**CHAPTER 4**

**TRYPANOSOME SINGLE AND COINFECTIONS IMPACT COMMUNITY STRUCTURE OF THE TRIATOMINE BUG *RHODNIUS PALLESCENS***

**4.1 Abstract.**

The co-occurrence of parasites within a vector can influence transmission dynamics at the population level by altering host and vector demographics and immunity. *Rhodnius* species are often coinfected with both *Trypanosoma* *cruzi* and *Trypanosoma* *rangeli*. Individually, both parasites can reduce the fitness of *Rhodnius*, however, coinfection with the two parasites appears to ameliorate the negative fitness costs of each of the parasites. Here, we examine relationships between single and coinfected trypanosome parasites and the gut microbial communities of wild-caught *Rhodnius pallescens* from rural areas of central Panama.

Coinfected early-stage triatomine nymphs displayed greater gut microbial diversity than uninfected early-stage nymphs, but there was no difference in gut microbial diversity observed among coinfected and uninfected late-stage nymphs and adults. Across both age groups, single and coinfected individuals had similar gut microbial communities, but they differed in community composition and structure from uninfected triatomines. In coinfected bugs, there were shifts in the composition of rare and abundant bacterial taxa, but these changes did not result in changes in overall gut microbial diversity within late-stage nymphs or adults.

Our findings suggest that trypanosome single and coinfection dynamics within the triatomine guts differ between developmental stages, and that single infection and coinfection with trypanosomes similarly modify the gut communities of triatomines, particularly in late-stage triatomines.

**4.2 Introduction.**

Gut microbes of disease vectors can directly and indirectly influence vector competence, which is the vector’s ability to acquire, maintain, and transmit pathogens. Gut microbes of insect vectors of infectious pathogens, such as trypanosomes that infect triatomine bugs, can directly interfere with pathogens by competing for resources within the gut, or indirectly by inducing the vector’s anti-parasitic activity and humoral immune defense responses. *Serratia* *marcescens*, for example, are Gram-negative bacteria often found in the midgut of triatomine and can elicit trypanolytic activity on several strains of *Trypanosoma cruzi*1. Furthermore, parasites elicit antimicrobial peptides (AMPs) once they invade the midgut, favoring parasite survival, which may cause an initial reduction in gut bacteria.

Many studies have closely examined the relationship between *T. cruzi* infection and gut microbial communities within their triatomine vectors2–8 to better understand factors that influence successful parasite establishment within the gut, and ultimately vector competence. **ONE SENTENCE**. Because many species of triatomines in sylvatic settings may also be co-infected with one or more species of trypanosomes in addition to *T. cruzi,* it is also important to understand relationships of triatomine single and co-infection with trypanosomes and triatomine gut microbial diversity- an avenue of research that is yet to be explored.

Studies have shown that both *T. rangeli* and *T. cruzi* can negatively impact vector fitness, but one study found that experimentally coinfected *R. prolixus* had higher survival than insects that were infected with only *T. cruzi* or only *T. rangeli* 9. These results suggest that coinfection mitigates some of the adverse effects of single trypanosome infection on the vector immune system. However, further examinations are required to better understand these mechanisms.

The objective of this study is to study relationships between single and coinfections of trypanosome parasites and the gut bacteria of *R. pallescens* vectors collected from central Panama. Specifically, we evaluate if there are differences in the gut microbial diversity and composition in vectors that are singly infected with *T. cruzi*, coinfected with *T. cruzi* and *T. rangeli*, and not infected. This present study builds upon the previous chapter by further investigating patterns that drive gut microbial diversity in wild-caught triatomines. Broadly, we predict that trypanosome infection status would be a strong driver of triatomine gut microbiome diversity and community structure. We expect infected samples to have significantly greater bacterial diversity and distinct communities compared to uninfected triatomines. Finally, we expected coinfected triatomines to have the greatest gut bacterial diversity, as coinfected triatomines tend to have greater survival rates, and, as a more diverse gut microbiome may be reflective of a more robust immune system or higher survivorship.

**4.3 Materials and Methods**

**Sampling efforts and insect collection.**

*Rhodnius pallescens* (N = 274) were opportunistically collected from 41 *Attalea butyracea* palm crowns across central Panama using Noireau traps (Noireau et al., 2002) during the summer of 2017. The samples consisted of adults (N = 54), nymphal stage 5 (N = 27), nymphal stage 4 (N = 14), nymphal stage 3 (N = 60), nymphal stage 2 (N = 60), and nymphal stage 1 (N = 74).

**Sample preparation and DNA extraction.**

After capture, each triatomine was placed in 70% ethanol and kept at 4˚C. The body surface of each individual was rinsed in sterile water, and then the midgut guts were dissected using sterile techniques. Between each dissection, forceps and scalpels were rinsed in 70% ethanol and then sterilized using a glass beads sterilizer. Each triatomine gut sample was macerated and digested overnight in digest ATL buffer with Proteinase K and extracted using phenol-chloroform-isoamyl alcohol. Extractions were reconstituted in TLE buffer (10 mM Tris, pH 8; 0.1 mM EDTA), and impurities were removed with Sera-Mag SpeedBeads™ (Thermo Fisher Scientific, Waltham, MA, USA; Faircloth and Glenn10) with a final reconstitution of 30 μL TLE. In addition, 14 negative samples were included during extractions, *16S* rRNA amplification, and library sequencing for a final total of 288 samples.

**DNA amplification, *16S,* and *12S* rRNA sequencing.**

We amplified bacterial *16S* rRNA DNA using the S-D-Bact-0341-b-S-17 (5′-CCT ACG GGN GGC WGC AG-3′) forward and S-D-Bact-0785-a-A-21 (5′-GAC TAC HVG GGT ATC TAA TCC-3′) reverse primer pair following the protocol from Kieran et al.11. All libraries were sent to the Georgia Genomics and Bioinformatics Core (http://dna.uga.edu) for sequencing on an Illumina MiSeq using a v3 PE300 kit (Illumina, San Diego, CA, USA). Each sample was screened for the presence of *T. cruzi* by amplifying the kinetoplastid minicircle using 121/122 primers12 and screened for *T. rangeli* by amplifying a specific repetitive DNA sequence for the parasite named P54213. Amplification results for each sample were visualized on a 1.5% agarose gel.

**Bioinformatic processing.**

Amplicon indices were demultiplexedusing Mr. Demuxy 1.20 (https://pypi.org/project/Mr\_Demuxy/), and the resulting files were imported into QIIME2 v. 2022.214. Using the QIIME2 plugin *q2-cutadapt*15, primers were trimmed, paired, and reads were merged. The quality of the sequences was verified, the sequences were filtered, and any chimera sequences were removed using the QIIME2 plugin *q2-demux,* followed by denoising with DADA2 via the *q2-dada2* plugin16 to a final set of amplicon sequence variants (ASVs). All ASVs were aligned using mafft via the *q2-alignment* plugin17 and then used to construct a phylogeny with fasttree2 via the *q2-phylogeny* plugin18. Taxonomy was assigned the *q2-feature-classifier* plugin19 against the Naive Bayes classifier Geengenes 13\_8 99% OTUs20 that was trained on this sample dataset. We filtered out non-bacterial sequences (i.e., chloroplast and mitochondria) and Wolbachia sequences. Relative abundance plots were created using the R package *phyloseq*21 to calculate the bacterial community composition across infection type and triatomine age group and visualized using *ggplot2*22 in R v.4.2.023.

**Statistical Analyses.**

***Alpha (within-sample) diversity.*** Statistical analyses were completed using QIIME2 and R. For alpha and beta diversity analyses, samples were rarefied to 2133 sequences. Results from Chapter 3 (Fig. S2) and several studies7,24 have demonstrated significant differences in microbial diversity between early-stage nymphs and older triatomines. For this reason, the data for all analyses were split into two age groups: nymphal stages N1 – N3, and nymphal stages N4 – N5 plus adults. Four alpha diversity metrics (ASV observed richness, Shannon diversity25, Faith’s phylogenetic diversity26, and Pielou’s evenness27 were calculated using *phyloseq* and QIIME2. Each test was compared among groups using Pairwise Wilcoxon Rank Sum Tests with Benjamini-Hochberg adjustments (for groups of two) or a Kruskal-Wallis analysis of variance (for more than two groups) followed by Pairwise Wilcoxon Rank Sum Tests with Benjamini-Hochberg adjustments28 to test for pairwise significance. Boxplots were visualized using *ggplot2*. We used linear mixed models (LMMs) that were implemented using the *lme4*29 and *lmerTest*30 packages in R to evaluate the associations between infection type and bacterial alpha diversity. Log-transformed alpha diversity metrics were used as the response variables (ASV richness, Shannon diversity, Faith’s phylogenetic diversity, and Pielou’s evenness), and model predictors included sampling region, habitat type, dominant blood meal, blood meal richness, and infection type, and palm identity was treated as a random effect. R2 values were calculated using *partR2* in R31.

***Beta (between-sample) diversity.*** All analyses were again split by the two age groups. Beta diversity calculations were used to measure community structure and composition differences across infection types. Differences in beta diversity between samples were estimated using a permutational multivariate analysis of variance (PERMANOVA) on unweighted and weighted UniFrac distance matrices with 999 permutations using the QIIME2 *q2-diversity* plugin and with Benjamini-Hochberg adjustments and were supported with beta-dispersion. Unweighted UniFrac (community composition) measures the presence/absence of ASVs that incorporates phylogenetic distances, while weighted UniFrac (community structure) considers the phylogenetic distances between observed ASVs in addition to the relative abundance of ASVs. Palm identity, sampling region, habitat type, blood meal type, and blood meal richness were included as model predictors and included first in each full model to specifically test the effects of infection type. Principal coordinate analysis ordinations (PCoA) were calculated based on unweighted and weighted UniFrac distance matrices with 999 permutations using the *q2-diversity* plugin in QIIME2 and visualized using *ggplot2*.

**4.4 Results**

**Infection Results.**

Across all stages, 11.7% (N = 32/274) of our triatomines samples were coinfected with *T. cruzi* and *T. rangeli*, 32.1% (N = 88/274) infected with only *T. cruzi*, 7/274 were infected with only *T. rangeli*, and 56.2% (N = 154/274) were uninfected. Due to the low number of triatomines infected with only *T. rangeli* we did not include those samples for the remainder of the study. After splitting by age groups, 7.0% of N1 – N3 triatomine nymphs were coinfected, 20.5% were singly infected with *T. cruzi*, and 72.4% were uninfected, while 21.3% of N4 – adult triatomines were coinfected, 56.2% were infected with only *T. cruzi*, and 22.5% were uninfected (Fig. 1).

**Relative abundances of infected and uninfected triatomines**

Across all infection types, the bacterial communities of the triatomines across age groups were dominated by the genera *Sphingomonas* (mean relative abundance = 74.92% ± standard deviatioN = 6.40%), *Erwinia* (5.60% ± 5.30%), *Stenotrophomonas* (5.58% ± 0.816%), *Delftia* (3.43% ± 0.45%), and *Dietzia* (3.23% ± 4.30%) (Fig. 2). For N1 – N3 stages, there were few differences in the mean relative abundance of the dominant bacterial genera across infection status and only *Sphingomonas* contributed to more than an average of 10% of sequences (72-79%) (Fig. 2a). Conversely, N4 – adult triatomines displayed greater variation in composition across infected and uninfected samples. *Sphingomonas* contributed to an average of 60.7% sequences in coinfected N4 - adult triatomines, 67.3% in *T. cruzi-singly infected* samples, and 72.1% in uninfected samples. Furthermore, *Erwinia* contributed to an average of 12-15% of sequences in coinfected and *T. cruzi-singly* infected samples, but only 7.4% in uninfected samples, and *Dietzia* contributed to an average of 12.4% of sequences in coinfected samples but only an average of 2.1% in uninfected samples (Fig. 2b).

Venn diagrams comparing the overlap of AVS between infection type revealed that 125 ASVs were shared by N1 – N3 nymphs (Fig. 3a) and 98 ASVs were shared by N4 – adult triatomines (Fig. 3b). There were 9 shared ASVs between coinfected and *T. cruzi*-singly infected N1 – N3 nymphs and 74 shared ASVs between coinfected and *T. cruzi-*singlyinfected N4 – adult triatomines.

**Variation in triatomine diversity and composition across infection status.**

For within-sample microbial diversity (alpha diversity), N1-N3 coinfected individuals had significantly greater diversity as measured by Shannon diversity (*p* = 0.021) and Pielou’s evenness (*p* = 0.040) compared to negative samples. There were no differences between *T. cruzi-*mono*-*infected samples and coinfected samples, nor between *T. cruzi* samples and uninfected samples (Table 1, Fig. 3). Infection type did not have any significant association with alpha diversity for N1-N3 nymphs (LMM: R2 range = 0.0037 – 0.0355, p ≥ 0.1164) (Table 2). N4 nymphs to adults showed no differences in alpha diversity and no significant effects on alpha diversity across any metric (Tables 1 & 3, Fig. 4).

For between sample diversity (beta diversity), infection status was significantly associated with N1 – N3 triatomine nymph gut communities for both measures of beta diversity (PERMANOVA: UniFrac, pseudo-F = 3.25, *p* = 0.001; wUniFrac, pseudo-F = 4.44, *p* = 0.003) (Table 4). Unweighted UniFrac (community composition) showed some distinct clustering between infected samples and uninfected samples, with *T. cruzi-*singly infected samples and coinfected samples having considerable overlap (Fig. 6b), whereas weighted UniFrac (community structure) showed complete overlap across all samples with differences in dispersion (Fig. 6d). After accounting for model predictors, infection status alone did not significantly contribute to variation in community composition or structure (Table 5).

In N4 – adult triatomines, infection status was a significant predictor for community composition (PERMANOVA: UniFrac, pseudo-F = 1.59, *p* = 0.021) but not community structure (PERMANOVA: wUniFrac, pseudo-F = 1.32, *p* = 0.185) (Table 6). After accounting for model predictors, infection status alone significantly contributed to about 2.3% of variation in community composition (R2 = 0.0231, *p* = 0.017), and, albeit not significantly, infection status contributed to about 1.5% of variation in community structure (R2 = 0.0153, *p* = 0.533) (Table 7). Similar to N1 – N3 triatomine nymphs, unweighted UniFrac showed some clustering between infected N4 – adult triatomines and uninfected N4 – adult triatomines but no clustering between coinfected and infected samples (Fig. 7b). Weighted UniFrac did not show any distinct clustering across infection type (Fig. 7d), however, there were differences in within-group variability for both measures of beta diversity, particularly in infected samples.

After further examining the within-group variability across infection status and age group, we found that across both age groups, our *T. cruzi*-singly infected samples and coinfected samples were not dissimilar from each other and displayed the lowest within-group variability as measured by unweighted UniFrac. The within-group variability of uninfected samples was significantly greater than the infected samples (Fig. 6a, 7a, Table 8; coinfected vs. *T. cruzi-*singlyinfected samples Pairwise Wilcoxon rank sum test: UniFrac distance, *p* = 0.14, coinfected vs. negative pairwise UniFrac distance *p* < 0.001, *T. cruzi-*singlyinfected vs negative pairwise UniFrac distance, *p* < 0.001). Conversely, across both age groups, our uninfected samples displayed the lowest inter-group variability as measured by weighted UniFrac and were significantly different from single and coinfected samples. Additionally, across both age groups, inter-group variability was greatest for coinfected individuals, with coinfected individuals significantly greater than both *T. cruzi-*singly infected and uninfected samples (Fig. 6c, 7c, Table 8; wUniFrac distance, *p* < 0.001, coinfected vs. negative pairwise wUniFrac distance *p* < 0.001, *T. cruzi-*singlyinfected vs. negative pairwise wUniFrac distance, *p* < 0.001).

**4.5 Discussion**

Here, we characterized the gut microbial communities of *T. cruzi-*singlyinfected, coinfected, and uninfected *Rhodnius pallescens* across early-stage nymphs and late-stage triatomines. Our results showed (i) greater alpha diversity in coinfected N1 – N3 nymphs compared to uninfected N1 – N3 nymphs, (ii) similar diversity and composition in single and coinfected individuals, (iii) variation in community composition and structure between infected and uninfected triatomines and (iii) differences in within-group variability between infected and uninfected triatomines. Our findings allow for further investigations into wild-caught triatomines and associations between infection status and gut microbial communities.

***T. cruzi-*singlyinfected triatomines and coinfected triatomines have similar gut diversity.** We found substantial similarities in bacterial composition and structure between *T. cruzi-*singlyinfected and coinfected samples across both age groups. These results may reflect the different parasite lifestyles. Although both parasites multiply in the midgut, *T. rangeli* will then invade the hemocoel and salivary glands while *T. cruzi* remains and continues to multiply in the midgut. Although we do not have data on the quantity of each parasite within the samples, it is very likely that there are more *T. cruzi* within the guts of our samples than *T. rangeli*, which may result in the similarities between singly-*T. cruzi* infected samples and coinfected samples.

Furthermore, studies have demonstrated that both *T. cruzi* and *T. rangeli* can induce a reduction of cultivable gut bacteria, likely by eliciting antimicrobial peptides that reduce bacteria in the midgut to support parasite survival32,33. As triatomines are likely to be coinfected by one parasite at a time, we expect the effects of one parasite to be similar to the impact of a second parasite that has invaded after the first parasite has established in the gut. Another possible explanation may be that we have unknown parasite loads in the gut. If our triatomine samples have detectable but low amounts of *T. rangeli*, the gut bacteria of single and coinfected samples would presumably look similar. And finally, it is important to note that *T. cruzi* reproduces in the guts while *T. rangeli* reproduces in the hemolymph and salivary glands. Thus, *T. rangeli* may very likely have lower loads than *T. cruzi* when we only screen the guts of our samples.

**Coinfected early-stage nymphs have greater alpha diversity than early-stage uninfected nymphs, but there were no differences in *T. cruzi-*singlyinfected samples and uninfected across all developmental stages.** Our results showed that our samples displayed greater Shannon diversity and Pielou’s evenness in coinfected N1 – N3 nymphs compared to uninfected nymphs, although we did not find this pattern in the older triatomines. Conversely, we also found no differences in alpha diversity between *T. cruzi-*singlyinfected and uninfected samples across either age group.

Previous studies that examined the relationship between *T. cruzi* infection and triatomine gut bacteria had conflicting results. Some studies have found that infection is associated with greater bacterial diversity7, one study found lowered diversity associated with *T. cruzi* infection3, whereas other studies found that there were no differences between gut bacterial diversity and infection4,5,8. These discrepancies may reflect genetic differences across triatomine species5,24,34,35, sequencing and bioinformatic methods, collection origin, or duration of infection.

In our current study, the duration of infection was unknown as these were wild-caught triatomines. *Rhodnius* nymph development varies by species and individual, but nymphs can develop to N3 instars within 35 days (based on laboratory conditions)36, which would indicate that infection with two parasites, which likely happened in successive feeding events, would have occurred shortly prior to field collections. Therefore, early-stage nymphs were likely coinfected for a shorter duration than the later-stage triatomines, with less time for the parasites to become established in the guts, which may be another explanation to why we only see greater gut bacterial diversity in coinfected early-stage nymphs.

**Within- and between-group comparisons reveal differences between infected and uninfected triatomines.**

Across both age groups, less abundant taxa influenced dissimilarities in community composition between infected and uninfected samples, and infection status significantly contributed to these differences in late-stage triatomines. Further, the less abundant taxa in infected samples are more phylogenetically similar (lower variability) to each other compared to the less abundant taxa found in uninfected samples.

Across both samples, the dominant taxa in infected samples are phylogenetically similar to the dominant taxa in uninfected ones, however, there is more variability in phylogenetic relatedness between the dominant taxa of negative samples than the dominant taxa found both infected groups. Although our data cannot tell us information about functional diversity, microbes that are more phylogenetically similar are more likely to be functionally similar. Any loss of related microbes is less detrimental than the loss of unrelated microbes, unrelated microbes may perform unique biological functions in the gut. Furthermore, any loss of rare taxa is more detrimental than the loss of abundant taxa because there are more abundant taxa. Ultimately, these results emphasize the importance to consider both rare and abundant taxa, as they may show different biologically relevant patterns.

Our results demonstrate various patterns between infected and uninfected triatomines across the two age groups. Consistent with several studies7,24 and Chapter 3, the results of the present study show differences in the gut microbial communities between early-stage triatomine nymphs and later-stage nymphs and adults. In general, our N1 – N3 nymphs displayed lower within-sample bacterial diversity compared to older triatomines (See Chapter 3 Supplementary figure 2), suggesting that triatomines acquire more bacteria through development, whether due to multiple feeding events, increased mobility, or simply a feature of aging (See Chapter 3). Additionally, these patterns may also reflect infection timing. As stated earlier, *Rhodnius* nymphs can develop to N3 within 35 days, while adults can live over a year. Early-stage nymphs may have been infected more recently than later-stage nymphs and adults, and thus these results may also reflect patterns associated with infection duration.

**4.6 Conclusions**

We demonstrated that singly and coinfected triatomines have similar within-sample diversity, between-group composition and structure, and within-group variability. We also revealed differences in the influence of trypanosome parasites between early-stage nymphs and older triatomines. We found that coinfected samples exhibited greater microbial diversity in early-stage nymphs only and that infection status was a significant predictor of differences in community composition only in late-stage nymphs and adults. The results of this study add to the increasing literature of triatomine microbial communities and their relationships with trypanosome parasites.

Ultimately, we found that while specific abundant taxa, like *Sphingomonas*, *Delftia*, *Dietzia*, and *Erwinia*, were conserved across infection status, variation in the less abundant bacteria differentiated many of the infection-specific patterns. These results may reflect the resiliency of triatomine microbiomes, such that diversity may increase immediately after infection and less abundant microbes will shift the composition of the gut community, but after a period of time, the bacterial communities will return to their previous stable state of lowered bacterial diversity with a new composition of bacteria. Further investigations will be required to explore the stability of the triatomine microbiome and to identify microbiome-based vector control strategies that consider differences in community structuring across developmental stages.

4.7 References

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**4.8 Tables**

**Table 4.1** **Pairwise Wilcoxon rank-sum test comparisons of four different alpha diversity metrics across infected, coinfected, and uninfected samples**

Results highlighted in boldface indicate p-values of less than 0.05. All p-values were corrected with Benjamini-Hochberg adjustments.

|  |  |  |  |
| --- | --- | --- | --- |
| **N1 - N3** | Tcruzi:Coinfection | Tcruzi:Negative | Coinfection:Negative |
| Richness | 0.64 | 0.76 | 0.33 |
| Shannon | 0.279 | 0.081 | **0.021** |
| Faith's PD | 0.87 | 0.62 | 0.62 |
| Evenness | 0.23 | 0.09 | **0.040** |
|  |  |  |  |
| **N4 - Adult** |  |  |  |
| Richness | 0.66 | 0.21 | 0.21 |
| Shannon | 0.33 | 0.33 | 0.33 |
| Faith's PD | 0.77 | 0.26 | 0.26 |
| Evenness | 0.67 | 0.67 | 0.67 |

**Table 4.2 Linear mixed models testing the effects of infection status in early-stage triatomines on alpha diversity across after accounting sampling region, habitat type, and blood meal data**

Alpha diversity values were log-transformed prior to fitting and palm identity was included as a random effect.

Marginal R2 values were reported for full models and partial R2 values were reported for each covariate. Significant relationships are shown in bold.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **R^2** | **Sum SQ** | **Mean SQ** | **NumDF** | **DenDF** | **F Statistic** | **p-value** |
| **Richness** |  |  |  |  |  |  |  |
| Sampling Region | 0.0491 | 0.23792 | 0.118959 | 2 | 45.65 | 1.9167 | 0.1587 |
| Habitat Type | 0 | 0.00357 | 0.001785 | 2 | 25.723 | 0.0288 | 0.9717 |
| Blood meal richness | 0.0209 | 0.29031 | 0.096771 | 3 | 158.074 | 1.5592 | 0.2015 |
| Dominant Blood meal | 0.0314 | 0.50513 | 0.050513 | 10 | 159.364 | 0.8139 | 0.6156 |
| Infection Type | 0 | 0.06251 | 0.031255 | 2 | 162.549 | 0.5036 | 0.6053 |
| Full Model | 0.1519 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |
| **Shannon** |  |  |  |  |  |  |  |
| Sampling Region | 0.0394 | 0.130449 | 0.065225 | 2 | 38.731 | 1.8283 | 0.17428 |
| Habitat Type | 0.0248 | 0.062922 | 0.031461 | 2 | 23.103 | 0.8819 | 0.42748 |
| Blood meal richness | 0.031 | 0.283766 | 0.094589 | 3 | 160.854 | 2.6514 | **0.05061** |
| Dominant Blood meal | 0.0449 | 0.279062 | 0.027906 | 10 | 160.757 | 0.7822 | 0.6458 |
| Infection Type | 0.0004 | 0.11799 | 0.058995 | 2 | 164.705 | 1.6537 | 0.1945 |
| Full Model | 0.2486 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |
| **Faith’s PD** |  |  |  |  |  |  |  |
| Sampling Region | 0.014 | 0.08591 | 0.042956 | 2 | 41.575 | 0.5616 | 0.57456 |
| Habitat Type | 0.0205 | 0.12843 | 0.064213 | 2 | 21.871 | 0.8395 | 0.44537 |
| Blood meal richness | 0.016 | 0.45694 | 0.152313 | 3 | 154.826 | 1.9913 | 0.11757 |
| Dominant Blood meal | 0.0848 | 1.49606 | 0.149606 | 10 | 156.303 | 1.9559 | **0.04164** |
| Infection Type | 0.0018 | 0.00396 | 0.001982 | 2 | 160.627 | 0.0259 | 0.97443 |
| Full Model | 0.1534 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |
| **Evenness** |  |  |  |  |  |  |  |
| Sampling Region | 0.0287 | 0.054225 | 0.027113 | 2 | 31.118 | 1.7958 | 0.18278 |
| Habitat Type | 0.1088 | 0.163296 | 0.081648 | 2 | 19.603 | 5.4078 | **0.01348** |
| Blood meal richness | 0.0366 | 0.136724 | 0.045575 | 3 | 163.962 | 3.0186 | **0.03148** |
| Dominant Blood meal | 0.07 | 0.178938 | 0.017894 | 10 | 158.559 | 1.1852 | 0.30462 |
| Infection Type | 0.0147 | 0.035863 | 0.017932 | 2 | 163.531 | 1.1877 | 0.30754 |
| Full Model | 0.2726 |  |  |  |  |  |  |

**Table 3. Linear mixed models testing the effects of infection status in late-stage triatomines on alpha diversity across after accounting sampling region, habitat type, and blood meal data**

Alpha diversity values were log-transformed prior to fitting and palm identity was included as a random effect.

Marginal R2 values were reported for full models and partial R2 values were reported for each covariate. Significant relationships are shown in bold.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **R^2** | **Sum SQ** | **Mean SQ** | **NumDF** | **DenDF** | **F Statistic** | **p value** |
| **Richness** |  |  |  |  |  |  |  |
| Sampling Region | 0.0332 | 0.07612 | 0.038058 | 2 | 41.156 | 1.0403 | 0.3624 |
| Habitat Type | 0 | 0.08264 | 0.04132 | 2 | 19.556 | 1.1295 | 0.3434 |
| Blood meal richness | 0.01 | 0.08683 | 0.021707 | 4 | 66.497 | 0.5934 | 0.6686 |
| Dominant Blood meal | 0.0824 | 0.32042 | 0.040052 | 8 | 59.951 | 1.0948 | 0.3795 |
| Infection Type | 0.0355 | 0.16236 | 0.081182 | 2 | 68.614 | 2.2191 | 0.1164 |
| Full Model | 0.2612 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |
| **Shannon** |  |  |  |  |  |  |  |
| Sampling Region | 0.0568 | 0.113098 | 0.056549 | 2 | 41.23 | 2.5805 | 0.08792 |
| Habitat Type | 0 | 0.005268 | 0.002634 | 2 | 17.916 | 0.1202 | 0.88745 |
| Blood meal richness | 0.0397 | 0.126016 | 0.031504 | 4 | 68.089 | 1.4376 | 0.23104 |
| Dominant Blood meal | 0.0574 | 0.106719 | 0.01334 | 8 | 59.215 | 0.6087 | 0.7669 |
| Infection Type | 0.0037 | 0.006957 | 0.003478 | 2 | 66.336 | 0.1587 | 0.85355 |
| Full Model | 0.3505 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |
| **Faith's PD** |  |  |  |  |  |  |  |
| Sampling Region | 0.0312 | 0.04719 | 0.023595 | 2 | 36.686 | 0.5359 | 0.5897 |
| Habitat Type | 0.0011 | 0.0255 | 0.012752 | 2 | 18.221 | 0.2896 | 0.7519 |
| Blood meal richness | 0.015 | 0.06557 | 0.016393 | 4 | 60.991 | 0.3723 | 0.8275 |
| Dominant Blood meal | 0.1256 | 0.56193 | 0.070241 | 8 | 57.693 | 1.5953 | 0.1463 |
| Infection Type | 0.0164 | 0.15566 | 0.077831 | 2 | 69.976 | 1.7676 | 0.1783 |
| Full Model | 0.2824 |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |
| **Evenness** |  |  |  |  |  |  |  |
| Sampling Region | 0.0304 | 0.038577 | 0.019288 | 2 | 36.956 | 1.5387 | 0.2281 |
| Habitat Type | 0 | 0.002576 | 0.001288 | 2 | 13.167 | 0.1028 | 0.90306 |
| Blood meal richness | 0.0882 | 0.111631 | 0.027908 | 4 | 68.749 | 2.2263 | 0.07508 |
| Dominant Blood meal | 0.055 | 0.069128 | 0.008641 | 8 | 55.279 | 0.6893 | 0.69911 |
| Infection Type | 0.0045 | 0.006017 | 0.003009 | 2 | 61.014 | 0.24 | 0.78736 |
| Full Model | 0.1991 |  |  |  |  |  |  |

**Table 4.4 PERMANOVA results of early-stage triatomines with unweighted and weighted UniFrac distance matrix**

Results highlighted in boldface indicate p-values of less than 0.05. All p-values were corrected with Benjamini-Hochberg adjustments

|  |  |
| --- | --- |
| Unweighted |  |
| **PERMANOVA results** |  |
| **method name** | PERMANOVA |
| **test statistic name** | pseudo-F |
| **sample size** | 187 |
| **number of groups** | 3 |
| **test statistic** | 3.253925 |
| **p-value** | **0.001** |
| **number of permutations** | 999 |
|  |  |
| Weighted |  |
| **PERMANOVA results** |  |
| **method name** | PERMANOVA |
| **test statistic name** | pseudo-F |
| **sample size** | 187 |
| **number of groups** | 3 |
| **test statistic** | 4.444095 |
| **p-value** | **0.003** |
| **number of permutations** | 999 |

**Table 4.5 Adonis test results on unweighted and weighted Unifrac distance matrix from 16S rRNA amplicon bacterial sequencing of early-stage triatomines to test the effects of infection status**

Results highlighted in boldface indicate p-values of less than 0.05. All p-values were corrected with Benjamini-Hochberg adjustments

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Df** | **SumsOfSqs** | **MeanSqs** | **F.Model** | **R2** | **Pr(>F)** |
| **Unweighted** |  |  |  |  |  |  |
| **Palm Identity** | 34 | 14.746741 | 0.433728 | 2.774669 | 0.381897 | **0.001** |
| **Dominant Blood meal** | 10 | 1.587268 | 0.158727 | 1.015417 | 0.041106 | 0.424 |
| **Blood meal richness** | 3 | 0.555297 | 0.185099 | 1.184126 | 0.014381 | 0.12 |
| **Infection Type** | 2 | 0.309681 | 0.154841 | 0.990555 | 0.00802 | 0.436 |
| **Residuals** | 137 | 21.415417 | 0.156317 | NaN | 0.554597 | NaN |
| **Total** | 186 | 38.614403 | NaN | NaN | 1 | NaN |
|  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |
| **Weighted** |  |  |  |  |  |  |
| **Palm Identity** | 34 | 1.70877 | 0.050258 | 3.453908 | 0.429082 | **0.006** |
| **Dominant Blood meal** | 10 | 0.201042 | 0.020104 | 1.381636 | 0.050483 | 0.149 |
| **Blood meal richness** | 3 | 0.052507 | 0.017502 | 1.202832 | 0.013185 | 0.255 |
| **Infection Type** | 2 | 0.026579 | 0.013289 | 0.91329 | 0.006674 | 0.461 |
| **Residuals** | 137 | 1.993492 | 0.014551 | NaN | 0.500577 | NaN |
| **Total** | 186 | 3.98239 | NaN | NaN | 1 | NaN |

**Table 4.6 PERMANOVA results of late-stage triatomines with unweighted and weighted UniFrac distance matrix**

Results highlighted in boldface indicate p-values of less than 0.05.

|  |  |
| --- | --- |
| **Unweighted** |  |
| **PERMANOVA results** |  |
| **method name** | PERMANOVA |
| **test statistic name** | pseudo-F |
| **sample size** | 100 |
| **number of groups** | 3 |
| **test statistic** | 1.590809 |
| **p-value** | **0.021** |
| **number of permutations** | 999 |
|  |  |
|  |  |
|  |  |
| **Weighted** |  |
| **PERMANOVA results** |  |
| **method name** | PERMANOVA |
| **test statistic name** | pseudo-F |
| **sample size** | 100 |
| **number of groups** | 3 |
| **test statistic** | 1.320066 |
| **p-value** | 0.185 |
| **number of permutations** | 999 |

**Table 4.7 Adonis test results on unweighted and weighted Unifrac distance matrix from 16S rRNA amplicon bacterial sequencing of late-stage triatomines to test the effects of infection status**

Results highlighted in boldface indicate p-values of less than 0.05. All p-values were corrected with Benjamini-Hochberg adjustments.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Df** | **SumsOfSqs** | **MeanSqs** | **F.Model** | **R2** | **Pr(>F)** |
|  |  |  |  |  |  |  |
| **Unweighted** |  |  |  |  |  |  |
| **Palm Identity** | 33 | 10.730884 | 0.325178 | 2.184134 | 0.516156 | **0.001** |
| **Dominant Blood meal** | 7 | 1.01502 | 0.145003 | 0.973945 | 0.048823 | 0.542 |
| **Blood meal richness** | 4 | 0.672909 | 0.168227 | 1.129937 | 0.032367 | 0.203 |
| **Infection Type** | 2 | 0.480431 | 0.240216 | 1.613463 | 0.023109 | **0.017** |
| **Residuals** | 53 | 7.890748 | 0.148882 | NaN | 0.379545 | NaN |
| **Total** | 99 | 20.789992 | NaN | NaN | 1 | NaN |
|  |  |  |  |  |  |  |
| **Weighted** |  |  |  |  |  |  |
| **Palm Identity** | 33 | 1.881464 | 0.057014 | 1.613897 | 0.465975 | **0.037** |
| **Dominant Blood meal** | 7 | 0.107123 | 0.015303 | 0.433191 | 0.026531 | 0.962 |
| **Blood meal richness** | 4 | 0.114707 | 0.028677 | 0.811752 | 0.028409 | 0.62 |
| **Infection Type** | 2 | 0.062072 | 0.031036 | 0.878532 | 0.015373 | 0.533 |
| **Residuals** | 53 | 1.872329 | 0.035327 | NaN | 0.463712 | NaN |
| **Total** | 99 | 4.037695 | NaN | NaN | 1 | NaN |

**Table 4.8 Pairwise Wilcoxon rank-sum test with Bonferroni correction across infected status for unweighted and weighted UniFrac distances**

Results highlighted in boldface indicate p-values of less than 0.05. All p-values were corrected with Benjamini-Hochberg adjustments.

|  |  |
| --- | --- |
| **N1 - N3** |  |
| **Unweighted** | p-value |
| Coinfected:Tcuzi | 0.14 |
| Coinfected:Negative | **5.70E-16** |
| Tcuzi:Negative | **< 2e-16** |
|  |  |
| **Weighted** |  |
| Coinfected:Tcuzi | **0.00026** |
| Coinfected:Negative | **< 2e-16** |
| Tcuzi:Negative | **< 2e-16** |
|  |  |
|  |  |
| **N4 - Adult** |  |
| **Unweighted** |  |
| Coinfected:Tcuzi | 0.68 |
| Coinfected:Negative | **8.60E-10** |
| Tcuzi:Negative | **< 2e-16** |
|  |  |
| **Weighted** |  |
| Coinfected:Tcuzi | **0.00236** |
| Coinfected:Negative | **1.40E-06** |
| Tcuzi:Negative | **0.00037** |

**Table 4.9 Pairwise beta dispersion results of early-stage and late-stage triatomines using unweighted and weighted UniFrac distance matrix**

Results highlighted in boldface indicate p-values of less than 0.05. All p-values were corrected with Benjamini-Hochberg adjustments.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Group 1** | **Group 2** | **Sample size** | **Permutations** | **F-value** | **p-value** | **q-value** |
|  |  |  |  |  |  |  |
| **N1 - N3** |  |  |  |  |  |  |
| **Unweighted** |  |  |  |  |  |  |
| Coinfection | Negative | 147 | 999 | 0.087779 | 0.798 | 0.921 |
| Coinfection | Tcruzi | 53 | 999 | 0.055611 | 0.853 | 0.921 |
| Negative | Tcruzi | 174 | 999 | 0.010573 | 0.921 | 0.921 |
|  |  |  |  |  |  |  |
| **Weighted** |  |  |  |  |  |  |
| Coinfection | Negative | 147 | 999 | 2.123222 | 0.097 | 0.1455 |
| Coinfection | Tcruzi | 53 | 999 | 0.002385 | 0.972 | 0.972 |
| Negative | Tcruzi | 174 | 999 | 4.921809 | 0.03 | 0.09 |
|  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |
| **N4 - Adult** |  |  |  |  |  |  |
| **Unweighted** |  |  |  |  |  |  |
| Coinfection | Negative | 41 | 999 | 0.990381 | 0.317 | 0.602 |
| Coinfection | Tcruzi | 79 | 999 | 0.320101 | 0.602 | 0.602 |
| Negative | Tcruzi | 80 | 999 | 0.365532 | 0.589 | 0.602 |
|  |  |  |  |  |  |  |
| **Weighted** |  |  |  |  |  |  |
| Coinfection | Negative | 41 | 999 | 0.61908 | 0.454 | 0.735 |
| Coinfection | Tcruzi | 79 | 999 | 0.041723 | 0.865 | 0.865 |
| Negative | Tcruzi | 80 | 999 | 0.548689 | 0.49 | 0.735 |

4.9 Figures

**Figure 4.1 The relative frequencies of single and coinfection of *Rhodnius pallescens* with *Trypanosoma cruzi* and *Trypanosoma rangeli*, separated by N1 – N3 nymphs and N4 – N5 nymphs plus adults.**

Chart, bar chart

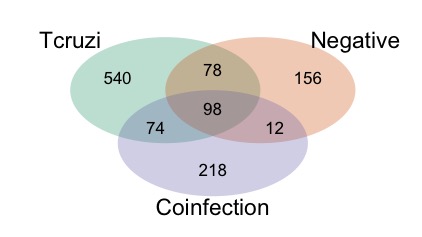
Description automatically generated

**Figure 4.2 Average relative abundances of the top 10 bacterial genera for all sampled *R. pallescens* (N = 274) split by age groups (N1 – N3 nymphs, and N4 – N5 nymphs and adults) as determined by *16S* rRNA metabarcoding.**

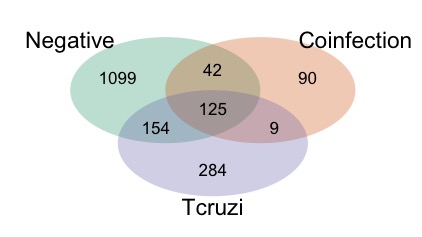
Chart, bar chart

Description automatically generated

**Figure 3.3 Venn diagram of unique and shared amplicon sequence variants (ASVs) in a) N1 – N3 triatomine nymphs, and b) N4 – N5 triatomines nymphs and adult**



b)



a)

**Figure 4.4 Boxplots of the four alpha diversity metrics (ASV richness, Pielou’s evenness, Shannon diversity, and Faith’s phylogenetic diversity), averaged across all N1 – N3 triatomine nymph samples that were single T. cruzi infected, coinfected, and uninfected.** n = Number of bugs sampled. Horizontal bars indicate two infection types that were compared via Pairwise Wilcoxon Rank Sum Tests to test for pairwise significance. Asterisks designates Benjamini-Hochberg-corrected p-values (\* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001; ns, not significant).

Diagram, box and whisker chart

Description automatically generated

**Figure 4.5 Boxplots of the four alpha diversity metrics (ASV richness, Pielou’s evenness, Shannon diversity, and Faith’s phylogenetic diversity), averaged across all N4 triatomine nymph and adult samples that were single T. cruzi infected, coinfected, and uninfected.** n = Number of bugs sampled. Horizontal bars indicate two infection types that were compared via Pairwise Wilcoxon Rank Sum Tests to test for pairwise significance. Asterisks designates Benjamini-Hochberg-corrected p-values (\* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001; ns, not significant).

Diagram, box and whisker chart

Description automatically generated

**Figure 4.6 (a-b) Unweighted UniFrac and (c-d) weighted UniFrac distances for N1 – N3 triatomine nymphs.** (a,c) Comparisons of pairwise distances between infection status with statistical results of Pairwise Wilcoxon Rank Sum Tests with Benjamini-Hochberg adjustment (p < 0.001 \*\*\*). (b,d) Principle component analysis (PCoA) with points colored by infection status shows clustering by infection type in coordinate space with 95% confidence interval ellipses.

Diagram

Description automatically generated

**Figure 4.7 (a-b) Unweighted UniFrac and (c-d) weighted UniFrac distances for N4 – N5 triatomine nymphs and adult triatomines.** (a,c) Comparisons of pairwise distances between infection status with statistical results of Pairwise Wilcoxon Rank Sum Tests with Benjamini-Hochberg adjustment (p < 0.001 \*\*\*). (b,d) Principle component analysis (PCoA) with points colored by infection status shows clustering by infection type in coordinate space with 95% confidence interval ellipses.

Chart, diagram

Description automatically generated