Abdominal microbiome composition and diversity of two *Heliconius* species (Lepidoptera: Nymphalidae) in the Colombian Andes

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Abstract: Internal insect microbial communities, that is microbe taxa that live within an organism, play important roles in digestion, protection from pathogens, and fitness of their insect hosts. Recent expansion of research in this field has highlighted the importance of endosymbiotic communities to their hosts and elucidated microbial community patterns based on host life history. Here, we document the bacterial microbiome of two species of the butterfly genus *Heliconius* (Nymphalidae), each from two fragmented populations, by sequencing the V4 region of the 16S rRNA gene. We used 14 individual adult butterflies from two species, *Heliconius cydno* (n = 10) and *H. clysonymus* (n = 4), from two forest reserves in the Central Colombian Andes. *Commensalibacter* (Acetobacteraceae) was the most common bacterial genus across all samples, although relative abundance varied across groups. Notably, we also observed the bacterial genera *Spiroplasma* (Spiroplasmataceae) and *Wolbachia* (Ehrlichiaceae). While we did not find distinct spatial or species-level patterns in bacterial composition of microbiomes, we did find disparate bacterial diversity across the two butterfly species, with *H. cydno* harboring higher diversity than *H. clysonymus*. The microbiome composition of the two butterfly species did not differ, but that of *H. cydno* was distinct from the microbiome composition of environmental/butterfly trap bait samples. These findings contribute to the documented diversity of insect microbiomes and inform future experimental and sampling efforts.

Keywords: butterfly, Central Cordillera, Colombia, *Heliconius clysonymus*, *Heliconius cydno*, *Spiroplasma*, *Wolbachia*.

Resumen: La microbiota que reside dentro de los insectos contribuye a la digestión, protección contra patógenos, e influye en la aptitud darwiniana del huésped. Estudios recientes han destacado la importancia que tienen las comunidades bacterianas dentro sus hospederos, además de dilucidar sus patrones de diversidad y cómo estas están relacionadas a la historia natural del organismo que las alberga. En este trabajo, exploramos y documentamos las comunidades de bacterias presentes en mariposas del género *Heliconius*, mediante la secuenciación de la región V4 del gen ARNr 16S. En total se capturaron 14 individuos que corresponden a las especies, *Heliconius cydno* (n=10) y *Heliconius clysonymus* (n=4), presentes en dos reservas naturales (poblaciones) de la Cordillera Central de los Andes colombianos. *Commensalibacter* fue el género de bacteria más abundante en todas las muestras, pero su abundancia relativa fue variable entre los grupos. Adicionalmente, observamos la presencia de otras bacterias como los simbiontes, *Spiroplasma* y *Wolbachia.* Aunque no encontramos un patrón particular en cuanto a la composición bacteriana en los microbiomas a nivel de especie ni sitio, la diversidad de bacterias de los microbiomas entre ambas especies de mariposa fue distinta - *H. cydno* tenía más diversidad que *H. clysonymus.* Las composiciones de los microbiomas de las dos especies de mariposa fueron muy parecidas, aunque la de *H. cydno* fue distinta de la composición bacteriana del microbioma de muestreos del ambiente/cebo de trampa. Esta investigación contribuye a la diversidad documentada de los microbiomas en insectos, además de informar futuros experimentos y métodos de muestreo.

Palabras Claves: Colombia, Cordillera central, *Heliconius clysonymus*, *Heliconius cydno*, mariposa, *Spiroplasma*, *Wolbachia*.

INTRODUCTION

Host-associated microbiomes are the characteristic microbial community associated with an organism (Whipps *et al.*, 1988) as well as that community's dynamic function in time and space (Berg *et al.*, 2020). Microbiomes, specifically bacterial communities, play important roles in the ecology, life history, digestion, and behavior of their hosts (Majumder, 2019; Krishnan *et al.*, 2014; Zytynska & Meyer, 2018). Attention to insect microbiomes has increased in recent years, generating research documenting the diversity of insect-associated microbes, studying effects of diet on microbiomes, and tying insect development to the microbiome across distinct life stages (e.g., Chandler, 2011; Hammer, 2019; van Schooten *et*

al., 2018). Microbiomes can also directly affect host fitness, by, for example, altering sex ratios (Anbutsu & Fukatsu, 2011; Saridaki & Bourtzis, 2010).

Lepidoptera can be an ideal group with which to examine microbiomes because of their ties to food plants, and diversity in form and function. Butterfly microbiomes have been studied, for example, within the context of metamorphosis, demonstrating that the microbiome transforms in congruence with the dietary and morphological changes associated with metamorphosis (Hammer *et al.*, 2014). Long-wing butterflies, members of the genus *Heliconius* Kluk (Lepidoptera: Nymphalidae), are a well-studied insect group with a high degree of wing pattern divergence (Joron *et al.*, 2006), including 48 described species at present (Jiggins, 2017). *Heliconius* butterflies are famed for their diversity of wing variants, ability to collect and use pollen as a protein source as adults (Gilbert, 1972), and larval fidelity to plants of Passifloraceae. While recent studies (e.g., van Schooten *et al.*, 2018; Hammer *et al.*, 2020; Ravenscraft *et al.*, 2019) have increased our understanding of how microbiomes vary across several *Heliconius* species, much detailed information is biased to specific locations (primarily Gamboa, Panama), and species.

In this study we document the bacterial microbial diversity of two *Heliconius* species from two populations in Colombia: *Heliconius cydno* (Doubleday, 1847), with nine described subspecies in that country, and *Heliconius clysonymus* Latreille, 1817, which has two subspecies within Colombia and displays little wing color pattern variation (Holzinger & Holzinger, 1970). These two species are sympatric and likely overlap significantly in their trophic niches (Young & Montgomery, 2020). Here we add to the body of knowledge on insect microbiomes and to the geographic extent of sequenced *Heliconius* microbiomes. We do so by examining the structure and variation of bacterial microbiomes across *Heliconius cydno cydnides* Staudinger, 1885 and *H. clysonymus clysonymus* from two geographically isolated forest fragments in the Colombian Central Andes.

MATERIALS AND METHODS

Microbiome sample collection

We sampled adult *Heliconius cydno cydnides* and *H. clysonymus clysonymus* using entomological nets and baited traps in two forested sites in Colombia: a fragment called El Águila in Manizales, Caldas (5.10655 N, 75.50636 W), and Bremen Reserve in Filandia, Quindío (4.672131 N, 75.64066 W; Figure 1). Trap baits consisted of one cup containing fermented fruit mixed with urine and one cup containing blended shrimp, fish, and urine. Traps were deployed for 10 days at each site and butterflies were collected from traps every 24-48 hours. Both sites are in the Colombian Central Andean mountain range at approximately 1,800 m above sea level. We collected 10 *H. cydno* (five from both El Águila and Bremen) and 4 *H. clysonymus* (two from both El Águila and Bremen) in May and June of 2019. Butterflies were collected under permits issued to C.S. The small sample size is a byproduct of logistical constraints of the field research team and cost constraints for sequencing.

We excised abdomens of each butterfly under sterile conditions and preserved them in RNA*later* (ThermoFisher) in Eppendorf tubes in a conventional freezer at approximately -15°C. Butterfly samples therefore included all components of the gastro-intestinal tract (save for the foregut component present in the head and thorax), genitalia, and cuticle. We collected samples of the environment and/or trap bait to document background bacteria and control for effects of bait on butterfly microbiome diversity. In other words, samples collected in traps could be compared with the bacterial content of the bait, and those caught with a hand net could be compared with environmental bacteria samples collected passively. Control sample collection for trapped butterflies involved stirring bait, adding a tiny drop of bait to the RNA*later*, and placing a tube with RNA*later* open next to the bait for at least 15 minutes. Samples that served as controls for butterflies caught with a net were collected by placing a tube with RNA*later*

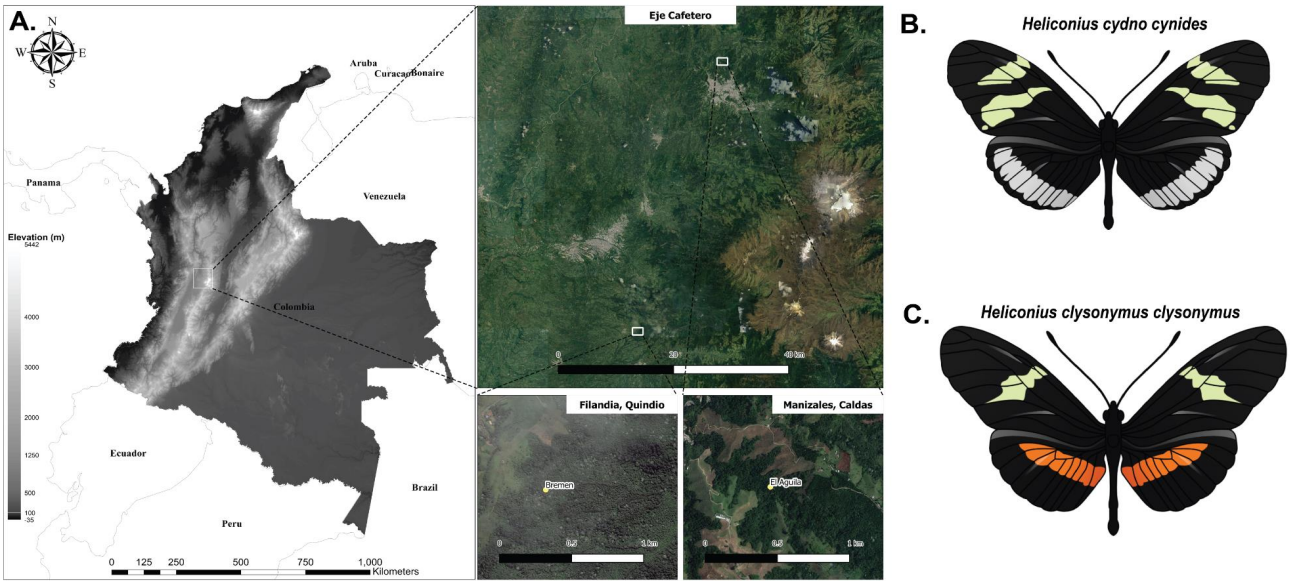

Figure 1. A. Butterfly sampling locations in the central cordillera of the Colombian Andes. Forest fragments Bremen and El Águila are in the departments of Quindío and Caldas, respectively. B. *Heliconius cydno cydnides*, and C. *Heliconius clysonymus clysonymus*.

open in the environment for at least 15 minutes. We collected control samples for each of the sampling events represented by butterflies, i.e., if two samples were collected from one trap, the sample from that trap's bait was the control associated with both butterflies.

DNA extraction, 16S rRNA gene amplification and sequencing

Before DNA extraction of microbiome bacteria, we vertically cut each abdomen, leaving half as a voucher specimen. Similarly, we processed half of each control sample. We performed extractions using the DNeasy PowerSoil Extraction Kit (Qiagen, Germantown, MD) for soil bacteria following the manufacturer's protocol. We quantified the concentration and purity of the DNA with a Nanodrop 2000 (ThermoScientific) and assessed the presence of a band using a 1% agarose gels with SYBR Safe dye (ThermoFisher).

We sequenced the 16S rRNA gene from controls (environmental/bait samples, n = 3) and butterfly samples (n = 14), following the Earth Microbiome Protocol (Gilbert *et al.*, 2014; Meyer *et al.*, 2019). For each PCR reaction, we used 1.25 µL of each 5 µM of the V4 Earth Microbiome primers 515F (GTGYCAGCMGCCGCGGTAA) and 806RB (GGACTACNVGGGTWTCTAAT) (Gilbert *et al.*, 2014; Apprill *et al.*, 2015), 2 µL of DNA, 0.75 µL of dimethyl sulfoxide and 12.5 µL Phusion High-fidelity Master Mix (New England BioLabs, Ipswich, MA), and water, for a 25 µL reaction. These universal primers were used in part for comparison across different species as well as with other studies with butterflies (e.g., Hammer *et al.*, 2020; van Schooten *et al.*, 2018). PCR conditions were: 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 50 °C for 1 min, 72 °C for 90 s, and a final elongation step at 72 °C for 10 min. Each sample was amplified in triplicate. Negative controls (no PCR template) were run on a 1% agarose gel with Ethidium bromide to ensure no contamination but were not sequenced. The triplicate PCR products were combined, purified and concentrated with the MinElute PCR purification kit (QIAGEN) and purified products were quantified with a Denovix (Denovix,Wilmington, DE) before pooling the library. A final amplicon pool of 240 ng of each sample was submitted to the University of Florida, Gainesville for 150bp paired-end sequencing on an Illumina MiSeq. Raw sequences are in the NCBI SRA database with accession numbers SAMN28093385 - SAMN28093402.

Quality Control and ASVs generation

Using raw reads, we removed primers and adapters using cutadapt v. 1.8.1 (Martin, 2011) and then we used the DADA2 v. 1.14.1 pipeline (Callahan *et al.*, 2016) for filtering and combining the sequences into ASVs (Amplicon Sequence Variants). Reads were quality filtered and trimmed using the default parameters in dada2: *filterAndTrim (fnFs*, *filtFs*, *fnRs*, *filtRs*, *truncLen=c(150,150)*, *maxN=0*, *maxEE=c(2,2)*, *truncQ=2*, *rm.phix=TRUE*, *compress=TRUE*, *multithread=TRUE)*. We then estimated error rates for the forward and reverse reads and merged forward and reverse reads into ASVs. Following merging, we removed chimeras (PCR artifacts not associated with the 16S region). We then assigned taxonomy using the SILVA rRNA database (v. 138.1, Quast *et al.*, 2013) to the genus level. We used phyloseq for further data processing and analysis (v 1.30.0, McMurdie & Holmes, 2013), where we also removed chloroplasts, mitochondria, and eukaryotes from our dataset. To further classify taxa, we used NCBI BLASTn to identify sequences to species at their highest percent sequence identity (>99.5%).

Bacterial diversity analyses

To compare alpha diversity of all microbiome bacteria samples, we created rarefaction curves using observed ASV richness and used ASV richness to estimate Shannon diversity in phyloseq. Due to the uneven and non-normal nature of our data, we evaluated differences in alpha diversity indices between the controls and butterfly species, and between the two butterfly species, using Kruskal-Wallis tests followed by Dunn's *post hoc* tests.

We visualized the bacterial community composition between control samples and butterfly samples; between both butterfly species; and between sampling locations using principal coordinates analysis (PCoA) using Bray-Curtis distances of the ASV relative abundances. We accompanied ordinations with Permutational Multivariate Analysis of Variance (PERMANOVA) to statistically test for differences between groups. We tested for differences between butterfly species and butterfly species versus controls in two separate PERMANOVAs. Multivariate analyses were performed using the vegan package in R (Oksanen *et al.*, 2014; R version 3.6.1, R Core Team, 2019). We then followed this analysis by pairwise PERMANOVA analyses using the pairwise.adonis package in R (Martinez Arbizu, 2020) to elucidate which groups most differed in composition from the others. To further investigate patterns in microbiome composition, we used the top_taxa function in the package phyloseq (McMurdie & Holmes, 2013) to examine patterns of relative abundance in the top 15 most abundant bacterial ASVs across groups.

Table 1. Total read counts per sample.

SampleID	Site	Species	Sequences per sample
BBCntl22519	BB	Control	43633
BBJCntrl-261518	BB	Control	70257
BBJCntrl-281519	BB	Control	9
EABCont322019	EAB	Control	86297
DNA0528	BB	*Heliconius clysonymus*	116385
DNA0688	BB	*Heliconius clysonymus*	155774
ESK0781	EAB	*Heliconius clysonymus*	47860
DNA8797	EAB	*Heliconius clysonymus*	140219
ESK0571	BB	*Heliconius cydno*	122837
ESK0659	BB	*Heliconius cydno*	93743
ESK0666	BB	*Heliconius cydno*	74710
ESK0667	BB	*Heliconius cydno*	110715
ESK0702	BB	*Heliconius cydno*	114668
ESK0777	EAB	*Heliconius cydno*	112826
ESK0785	EAB	*Heliconius cydno*	126914
ESK0796	EAB	*Heliconius cydno*	129417
ESK0918	EAB	*Heliconius cydno*	98509
DNA0937	EAB	*Heliconius cydno*	70971
Total			1715744

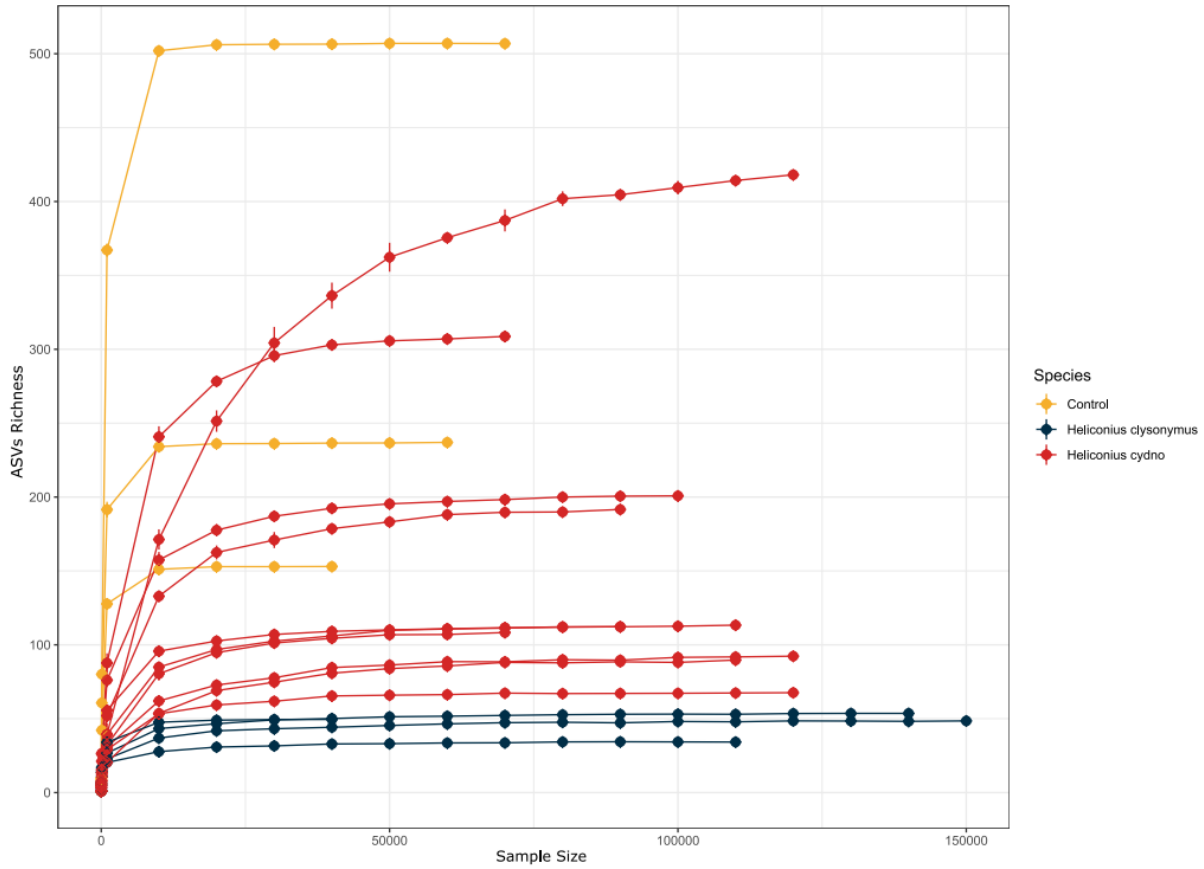

Figure 2. Rarefaction curves of observed ASV richness and of Shannon diversity index of microbial communities in *H. cydno*, *H. clysonymus*, and control samples.

RESULTS

Overall, we obtained 1,715,744 good-quality reads with an average of 95,319.11 (±38880.04, sd) reads per sample (Table 1). The asymptotic trend of the rarefaction curves demonstrates that we detected most of the microbial taxonomic richness present in our butterfly and control samples (Figure 2). As such, we used raw sequence abundance to estimate diversity and did not rarefy the data to an equal sequence depth. Observed ASV richness differed between butterfly species (K-W χ^2 = 6.51, p = 0.038) driven by the higher diversity harbored in *Heliconius cydno* (mean = 189.1 ± 39.0, se) compared to *H. clysonymus* (mean = 49.0 ± 4.34, se; Dunn Test Z = -2.406, p = 0.048). Control samples were variable (mean = 353 ± 192, se). The estimated Shannon diversity, however, did not differ among the three groups (Wilcoxon tests: control v. *H. cydno* (p = 0.14), control v. *H. clysonymus* (p=0.34), *H. cydno* v. *H. clysonymus* (p = 0.73)).

Despite differential observed alpha diversity, bacterial community structure did not significantly differ between the two butterfly species (PERMANOVA: F = 0.72, p = 0.86), nor by site (F = 1.07, p = 0.37; Figures 3, 4), a discrepancy likely stemming from our small sample size. Differences between control and butterfly microbiome composition reflected in the PCoA were corroborated by a PERMANOVA which demonstrated significant differences between the microbiome composition of the three groups (F = 1.62, p = 0.004; Figures 3, 4). Pairwise analyses showed the strongest community differentiation between *H. cydno* and environmental bacterial communities (F = 1.18; adjusted p = 0.012), while there was less distinction between *H. clysonymus* and environmental samples (F=1.23; adjusted p = 0.26). Pairwise PERMANOVA between the microbiome composition of the two butterfly species corroborated the pattern demonstrated in the ordination, and was non-significant (F=1.09; adjusted p = 0.37).

The discrepancies between microbiome composition are illustrated using the top 15 taxa as extracted by phyloseq (see above; Figure 3). Due to the fact that 16S rRNA datasets can harbor bias with respect to relative abundance, we did not run pairwise comparisons of relative abundance of these taxa; however, Figure 3 illustrates variation in bacterial composition.

We found *Wolbachia* (Hertig, 1936: Ehrlichiaceae). and *Spiroplasma* (Sagilo *et al.*, 1973: Spiroplasmataceae), ecologically relevant bacteria, in several samples. In two individuals, one *H. cydno* and one *H. clysonymus*, we observed those bacterial genera in high relative abundances (Figure 3). *Wolbachia*, matching to the strain *W. pipientis* (Hertig 1936) at 99.3% sequence identity, comprised 74% of the total reads of the *H. cydno* individual from El Águila. The single *H. clysonymus* from Bremen showed *Spiroplasma* in high abundance, 44% of that individual's total microbiome reads. *Spiroplasma* sequences recovered in our data were unable to be matched with any individual strain with certainty.

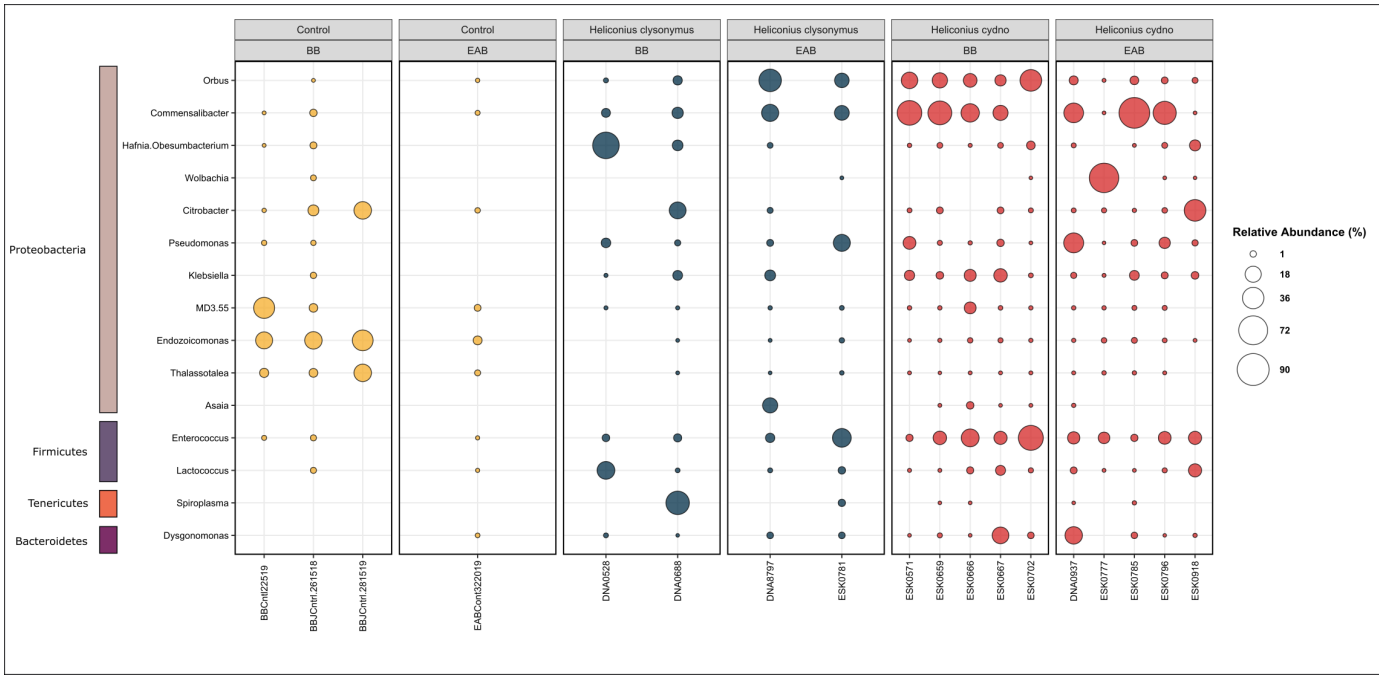

Figure 3. Relative abundance of the 15 most abundant genera of microorganisms found in both butterfly species and control samples at the two sampling sites (EAB = El Águila; BB = Bremen), all samples are represented. The size of the bubble indicates relative abundance. If a bubble is absent, that indicates that the bacterial taxon is not present in the sample.

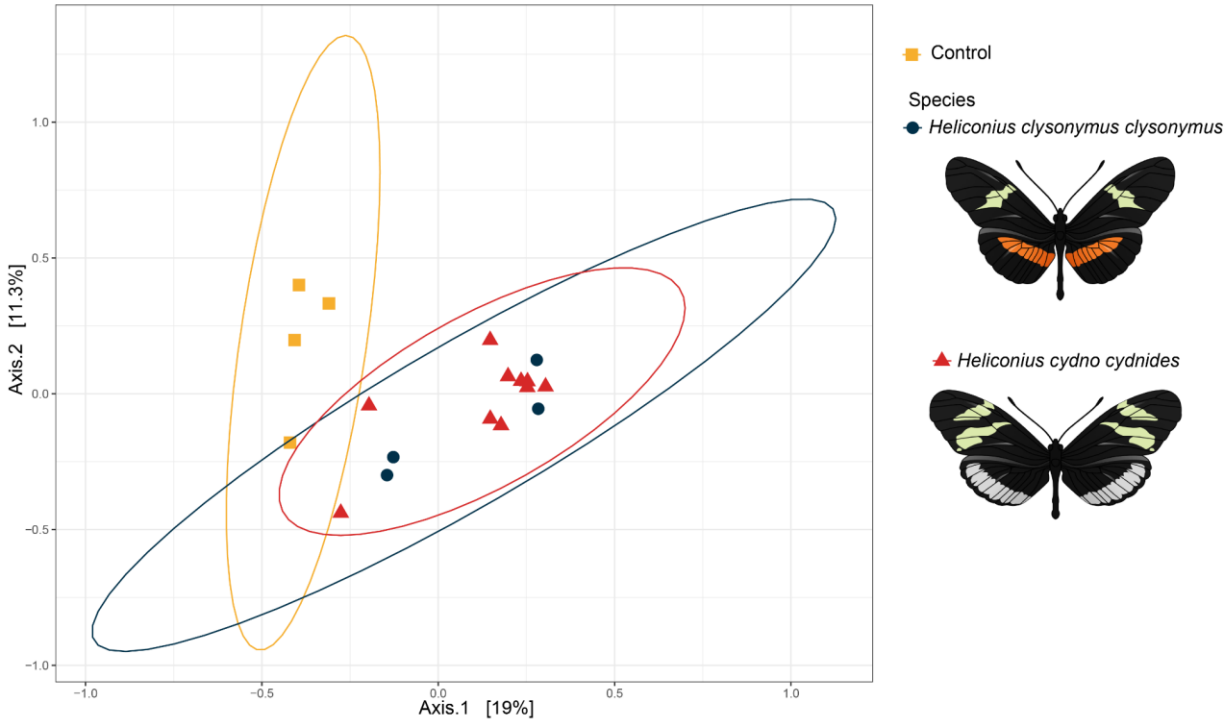

Figure 4. Principal coordinate (PCoA) biplot of all microbiome samples. Butterfly and control microbiomes differed in ASV (taxonomic) composition (PERMANOVA: F = 1.62, p = 0.004).

DISCUSSION

The purpose of this study is to describe the bacterial composition within two species of *Heliconius* butterfly not previously extensively studied, in a location not previously sampled for this purpose. The two focal *Heliconius* species shared similar microbial communities both within and across sampling sites. While we did not document adult behavior or foraging resource use, this similarity is likely due, in part, to overlapping trophic niches of the two butterfly species. Although communities were similar, we detected potentially biologically relevant differences in relative abundances of several bacterial genera between the species. The genus *Commensalibacter* (Rho *et al.*, 2008: Acetobacteraceae) was highly abundant in *H. cydno* but was present in low relative abundances in *H. clysonymus* (Figure 3). *Commensalibacter* has been documented in many insects with sugar-heavy diets like *Drosophila* (Fallén, 1823) (Drosophilidae) and honeybees (*Apis mellifera* Linnaeus, 1758; Apidae) and is known to have an important role in gut immune homeostasis in *Drosophila* (Chandler *et al.*, 2011; Siozios *et al.*, 2019.). The genus *Orbus* (Volkmann *et al.*, 2010) (Gammaproteobacteria) was also present in both species, but its relative abundance was extremely uneven across sites (Figure 3). It was not as prevalent in our two focal species as Hammer *et al.*, (2019) found it to be in *Heliconius erato* (Linneaus 1758) (Nymphalidae) in Panama, or as Ravenscraft *et al.*, (2019) found in fruit- and nectar-feeding butterflies in Costa Rica, suggesting general ubiquity but variability in density across sites. We found significant amounts of Proteobacteria and Firmicutes (see Figure 3), two bacterial phyla that dominate the microbiomes of several other *Heliconius* species (van Schooten *et al.*, 2018). Results from our study and those previously published on *Heliconius* species in other locations imply that the overall bacterial composition of the microbiomes of this group of butterflies is fairly similar despite some species and site variation, perhaps due to the similarities of foraging behaviors across the genus.

Wolbachia, a genus with diverse effects on hosts (Saridaki & Bourtzis, 2010), was found in high relative abundance in one individual of *H. cydno*, and present in low abundances in an additional three individuals. These bacteria, particularly *Wolbachia pipientis*, the strain found in our sample, can play important roles in reproduction of butterflies and other insects (Chandler *et al.*, 2011; Stouthamer *et al.*, 1999). Its effects on *Heliconius* butterflies specifically, however, remain to be examined, as well as how widely associated it is with *H. cydno*. We also found bacteria in the genus *Spiroplasma*, another bacterial group known for fitness effects on insect hosts, in *H. clysonymus*. *Spiroplasma* has been found in other *Heliconius* species (Hammer *et al.*, 2020; van Schooten *et al.*, 2018); this is the first record in microbiome samples of *H. clysonymus*, suggesting that the prevalence of this genus varies among host species and populations. *Spiroplasma* has been documented widely in the insect world, including in other butterflies (e.g., Jiggins *et al.*, 2000), and has been shown to influence survival rates of some *Drosophila* species (Xie *et al.*, 2010). The precise location within the butterfly and effects of these symbionts on *Heliconius* butterflies throughout their life cycle remains to be investigated. Additionally, although high relative abundances do not always translate into high cell counts of these bacteria, their detection in these samples are important to note.

While we found little distinct spatial or species-level patterns in the microbial composition of the individuals studied, perhaps due to the low power resulting from a small sample size, we did find higher observed bacterial diversity in the microbiome of *H. cydno* compared with *H. clysonymus.* The discrepancies in pattern between observed ASV diversity and estimated Shannon ASV richness is likely due to our low sample size. Increased sampling as well as detailed foraging data on the two species is needed to confirm and understand this observed pattern. The trend of higher bacterial diversity in *H. cydno* compared with *H. clysonymus* corresponds with the diversity of wing morphs of the two species; across its range, *H. cydno* has a greater diversity of phenotypes (Jiggins, 2017), which may influence foraging or other behaviors resulting in differential diversity of microbiomes. In this study, however, we only sampled one color morph of each species. The difference in bacterial composition of the microbiomes of *H. cydno* and that of the control samples collected may imply a possible disconnect between the microbiomes of *H. cydno* butterflies and that of their environment, and perhaps food sources. More robust patterns, including distinctions between environmental microbial diversity and butterfly-associated bacteria, may come to light with increased sampling, representation across sexes, species/subspecies, and morphological variants, and with detailed data on diet as collected by observing individuals as well as broad floristic surveys. With increased efforts across taxonomy and space, we will better understand patterns of microbial diversity, including intraspecific variation and geographic patterns. Likewise, more data on butterfly diet and plant inventories may further explain the structure and variability of microbiomes, and the interactions between an individual's diet and microbiome; and how bacteria are transferred via ecological interactions. These data add to the fast-growing library of microbiome sequences of *Heliconius* butterflies and advance the exploration of ecological and evolutionary patterns in insect microbiomes.

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LITERATURE CITED

Apprill, A., Mcnally, S., Parsons, R., Weber, L. 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology* 75: 129-137.

Anbutsu, H., Fukatsu, T. 2011. *Spiroplasma* as a model insect endosymbiont. *Environmental Microbiology Reports 3*(2): 144-153.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C. C., Charles, T., Xiaoyulong, C., Cocolin, L., Eversole, K., Corral, G H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J A., Maguin, E., Mauchline, T., McClure, R., Mitter, B., Ruan, M., Sarand, I., Smidt, H., Schelkle, B., Roume, H., Kiran, G. S., Selvin, J., de Souza, R. S. C., van Oberbeek, L., Singh, B. K., Wagner, M., Walsh, A., Sessitsch, A., Schloter, M. 2020. Microbiome definition re-visited: old concepts and new challenges. *Microbiome* 8(1): 1-22.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods* 13(7): 581-583.

Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A., Kopp, A. 2011. Bacterial communities of diverse *Drosophila* species: ecological context of a host-microbe model system. *PLoS Genetics* 7(9), e1002272.

Gilbert, J. A., Jansson, J. K., Knight, R. 2014. The Earth Microbiome project: Successes and aspirations. *BMC Biology* 12: 1-4.

Gilbert, L. E. 1972. Pollen feeding and reproductive biology of *Heliconius* butterflies. *Proceedings of the National Academy of Sciences* 69(6): 1403-1407.

Hammer, T. J., Dickerson, J. C., McMillan, W. O., Fierer, N. 2020. *Heliconius* butterflies host characteristic and phylogenetically structured adult-stage microbiomes. *Applied and Environmental Microbiology* 86(24).

Hammer, T. J., Dickerson, J. C., McMillan, W. O., Fierer, N. 2019. Pollen-feeding *Heliconius* butterflies host distinctive adult-stage microbiomes. *bioRxiv* 820829.

Hammer, T. J., McMillan, W. O., Fierer, N. 2014. Metamorphosis of a butterfly-associated bacterial community. *PloS One* 9(1): e86995.

Holzinger, H., Holzinger, R. 1970. *Heliconius hygianus fischeri* (Fassl) comb. nov., eine Subspecies aus West-Columbien (Lep. Nymph.). *Zeitschrift der Arbeitsgemeinschaft österreichischer Entomologen* 22(2): 33-41.

Jiggins, C. D. 2017. *The Ecology and Evolution of* Heliconius *Butterflies*. Oxford, Oxford University Press.

Jiggins F. M., Hurst G. D., Jiggins C. D., Schulenburg J. H., Majerus, M. E. 2000. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology* 120(5):439-446

Joron, M., Jiggins, C. D., Papanicolaou, A., McMillan, W. O. 2006. *Heliconius* wing patterns: an evo-devo model for understanding phenotypic diversity. *Heredity* 97(3): 157-167.

Kim, J. Y., Lee, J., Shin, N. R., Yun, J. H., Whon, T. W., Kim, M. S., Jung, M. J., Roh, S. W., Hyun, D. W., Bae, J. W. 2013. *Orbus sasakiae* sp. nov., a bacterium isolated from the gut of the butterfly *Sasakia charonda*, and emended description of the genus *Orbus. International Journal of Systematic and Evolutionary Microbiology* 63(5): 1766-1770.

Krishnan, M., Bharathiraja, C., Pandiarajan, J., Prasanna, V. A., Rajendhran, J., Gunasekaran, P. 2014. Insect gut microbiome-An unexploited reserve for biotechnological application. *Asian Pacific Journal of Tropical Biomedicine* 4: S16-S21.

Majumder, R., Sutcliffe, B., Taylor, P. W., Chapman, T. A. 2019. Next-Generation Sequencing reveals relationship between the larval microbiome and food substrate in the polyphagous Queensland fruit fly. *Scientific Reports* 9(1): 1-12.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *Advances in Environmental Biology* 7: 2803-2809.

Martinez, A. P. 2020. *pairwiseAdonis: Pairwise multilevel comparison using adonis*. R package version 0.4.

McMurdie, P. J., Holmes, S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*. 8(4): e61217.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, H. H. H., Sxoecs, E., Wagner, H., Barbour, M., BEdward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H. B. H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M. O., Lahti, L., McGlinn, D., Ouellette, M. H., Cunha, E. R., Smith, T., Stier, A., Braak, C. J. F. T., Weedon, J. 2019. *vegan: Community Ecology Package. R package version 2.5-6*. https://CRAN.R-project.org/package=vegan.

Quast, C, Pruesse, E , Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41(D1): D590-D596.

Ravenscraft, A., Berry, M., Hammer, T., Peay, K., Boggs, C. 2019. Structure and function of the bacterial and fungal gut microbiota of Neotropical butterflies. *Ecological Monographs* 89(2): e01346.

Saridaki, A., Bourtzis, K. 2010. *Wolbachia*: more than just a bug in insect genitals. *Current Opinion in Microbiology* 13(1): 67-72.

Siozios, S., Moran, J., Chege, M., Hurst, G. D., Paredes, J. C. 2019. Complete reference genome assembly for *Commensalibacter* sp. Strain AMU001, an Acetic Acid Bacterium isolated from the gut of Honey Bees. *Microbiology Resource Announcements* 8(1).

Stouthamer, R., Breeuwer, J. A., Hurst, G. D. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annual Reviews in Microbiology 53*(1): 71-102.

van Schooten, B., Godoy-Vitorino, F., McMillan, W. O., Papa, R. 2018. Conserved microbiota among young *Heliconius* butterfly species. *PeerJ* 6: e5502.

Whipps, J., Lewis, K., Cooke, R. 1988. *Mycoparasitism and plant disease control*, p. 161-187. *In*: Burge, M. N. (Ed.), *Fungi in Biological Control Systems*. Manchester, Manchester University Press.

Xie, J., Vilchez, I., Mateos, M. 2010. Spiroplasma bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp Leptopilina heterotoma. *PLOS ONE* 5: e12149.

Young, F. J., Montgomery, S.H. 2020. Pollen feeding in *Heliconius* butterflies: the singular evolution of an adaptive suite. *Proceedings of the Royal Society B* 287(1938): 20201304.

Zytynska, S. E., Meyer, S. T. 2019. Effects of biodiversity in agricultural landscapes on the protective microbiome of insects-a review. *Entomologia Experimentalis et Applicata* 167(1): 2-13.