE 1 | (Continued)

Subject	Group	Age	Sex	Status	Dates sampled	No. of samples	Variables
AM3Q	3	Adult	Male	Resident	2/7/20–3/3/20	3	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge
AM3R	3	Adult	Male	Resident	2/7/20–3/9/20	3	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge
AM3S	3	Adult	Male	Resident	2/10/20–3/20/20	5	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge
AF3T	3	Adult	Female	Resident	2/10/20–3/19/20	4	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge
AF3U	3	Adult	Female	Resident	2/11/20–3/16/20	3	location, substrate, weather, temperature, time to collection, enclosure, Bristol stool scale, group merge

American Society of Primatologists' Principles for the Ethical Treatment of Non-Human Primates. The chimpanzees were never food or water deprived, and no manipulations were made to their typical care routine for the purpose of this study. This research was reviewed and approved by the Lincoln Park Zoo Research Committee (study approval number: 2018–011), Chimp Haven's Sanctuary Chimpanzee Care Committee (SCCC), and the NIH Chimpanzee Research Use (CRU) Committee.

2.3 | Noninvasive and Opportunistic Sample Collection at Chimp Haven

Fecal samples were collected longitudinally from 13 individual chimpanzees in two social groups: Group 1 and Group 2. Sampling began in February 2019 when the first group arrived at Chimp Haven (with the second arriving in March 2019) and continued until March 2020 (Table 1). To the extent possible, samples were collected weekly for the first month of residency in the McGrath Welcoming Center and monthly thereafter when they were relocated to the sanctuary's long-term housing area. The intention of this timeline was to capture any rapid microbial change that might occur upon arrival and through the transition out of quarantine and into normal enclosures. Samples were also collected from eight chimpanzees in a third group (Group 3), who had resided at Chimp Haven since either 2014 or 2017, approximately every 2 weeks from January, 2020, through March, 2020, when this group was combined with Group 2. Our aim had been to study Group 2's acclimation to Chimp Haven and then any additional resultant, long-term changes in their microbiome following this social introduction, but data collection ended early in March 2020 to protect both chimpanzees and research staff at Chimp Haven in response to the COVID-19 pandemic.

Fecal samples were identified through a combination of in-person and remote camera behavioral monitoring. Location and subject were recorded, and samples were later collected into tubes containing 96% ethanol when husbandry accessed their enclosures for regularly scheduled cleaning. We only collected samples that were unmanipulated by the chimpanzees. When possible, we collected information describing where samples were collected (indoor/outdoor) and on what substrate (cement, metal, wood, hay, soil, grass, or wood shavings). We also recorded the outdoor ambient temperature when samples were collected and the weather (cloudy, sunny, and rainy). The time of defecation and the time of collection of the sample were recorded and used to calculate how long samples were in the environment before collection. Most samples were collected in the morning with an average time of 99.4 ± 92.8 min to collection following defecation (range 1–493 min).

We recorded information regarding a range of variables describing the chimpanzees. These included variables that did not change over time, such as age, sex, social group, and health status (healthy, compromised, or fragile, as determined by the veterinary staff and based on NIH categorizations [NIH 2019]). We also recorded variables that did change over time, including enclosure, Bristol stool scale rating, and administration of antiparasitics, antibiotics, and stool softener.

Samples stored in 96% ethanol were maintained at ambient temperature at Chimp Haven until shipping to the Amato Lab. There, they were kept at −80°C until processing.

2.4 | Behavioral Data Collection

When Group 2 was combined with Group 3, we collected behavioral data over the course of the first 3 months that the combined groups were housed together. All behavioral data were collected using the ZooMonitor application (Tracks Software, Salida, Colorado, the United States; M. R. Ross et al. 2016) on an Apple iPad mini (iOS 9.3.5, Cupertino, California, the United States) using 10-min focal follows with a data point recorded every minute. The ethogram we used was the same as that reported by S. Ross et al. (2021) and encompassed a broad range of species-typical behaviors. In addition to recording behavior, for each data point, we also recorded the focal animal's relative proximity to all other group members as "in contact" (physically touching another group member), "in proximity" (within arm's reach of another group member), or "distant" (farther than an arm's reach from the other group member). In total, we collected 1061 follows, with an average of 123.15 observation sessions for each of the 13 chimpanzees in Groups 2 and 3 over the 3-month observation period.

2.5 | Microbiome Data Generation

DNA was extracted from fecal samples using the Qiagen DNEasy PowerSoil kit with modifications as previously described (Moy et al. 2023). A two-step polymerase chain reaction (PCR) was used to amplify the V4-5 region of the 16S rRNA gene using the 515 F/926 R primers with Fluidigm linker sequences as previously described (Mallott and Amato 2018). Extraction and PCR negatives were used to control for contamination. PCR products were purified and normalized using a SequalPrep Normalization Plate and sequenced on the Illumina MiSeq V4 platform at the Rush University Microbiome and Genomics Core. Raw DNA sequences are available in the Sequence Read Archive (number to be provided upon acceptance).

Sequencing yielded 5,365,740 raw sequence reads. All controls had fewer sequence reads than the actual samples and were therefore discarded from subsequent analyses. Excluding controls, we had an average of 24,208 sequences per sample before quality filtering (range of 102–59,523 sequences per sample). Raw sequence data were trimmed, quality-filtered, and dereplicated; amplicon sequence variants (ASVs) were inferred; and paired reads were merged using the DADA2 plug-in (Callahan et al. 2016) for QIIME2 (v2023.2)(Bolyen et al. 2018). Taxonomy was assigned in QIIME2 using a Naive Bayes classifier trained on the GreenGenes2 database using the full 16S rRNA gene sequence lengths (McDonald et al. 2024). Mitochondria and chloroplast ASVs were filtered from the dataset. After quality filtering, there was an average of 7019 sequences per sample (range: 3–17,980 sequences per sample) and a total of 12,230 ASVs.

We generated α-rarefaction curves using the QIIME2 α-rarefaction command and, based on the output, chose to rarefy our data to 3000 reads per sample. This resulted in the loss of eight samples. We calculated the Shannon α diversity as well as weighted and unweighted UniFrac distances between samples using the core diversity plug-in in QIIME2. The analysis code is available on GitHub (https://github.com/Kramato-lab/ChimpHaven_monitoring).

2.6 | Statistical Analysis

After quality filtering, we had 192 samples from all three social groups for which we collected data describing technical factors. For Group 1 and Group 2, we had an average of 14.6 samples per individual (range 11–18 samples per individual). For Group 3, we had an average of 2.8 samples per individual (range 1–5). To test the extent to which these factors were associated with gut microbiome composition, we used all of the samples from all three groups in a series of permutational analyses of variance (PERMANOVA) using the adonis2 function in the package, vegan (Oksanen et al. 2018) with R software (v4.2.2), for both the unweighted UniFrac and weighted UniFrac distance matrices. To control for repeated measures, we included individual identity in each model. We tested for the effects of location, substrate, weather, temperature, and time to collection separately. Substrates were categorized as either solid (cement, metal, or wood) or loose (hay, soil, grass, or wood shavings). We also tested for an interaction between temperature and time to collection. To test for an association between these technical factors and microbial α diversity, we used a linear mixed effects model with individual identity included as a random effect (R package lme4) as well as generalized additive models (GAMs, R package mgcv). We used a series of Pearson correlations to test for significant associations between the relative abundances of individual microbial taxa and both outdoor temperature and time to collection. We corrected the p values for multiple comparisons using the fdrtool package. We also used GAMs to test for the effect of time to collection on the relative abundance of individual ASVs, including the effects of age, group, sex, location, substrate, weather, temperature, health status, Bristol stool scale rating, and accounting for repeated measurements. For the ASVs with relative abundances that changed in response to time to collection, we also clustered them for pattern identification. The average relative abundance over time to collection was first estimated using loess so that nonlinear patterns could be incorporated. The estimated mean relative abundance was standardized to have a mean of 0 and standard deviation of 1 across the samples so that we could identify their pattern over time, but not the magnitude of change. We then used hierarchical clustering to group the ASVs according to their patterns.

We also used PERMANOVA and linear regression to test for an association between gut microbiome composition and individual factors that were constant over time, including age, sex, social group, and health status (healthy, compromised, or fragile, as determined by the veterinary staff). We also used the ANCOM-BC package (Lin and Peddada 2020) to test for significant differences in the relative abundances of individual GM taxa using the ancombc2 function. However, given the strong effect of individuals on our data, to run these tests, we removed the repeated measures by calculating an average microbiome

composition for each individual over time. We also included only those individuals that were sampled for the full duration of the study (Groups 1 and 2) since the sampling of Group 3 included fewer samples and a temporal bias. To test the extent to which any detected effects could be identified with fewer time points, we also ran the same models on a single time point collected from each individual in Groups 1 and 2 in March/April, 2019, as well as a single time point collected from each individual in Groups 1 and 2 in February/March, 2020.

Using the repeated measures dataset for all chimpanzees in all three groups, we tested for associations between gut microbiome composition and two individual factors that changed over time—enclosure and Bristol stool scale rating—using PERMANOVA, linear mixed effects models, and Pearson correlations as described above. While we had data on the administration of antibiotics, antiparasitics, and stool softeners, there were not sufficient data points with these influences for us to evaluate the impacts statistically. Models were run on the reduced dataset of 186 samples for which we had full technical data and controlled for individual identity, outdoor temperature, and time to collection.

To test whether the introduction of Groups 2 and 3 led to a convergence of their gut microbiome composition, we analyzed a dataset that included samples from only those two groups collected starting in January, 2020, when the groups were combined and ending in March, 2020 ($n = 35$ samples). We ran a PERMANOVA to test whether the effect of the original social group interacted with time. We ran these models as an interaction between social group and month as well as social group and sample number. We ran models that controlled for individual identity, temperature, and time to collection. We also ran these models without controls to test if there was an effect without partitioning out other variations in the data. Additionally, we analyzed the social interactions between the members of Groups 2 and 3 when first combined by creating a composite sociality score that included the proportion of observation in which each possible dyad was either in contact or in proximity with each other. To visualize the chimpanzees' social interactions, we used the "heatmap" function in R. We then tested for a correlation between pairwise sociality data and pairwise microbiome data using a Mantel test (ade4 package).

Finally, to examine change in the gut microbiome over time, we used GAMs to identify microbial ASVs that shifted in relative abundance as described above for time to collection, using days since baseline instead of time to collection as the independent variable of interest. We used the same hierarchical clustering approach to group ASVs by patterns over time.

The analysis code is available on GitHub (https://github.com/Kramato-lab/ChimpHaven_monitoring). All variables and samples are summarized in Figure 1.

3 | Results

3.1 | Technical Factors Associated With Microbiome Patterns

PERMANOVA demonstrated that overall sample microbial composition varied slightly depending on the location (i.e., the specific enclosure in which the chimpanzees were housed; unweighted UniFrac: $F_{1,171} = 1.5$, $r^2 = 0.01$, $p = 0.02$) and substrate from which the sample was collected (unweighted UniFrac: $F_{1,191} = 1.5$, $r^2 = 0.01$, $p = 0.01$) with respect to the presence/absence of microbial taxa, but not their relative abundance (weighted UniFrac: $p > 0.05$). Weather at the time of sample collection had no effect on sample microbial composition, but outdoor (ambient) temperature had a small effect (unweighted UniFrac: $F_{1,191} = 1.3$, $r^2 = 0.01$, $p < 0.001$; weighted UniFrac: $F_{1,191} = 2.4$, $r^2 = 0.01$, $p = 0.01$). Time to collection post-defecation also had a small effect on sample microbial composition (unweighted UniFrac: $F_{1,185} = 3.2$, $r^2 = 0.015$, $p < 0.001$; weighted UniFrac: $F_{1,185} = 4.2$, $r^2 = 0.02$, $p < 0.001$), but there was no interaction between temperature and time to collection.

Given the effects of outdoor temperature and time to collection on overall microbial community composition, we used a linear mixed effects model to test for a relationship between these variables and microbial α diversity. Time to collection, but not outdoor temperature, was significantly negatively associated with Shannon diversity ($c^2 = 6.7$, $\mathrm{df} = 1$, $p = 0.009$). A GAM model also showed a significant nonlinear relationship of time to collection with Shannon diversity ($F_{1,191} = 10.63$, $p = 0.001$). When considering these variables only, Pearson correlations revealed 655 taxa whose relative abundances were significantly correlated with time to collection (Table S1) and 314 whose relative abundances were significantly correlated with outdoor temperature (Table S2). However, GAMs accounting for all variables of interest did not reveal any ASVs that shifted in relative abundance with increasing time to collection, and only

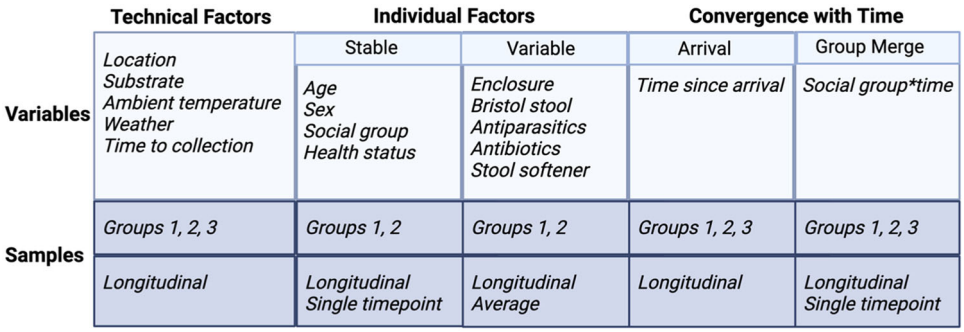

FIGURE 1 | Diagram outlining the variables tested, the social groups included, and the sample format used for each test. Single time point tests for the group merge variable were compared to each other to look for differences in effect size. (Figure created with Biorender. com).

one ASV, an unknown Lentisphaeria, increased in relative abundance with increasing temperature.

3.2 | Time-Stable Chimpanzee Factors Associated With Microbiome Patterns

When we calculated an average microbiome composition for each individual to explore the effect of stable individual traits on the microbiome, we detected differences in overall microbiome composition as evaluated using presence/absence of microbial taxa for both age (unweighted UniFrac: $F_{1,12} = 1.6$, $r^2 = 0.13$, $p = 0.04$; Figure S1) and social group (unweighted UniFrac: $F_{1,12} = 2.4$, $r^2 = 0.18$, $p = 0.004$; Figure 2). Differences in α diversity were not associated with either of these factors. In contrast, sex (unweighted UniFrac: $F_{1,12} = 1.6$, $r^2 = 0.13$, $p = 0.04$; weighted UniFrac: $F_{1,12} = 2.0$, $r^2 = 0.16$, $p = 0.04$; Figure S2) and health status (unweighted UniFrac: $F_{2,12} = 1.6$, $r^2 = 0.24$, $p = 0.009$; weighted UniFrac: $F_{2,12} = 1.7$, $r^2 = 0.26$, $p = 0.05$; Figure 3) were associated with differences in overall microbiome composition. Both of these factors were also associated with differences in Shannon diversity ($F_{1,12} = 8.9$, $p = 0.01$; $F_{2,12} = 6.4$, $p = 0.02$) using a linear mixed effects model, but not GAMs. ANCOM-BC identified six ASVs that differed significantly in relative abundance between healthy,

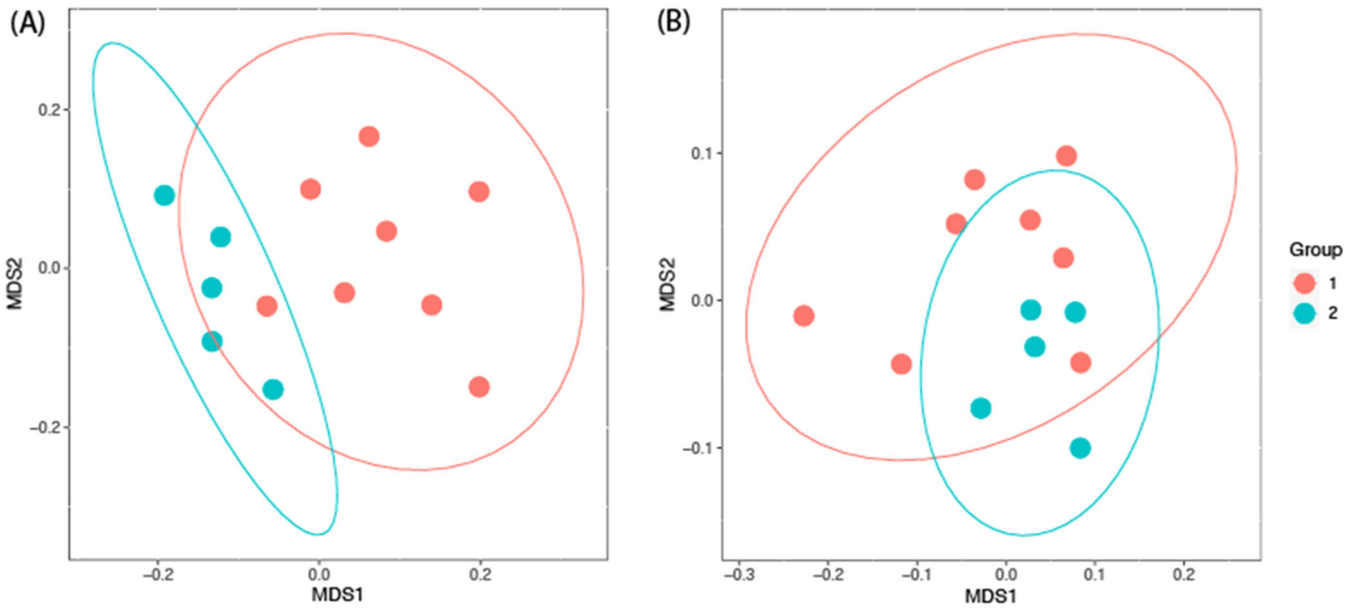

FIGURE 2 | Nonmetric multidimensional scaling (NMDS) plot demonstrating clustering of chimpanzee gut microbiomes by incoming chimpanzees' social group as measured using (A) unweighted UniFrac distances and (B) weighted UniFrac distances.

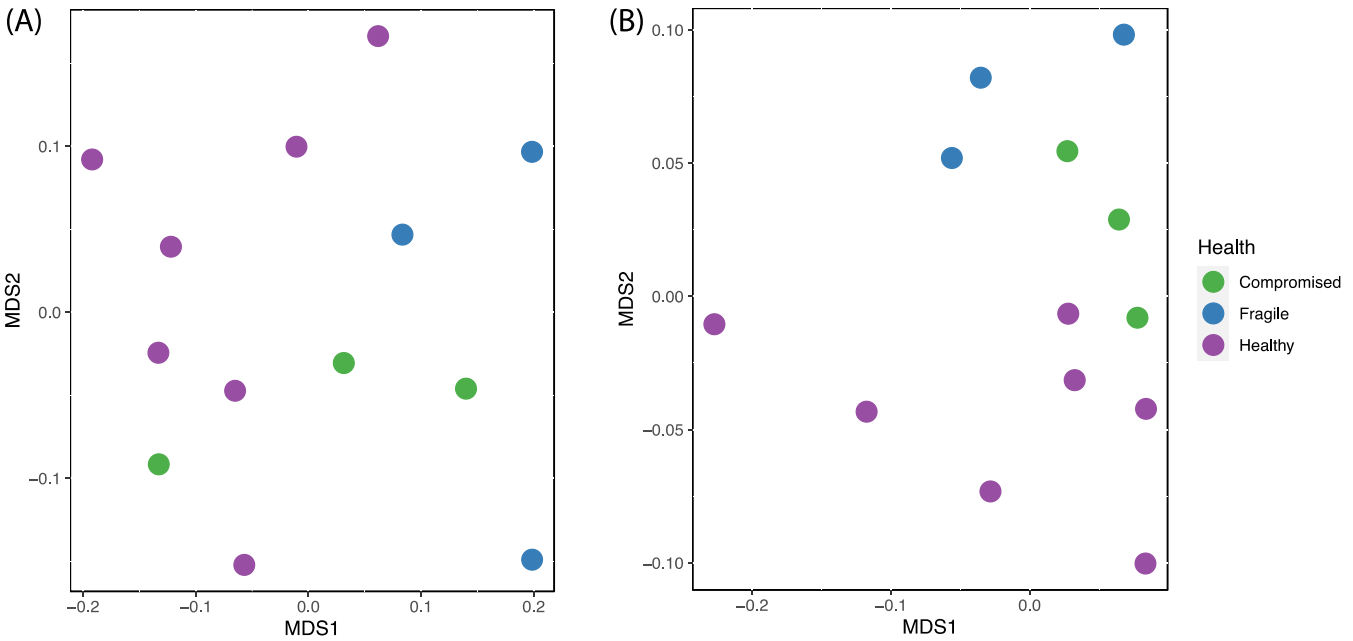

FIGURE 3 | Nonmetric C scaling (NMDS) plot demonstrating clustering of chimpanzee gut microbiomes by NIH health status ("healthy," "fragile," or "compromised") as measured using (A) unweighted UniFrac distances and (B) weighted UniFrac distances.

compromised, and fragile individuals. Specifically, there were higher relative abundances of a *Duodenibacillus* and a Lachnospiraceae genus 1XD8-76 ASV in "fragile" versus "compromised" individuals and a higher relative abundance of a Bacteroidaceae genus UBA4334 ASV in "compromised" versus "fragile" individuals. An unknown Lachnospiraceae ASV and an unknown Christenellaceae ASV had higher relative abundances in compromised individuals compared to both healthy and fragile individuals. An ASV from Lachnospiraceae genus G11 was lower in relative abundance in "compromised" individuals compared to both "healthy" and "fragile" chimpanzees. There were no ASVs that differed significantly in relative abundance between males and females using ANCOM-BC, but a GAM showed that males had higher relative abundances of *Prevotella copri*. In general, these effects were only apparent in the averaged data set, and not when we ran the same models on single time point data from either the beginning or end of the study period.

3.3 | Time-Variable Chimpanzee Factors Associated With Microbiome Patterns

When we considered factors that varied over time for individuals, PERMANOVA indicated a weak association between overall microbial community composition and the enclosure the chimpanzee lived in at the time of sampling (unweighted UniFrac: $F_{8,185} = 1.7$, $r^2 = 0.07$, $p < 0.001$; weighted UniFrac: $F_{8,185} = 1.8$, $r^2 = 0.06$, $p < 0.001$). However, enclosure was not significantly associated with differences in Shannon diversity. 35 ASVs varied in relative abundance across the enclosures (Table S3), many of which belonged to the family Lachnospiraceae and the genus *Treponema*. Compared to enclosure PY31, PY33 had 22 ASVs with different relative abundance, PY41 had one, PY42 had three, PY43 had 14, and quarantine had 40 (Table S4). Patterns in the differentiating ASVs suggest enclosures PY31, PY41, and PY42 were associated with similar gut microbiome composition. PY33 and PY43 were also similar, and quarantine was different from all non-quarantine enclosures.

Using linear mixed effects models, overall microbiome composition was weakly but significantly correlated with the Bristol stool scale rating (unweighted UniFrac: $F_{2,185} = 1.4$, $r^2 = 0.015$, $p = 0.003$; weighted UniFrac: $F_{2,185} = 1.6$, $r^2 = 0.015$, $p = 0.04$), and Shannon diversity of samples was also significantly associated with variation in Bristol stool scale ratings ($c^2 = 7.7$, df = 2, $p = 0.02$; Figure 4). Our GAM also showed a significant relationship between Bristol stool scale and Shannon diversity (3 and below compared to 4: $t = -2.4$, $p = 0.012$; 3 and below compared to 5: $t = -3.0$, $p = 0.003$). There was one Acutalibacteraceae ASV with relative abundances that were significantly different among Bristol stool scale indices when using

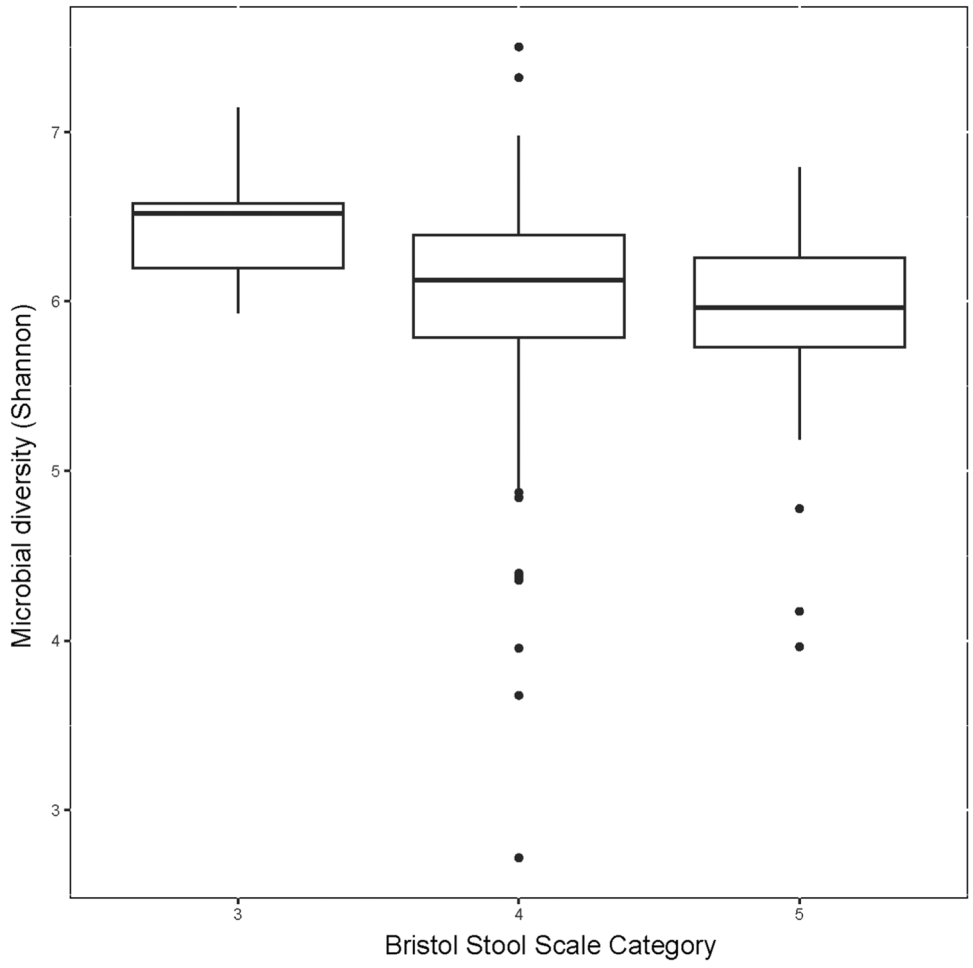

FIGURE 4 | Box plots showing significant differences in gut microbial diversity (Shannon index) among chimpanzees with different Bristol stool scale scores.

ANCOMBC, and 23 when using GAMs. Of these 23, 18 including the genera *Cryptobacteroides, Sodaliphilus, Dysosmobacter*, and *Prevotella*, exhibited increased relative abundances in samples with lower-than-normal Bristol indices that are associated with harder stools. Four, from the genera *Phascolarctobacterium, Oribacterium, Methanobrevibacter*, and *Gemmiger*, exhibited increased relative abundances in samples with higher-than-normal Bristol indices that are associated with looser stools. One, from the genus *Blautia*, exhibited higher relative abundances in normal stools.

3.4 | Chimpanzee Social Group Changes

Using PERMANOVA, we tested the extent to which the gut microbiome composition of the two social groups that merged in January 2020 (Groups 2 and 3) became more similar to each other over a period of the first three months that the chimpanzees lived together. After we accounted for repeated measures of individuals, there was no strong effect of the original social group on gut microbiome composition, and this pattern did not change over time, suggesting that the groups had similar gut microbiomes to begin with and did not become more or less similar over the three month period they were studied. Indeed, gut microbiome composition was generally similar across all groups even at the last time point of the study (Figure S3).

The majority of the chimpanzees in Groups 2 and 3 interacted with one another once the groups were merged: over the first three months the chimpanzees were cohoused, we observed that of the possible 78 pairwise combinations of the 13 chimpanzees, 72 dyads were observed in contact or in proximity at least once. However, the chimpanzees preferentially spent more time within proximity of their former group members (Figure S4). Indeed, of the possible pairwise combinations of the 13 chimpanzees, the six dyads that we never recorded within contact or proximity to each other were all comprised of one member from Group 2 and one member from Group 3. Specifically, AF2J was never observed within proximity of AF3U, AF30, or AF3N; AF2M was never observed in proximity with AFT3, while both AM21 and AF2L were never observed in proximity of AF3O. Accordingly, we found no significant correlation between pairwise measures of sociality and either unweighted or weighted UniFrac indices of microbiome dissimilarity.

3.5 | Overall Microbial Change Over Time

Overall gut microbiome composition and gut microbial α diversity did not change uniformly in individuals over time (Figures 5 and 6). Accordingly, our GAM for Shannon diversity revealed a nonlinear, significant interaction between individual and time ($F = 1.24$, $p = 0.04$). In contrast, examinations of patterns in microbiome composition and diversity for each individual revealed acute, short-term changes in microbiome composition and diversity in four individuals—AF1C, AM1D, AF1F, and AF1E—that were not observed in other individuals during the study period. In some cases, these patterns were linked to veterinary and behavioral observations. For example, AM2I and AF1E arrived at Chimp Haven with normal microbial diversity levels but experienced a drop in diversity approximately one month after their arrival that then rebounded quickly. This timing lines up with sedation and transfer out of quarantine. However, AF1F, AF1C, and AM1D were also sedated and transferred out of quarantine at the same

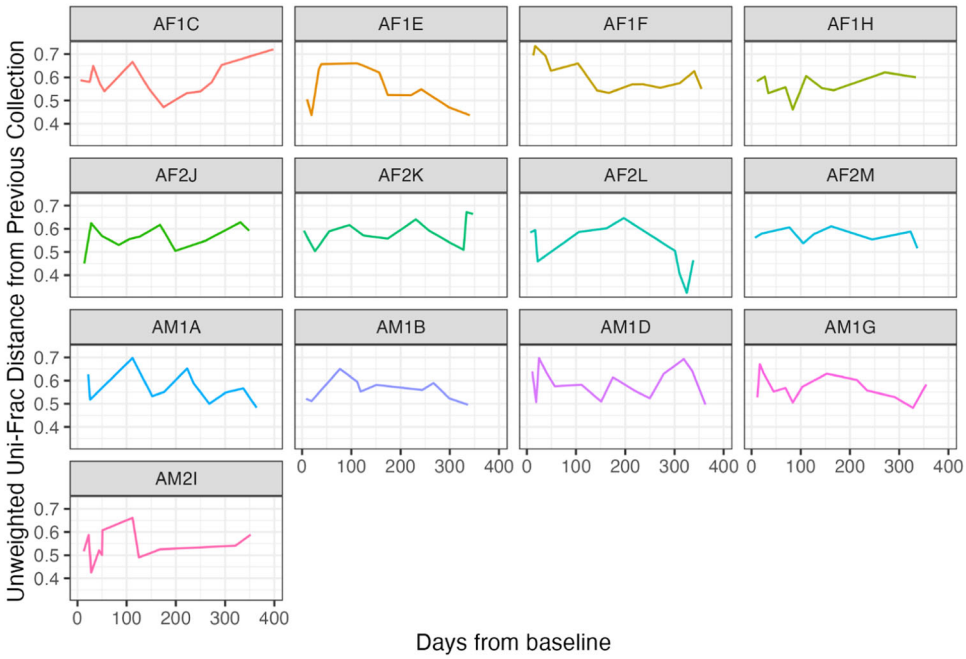

FIGURE 5 | Line graphs demonstrating longitudinal changes in overall gut microbiome composition in each individual incoming chimpanzee. For a given individual, each point represents the unweighted UniFrac distance between the current sample and the previous sample. *Note:* AM = "adult male," AF = adult female, and the number reflects the social group in which the animal lived, such that AF1C is adult female "C" who lived in Group 1.

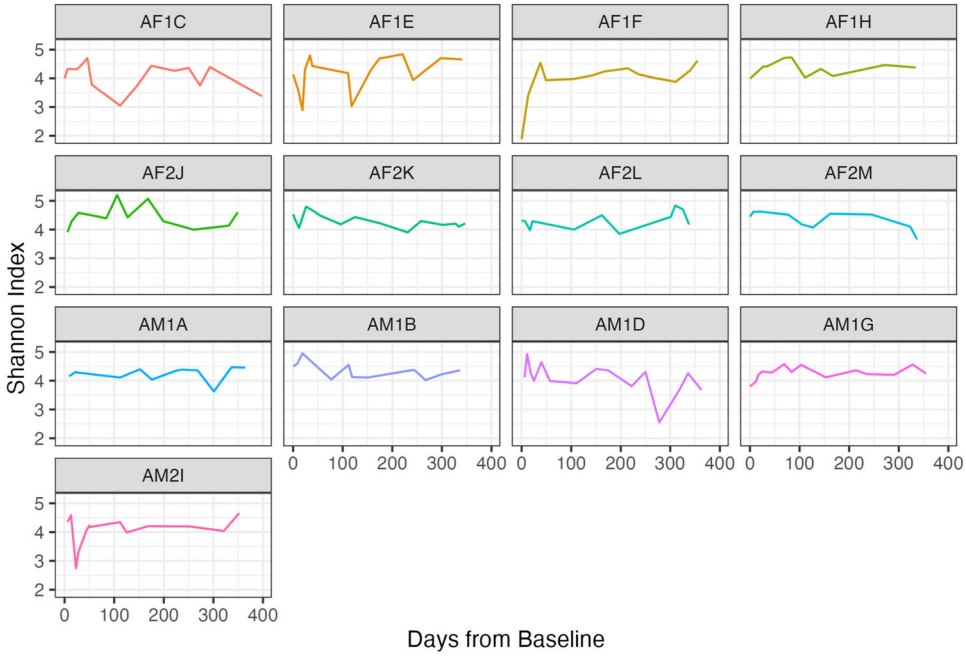

FIGURE 6 | Line graphs demonstrating longitudinal changes in gut microbial diversity in each individual incoming chimpanzee. For a given individual, each point represents the Shannon diversity index calculated for that sample. *Note:* AM = "adult male," AF = adult female, and the number reflects the social group in which the animal lived, such that AF1C is adult female "C" who lived in Group 1.

time and did not exhibit the same disruption in microbial diversity. These differences could be a result of individual treatments during the physical exam. For example, AM2I received antibiotics for a tooth infection, which likely contributed to the reduction in microbial diversity (Dethlefsen et al. 2008). Notably, AM1D also received antibiotics for social wounds at approximately nine months and also exhibited a rapid drop in microbial diversity, followed by a recovery. In contrast, AF1F had extremely low microbial diversity when she first arrived at Chimp Haven, which increased and stabilized during her first two months living at the sanctuary, but this change was not associated with a clear medical or behavioral factor. AF1F was diagnosed and treated for strongyloides parasites upon arrival at Chimp Haven, but so were AF1C and AM1D, and they did not exhibit similar microbial patterns. It is possible that the stress of transport and/or a pre-transport treatment contributed to this pattern, but we do not have data to verify this. Finally, AF1C, AM1D, and AF1E were all diagnosed with liver and/or cardiac conditions during their initial exams that required monitoring and treatment over time. These individuals also had the least stable microbiomes during the study period. While there are no direct links in our records between symptoms or treatments and microbial shifts, it is possible that physiological changes associated with these conditions led to microbial changes.

When we examined individual microbial taxa, GAMs indicated that 32 ASVs shifted in relative abundance as days since baseline increased during the study period. The ASVs that shifted temporally across the study could be grouped into five main clusters (Figure 7). One cluster showed increased relative abundances as time to collection increased and included mainly Firmicutes from the family Lachnospiraceae (Table S5). Another cluster showed decreased relative abundances over time and consisted of *Sodaliphus, Lachnospira, Treponema, Faecalibacterium, Prevotella*, and *Catenibacterium* (Table S5). The last three clusters showed nonlinear relationships with time. The first included *Cryptobacteroides, Succinivibrio, Egerieousia, Ruminococcus, Treponema*, and *Prevotella*. The last two clusters included only one (*Bruticola*) and two taxa (*Phascolarctobacterium*, UBA1436), respectively (Table S5).

4 | Discussion

In this study, we used longitudinal microbiome sampling of newly arrived chimpanzees at Chimp Haven to explore the feasibility of using the gut microbiome as a health and welfare biomarker in a sanctuary environment. We also tested the hypothesis that a transition to a new living environment, and integration into new social groupings, would result in temporal changes in gut microbiome composition. Although our hypothesis regarding temporal microbiome changes was not supported, we did identify important environmental and health factors that were associated with microbiome variation. We also generated important baseline data regarding microbiome dynamics in this population. Our findings also demonstrate that the microbiome has potential as a noninvasive biomarker of health and welfare for captive primate populations more broadly.

To begin with, contrary to expectations, our data revealed limited longitudinal change in the gut microbiomes of chimpanzees arriving at Chimp Haven. Because this transition involves a range of potential changes in environment reported to affect gut microbiomes in other host species and contexts (Amato et al. 2015; Bisanz et al. 2019; David et al. 2014; Hicks et al. 2018; Meehan et al. 2018; Perofsky et al. 2017; Roslund

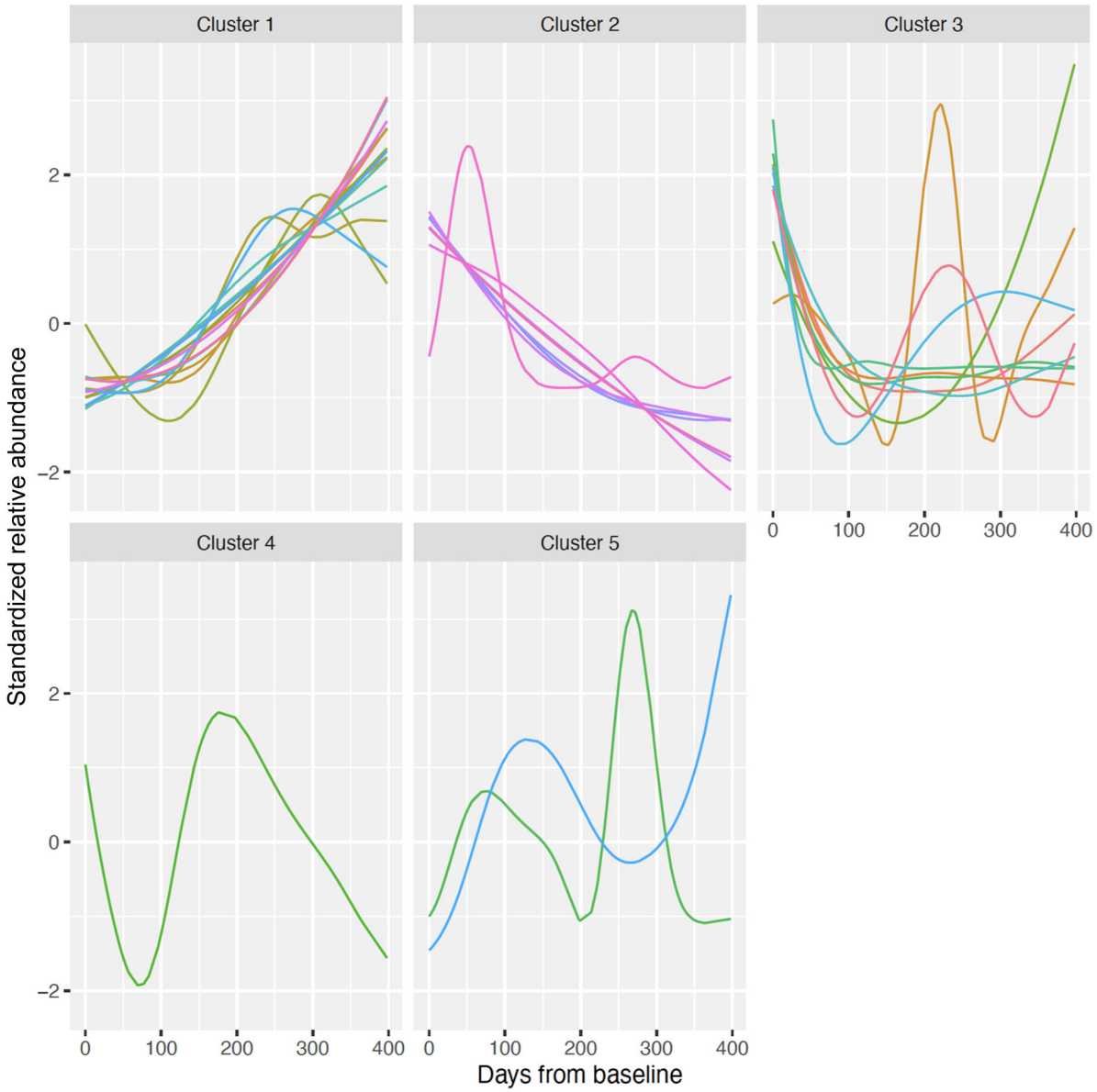

FIGURE 7 | Line graphs demonstrating average longitudinal changes in gut microbial taxa abundances across all chimpanzees. Each line represents a single microbial taxon, and each point is the average relative abundance of that taxon in all samples at that time point. Each panel represents a group of taxa whose relative abundances shifted in similar ways, as assessed using hierarchical clustering.

et al. 2020; Tung et al. 2015), including access to different substrates and local ecology, social interactions, and some aspects of diet, we expected to see substantial shifts in gut microbiome composition as chimpanzees spent more time at Chimp Haven. In wild chimpanzees, the environment has a strong effect on the gut microbiome, with variations in host genetics, diet, social interactions, and, to some extent, tool use, showing associations with gut microbiome composition (Bueno de Mesquita et al. 2021; Moeller et al. 2016). Wild versus zoo-housed chimpanzees have also been reported to exhibit environment-associated differences in gut microbiome composition (Frankel et al. 2019; McKenzie et al. 2017; Narat et al. 2020). The lack of any strong convergence of microbiomes from chimpanzees moved from other institutions to a shared environment in our dataset suggests that both the transition to Chimp Haven as well as the overall environment at Chimp Haven are stable for chimpanzees. Although there appeared to be some stochastic change in individual gut microbiomes over time, there were no strong universal changes that could be associated with the environment. In some ways, these findings are not surprising since a goal in transferring animals between institutions is to maintain stability and ensure continuity of care. Individuals are maintained on extremely similar diets and generally do not undergo changes in medications and other health interventions unless necessary for the individuals' health and well-being. They are also maintained in familiar social groups to reduce social stress. Therefore, while newly arriving chimpanzees have access to new and different environments at Chimp Haven, many microbiome-relevant environmental factors remain similar, something that was reflected in our data.

While overall gut microbiome composition did not change substantially over time, we did observe some temporal patterns in individual microbial ASVs. Some of these patterns suggested

potential influences on health. For example, many of the taxa that increased over time were Lachnospiraceae, which degrade carbohydrates and can produce beneficial metabolites such as short-chain fatty acids as by-products. In contrast, many of the taxa that decreased over time were *Treponema*. This genus is believed to be beneficial to human health, and its absence in populations with high processed food intake and antibiotic use is often interpreted as a health risk (Angelakis et al. 2019; Obregon-Tito et al. 2015). Whether this is a health risk in our sanctuary chimpanzee population is unclear. A previous study including wild and zoo-housed chimpanzees detected high relative abundances of *Treponema* in both populations but interpreted these patterns as a potential effect of diet (Campbell et al. 2020). Whether the reduced relative abundances of this genus that we observed over time in our sanctuary population are related to diet and/or health remains to be determined.

While time itself was not associated with many gut microbiome differences in our study, we did identify chimpanzee traits that were. First, sex had an effect on gut microbiome composition. Minor sex differences in gut microbiome composition have been reported previously in primate studies (Amato et al. 2014; Bennett et al. 2016; Pafčo et al. 2019; Tung et al. 2015), suggesting that this is an important variable to consider, particularly if the gut microbiome is being used as a health biomarker, since baseline values may vary between males and females. In wild chimpanzees, sex is significantly associated with microbiome composition in some studies (Degnan et al. 2012; Reese et al. 2021) but not in others (Bueno de Mesquita et al. 2021). Importantly, sex effects often only emerge with large sample sizes and/or temporal datasets in which average gut microbiome compositions can be calculated. For example, in this study, using a single time point resulted in no sex differences in the gut microbiome. Therefore, multiple samples may be necessary to establish baseline male and female values, highlighting the value and importance of longitudinal sampling as we performed here and the importance of considering sex as a biological variable (Shansky and Murphy 2021).

Enclosure also had a small but significant effect on gut microbiome composition in this study. These findings mirror others that consider the role of the built environment in shaping host microbiomes in both humans and captive animals (Hildebrand et al. 2013; Hufeldt et al. 2010; Hyde et al. 2016; Lax et al. 2014; Nishida and Ochman 2021; Singh et al. 2021). For example, in mouse studies, cage effects on the gut microbiome are commonly reported (Hildebrand et al. 2013; Hufeldt et al. 2010; Singh et al. 2021). Interestingly, in contrast to most other studies, in this study, the chimpanzees were not exclusively housed in one enclosure. Instead, they rotated through different enclosures during our year-long sampling efforts. Nevertheless, we still detected an effect of enclosure, suggesting that even short-term tenures in different enclosures can result in shifts in gut microbiome composition. Often, built environment effects are at least partially explained by social contact as well as microbial seeding of built environments by the hosts themselves. In this case, such an effect is less likely as the chimpanzees moved between different enclosures with their social group. Instead, it seems that distinct environmental microbial pools associated with different enclosures may have resulted in different chimpanzee gut microbiomes, even across enclosures at this single facility. More exploration of these dynamics is warranted.

In contrast to sex and enclosure, both age and social group were associated with differences only in the presence and absence of microbial taxa. In many other primate and human microbiome studies, these variables have relatively strong effects (Amato et al. 2014; Bennett et al. 2016; Gogarten et al. 2018; Grieneisen et al. 2017; Janiak et al. 2021; Perofsky et al. 2021; Reese et al. 2021). The somewhat limited effects we observed are likely a result of the lack of variation in age and social interactions in this population. Studies of other nonhuman primates and humans indicate the largest effects of age when multiple age classes are included in the study. For example, two studies of wild chimpanzees found a strong effect of age, but most reported differences were observed in individuals aged less than 2 years (Degnan et al. 2012; Reese et al. 2021). In our study, all chimpanzees were adults. Similarly, multiple studies of wild chimpanzees report strong effects of social group on gut microbiome composition (Degnan et al. 2012; Gogarten et al. 2018; Reese et al. 2021). However, in the population we studied, social groups have frequent contact with each other, both as a result of proximate housing conditions and occasional transfers of individuals between social groups.

Proximity and frequent social interactions have been previously reported to lead to convergence of chimpanzee gut microbiomes among individuals (Moeller et al. 2016) and even across host species (Moeller, Peeters, et al. 2013). Therefore, unlike what is observed in many wild primate populations with more fixed territories and limited patterns of interaction, social group may not be a strong indicator of gut microbiome composition in this sanctuary environment where proximity and social interactions between groups are high. A key example of this was that even when two social groups merged during our study, we did not observe an effect on microbiome composition. It is possible that our weekly sampling across 3 months during this group merge was not frequent enough or long enough to uncover more subtle changes in gut microbiome composition. Unfortunately, due to safety precautions due to COVID-19, we were unable to study the merged group for longer than 3 months, which may have also revealed greater convergence of the groups' members via increased social interactions between chimpanzees originating from Groups 2 and 3. However, our data suggest that the differences between social group gut microbiomes were relatively small to begin with. This pattern may be a result of shared environmental exposures at the sanctuary and indirect microbial transmission between social groups as they are moved between enclosures at the sanctuary.

Beyond providing insight into chimpanzee biological and environmental variations, our data provide important baseline information demonstrating the potential utility of gut microbiome analyses as a noninvasive health biomarker. Although the scope of this initial study did not include the collection of paired microbiome and physiological data, our results suggest that patterns in microbiome composition are linked to physiology. First and foremost, individual general health status had the strongest relationship with gut microbiome composition compared to all other measured variables in this study. This finding is striking given that an individual's status classified as

"healthy," "compromised," or "fragile" was independent of any specific disease exposure and instead represented a holistic assessment of well-being based on both quantitative and qualitative measures (NIH 2019). The fact that we could identify a relationship between these categories and gut microbiome composition indicates that gut microbiome analyses can be powerful and versatile biomarkers of health. Two previous studies of a wild chimpanzee population reported some associations of gut microbiome composition with viral infections, such as simian immunodeficiency virus (SIV), although patterns depended on disease stage (Barbian et al. 2018; Moeller, Shilts, et al. 2013). Parasite infections have also been associated with gut microbial differences in another population of wild chimpanzees (Renelies-Hamilton et al. 2019). To further increase their specificity, future studies should test for relationships with specific symptoms and health conditions. With these data, it would be possible to develop a dual approach, in which overall microbiome changes indicate a general health problem and more targeted microbial analyses can be used to narrow to specific diseases.

Our data also highlighted the potential for microbiome analyses to detect subtle physiological changes with no clear causes or symptoms. While we did not have the ability to collect temporal physiological data for this study, we noted temporal changes in the gut microbiome that were unique to individuals and, in some cases, were associated with known health interventions. For example, treatment with antibiotics occurred around the same time as marked changes in microbial diversity for two individuals, and a third experienced changes in microbial diversity concurrent with antiparasitic treatments. Although other individuals were also treated for parasites, it is possible these patterns were revealing a distinct impact of the treatment on the individual in question. It is also possible there was another unknown cause driving microbial shifts. In a situation where microbiome data were being used in real time to improve animal care, further assessments of this individual's behavior and physiology would be warranted. Essentially, microbiome data could provide an initial warning spanning multiple body systems, and more targeted biomarkers could then be used as a follow-up.

Admittedly, generating longitudinal microbiome data requires time and resources that may not be readily available in some captive settings, both in terms of sample collection and analysis. However, the relationship we uncovered between measures on the Bristol stool scale and gut microbiome composition offers a potential practical solution. Since assessing the Bristol stool scale is both quick and cheap, it could be used as a preliminary assessment of the gut microbiome and health that could be employed more frequently than direct microbiome analyses. This approach is currently used by some facilities in conjunction with other health biomarkers but could also be used to indicate key times to assess animals using more complex microbiome analyses.

For those institutions where it is possible to do more regular microbiome analyses, either directly or via institutional collaborations such as this one, the results of this study can be used to help design and implement effective microbiome monitoring programs in captive populations. Because of the breadth and duration of sampling, we were able to test the effects of a range of technical factors on microbiome results. We found that only two of these factors were associated with meaningful variation in the data: outdoor temperature and time to collection. Because animal care staff have busy schedules with many responsibilities, monitoring programs that require careful control of environmental factors can be overly burdensome. The fact that most of our measured environmental factors had a negligible influence makes it more feasible to incorporate this type of sampling into regular care routines. Additionally, the two factors that had the most influence are the most difficult to control. As a result, we suggest that institutions record these variables rather than try to control them. However, given that warmer temperatures and longer times to collection led to detectable differences in microbiome composition, we recommend that samples are collected as quickly as possible after defecation (within an hour if possible) and that collections are prioritized on cooler days, particularly in locations with seasonally high daily temperatures.

We also found that multiple samples collected from the same individuals over time provided a better understanding of the overall microbiome composition, and likely health status, compared to single time point snapshots. However, it remains unclear what minimum frequency of sampling is necessary. Conversely, our study did not have enough fine-scale sampling around events such as administration of antibiotics, antiparasitics, and stool softeners. These interventions have been reported to have effects on gut microbiome composition in other studies (Dethlefsen et al. 2008; He et al. 2018; Ma et al. 2023), and while we have some qualitative evidence that treatments such as antibiotics affected the microbiomes of some individuals in our study, we could not detect them reliably. Future studies can test different sampling schemes more systematically to identify minimum sampling frequency to address different aspects of health and medical care.

In conclusion, the primary goal of captive primate management is to ensure optimal health and welfare of the species in our care. Because health and welfare can be measured across many dimensions, management teams often rely on a range of tools to assess them. Given that the gut microbiome interacts closely with host metabolism, immunity, and even cognition, it represents a potentially powerful tool for identifying changes in health status that does not rely on prior knowledge or assumptions about which body system should be targeted. It is also sensitive enough to respond to changes in physiology that may not be obvious to caregivers due to a lack of symptoms or gradual shifts over time (Diaz and Reese 2021). Once data are generated, caregivers can then focus efforts on individuals of note, employing other non-microbiome tools to improve diagnoses. Microbiome data may also reveal environmental impacts on physiology that can affect welfare even when there are no clear and immediate risks to health (Diaz and Reese 2021). Again, this information may trigger additional evaluations of day-to-day care that would otherwise not be completed. Overall, we show that the gut microbiome has the potential to be effectively deployed as a tool for health monitoring and assessing environmental stability in a population of sanctuary chimpanzees. Together this information can be extremely useful for making decisions about care in this environment. We

encourage other institutions to apply these approaches and to integrate more behavioral and physiological data to build on the utility of gut microbiome analysis to ensure the welfare of captive primates in a range of contexts.

Author Contributions

Katherine R. Amato: conceptualization (equal), methodology (equal), project administration (equal), resources (equal), supervision (equal), writing – original draft (lead). **Benjamin R. Lake:** data curation (equal), methodology (equal), writing – review and editing (equal). **Samuel Ozminkowski:** formal analysis (equal), visualization (equal), writing – review and editing (supporting). **Hongmei Jiang:** formal analysis (equal), visualization (equal), writing – review and editing (supporting). **Madelyn Moy:** formal analysis (equal), methodology (equal), writing – review and editing (equal). **Maria Luisa Savo Sardaro:** methodology (equal), project administration (equal), writing – review and editing (equal). **Amy Fultz:** conceptualization (equal), funding acquisition (equal), methodology (equal), project administration (equal), writing – review and editing (equal). **Lydia M. Hopper:** conceptualization (equal), funding acquisition (equal), methodology (equal), project administration (equal), writing – review and editing (equal).

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Disclosure

The views and opinions expressed in this publication, presentation, or abstract represent the authors' views alone and do not express or imply the views, endorsement, or financial support of the Federal government or any of its agencies, including the National Institutes of Health, unless otherwise stated by an authorized representative thereof.

Data Availability Statement

The analysis code is available on GitHub (https://github.com/Kramato-lab/ChimpHaven_monitoring). The ASV table and metadata are included in the supplemental material (Tables S1, S2). Raw DNA sequences are available upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.