

INVESTIGATING THE ROLE OF THE CLOACAL MICROBIOME IN AVIAN
MALARIA SUSCEPTIBILITY IN HAWAIIAN HONEYCREEPERS

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By
Amanda K. Navine

Thesis Committee:
Patrick Hart
Kristina Paxton
Eben Paxton
Jonathan Awaya

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ABSTRACT

Anthropogenic disturbance has led to an increase in the frequency and severity of infectious wildlife diseases in naive ecosystems, posing major threats to global biodiversity. For a poignant example, of the 55 Hawaiian honeycreeper species (subfamily Carduelinae) that have been documented across the Hawaiian archipelago, only 17 remain, and 9 of them are endangered. Among the most pressing threats to honeycreeper survival is avian malaria, caused by the introduced blood parasite *Plasmodium relictum*, which is expanding in distribution in Hawai‘i as a result of climate change. Recent research on mammals has revealed strong connections between gut microbiome composition and malaria susceptibility, illuminating a potential novel approach to malaria control through the administration of antimalarial probiotics. One honeycreeper species, the Hawai‘i ‘Amakihi (*Chlorodrepanis virens*), persists in some areas of high malaria prevalence, indicating they have acquired some level of immunity to this disease.

To investigate if avian host-specific microbes may be associated with malaria survival, I characterized cloacal microbiomes and malaria infection for 174 ‘amakihi and 171 malaria-resistant Warbling White-eyes (*Zosterops japonicus*) from Hawai‘i Island using 16S rRNA gene sequencing and qPCR. Neither microbiome alpha nor beta diversity covaried with malaria infection, but 71 microbes showed positive associations with survivors of malaria, revealing promising targets for future probiotic research. Among these were *Escherichia* and *Lactobacillus* spp., which have supported potential to mitigate malaria severity in mammalian hosts, and *Pseudomonas* spp., which have been linked to *Plasmodium* suppression in mosquito vectors. My findings suggest targeted probiotics warrant further investigation for their potential to provide practical and effective methods of augmenting immunity in malaria sensitive species to prevent imminent extinctions.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
ABSTRACT	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
INTRODUCTION	viii
Hawaiian honeycreepers and avian malaria	1
Malaria and the microbiome	3
The microbiome and Hawaiian honeycreeper conservation	4
Research objectives, questions, and predictions	5
METHODS	8
Study species	8
Study sites	9
Sample collection	9
Determining malaria infection status and intensity	10
Cloacal microbiome amplicon sequencing	10
Bioinformatics and Statistical Analysis	12
RESULTS	14
Cloacal microbiome diversity does not covary with malaria infection	14
Certain cloacal ASVs differ in abundance between host species and between malaria infected and uninfected birds	14
DISCUSSION	15
APPENDIX A: TABLES	20
APPENDIX B: FIGURES	25
LITERATURE CITED	30

LIST OF TABLES

Table 1. Number of avian malaria infected and uninfected Hawai‘i ‘Amakihi and Warbling White-eyes at sampling sites across Hawai‘i Island.....20

Table 2. Pairwise comparisons of ASV abundances between the cloacal microbiomes of Hawai‘i ‘Amakihi chronically infected with avian malaria and uninfected ‘amakihi.....21

Table 3. Pairwise comparisons of ASV abundances between the cloacal microbiomes of Warbling White-eyes uninfected with avian malaria and uninfected Hawai‘i ‘Amakihi.....22

Table 4. Pairwise comparisons of ASV abundances between the cloacal microbiomes of Warbling White-eyes uninfected with malaria and chronically infected Hawai‘i ‘Amakihi.....23

LIST OF FIGURES

Figure 1. Map of sampling sites on Hawai‘i Island	25
Figure 2. Plots of Hawai‘i ‘Amakihi and Warbling White-eye cloacal microbiome alpha diversity.....	26
Figure 3. Principal coordinate analyses of Hawai‘i ‘Amakihi and Warbling White-eye cloacal microbiome beta diversity.....	27
Figure 4. Relative abundances of the 10 most common phyla in the cloacal microbiomes of Hawai‘i ‘Amakihi and Warbling White-eyes	28
Figure 5. Log ₂ fold increase of ASV abundance between the cloacal microbiomes of different groups of malaria infected and uninfected Hawai‘i ‘Amakihi and Warbling White-eyes	29

LIST OF ABBREVIATIONS

Adenine and thymine - AT
Adjusted p value - p -adj
Amplicon sequence variant - ASV
Benjamini and Hochberg correction - BH
Base pairs - bp
Bovine serum albumin - BSA
Deoxyribonucleic acid - DNA
Double stranded deoxyribonucleic acid - dsDNA
Galactose- α -1,3-galactose - α -gal
Guanine and cytosine - GC
Linear model - LM
Negative binomial generalized linear model - nbGLM
Operational taxonomic unit - OTU
Pantoea agglomerans - *P. agglomerans*
Permutational multivariate analysis of variance model - PERMANOVA
Plasmodium relictum - *P. relictum*
Polyethylene glycol-sodium chloride -PEG/NaCl
Polymerase chain reaction - PCR
Principal coordinate analysis - PCoA
Quantitative polymerase chain reaction - qPCR
Species - spp.
Threshold cycle - Ct
Tris-acetate-ethylenediaminetetraacetic acid - TAE
United States Geological Survey - USGS
16S ribosomal ribonucleic acid - 16S rRNA

INTRODUCTION

Hawaiian honeycreepers and avian malaria

Anthropogenic disturbance has directly or indirectly influenced nearly every ecosystem on earth, often inducing profoundly negative changes to the ecological integrity and biodiversity of these ecosystems (Foley et al. 2005). Habitat destruction, globalization, climate change, pollution, over harvesting of natural resources, and other human impacts have disrupted species interactions, altered biogeochemical cycles, and exposed countless ecosystems to invasion by alien species (Vitousek et al. 1996, Perrings et al. 2010, Zarnetske et al. 2012, Sen & Peucker-Ehrenbrink 2012, Young et al. 2017). Of the many human-introduced alien species that lead to a loss of biodiversity, pathogens have been responsible for myriad local extinction events in wildlife populations (Warner 1968, Daszak et al. 2000, Daszak et al. 2001, Dobson & Foufopoulos 2001, Harvell et al. 2002, Plowright et al. 2008, Rogalski et al. 2017). Island species are particularly vulnerable to annihilation by introduced diseases, as they lack the immunological adaptations that their mainland counterparts have evolved through historical exposure to these pathogens (Warner 1968, van Riper et al. 1986, Daszak et al. 2000, Wikelski et al. 2004, Russell et al. 2017).

The devastating decline in Hawaiian honeycreeper (subfamily Carduelinae) diversity due to the introduction of *Plasmodium relictum*, a blood parasite that causes avian malaria, and its mosquito vector *Culex quinquefasciatus* is a prime example of how anthropogenically introduced disease to naïve ecosystems can lead to modern extinctions (Warner 1968, van Riper et al. 1986, Fonseca et al. 2000, van Riper & Scott 2001, Harvell et al. 2002, Fonseca et al. 2006, Plowright et al. 2008). Of the 55 honeycreeper species that have been documented across the Hawaiian archipelago (Pratt et al. 2009, Lerner et al. 2011), only 17 remain, 9 of which are endangered, and 6 are on the verge of extinction (IUCN 2021). Most of the extant honeycreeper species can only persist in high-elevation refugia, where mosquito population dispersal and malarial sporozoite (the infectious life phase of *P. relictum*) development are limited by cooler temperatures (LaPointe et al. 2010). As global temperatures rise due to climate change, *P. relictum* is expected to expand into higher elevations, likely leading to a rapid loss of such avian refugia (Benning et al. 2002, Paxton et al. 2018). Avian community collapse has already been observed at high-elevation sites on the island of Kaua‘i (Paxton et al. 2016) where dramatic declines of honeycreeper populations are correlated with increased temperatures and disease

pressure (Atkinson et al. 2014). Without intervention, several more extinctions can be expected in the coming decades (Benning et al. 2002, Paxton et al. 2016, Liao et al. 2017, Paxton et al. 2018). Protecting Hawaiian avifauna from extinction will require conservation strategies that combat avian malaria at multiple stages, including reforesting high-elevation habitats, controlling the distribution of its mosquito vector, and facilitating honeycreeper persistence in environments with increased malaria prevalence (Liao et al. 2017, Paxton et al. 2018, Dahlin & Feng 2019, Samuel et al 2020).

The non-endangered Hawai'i 'Amakihi (*Chlorodrepanis virens*) is among the two honeycreeper species of least conservation concern (IUCN 2021) and is extant in high densities throughout its historical range, including some areas of high malaria prevalence (Woodworth et al. 2005, Spiegel et al. 2006, Gorresen et al. 2009, Camp 2019, McClure et al. 2020). 'Amakihi display differential malaria induced mortality rates along an elevational gradient, with low-elevation populations suffering significantly lower mortality rates than high-elevation populations (Woodworth et al. 2005, Spiegel et al. 2006, Eggert et al. 2008, Atkinson et al. 2013, Samuel et al. 2015). Regardless of variable mortality rates, 'amakihi like all honeycreepers are highly susceptible to infection with avian malaria, and individuals that survive infection have been shown to maintain chronic low levels of the parasite in their blood post exposure (Atkinson et al. 2001). Chronic infection does not appear to hinder reproductive success, in fact one study showed that 'amakihi that survived malaria had higher nesting success than uninfected individuals (Kilpatrick et al. 2006). Experimental infections with *P. relictum* suggest that the increased survival rate of low-elevation populations may be partially due to the evolution of malaria tolerance genes (Atkinson et al. 2013), which has permitted population persistence despite elevated disease levels, and demonstrates how selection pressure from disease can promote rapid adaptation (Kilpatrick et al. 2006, Eggert et al. 2008). It has been proposed that if specific genes can be identified as conferring immunity to malaria, targeted translocations of individual birds possessing those genes (Paxton et al. 2018), or even gene editing (Samuel et al. 2020), could be used to facilitate adaptation in malaria sensitive species. However, these strategies bear significant technical challenges and cost (Samuel et al. 2020), and public support for the use of gene editing in wildlife is historically lacking (Kohl et al. 2019). An alternative hypothesis to explain reduced 'amakihi malaria mortality that has not been assessed, but has significant conservation implications, is the possibility that the gut microbiomes, the microbial

communities within the gastrointestinal tracts, of these bird populations can confer malaria immunity.

Malaria and the microbiome

There is building evidence to suggest microbiomes have a strong influence over host health and fitness (Sekirov et al. 2010, Suzuki 2017, Desselberger 2018, Ippolito et al. 2018, Waide & Schmidt 2020), and can shape various ecological and evolutionary processes like behavior, adaptation, sexual selection, host-parasite interactions, and disease susceptibility or severity (Koch & Schmid-Hempel 2011, Honda & Littman 2012, Ezenwa et al. 2012, Yilmaz et al. 2014, Yooseph et al. 2015, Villarino et al. 2016, Stough et al. 2016, Brooks et al. 2016, Broom & Kogut 2018, Jacobson et al. 2018, Morffy Smith et al. 2019, Daisley et al. 2020, Rowe et al. 2020, Bragg et al. 2020, Charania et al. 2020, Videvall et al. 2021, Lutz et al. 2021). Microbiome research is rapidly advancing with the improvement of sequencing capabilities such as 16S ribosomal RNA (16S rRNA) gene sequencing for bacterial identification. Sequencing the prokaryotic 16S rRNA gene is a powerful method for characterizing bacterial communities because of its large size (1,500 bp), presence in nearly all bacteria, and slow rate of evolution (Patel 2001). Such techniques allow for the study of microbial gut community composition from a wide range of samples, including fecal, anal, or cloacal swabs that can be collected from wild animal populations. With the recent surge in animal microbiome data and as the importance of microbiomes to host health comes to light there has been a call to incorporate microbiota into conservation planning (West et al. 2019, Trevelline et al. 2019), and honeycreepers are posed to potentially benefit exponentially from conservation strategies that manipulate microbiota to manage disease risk.

Recent experiments with mice revealed a strong connection between gut microbiome composition and malaria parasite resistance and disease severity. Mice that are genetically similar but differ in their gut bacterial communities have been shown to differ significantly in their parasite susceptibility, burden, and mortality rates after exposure to *Plasmodium* spp. (Martínez-Gómez et al. 2006, Yilmaz et al. 2014, Villarino et al. 2016, Stough et al. 2016, Morffy Smith et al. 2019). Additionally, cecal content transplants from the guts of mice that displayed malaria resistance to mice with sterilized guts resulted in lower severity infection in the recipient mice than in those that received transplants from malaria susceptible mice

(Villarino et al. 2016, Morffy Smith et al. 2019). One proposed mechanism explaining how gut microbiota can increase malaria resistance centers around certain bacterial species' ability to prime host immune responses. In lab mice, experimental gut colonization with *Escherichia coli* spp., which produce high levels of an oligosaccharide expressed by *Plasmodium* sporozoites, galactose- α -1,3-galactose (α -gal), stimulated the production of glycan-specific antibodies by the host's immune system (Yilmaz et al. 2014). Elevated levels of circulating anti- α -gal antibodies enabled the rapid identification of *Plasmodium* parasites by the host's immune system during exposure via mosquito bite and reduced malaria transmissibility from skin to liver. *Lactobacillus* spp. are common vertebrate symbionts with diverse functions and documented abilities to modulate host immune responses, and have been the focus of numerous probiotic studies, including many exploring malaria control strategies (Martínez-Gómez et al. 2006, Villarino et al. 2016, Cabezas-Cruz & de la Fuente 2017, Omeiza et al. 2020, Mahajan et al. 2021, Toukam et al. 2021). In model mouse systems, supplementation of *Lactobacillus* spp., both prophylactically and as post infection treatment, has significantly attenuated the severity of malaria in susceptible mice (Villarino et al. 2016, Toukam et al. 2021), potentially by increasing nitric oxide concentrations in the host's blood, thereby inhibiting *Plasmodium* growth during the erythrocytic stage of infection (Balmer et al. 2000, Martínez-Gómez et al. 2006).

The microbiome and Hawaiian honeycreeper conservation

The discovery of encouraging relationships between malaria and the microbiome in mammals illuminates a novel potential approach to conservation of wildlife species sensitive to malaria infection, namely the development of antimalarial probiotics. Prophylactic probiotics have recently garnered scientific attention for their potential to lessen the burden of malaria on human health (Travers et al. 2011, Ngwa & Pradel 2015), but they are also an attractive option for treating wild animal diseases for several reasons (McKenzie et al. 2018, Stedman et al. 2020). Orally delivered probiotics have been shown to establish in the gut, increase circulating anti- α -gal antibodies, effectively mitigate disease severity in experimental mice and poultry models, and are used extensively in livestock operations (Posekany et al. 2002, Villarino et al. 2016, Broom & Kogut 2018, Hodžić et al. 2020, Mahesh et al. 2021, Toukam et al. 2021), and have potential applications in wild populations. Targeted probiotics that could be passively administered in food supplementations to honeycreeper populations in the wild (van Riper 1984)

as well as in captive breeding programs may be an efficient, cost-effective, and practical approach for bolstering malaria immunity in endangered birds. However, probiotics have never been tested in wildlife, and while the link between the gut microbiome and malaria immunity has been established for mice in a laboratory setting, little is known about the potential connection in other species or in natural environments (but see Lutz et al. 2021, Videvall et al. 2021). Further, extrapolating insights gained from mammalian studies to avian systems may be challenging or inappropriate as current research suggests bird-microbe symbiosis dynamics are distinct from those of other vertebrates (Bodawatta et al. 2021).

There are numerous introduced bird species to Hawai'i Island with historical exposure to avian malaria that do not suffer malaria induced mortality, but serve as reservoirs for disease (Palinauskas et al. 2008, McClure et al. 2020). One such species is the Warbling White-eye (*Zosterops japonicus*), which was introduced in the 1920s and is now ubiquitous throughout Hawai'i forests and suffers negligible malaria mortality (van Riper et al. 1986, van Riper & van Balen 2020, McClure et al. 2020). White-eye exposure to *Plasmodium* spp. in its historic range over evolutionary time likely favored the adaptation of a suite of immunological strategies which allows them to clear parasites from their bloodstream (Cellier-Holzem et al. 2010), potentially including harboring a microbiome that can confer malaria immunity. Once introduced to Hawai'i, antimalarial microbes may have spread through habitats shared by white-eyes and honeycreepers and colonized 'amakihi microbiomes, imparting their benefits to their new hosts. It is also possible that 'amakihi have developed unique strains of protective microbes to overcome disease. Elucidating a relationship between the microbiome and malaria susceptibility in Hawaiian honeycreepers is an essential first step in assessing the feasibility of mitigating the honeycreeper extinction crisis through the development of antimalarial probiotics. Therefore, for my thesis research, I investigated correlates between microbiome composition and malaria infection in Hawai'i 'Amakihi and Warbling White-eyes.

Research objectives, questions, and predictions

In order to assess if microbiota may be conferring malaria immunity to 'amakihi, I focused on the following objectives: (1) determine if variation in cloacal microbiome composition correlates with *P. relictum* infection, and (2) identify which, if any, bacterial taxa

are associated with malaria infection survival in honeycreepers. To address these objectives, I had three research questions:

Q₁: Does cloacal microbiome diversity covary with *P. relictum* infection?

H_{a1}: Cloacal microbiome diversity will vary between infected and uninfected birds.

Prediction₁: Experimental infections have demonstrated response to *P. relictum* infection varies widely across individual honeycreepers (Atkinson et al. 1995, Yorinks & Atkinson 2000, Atkinson et al. 2013) and I hypothesize that this variation in response to infection correlates at least in part with individual microbiome diversity. Disease and inflammation have strong influence over microbiome composition (Sekirov et al. 2010, Honda & Littman 2012, Mooney et al. 2015, Taniguchi et al. 2015, Ippolito et al. 2018, Muletz-Wolz et al. 2019, Bamgbose et al. 2021), thus I expect to see significant differences in cloacal bacterial diversity associated with *P. relictum* infection. As I stated earlier, multiple studies have established that the vertebrate gut microbiome may modulate *Plasmodium* spp. infection response (Martínez-Gómez et al. 2006, Yilmaz et al. 2014, Yooseph et al. 2015, Villarino et al. 2016, Stough et al. 2016, Morffy Smith et al. 2019), but other studies have indicated that acute malaria infection can reversibly reduce microbial diversity (Mooney et al. 2015, Taniguchi et al. 2015). While gut microbiomes have not been explicitly examined in relation to malaria in birds, it is reasonable to suggest that this connection may extend to avian host-parasite interactions, thus I expect to see lower microbial diversity in the cloacas of *P. relictum* infected ‘amakihi than in uninfected ‘amakihi, especially those with high parasitemia. Because white-eyes do not suffer malaria morbidity (van Riper et al. 1986), I expect to see less significant difference in cloacal microbiome diversity between infected and uninfected white-eyes.

Q₂: Are specific bacterial taxa within the ‘amakihi cloacal microbiome associated with malaria survivors?

H_{a2}: Certain bacterial species will vary in presence or abundance in ‘amakihi cloacal microbiomes in association with malaria infection status.

Prediction₂: Several studies have demonstrated that acute malaria infection can alter the relative abundance of certain members of the gut microbiome, particularly members of the Firmicutes and Proteobacteria phyla, but these changes typically revert back to baseline upon recovery (Mooney et al. 2015, Taniguchi et al. 2015, Ippolito et al. 2018, Bamgbose et al. 2021). Therefore, even if overall microbial diversity does not covary with infection status, I predict

microbial abundances will differ substantially between birds with high parasitemia intensity, and those with no or low-level parasitemia. If specific microbes, such as those that secrete α -gal like *Escherichia coli* spp., are modulating malaria severity in ‘amakihi, as was found to be the case in mice (Yilmaz et al. 2014), I expect they would be more abundant in birds that survived acute malaria. I also expect potentially beneficial microbes would scale in abundance with malaria prevalence in ‘amakihi habitats, as selective disease pressure would favor the evolution of immune strategies.

In addition to *Escherichia* spp., other bacteria with ties to malaria that were targets for closer inspection in my study were members of the *Lactobacillus*, *Bifidobacterium*, *Pseudomonas*, and *Pantoea* genera, which can be found in avian microbiomes (Cabassi et al. 2004, Kohl 2012, Grond et al. 2018). Increased abundance of *Lactobacillus* and *Bifidobacterium* spp. were also found to be associated with increased malaria resistance in mice (Martínez-Gómez et al. 2006, Villarino et al. 2016), and *Pseudomonas putida* and a *Pantoea* sp. were shown to block *Plasmodium* development both in vitro and in vivo in mosquito vectors (Bahia et al. 2014). Though no connection between *Pseudomonas* spp. in host microbiomes and *Plasmodium* infection has been assessed, *Pantoea agglomerans*, was found to be strongly associated with infection with malarial parasites in the mouths of wild miniopterid bats (Lutz et al. 2021). The broad-spectrum antibiotic, ‘Immunopotentiator from *P. agglomerans* 1,’ produced by *P. agglomerans* has been of particular interest in the study of pathogen control, however whether it is effective against malarial parasites, and whether this bacteria is a common member of the avian microbiome is unknown (Dutkiewicz et al. 2016).

Q₃: Are microbes with supported potential to modulate malaria severity in higher abundance in white-eye than in ‘amakihi cloacal microbiomes?

H_{a3}: White-eye microbiomes will harbor a greater abundance of potentially beneficial microbes than ‘amakihi microbiomes.

Prediction₃: If malaria immunity conferring microbes, such as those of interest outlined above, are aiding white-eyes in survival it is reasonable to expect that those microbes will be in greater abundance in white-eye samples than in ‘amakihi samples. Some beneficial microbes introduced from non-native birds may have colonized ‘amakihi cloacal microbiomes, however even ‘amakihi that survive acute malaria suffer morbidity (Atkinson et al. 2013), whereas white-eyes are nearly entirely resistant (van Riper et al. 1986). This is potentially because these

recently introduced beneficial microbes are in lower abundance in the native host species. Because honeycreepers are endemic to isolated oceanic islands and evolved separately from mainland species for millions of years (Pratt et al. 2009, Lerner et al. 2011), it is plausible that a large proportion of their gut microbiota may be absent in the non-native white-eyes and vice versa (Brooks et al. 2016). It is also possible that some of these unique species contribute toward increased malaria immunity in certain ‘amakihi populations, however I predict that white-eye cloacal microbiomes will have higher abundance of microbes that have established ties to malaria immunity.

METHODS

Study species

The Hawai‘i ‘Amakihi is the most common honeycreeper species at low- and mid-elevations (< 1,500 m; Woodworth et al. 2005, Spiegel et al. 2006, Gorresen et al. 2009, Camp 2019, McClure et al. 2020), likely due to its much lower malaria induced mortality rate compared to other honeycreeper species (Samuel et al. 2015). Recent evidence suggests ‘amakihi may undergo elevational movements more frequently than previously thought to track seasonal food resources (Paxton et al. 2020). Despite these movements and potential increased exposure to malaria parasites, an experimental infection study showed ‘amakihi sourced from a high-elevation population had a mortality rate of 50.0%, while low-elevation birds showed only 16.7% mortality (Atkinson et al. 2013), but some studies estimate low-elevation mortality rates to be as low as 3% (Samuel et al. 2015). ‘Amakihi can be found in native xeric, mesic, and wet forests of Hawai‘i, Maui, and Moloka‘i (Spiegel et al. 2006, Gorresen et al. 2009, Camp 2019, Lindsey et al. 2020), and have one of the most generalist feeding strategies amongst the honeycreepers, foraging on nectar, mainly from native ‘ōhi‘a lehua (*Metrosideros polymorpha*) and māmane (*Sophora chrysophylla*) blossoms, insects gleaned from plants, and the pulp of fruit (Baldwin 1953, Richards & Bock 1973).

Warbling White-eyes are abundant on all main Hawaiian Islands in nearly every ecosystem type from high-elevation forests to urban environments (van Riper & van Balen 2020). White-eyes do not suffer mortality from *P. relictum* infection (van Riper et al. 1986), but have been indicated as an important reservoir for malaria parasites in Hawaiian forests (McClure 2020). In Hawai‘i, this species is non-migratory, generally does not disperse far from its natal

territory, and is not known for interisland flight, despite some populations in Japan showing migratory behavior (van Riper & van Balen 2020). Similar to ‘amakihi, White-eyes are generalist feeders, consuming mainly nectar, arthropods, and fruit (Guest 1973, van Riper & van Balen 2020).

Study sites

‘Amakihi and white-eye samples used for my analysis were collected from 16 sampling sites that were selected based on ‘amakihi capture rates and malaria prevalence (E. H. Paxton unpublished data) and varied in elevation, annual rainfall, and forest structure. Each site was assigned a 4 letter code and organized into one of six regions across Hawai‘i Island based on their geographic proximity (Fig. 1). The Ka‘ū region had four sampling sites (KAHU, KAUF, KIOL, MANU) ranging from 584-1,275 m, and malaria prevalence from 13-92%. Despite being more proximal to the Kīlauea region, KAUF was assigned to the Ka‘ū region because of the large lava flow separating it from Kīlauea sites and the high forest continuity within the Ka‘ū Forest Reserve. Kona consisted of one site (KONA) at 1,550 m with 55% malaria prevalence. The Maunakea region also had one site (MAUL) located at 1,610 m within Hakalau Forest National Wildlife Refuge. Maunakea had the lowest malaria prevalence at 8%. Puna consisted of two sites (LAMA, MARC), at 50 and 100 m in heavily anthropogenically altered areas. Very few native birds persist in Puna as malaria prevalence is 93% in this region. The two Pu‘u Wa‘awa‘a sites (PFBS, PWWA) were located at 790 and 1,230 m and had 18 and 26% malaria prevalence, respectively. Kīlauea had six sites (AIRA, BIPA, BYLE, CRBX, KIVO, UPKE) located within Hawai‘i Volcanoes National Park ranging from 1,050-1757 m. Most sites within the Kīlauea region had 51% malaria prevalence, however prevalence was only 18% at the highest elevation site (UPKE) and at another (KIVO) prevalence was based on one sample, which was infected.

Sample collection

To assess ‘amakihi and white-eye *P. relictum* parasitemia levels and cloacal microbiomes, blood from the brachial wing vein and cloacal swab samples were collected from birds passively captured in mist-nets between February to July in 2019 and 2020 by the U. S. Geological Survey (USGS) Avian Malaria Genomics Research Project crew and myself. Blood samples were stored in Queen’s lysis buffer and cloacal swabs were stored dry in 2 ml tubes at -

20 °C. Only one sample was retained for analysis in instances where a bird was captured and resampled in subsequent banding efforts. At the time of capture, each bird was banded with a unique USGS aluminum numerical band and basic morphological measurements were taken as part of a larger project studying the genomics of avian malaria in Hawai'i forest birds. All research was conducted under federal (U. S. Banding Permit # 23064) and state (Hawai'i Protected Wildlife Permit # WL19-20) research permits and approved by the University of Hawai'i's Animal Care and Use Committee (protocol # 18-2916).

Determining malaria infection status and intensity

DNA was extracted from blood samples using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol at the Pathogen and Microbiome Institute at Northern Arizona University. Samples were then evaluated for *P. relictum* parasitemia via quantitative polymerase chain reaction (qPCR) assay with a hydrolysis probe (K. L. Paxton unpublished data) and adapted from primers targeting the cytochrome B region (Zehindjiev et al. 2008). Each sample was tested in duplicate and threshold cycle (Ct) values were averaged between the two runs. Samples with an average Ct greater than 38 were classified as uninfected for this study because repeatability decreased substantially after cycle 38 (data not shown). Relative *P. relictum* parasitemia intensity was calculated using the formula $1/(2^{(\text{avgCt}-25)})$. Bird species with evolutionary histories of exposure to *Plasmodium* spp. may be able to clear circulating parasites to levels undetectable by qPCR (Cellier-Holzem et al. 2010). Therefore, uninfected white-eyes in my study could comprise individuals either naive to *P. relictum* exposure or survivors of an infection prior to capture, while infected white-eyes were likely recently exposed individuals. In contrast, 'amakihi are unable to clear *P. relictum* and, if they survive the acute phase of malaria, maintain chronic low levels of parasitemia post exposure (Atkinson et al. 2001). Consequently, uninfected 'amakihi in my study were most likely naive to *P. relictum* exposure, and birds with low-level infections (average Ct values greater than 30) were assumed to be those that survived an infection prior to capture.

Cloacal microbiome amplicon sequencing

I extracted DNA from the cloacal swabs and from negative control swabs using the QIAamp PowerFecal Pro DNA Kit (Qiagen), then quantified extracted DNA concentrations

using the Qubit dsDNA High Sensitivity Assay Kit and Qubit 2.0 Fluorometer (Invitrogen, Waltham, Massachusetts, USA) at the Smithsonian Conservation Biology Institute's Center for Conservation Genomics. I prepared DNA libraries for 16S rRNA gene sequencing at the University of Hawai'i at Hilo Core Genetics Facility by amplifying the V3-V5 regions of the 16S rRNA gene, ~570 base pairs (bp), using a slightly modified version of the 16S Illumina Amplicon Protocol (Illumina Inc., Foster City, California, USA). For duplicate 25 μ l amplicon PCR, I used 12.5 μ l of KAPA HiFi HotStart ReadyMix (Roche Holding AG, Basel, Switzerland), 0.5 μ l of each 10 μ M universal primer, 515F (GTGCCAGCMGCCGCGGTAA) and 939R (CTTGTGCGGGCCCCGTCAATTC; Gilbert et al. 2014), 1 μ l of BSA to boost amplified DNA yields (Farell & Alexandre 2012), and up to 10.5 μ l of sample (or a maximum of 12.5 ng brought to 10.5 μ l with sterile water) or 1 μ l of extraction control sample, including PCR negative controls for each batch. Cycling conditions for the amplicon PCR were: activation at 95 $^{\circ}$ C for 3 min, followed by 30 cycles of denaturation at 95 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 30 s, and extension at 72 $^{\circ}$ C for 30 s, followed by a final extension at 72 $^{\circ}$ C for 5 min before holding at 4 $^{\circ}$ C using an Eppendorf Mastercycler Pro (Eppendorf, Hamburg, Germany). To verify all samples amplified, I screened the amplicon PCR products using 1.5% agarose gel electrophoresis in a TAE buffer. Amplicon duplicates were combined and cleaned using Speed-beads in a PEG/NaCl buffer (Rohland & Reich 2012) and quantified via Qubit.

After the 16S rRNA gene was amplified from all samples, I used the KAPA HiFi kit to attach unique dual Nextera indices (Illumina Inc.) to each amplicon via PCR. Each 25 μ l indexing reaction had 12.5 μ l of KAPA HiFi, 1 μ l BSA, 1 μ l each of 10 μ M I5 and I7 primers in a unique combination, 3 μ l of sample, and 6.5 μ l of sterile water. Cycling conditions for the indexing PCR were: activation at 95 $^{\circ}$ C for 3 min, followed by 8 cycles of denaturation at 98 $^{\circ}$ C for 20 s, annealing at 62 $^{\circ}$ C for 15 s, and extension at 72 $^{\circ}$ C for 30 s, followed by a final extension at 72 $^{\circ}$ C for 1 min before holding at 4 $^{\circ}$ C. Again, I used gel electrophoresis to verify, magnetic beads to clean, and Qubit to quantify the PCR products. I pooled the cleaned, indexed amplicons in equimolar amounts in 4 batches of roughly 100 samples per pool and combined the four batches into an equimolar master pool. I dried 1 μ g of pooled DNA down to a 30 μ l sample for size selection to remove non-target DNA using the BluePippin protocol (Sage Science, Massachusetts, USA) trained on 500-640 bp fragment collection and using a 2% agarose cassette with internal standards. After size selection, I used Qubit to quantify the final pool and prepared

4 nM DNA libraries that were sequenced at the Center for Conservation Genomics on a single Illumina MiSeq run using MiSeq Reagent Kit v3 with a 25% PhiX Control v3 spike to balance AT/CG content.

Bioinformatics and Statistical Analysis

I used FastQC (Andrews 2010) to quality screen the sequenced reads, and MultiQC (Ewels et al. 2016) to compile the FastQC results into one report. I then used Trimmomatic (v. 0.39, Bolger et al. 2014) to remove primer sequences, and DADA2 (v. 2020.11, Callahan et al. 2016) to denoise and quality trim the reads in QIIME2 (v. 2020.11, Bolyen et al. 2019). Amplicon sequence variants (ASVs, frequently referred to as operational taxonomic units or OTUs; Callahan et al. 2017) were taxonomically assigned using a Naive Bayes classifier trained on my dataset using SILVA (v. 138) full-length reference sequences (Quast et al. 2013). Using MAFFT (Kato & Standley 2013) and FastTree2 (Price et al. 2010) I generated a phylogenetic tree of the identified ASVs within QIIME2. Further processing and all statistical analyses were conducted in statistical framework R (v. 3.6.3, R Development Core Team 2021). I used the R package decontam (v. 1.6.0, Davis et al. 2018) set at the default decontamination probability threshold of 0.1 to filter out ASVs identified in the negative control samples. Next, I filtered out reads identified as mitochondrial, chloroplastic, or eukaryotic DNA, reads that could not be assigned to the kingdom level, as well as ASVs with mean relative abundance less than 10^{-5} using the package phyloseq (v. 1.30.0, McMurdie & Holmes 2013). I removed samples that had fewer than 500 reads remaining after filtering. All data visualizations were generated using ggplot2 (v. 3.3.3, Wickham 2016).

To address my first research question, whether cloacal microbiome diversity covaries with malaria infection, I assessed microbial alpha diversity, within sample variation, and beta diversity, between sample variation. I measured alpha diversity with Shannon diversity indices, which accounts for species richness and evenness (Shannon 1948), using both rarefied and non-rarefied reads and found no difference in results. To avoid discarding reads unnecessarily I proceeded with analyses using non-rarefied reads (McMurdie & Holmes 2014). I assessed differences in Shannon diversity indices between host species ('amakihi, white-eye), sampling regions (6 regions described above; Fig. 1), malaria infection status (infected, uninfected), and relative parasitemia intensity (relative values between 0-1) using a negative binomial generalized

linear model (nbGLM) to account for overdispersion in indice values with the R package MASS (v. 7.3-51.5, Venables & Ripley 2002). Cloacal microbiome beta diversity was measured using Bray-Curtis dissimilarity, which measured the differences in ASV abundance and richness between each pair of samples (Bray & Curtis 1957), and visualized with a principal coordinate analysis (PCoA) using phyloseq's ordinate function. I analyzed Bray-Curtis dissimilarities with a permutational multivariate analysis of variance model (PERMANOVA) using the adonis function from the R package vegan (v. 2.5-7, Oksanen et al. 2020), again comparing host species, sampling region, malaria infection status, as well as relative parasitemia intensity.

For my second and third research questions, assessing if there are specific microbes associated with malaria survival in 'amakihi or white-eye cloacal microbiomes, I used phyloseq functions to visualize variation in the relative abundances of ASVs at the phylum level between sampling regions, host species, and infection statuses. To identify any bacteria in 'amakihi microbiomes positively associated with disease pressure, I ran a linear model (LM) for each ASV, using abundance counts normalized to each sample's library size against malaria prevalence at the site where the sample was collected (E. H. Paxton unpublished data). The Benjamini-Hochberg (BH) correction was used to adjust p values (p -adj) for multiple comparisons with a significance cutoff of less than a 5% false discovery rate. I used DESeq2 (v. 1.26.0, Love et al. 2014) to test for differentially abundant ASVs between different groups while controlling for sampling site, again using the BH correction to generate p -adj. I compared 'amakihi with low-level *P. relictum* infection intensity, i.e. individuals that likely survived acute malaria and are persistently infected, to uninfected 'amakihi, which were likely naive to exposure (Atkinson et al. 2001). I then compared uninfected white-eyes, all assumed to be capable of surviving malaria infection (van Riper et al. 1986, Cellier-Holzem et al. 2010), to chronically infected and uninfected 'amakihi, respectively.

'Amakihi with high-level parasitemia (average Ct values less than 30) and all infected white-eyes were excluded from the LM and differential abundance analysis to avoid the potential confounding factor of transient microbial imbalances, particularly within members of the Firmicutes and Proteobacteria phyla, associated with the acute phase of malaria infection (Ippolito et al. 2018, Bamgbose et al. 2021). I assumed that 'amakihi with low-level infections were likely to have recovered their normal microbial communities at the time of capture for two reasons. First, malaria induced microbial imbalances appear to revert back to baseline within 30

days post infection in mice (Mooney et al. 2015, Taniguchi et al. 2015). Second, in ‘amakihi that survive malaria, parasitemia drops to chronic low levels within 30 days post infection (Atkinson et al. 2001).

RESULTS

Cloacal microbiome diversity does not covary with malaria infection

After all processing and filtering steps 174 ‘amakihi and 171 white-eye samples were retained for analyses. Of those, *P. relictum* infection was detected in 49 ‘amakihi (28%) and 39 white-eyes (23%; Table 1). Within the infected groups, 9 ‘amakihi and 3 white-eyes had high parasitemia levels indicative of the acute phase of malaria. The other 40 infected ‘amakihi were classified as malaria survivors with persistent low-level parasitemia. Cloacal microbiome alpha diversity was strongly influenced by sampling region (nbGLM: $\chi^2(5) = 22.057$, $p < 0.001$; Fig. 2a), but did not differ between ‘amakihi and white-eyes ($\chi^2(1) = 0.172$, $p > 0.1$; Fig. 2), between infected and uninfected birds ($\chi^2(1) = 0.228$, $p > 0.1$; Fig. 2b), nor covary with relative parasitemia intensity ($\chi^2(1) = 0.046$, $p > 0.1$; Fig. 2c). The majority of the variation in cloacal microbiome beta diversity was explained by the region where the sample was collected (PERMANOVA: $R^2 = 0.075$, $F_{5,337} = 5.600$, $p = 0.001$; Fig. 3a), followed by bird species ($R^2 = 0.018$, $F_{1,337} = 6.574$, $p = 0.001$; Fig. 3). Beta diversity did not vary between infected and uninfected birds ($R^2 = 0.002$, $F_{1,337} = 0.874$, $p > 0.1$; Fig. 3b), nor covary with relative infection intensity ($R^2 = 0.001$, $F_{1,337} = 0.379$, $p > 0.1$; Fig. 3c).

Certain cloacal ASVs differ in abundance between host species and between malaria infected and uninfected birds

From the 345 cloacal samples, I identified 3586 ASVs. For both ‘amakihi and white-eye microbiomes, the most abundant phyla were Proteobacteria (77% and 62%, respectively), Firmicutes (8% and 21%, respectively), and Actinobacteria (10% and 13%, respectively; Fig. 4). In both species, infected birds had slightly higher Proteobacteria and slightly lower Firmicutes and Actinobacteria relative abundances than uninfected birds (Fig. 4b). The most abundant ASV genera differed between the two host species. The top genera in ‘amakihi microbiomes were *Pseudomonas* (32%), *Cutibacterium* (9%), *Thermomonas* (4%), and *Blastomonas* (4%), while the top genera of white-eye samples were *Lactobacillus* (19%), *Pseudomonas* (16%),

Escherichia/Shigella (11%; these genera are closely related and difficult to distinguish (Devanga Ragupathi et al. 2017) thus were grouped together), and *Cutibacterium* (9%).

Of the 3586 ASVs in my study, 126 were identified in the differential abundance analysis, and 71 of these were positively associated with birds likely to survive malaria infection ($p\text{-adj} < 0.05$; Tables 2, 3, 4; Fig. 5). Additionally, three ASVs, an Alphaproteobacterium that could not be identified beyond the class level (LM: $R^2_{\text{adj}} = 0.071$, $F_{1,163} = 13.547$, $p\text{-adj} = 0.027$) and two *Pseudomonas* spp. ($R^2_{\text{adj}} = 0.075$, $F_{1,163} = 14.392$, $p\text{-adj} = 0.020$; $R^2_{\text{adj}} = 0.121$, $F_{1,163} = 23.496$, $p\text{-adj} = 0.001$), increased in abundance with increasing malaria prevalence at sampling sites in ‘amakihi microbiomes. My comparison between chronically infected ‘amakihi (malaria survivors) and uninfected ‘amakihi (malaria naive) revealed 33 differentially abundant ASVs, 14 of which were more abundant in the infected birds (Table 2; Fig. 5a). In my interspecific comparisons, 45 and 84 ASVs were differentially abundant between uninfected white-eyes and uninfected and infected ‘amakihi, respectively. In both of these comparisons roughly half of the differentially abundant ASVs, 25 and 48, respectively, were positively associated with the white-eyes (Tables 3, 4; Fig. 5b, 5c).

DISCUSSION

Hawaiian honeycreepers are in the midst of an extinction crisis and investing in research and development of innovative conservation strategies needs to be of top priority (Atkinson et al. 2014, Paxton et al. 2016, Liao et al. 2017, Paxton et al. 2018, Dahlin & Feng 2019, Samuel et al. 2020). The volume of research supporting critical connections between microbiomes and malaria susceptibility and severity in mammals is steadily growing (Ippolito et al. 2018, Bamgbose et al. 2021), however little homologous research has been conducted in other host taxa or in wild environments (Trevelline et al. 2019, but see Lutz et al. 2021, Videvall et al. 2021). My study is among the first to assess correlates between malaria infection and microbiome composition in avian hosts sampled in a natural system. I found no difference in cloacal microbiome alpha diversity between Hawai‘i ‘Amakihi and Warbling White-eyes, only a small, yet significant, difference in beta diversity, and that host species effects on diversity are greatly overshadowed by the influence of sampling location. While microbiome diversity did not covary with malaria infection, I identified 71 bacterial ASVs that were more abundant in groups that likely possess some immunity to malaria mortality. These candidate bacteria may be capable of modulating

malaria severity in wild birds and are valuable targets for future studies aimed at developing antimalarial probiotics for Hawaiian honeycreepers.

There are numerous variables besides disease that shape each individual's gut microbiomic profile (Waite & Taylor 2014, Bodawatta et al. 2021), including host taxonomy, geographic location, diet, age, and other host-specific factors (Klomp et al. 2008, Banks et al. 2009, Benskin et al. 2010, Hammons et al. 2010, van Dongen et al. 2013, Hird et al. 2014, Hird et al. 2015, van Veelen et al. 2017, Grond et al. 2019, Bragg et al. 2020, Costantini et al. 2021). A study on Spotted Towhees (*Pipilo maculatus*) revealed significant differences among the cloacal microbiomes of birds from different sites less than 3.1 km apart, suggesting small geographical variations can have a substantial impact on avian microbiome composition (Klomp et al. 2008). Thus, I was not surprised to find significant levels of variation in the cloacal microbiomes of birds from different sampling sites that ranged from 50-1750 m in elevation and from heavily anthropogenically disturbed to nearly pristine native forest. These environmental factors also influence mosquito distribution and malarial parasite development, and therefore selective disease pressure (LaPointe et al. 2010), at each site, making it more challenging to disentangle environmental and disease effects on the microbiome. The Maunakea region had the lowest rate of infected 'amakihi (0% of the birds in my study, 8% of birds sampled by USGS) while the Puna region had the highest (50% and 93%), as expected given their elevations (1,610 m and ≤ 110 m, respectively). However, the Kona region had the second highest malaria prevalence (42% and 55%) despite being relatively high-elevation (1,550 m). Kona's unexpectedly high rate of infection highlights that malaria is encroaching on habitats believed to be avian refugia from disease and the need for urgency in our development and implementation of conservation strategies with high chances of success.

More surprising than the large differences observed between sites was the modest difference in cloacal microbiome diversity between the two host species, given that several studies suggest host taxonomy has a heavy influence over microbiome composition (Waite & Taylor 2014, Hird et al. 2015, Costantini et al. 2021). However, my findings align with other studies that have found locality to be more influential than host species in predicting gut microbiota (Hird et al. 2014, van Veelen et al. 2017, Grond et al. 2019). The similarity in microbial diversity between 'amakihi and white-eyes may be partially explained by the similarities in their diets. Both species are generalists that mainly forage on nectar, arthropods,

and some fruit, although fruit may be more heavily exploited by white-eyes (Lindsey et al. 2020, van Riper & van Balen 2020). Both ‘amakihi and white-eyes positive for *P. relictum* showed higher abundance of Proteobacteria, which is congruent with gut microbiota imbalances seen in mammals with malaria (Ippolito et al. 2018, Bamgbose et al. 2021). Proteobacteria are frequently among the most abundant bacteria in bird microbiomes (Bodawatta et al. 2021), and their higher abundance in infected birds may be due to opportunistic expansion in compromised hosts (Shin et al. 2015). More interesting are the patterns that I detected in the differential abundance of certain ASVs between birds of variable susceptibility to malaria mortality.

Three bacterial genera of interest were prominent among candidate ASVs, *Escherichia*, *Lactobacillus*, and *Pseudomonas*. *Escherichia* and *Lactobacillus* spp. are well supported in their potential to modulate malaria susceptibility and severity in mammalian species (Martínez-Gómez et al. 2006, Yilmaz et al. 2014, Villarino et al. 2016, Stough et al. 2016, Morffy Smith et al. 2019, Toukam et al. 2021), and were among the top genera in white-eye microbiomes, accounting for 11% and 19% ASV relative abundance respectively, but both were much less abundant, only 1-2%, in ‘amakihi. Further, an *Escherichia/Shigella* spp. (as well as three ASVs in the same family, Enterobacteriaceae, that could not be identified to the genus level) and five *Lactobacillus* spp. were significantly more abundant in white-eyes than in ‘amakihi. Elevated immune function stimulated by these microbes may be working synergistically with white-eye genetics and behaviors to protect them from malaria mortality. *Escherichia* and *Lactobacillus* spp. were absent in the comparison between ‘amakihi that survived malaria and ‘amakihi naive to *P. relictum* exposure, suggesting they may not be a factor contributing to increased ‘amakihi survival rates. Even so, it may be interesting to experimentally introduce them into honeycreeper guts and assess how well they establish and if they increase malaria resistance.

Pseudomonas was the top genera in ‘amakihi microbiomes, accounting for 27% ASV relative abundance in uninfected naive ‘amakihi, and 46%, nearly double, in chronically infected survivors. Moreover, two *Pseudomonas* spp. found in ‘amakihi microbiomes were positively associated with malaria pressure at sampling sites. *Pseudomonas* spp. colonization of the midguts of mosquitoes has been implicated in *Plasmodium* suppression, potentially by upregulating the expression of certain anti-*Plasmodium* immune genes (Bahia et al. 2014). It is possible that *Pseudomonas* spp. can similarly mitigate malaria severity by stimulating the expression of immune genes in host birds, thus harboring a greater abundance of them increases

chances of survival. However, it is important to note that *Pseudomonas* is the largest and most diverse gram-negative bacterial genus, with species that inhabit a wide range of environments (Lalucat et al. 2020), and few ASVs in my study were identified to the species level. Teasing apart the role of various *Pseudomonas* spp. in malaria susceptibility in honeycreepers may require culturing bacterial isolates from gut microbiome samples for deeper taxonomic identification and functional characterization.

Few patterns emerged around the other bacteria with potential links to malaria immunity that I identified from the scientific literature. *Bifidobacterium* spp. were found to be enriched in mice that showed resistance to malaria (Martínez-Gómez et al. 2006, Villarino et al. 2016), however no such connection was evident in my study. Similar to *Pseudomonas* spp., *Pantoea* spp. have been implicated in blocking *Plasmodium* development in mosquitoes (Bahia et al. 2014), and have been linked to malaria in bat hosts (Lutz et al. 2021). While I did not detect *Pantoea* spp. in any of the cloacal microbiome samples, one ASV identified to the same family level, Erwiniaceae, was more abundant in chronically infected than uninfected ‘amakihi. ‘Amakihi microbiomes had slightly higher relative abundance of Erwiniaceae than white-eyes’ (4% and 1%, respectively), but with only these observations, support for a connection between Erwiniaceae and malaria in birds is weak.

Though the number of avian microbiome studies is exponentially growing (Bodawatta et al. 2021), the current lack of characterized avian microbes in taxonomic databases poses barriers to the interpretation of the data generated by studies such as mine. Even at the phylum level, I was unable to taxonomically assign a notable number of ASVs, which has been highlighted as a prevailing limitation to microbiome studies (Levin et al. 2021). Microbes that have been found to be influential in malaria immunity have mainly been members of mammalian intestinal communities (Yilmaz et al. 2014, Villarino et al. 2016, Stough et al. 2016, Morffy Smith et al. 2019), however I used cloacal microbiome samples because they are substantially less invasive to collect and were logistically practical. Cloacal and fecal microbiomes are not perfect representatives of the microbial community in other regions of the gut (Videvall et al. 2018), and despite the added challenge, sampling the ileum, cecum, or colon in laboratory experiments may reveal stronger associations between commensal microbes and infection response. Controlled laboratory experiments would also allow us to sample the microbiomes of birds that succumb to malaria mortality, which was not possible for my study. I could not directly compare survivor

‘amakihi microbiomes to those of fatalities, instead I used comparisons between chronically infected survivors and uninfected, malaria-naïve birds to identify microbes that may be augmenting malaria immunity. My pool of naïve ‘amakihi likely consists of a mixture of birds that would either survive or die upon exposure; a heterogeneity which may weaken my ability to detect if beneficial microbes are in greater abundance in survivors.

Through this research I have shown that the malaria-resistant white-eye harbors microbes with established connections to malaria immunity in greater abundance than ‘amakihi, and that there are certain bacteria associated with malaria survival in ‘amakihi microbiomes. If beneficial bacteria within ‘amakihi cloacal microbiomes confer malaria immunity, it is possible, though speculative, that they may be transferred from parents to nestlings. Higher reproductive success has been observed in chronically infected ‘amakihi over uninfected individuals, and it was hypothesized that this is attributable to heritable malaria resistance genes (Kilpatrick et al. 2006). However, some research suggests that gut microbiome composition may transcend genetic determinants of malaria susceptibility and severity (Stough et al. 2016, Morffy-Smith et al. 2019), and I postulate that variation in nestling survival may potentially be explained, in part, by colonization by antimalarial microbes sourced from parents. It may be worthwhile to test the hypothesis that microbes can be vertically transmitted by supplementing captive birds with probiotics then testing their nestlings for those microbes. The candidate microbes I have outlined here warrant closer study to elucidate the potential value of supplementing malaria-susceptible Hawaiian honeycreepers with immune-modulating bacteria. *Lactobacillus* spp. in particular show promise as they are frequently used as probiotics in other systems and are less likely than *Escherichia* spp. to become opportunistic pathogens (Stedman et al. 2020, Bamgbose et al. 2021). *Pseudomonas* spp. may also be valuable candidates for probiotics as they appear to be naturally abundant in ‘amakihi microbiomes, may inhibit *P. relictum* development (Bahia et al. 2014), and could represent a unique adaptation evolving in ‘amakihi. To conclude, my research suggests further investigation into probiotics may yield a feasible and effective conservation strategy for bolstering malaria immunity in the face of increasing disease pressure and imminent extinctions in Hawai‘i.

APPENDIX A: TABLES

Table 1. Elevation, number of Hawai'i 'Amakihi (HAAM) and Warbling White-eye (WAVE) infected and uninfected with *Plasmodium relictum*, and infection rate for each region and sampling site on Hawai'i Island.

Region	Site	Elevation (meters)	Uninfected HAAM	Infected HAAM	HAAM Infection Rate	Uninfected WAVE	Infected WAVE	WAVE Infection Rate	Combined Infection Rate
Ka'u	KAHU	1275	11	6	35.3%	2	0	0.0%	31.6%
	KAUF	1094	6	2	25.0%	5	2	28.6%	26.7%
	KIOL	584	0	3	100.0%	2	3	60.0%	75.0%
	MANU	603	9	3	25.0%	29	4	12.1%	15.6%
	Region Total	889	26	14	35.0%	38	9	19.1%	26.4%
Kona	KONA	1550	15	11	42.3%	6	2	25.0%	38.2%
	Region Total	1550	15	11	42.3%	6	2	25.0%	38.2%
Maunakea	MAUL	1610	18	0	0.0%	2	2	50.0%	9.1%
	Region Total	1610	18	0	0.0%	2	2	50.0%	9.1%
Puna	LAMA	110	0	1	100.0%	2	1	33.3%	50.0%
	MARC	50	1	0	0.0%	2	1	33.3%	25.0%
	Region Total	80	1	1	50.0%	4	2	33.3%	37.5%
Pu'u Wa'awa'a	PFBS	1230	3	1	25.0%	1	0	0.0%	20.0%
	PWWA	790	13	1	7.1%	17	3	15.0%	11.8%
	Region Total	1010	16	2	11.1%	18	3	14.3%	12.8%
Kīlauea	AIRA	1050	30	20	40.0%	14	3	17.6%	34.3%
	BIPA	1222	0	0	-	24	12	33.3%	33.3%
	BYLE	1203	0	0	-	1	0	0.0%	0.0%
	CRBX	1166	1	0	0.0%	0	0	-	0.0%
	KIVO	1168	0	1	100.0%	13	5	27.8%	31.6%
	UPKE	1757	18	0	0.0%	12	1	7.7%	3.2%
	Region Total	1261	49	21	30.0%	64	21	24.7%	27.1%
Grand Total			125	49	28.2%	132	39	22.8%	25.5%

Table 2. The results of pairwise DESeq2 comparisons of ASV abundances between the cloacal microbiomes of Hawai'i 'Amakihi chronically infected with avian malaria (N = 40) and uninfected 'amakihi (N = 125). Only ASVs with significant differences (p -adj < 0.05) are given.

ASV	Base mean	Log2 fold change	Standard error	Wald statistic	p -adj	Kingdom	Phylum	Class	Order	Family	Genus	Species
d7a30610ff1ab3cc697d7e16548b43dd	4.31	28.20	3.89	7.25	4.01E-10	Bacteria						
87096b71d1655d5bc758daba33e57ad1	2.52	25.83	3.89	6.64	1.98E-08	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Lysinibacillus</i>	<i>Bacillus subtilis</i>
f884ac1ea3e305c4bf202a58e27a1757	1.32	25.63	3.89	6.59	2.10E-08	Bacteria						
76befea3e4808b0a3d571f4bbaf13bc8	1.28	25.02	3.89	6.43	4.81E-08	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Lysinibacillus</i>	<i>Bacillus subtilis</i>
c621ee1960388b8f94fa7103b6eac118	0.66	24.40	3.89	6.27	9.05E-08	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Lysinibacillus</i>	<i>Bacillus subtilis</i>
f3943580798fb6f845d81e76c1f9500e	0.83	24.36	3.89	6.26	9.05E-08	Bacteria	Firmicutes	Bacilli	Paenibacillales	Paenibacillaceae	<i>Paenibacillus</i>	
67935f30a7e80785aa1a688d0bac9026	0.48	23.69	3.89	6.09	2.37E-07	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	
95d6524846634dea5068aaab6fb79684	2.84	22.79	3.88	5.87	7.68E-07	Bacteria	Firmicutes	Bacilli	Bacillales			
e650b5c5853388383aaa908a8cb986c5	7.58	18.75	3.88	4.83	1.70E-04	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	<i>Endobacter</i>	
19c9618e780523a10142373d2d5b7a83	1.93	16.93	3.89	4.36	1.50E-03	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Lysinibacillus</i>	<i>Bacillus subtilis</i>
923a0d92b3e5db3c2fb9838ded4fe299	5.00	12.51	3.13	4.00	5.57E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Methylobacterium</i>	
60ec73d29d807cf742ccc732e0765989	754.58	8.83	1.50	5.89	7.37E-07	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Erwiniaceae		
8e2bb88fb2106027c76c54c718633812	9.36	6.77	1.83	3.69	1.53E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia sensu lato</i>	
0f00f691387551d56bb888cfc8072aa	197.42	6.41	1.29	4.95	1.01E-04	Bacteria						
31a6e32d8c0677a01ec6e345dcd8bc4	1.31	-12.97	3.89	-3.33	4.94E-02	Bacteria	Actinobacteriota	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	<i>Pseudonocardia</i>	
f832853cc04bd8a8d695419fa6b349a0	2.32	-13.45	3.89	-3.46	3.27E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Orbales	Orbaceae	<i>Gilliamella</i>	<i>Serratia symbiotica</i>
60de4bf68e5291641da842d5d09d4430	2.74	-13.58	3.89	-3.49	2.96E-02	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	<i>Lactococcus</i>	
1a191abfa35ad5e2978bc3f9a057529f	1.09	-13.91	3.89	-3.58	2.23E-02	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	
e67aeec9cccd62ffd7493fb8134057c	1.80	-14.16	3.89	-3.64	1.80E-02	Bacteria						
60283ea84f5507e446caad3e1857a830	2.85	-14.68	3.89	-3.77	1.14E-02	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	<i>Weissella</i>	<i>minor</i>
464d8c25f3c4cbe10714a28f04298cd6	1.08	-15.05	3.89	-3.87	8.05E-03	Bacteria						
c0892279a961c1acc5d6de1b2222ba80	1.19	-15.08	3.89	-3.88	8.05E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Methylobacterium</i>	
869ead1bc0d6f5d30cdbf12a4864b1ac	1.77	-15.34	3.89	-3.94	6.39E-03	Bacteria						
7635e59584ba803014e2ac7959bd8a07	1.87	-15.35	3.89	-3.94	6.39E-03	Bacteria						
57f4264c3c280a65e76d274e0006c0b2	2.80	-15.90	3.89	-4.09	3.92E-03	Bacteria						
f504627ef0c77fb913f8c03e76eedda	6.19	-16.09	3.89	-4.14	3.37E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
2e498fb11a0d6bdf6ee2aad8e6676e1e	9.01	-16.28	3.89	-4.18	2.87E-03	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	
d10db8b91ac18f4e0998f4e4c3370df7	2.70	-16.68	3.89	-4.29	1.90E-03	Bacteria	Proteobacteria	Gammaproteobacteria				
430319cf36872ee0ddef1068cc9beba	36.78	-16.94	3.89	-4.35	1.50E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
a5212853edb36798077c7565b2a5ca9a	5.29	-20.40	3.89	-5.25	2.29E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae		
4db8f8510d90e8a04bd309f0d69dfddd	405.37	-20.48	3.89	-5.27	2.19E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	
042a3e19d6145fdea381d6bd89dc4be1	15.74	-24.81	3.89	-6.38	5.50E-08	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae		
2d4809fa27caf67afd19a472bd66a424	2.87	-28.45	3.89	-7.32	4.01E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	

ASV: amplicon sequence variant; p -adj: Benjamini-Hochberg adjusted p value

Table 3. The results of pairwise DESeq2 comparisons of ASV abundances between the cloacal microbiomes of Warbling White-eyes uninfected with avian malaria (N = 132) and uninfected Hawai'i 'Amakihi (N = 125). Only ASVs with significant differences (p -adj < 0.05) are given.

ASV	Base mean	Log2 fold change	Standard error	Wald statistic	p-adj	Kingdom	Phylum	Class	Order	Family	Genus	Species
293a614ecb2cbaa200b31bc6cf465914	7.71	29.65	2.27	13.04	3.63E-36	Bacteria						
fed10ef04f87dbb10f0ea59b9cf48ee6	152.62	27.96	1.60	17.52	1.53E-65	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
8fd4c67e428055ea93ffc42522adc886	12.32	27.56	3.54	7.78	8.68E-13	Bacteria	Spirochaetota	Brachyspirae	Brachyspirales	Brachyspiraceae	<i>Brachyspira</i>	
d95cdd8d4bd541a8645ca337b4767c29	1.86	25.35	3.54	7.16	7.03E-11	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>1174-901-12</i>	Uncultured
2bc84f2b91e0dec574cd8bc8ea244667	28.99	23.22	2.09	11.09	5.20E-26	Bacteria	Spirochaetota	Spirochaetia	Spirochaetales	Spirochaetaceae		
eaca7e0da2a576d7893c36d9a6577601	2.74	19.00	3.54	5.36	4.89E-06	Bacteria	Firmicutes	Bacilli	Erysipelotrichales	Erysipelatoclostridiaceae	<i>Erysipelatoclostridium</i>	
eb6dec166c633b941a826f104a2ac624	2.68	18.99	3.54	5.36	4.89E-06	Bacteria	Deinococcota	Deinococci	Deinococcales	Deinococcaceae	<i>Deinococcus</i>	
6f7db326105dceea6831f85b2ab9bb2a	4.29	18.91	3.54	5.34	5.29E-06	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
91f86fb95392ed61af533a4b5c80f31	9.88	18.35	3.54	5.18	1.14E-05	Bacteria	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	<i>Corynebacterium</i>	<i>kroppenstedtii</i>
bd6ab08100624ed3ef1571d06f20db5e	3.79	17.71	3.54	5.00	2.91E-05	Bacteria	Campilobacterota	Campylobacteria	Campylobacterales	Helicobacteraceae	<i>Helicobacter</i>	
a4c60c605d7b4038fa3d2b0528385e64	34.61	17.67	3.54	4.99	2.96E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
1e1ca81f4c0e504533a3ae1f64eb00e3	3.37	17.57	3.54	4.96	3.32E-05	Bacteria	Firmicutes	Clostridia	Peptostreptococcales	Peptostreptococcales	<i>Peptoniphilus</i>	
c003ba4a8a08dffbcf5b0a35dff50259	15.06	17.29	3.54	4.88	4.84E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	
611826b48dfcb832359f517f75d1353f	1.27	17.24	3.54	4.86	5.06E-05	Bacteria						
c6b9d4d6f9b583be9f5770cc224f1910	5.34	17.21	3.54	4.86	5.09E-05	Bacteria	Firmicutes	Bacilli	Mycoplasmatales	Mycoplasmataceae	<i>Ureaplasma</i>	Gut metagenome
e225c15c97ef040170ba564d5d4839ab	8.21	16.70	3.54	4.71	1.01E-04	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Rhodocyclaceae	<i>C39</i>	
ce2e335b29da4aaa3d700d5ac9ec8e89	0.42	16.50	3.54	4.66	1.27E-04	Bacteria						
cae1942ec54a89711de1f4e2d0feab8c	2.17	14.18	3.54	4.00	2.23E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Rhodoblastus</i>	
19975f08aff6de0b18099c14796a64ef	94.01	13.76	0.95	14.52	6.27E-45	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Bartonella</i>	
617b8deefc2a9134fcd1e3802d65dbf3	2.44	12.49	3.54	3.53	1.43E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Thermomonas</i>	
b140aec5c1601d97b62965aeb3f2132	353.69	12.22	1.55	7.89	4.00E-13	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
68effa8db36b024c77f69333e6bbdb86e	6.18	11.25	3.54	3.18	4.83E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
393b30b526fc2aed0ef8ff085fcb8bf	1775.67	8.62	0.96	8.99	5.95E-17	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
79a0f8e6d541800e6cfbfa61c4ef7200	11.08	5.74	1.40	4.11	1.46E-03	Bacteria						
0210281598724d3a159e62cad0cf6760	1299.92	3.95	0.97	4.08	1.62E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Escherichia/Shigella</i>	
03109180f17100ca0df67692a14a7df4	53.62	-5.22	1.56	-3.34	2.80E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Ralstonia</i>	
03e63ddf91fb72af52b47c17571ebecb	103.04	-9.40	2.26	-4.16	1.25E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Blastomonas</i>	
2268dbabed6bdad7c710b68185e89594	23.19	-10.11	1.80	-5.62	1.19E-06	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Uncultured	Uncultured
0f00f691387551d566b888cfc8072aa	99.02	-11.96	1.19	-10.02	3.49E-21	Bacteria						
6da66831719903efc353b180f3184e00	4.28	-12.63	3.54	-3.57	1.26E-02	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Weeksellaceae	<i>Chryseobacterium</i>	
7635e59584ba803014e2ac7959bd8a07	1.40	-16.57	3.54	-4.68	1.19E-04	Bacteria						
6952e39b465769afee18e02ead034c12	1.77	-18.69	3.54	-5.28	7.14E-06	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	<i>Zymobacter</i>	Uncultured
1a191abfa35ad5e2978bc3f9a057529f	0.71	-20.90	3.54	-5.90	2.45E-07	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	
e67aeec9eccdc62ffd7493fb8134057c	1.24	-21.56	3.54	-6.08	8.21E-08	Bacteria						
0051a4a12d41fb3a7f101e8c44ac3c7d	1.47	-21.81	3.54	-6.15	5.49E-08	Bacteria						
60283ea84f5507e446caad3e1857a830	1.84	-22.84	3.54	-6.44	8.86E-09	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	<i>Weissella</i>	<i>minor</i>
54035dd0d7044b7eac8dd8c16c199666	1.26	-23.75	3.54	-6.70	1.63E-09	Bacteria	Proteobacteria					
f504627ef0c77fb913f8c03e76eeedda	3.72	-25.73	3.54	-7.26	3.49E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
2e498fb11a0d6bdf6ee2aad8e6676e1e	6.11	-26.21	3.54	-7.40	1.37E-11	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	
cf42a69dcca37f7e2b4882ddb0fa712	4.67	-26.75	3.54	-7.55	4.56E-12	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales			
57f4264c3c280a65e76d274e0006c0b2	1.96	-26.82	3.54	-7.57	4.26E-12	Bacteria						
430319cf36872ee0ddefd1068cc9beba	22.08	-28.10	3.54	-7.93	3.20E-13	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
9890b3d0c3cf39d06ca2b2a735696c11	9.20	-29.51	3.35	-8.81	2.56E-16	Bacteria	Spirochaetota	Spirochaetia	Spirochaetales	Spirochaetaceae		
e9eddffaf8544e6d4e7445394b5fd9fa	10.41	-29.60	3.54	-8.36	9.91E-15	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Bartonella</i>	
bc9feac252748f6d592a01a9cda80ee	47.50	-29.88	3.54	-8.44	5.94E-15	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	<i>Candidatus</i>	<i>Arthromitus</i>

ASV: amplicon sequence variant; p-adj: Benjamini-Hochberg adjusted p value

Table 4. The results of pairwise DESeq2 comparisons of ASV abundances between the cloacal microbiomes of Warbling White-eyes uninfected with avian malaria (N = 132) and chronically infected Hawai'i 'Amakihi (N = 40). Only ASVs with significant differences (p -adj < 0.05) are given.

ASV	Base mean	Log2 fold change	Standard error	Wald statistic	p-adj	Kingdom	Phylum	Class	Order	Family	Genus	Species
81930f2408877b5b41a5abd4dc5010c4	4.47	29.97	3.31	9.06	4.88E-17	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	<i>Granulicatella</i>	
2bc84f2b91e0dec574cd8bc8ea244667	43.97	29.70	2.75	10.79	2.11E-24	Bacteria	Spirochaetota	Spirochaetia	Spirochaetales	Spirochaetaceae		
03ae944a2c445095e2800498f72249ef	4.53	29.35	3.15	9.33	4.73E-18	Bacteria						
fed10ef04f87dbb10f0ea59b9cf48ee6	228.35	28.96	2.18	13.28	2.20E-37	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
19975f08aff6de0b18099c14796a64ef	143.89	28.11	1.34	20.98	2.20E-94	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Bartonella</i>	
79a0f8e6d541800ee6fbfa61c4ef7200	16.85	27.76	1.85	15.00	8.20E-48	Bacteria						
8fdc467e428055ea93ffc42522adc886	19.61	25.56	4.45	5.74	1.70E-06	Bacteria	Spirochaetota	Brachyspirae	Brachyspirales	Brachyspiraceae	<i>Brachyspira</i>	
e923ae81bf32c9d4f881530aa8c00a8c	1.78	25.43	4.45	5.71	1.85E-06	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Porphyromonadaceae	<i>Porphyromonas</i>	
f145532357f2d15ae9bf823687df66	25.30	18.65	4.46	4.18	1.49E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	
abe1baa0d26c0ba87b39735ba4ebf67d	6.31	18.64	4.45	4.19	1.49E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	
abcde5bb3f6f1065d36f55ff0c50b8f5d	15.71	18.43	4.46	4.14	1.78E-03	Bacteria	Bacteroidota	Bacteroidia	Cytophagales	Hymenobacteraceae	<i>Hymenobacter</i>	
eaca7e0da2a576d7893c36d9a6577601	4.13	18.37	4.45	4.12	1.78E-03	Bacteria	Firmicutes	Bacilli	Erysipelotrichales	Erysipelotrichaceae	<i>Erysipelatoclostridium</i>	
61f38e80ac7f8e44f2f9c295fd33b	13.36	18.37	4.46	4.12	1.78E-03	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Micrococaceae		
eb6dee166c633b941a826f1042ae624	4.04	18.35	4.45	4.12	1.78E-03	Bacteria	Deinococcota	Deinococci	Deinococcales	Deinococcaceae	<i>Deinococcus</i>	
a4c60c605d7b4038fa3d2b0528385e64	22.38	18.22	4.46	4.09	1.99E-03	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
239e53740b903f2e0ba0cac242aba7e9	2.61	18.10	4.45	4.06	2.19E-03	Bacteria						
08cee6ac79168ec959f1234425625b84	2.39	17.99	4.45	4.04	2.37E-03	Bacteria	Firmicutes	Bacilli	Erysipelotrichales	Erysipelatoclostridiaceae	<i>Erysipelatoclostridium</i>	Uncultured <i>Clostridium</i>
bc47de0bcdce53de65fad1855b6121d	2.07	17.94	4.45	4.03	2.45E-03	Bacteria	Chloroflexi	Ktedonobacteria	Ktedonobacteriales	Ktedonobacteraceae	<i>1959-1</i>	Uncultured
2a0c58669d33ee4546048aa5e29762a	4.57	17.88	4.45	4.01	2.49E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>	<i>endophytica</i>
59dba22d5f067b542076f4510f852671	4.49	17.68	4.46	3.97	3.01E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Methylobacterium</i>	<i>aerolatum</i>
293a614eb2cbaa200b31bc6cf465914	7.16	17.63	3.22	5.47	5.23E-06	Bacteria						
16d953c343ccaa30c3ef8baf4c26ae4	3.06	17.58	4.46	3.94	3.25E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>	
6952e39b465769afee18e0ead034c12	2.54	17.54	4.46	3.93	3.31E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	<i>Zymobacter</i>	Uncultured
eaafc175dbac6721c5284d069101b4d0	2.47	17.51	4.46	3.93	3.34E-03	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
b86525399f4e10367295c210b798109c	2.01	17.39	4.46	3.90	3.60E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	<i>Achromobacter</i>	<i>aloverae</i>
a63a94209217ad0baeaf9cdf55279c04	1.95	17.35	4.46	3.89	3.63E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Methylobacterium</i>	<i>aerolatum</i>
8bfa6ebcb3ff0fa59365ca3603de9d4a	1.54	17.19	4.46	3.86	4.09E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae		
45e9c55f5ce17f941fd8e43a4f6693c5	1.17	17.08	4.46	3.83	4.41E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
a5f8eb42f32b7a0001e19110715b7850	0.92	16.94	4.46	3.80	4.91E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>	
a5908484f3b2d76b291db73949e3d11f	4.42	16.94	4.46	3.80	4.91E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae		
54cc8e61e071d9d58efeb36ee1ab999	1.18	16.91	4.46	3.79	4.96E-03	Bacteria						
f8cc9a9795250b9d7002aa6d1bed6171	1.04	16.69	4.46	3.75	5.93E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacteriales	Acetobacteraceae	<i>Roseomonas</i>	
60de4bf68e5291641da842d5d09d4430	9.57	15.98	4.46	3.59	1.05E-02	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	<i>Lactococcus</i>	
bd6ab08100624ed3ef1571d06f20db5e	5.69	15.89	4.46	3.57	1.12E-02	Bacteria	Campilobacterota	Campylobacteria	Campylobacteriales	Helicobacteraceae	<i>Helicobacter</i>	
ce2e335b29da4aaa3d700d5ac9ec8e89	0.56	15.64	4.46	3.51	1.38E-02	Bacteria						
b140aec5c1601d97b629656aeb3f2132	526.35	15.36	2.26	6.78	3.84E-09	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
fc1fca5d139c81ebd9945fba6db5440	0.38	15.29	4.46	3.43	1.83E-02	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Roseiarcus</i>	Uncultured
e2490beb21cb8c66f6daad5cc5966232	3.84	15.27	4.46	3.43	1.84E-02	Bacteria						
4e8629595fa8f16d22b86dfface6f670	0.44	15.18	4.46	3.41	1.95E-02	Bacteria	Actinobacteriota	Actinobacteria	Micromonosporales	Micromonosporaceae	<i>Actinoplanes</i>	<i>cibodasensis</i>
f832853cc04bd8a8d695419fa6b349a0	6.31	14.61	4.46	3.28	3.06E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Orbales	Orbaceae	<i>Gilliamella</i>	<i>Serratia symbiotica</i>
c6b9d4d6f9b583be9f5770cc224f1910	8.32	14.06	4.46	3.15	4.51E-02	Bacteria	Firmicutes	Bacilli	Mycoplasmatales	Mycoplasmataceae	<i>Ureaplasma</i>	Gut metagenome
c003ba4a8a08dffbcf5b0a35dff50259	23.19	13.93	4.46	3.12	4.93E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	
c6470fce9dc00a1583f3f2291284b236	26.64	11.33	2.32	4.88	6.68E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Thermomonas</i>	
0be7b380831f7b2cbea901ae6080e440	5.91	10.91	2.98	3.67	7.99E-03	Bacteria	Firmicutes	Negativicutes	Veillonellales	Veillonellaceae	<i>Veillonella</i>	

Table 4. (Continued) The results of pairwise DESeq2 comparisons of ASV abundances between the cloacal microbiomes of Warbling White-eyes uninfected with avian malaria (N = 132) and chronically infected Hawai'i 'Amakihi (N = 40). Only ASVs with significant differences (p -adj < 0.05) are given.

3f04183891ca9fd70851b363e90f6ef1	13.11	9.94	2.01	4.94	5.10E-05	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	<i>Methylobacterium</i>	<i>aerolatum</i>
ce97a57162ab2d8cf6a5cff107f57d32	14.92	8.80	1.90	4.63	2.21E-04	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae	<i>Alloprevotella</i>	
393b30b526fc2aeced0ef8f085fcb8bf	2734.76	7.56	1.37	5.54	3.88E-06	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	<i>Lactobacillus</i>	
0210281598724d3a159e62cad0cf6760	1964.26	6.06	1.47	4.12	1.78E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	<i>Escherichia/Shigella</i>	
7b7bb6a669e64c4b7ba652c57d8aad03	30.45	-7.79	2.44	-3.19	4.05E-02	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae		
b9ace6d6b36697d9f1c48f6e7a85b27	10.45	-9.69	2.48	-3.91	3.57E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	<i>Endobacter</i>	
03109180f17100ca0df67692a14a7df4	51.24	-10.67	2.51	-4.26	1.14E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Ralstonia</i>	
6810521aa5f93ecd015c093e0a9daae0	8.09	-10.81	3.46	-3.12	4.93E-02	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales			
983e615811fea5d8dab41b059709736c	1.06	-14.19	4.46	-3.18	4.13E-02	Bacteria						
67935f30a7e80785aa1a688d0bac9026	0.69	-16.22	4.46	-3.64	8.75E-03	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	<i>Enterococcus</i>	
d5ab1712ad276a99e84c274f0fbc9623	8.16	-16.94	4.35	-3.90	3.63E-03	Bacteria	Actinobacteriota	Actinobacteria	Corynebacterales	Nocardiaceae	<i>Rhodococcus</i>	
abf26db7c87dc2d336a4991e452c5814	0.49	-17.26	4.46	-3.87	3.86E-03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Allorhizobium sensu lato</i>	
283cdd72da51caef595bf847dbb01bec	57.28	-17.89	4.45	-4.02	2.48E-03	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	
5e09dae51a1a68ae584c0d34b6d705a6	0.33	-19.25	4.46	-4.32	8.85E-04	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales			
cd96179eb2b0f03677d0685317865a57	0.36	-19.89	4.46	-4.46	4.69E-04	Bacteria	Proteobacteria					
2513529d06320f02c453d78dfe4a47c0	0.53	-20.09	4.46	-4.51	3.86E-04	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	
38679cd2ad94c9b80b0bd042233fc5	0.85	-20.95	4.46	-4.70	1.62E-04	Bacteria						
20caf3cf9849df9f8793b99d1fe9cccf	0.11	-22.25	4.46	-4.99	4.05E-05	Bacteria						
4bac56fd234c0e83315d1870b63ae0e	0.11	-22.25	4.46	-4.99	4.05E-05	Bacteria	Actinobacteriota	Actinobacteria				
2305b616011139821c33ac4e4fc12bdb	0.12	-22.26	4.46	-4.99	4.05E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Neisseriaceae	<i>Neisseria</i>	<i>oralis</i>
8e09ac2960d14c27acc938f25673ef7	1.14	-22.31	4.46	-5.01	4.05E-05	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales			
54035dd0d7044b7eac8dd8c16c199666	2.33	-22.37	4.46	-5.02	3.99E-05	Bacteria	Proteobacteria					
df6f60ee73d3261831d25c5c5370d6c1	0.14	-22.52	4.46	-5.05	3.45E-05	Bacteria	Actinobacteriota	Thermoleophilia	Gaiellales	Uncultured	Uncultured	<i>bacterium Ellin6517</i>
752dfab2a0f4e89477e7e8ab33411010	0.14	-22.52	4.46	-5.05	3.45E-05	Bacteria						
af8db7f27b3bc7a68af034291e6aaad	3.60	-22.58	4.46	-5.07	3.45E-05	Bacteria						
c75c92918a5438e635f488295ff4512c	5.10	-23.22	4.46	-5.21	1.68E-05	Bacteria						
9890b3d0c3cf39d06ca2b2a735696c11	0.34	-23.38	4.46	-5.25	1.43E-05	Bacteria	Spirochaetota	Spirochaetia	Spirochaetales	Spirochaetaceae		
f3943580798fb6f845d81e76c1f9500e	0.74	-23.77	4.46	-5.33	9.24E-06	Bacteria	Firmicutes	Bacilli	Paenibacillales	Paenibacillaceae	<i>Paenibacillus</i>	
fa787e4af751475adac0f46bdafad14f	0.14	-24.18	4.46	-5.43	5.79E-06	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	<i>Acetobacter</i>	
30337c074c4d1bbb19a6a8d49e8eb971	0.14	-24.18	4.46	-5.43	5.79E-06	Bacteria						
f47ab430884ebc5958b358303d5e8ee5	0.14	-24.18	4.46	-5.43	5.79E-06	Bacteria						
b5815501660b127568c577ad832c93fe	0.19	-24.37	4.46	-5.47	5.23E-06	Bacteria	Actinobacteriota	Actinobacteria	Propionibacteriales	Propionibacteriaceae	<i>Cutibacterium</i>	
0c6222d275610d344fd35e642fcfc522	0.55	-25.23	4.45	-5.66	1.99E-06	Bacteria	Cyanobacteria	Cyanobacteriia	Cyanobacteriales	Nostocaceae	<i>Nostoc PCC-7107</i>	Uncultured <i>Streptostemon</i>
f179fe6b236c82b74ee9f847ffb0134d	0.37	-25.26	4.46	-5.67	1.99E-06	Bacteria						
76befea3e4808b0a3d571f4bbaf13bc8	1.14	-25.26	4.46	-5.67	1.99E-06	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Lysinibacillus</i>	<i>Bacillus subtilis</i>
5cdd9ce50d6cf64017a1fae455ab7262	0.60	-25.77	4.46	-5.78	1.42E-06	Bacteria						
1a8d50b3dae35d753d5a55b7986c68e8	0.82	-26.13	4.46	-5.86	9.49E-07	Bacteria						
87096b71d1655d5bc758daba33e57ad1	2.24	-26.69	4.46	-5.99	4.85E-07	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	<i>Lysinibacillus</i>	<i>Bacillus subtilis</i>
ad46099e721c9edeff56ce9bbbff59a8	1.27	-26.71	4.46	-5.99	4.85E-07	Bacteria						
d7a30610f1ab3cc697d7e16548b43dd	4.25	-28.68	4.46	-6.43	3.56E-08	Bacteria						

ASV: amplicon sequence variant; p -adj: Benjamini-Hochberg adjusted p value

APPENDIX B: FIGURES

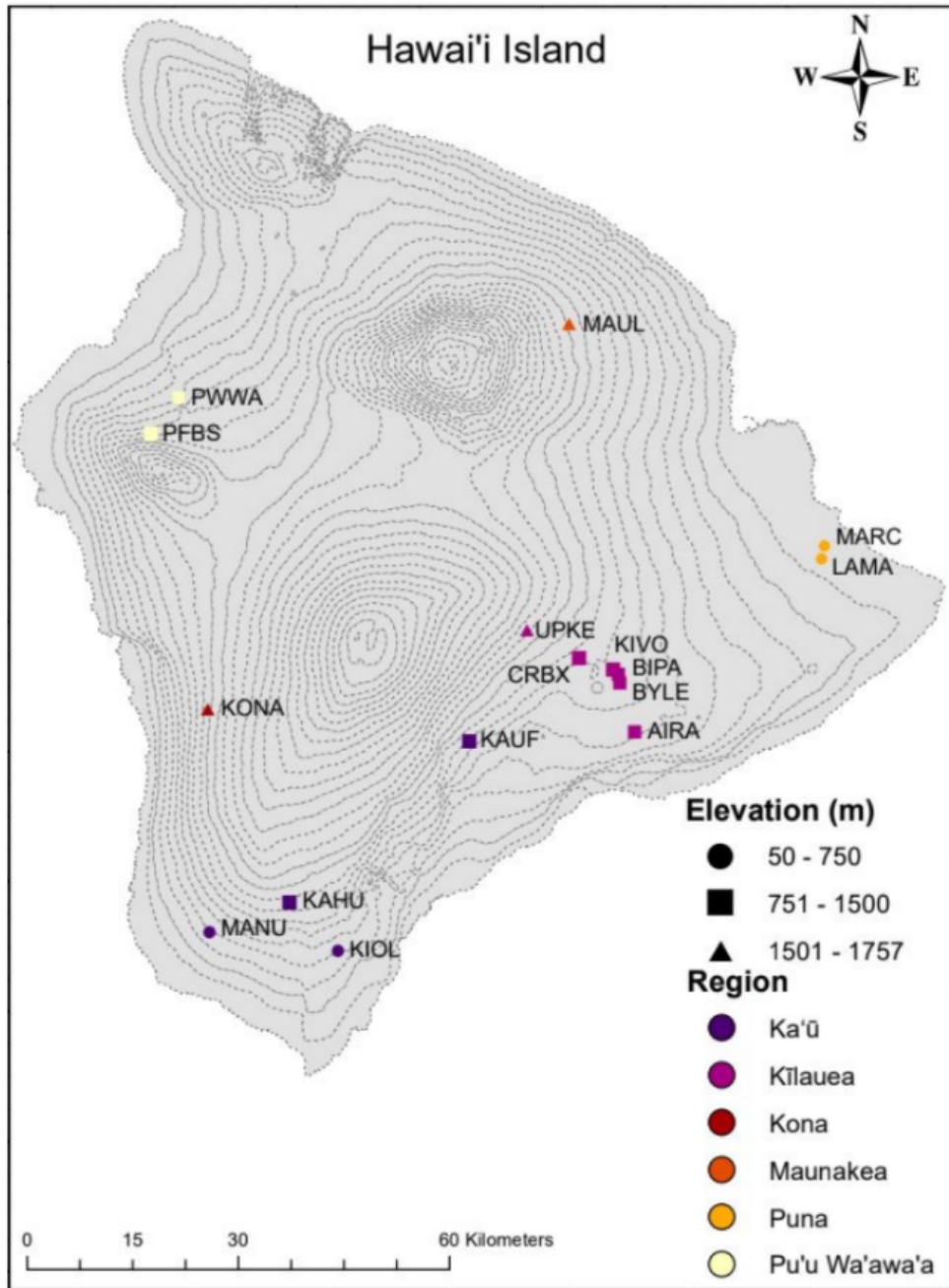


Figure 1. Topographic map of Hawai'i Island with 150 meter elevational contour lines and sampling locations. Elevation of each site is indicated by point shape and assigned region is indicated by point color. (Robert Lee Justice III. "Hawai'i Island." November, 2021. Using: ArcGIS. Version 10.8.1. Sources: Hawai'i Statewide GIS Program.)

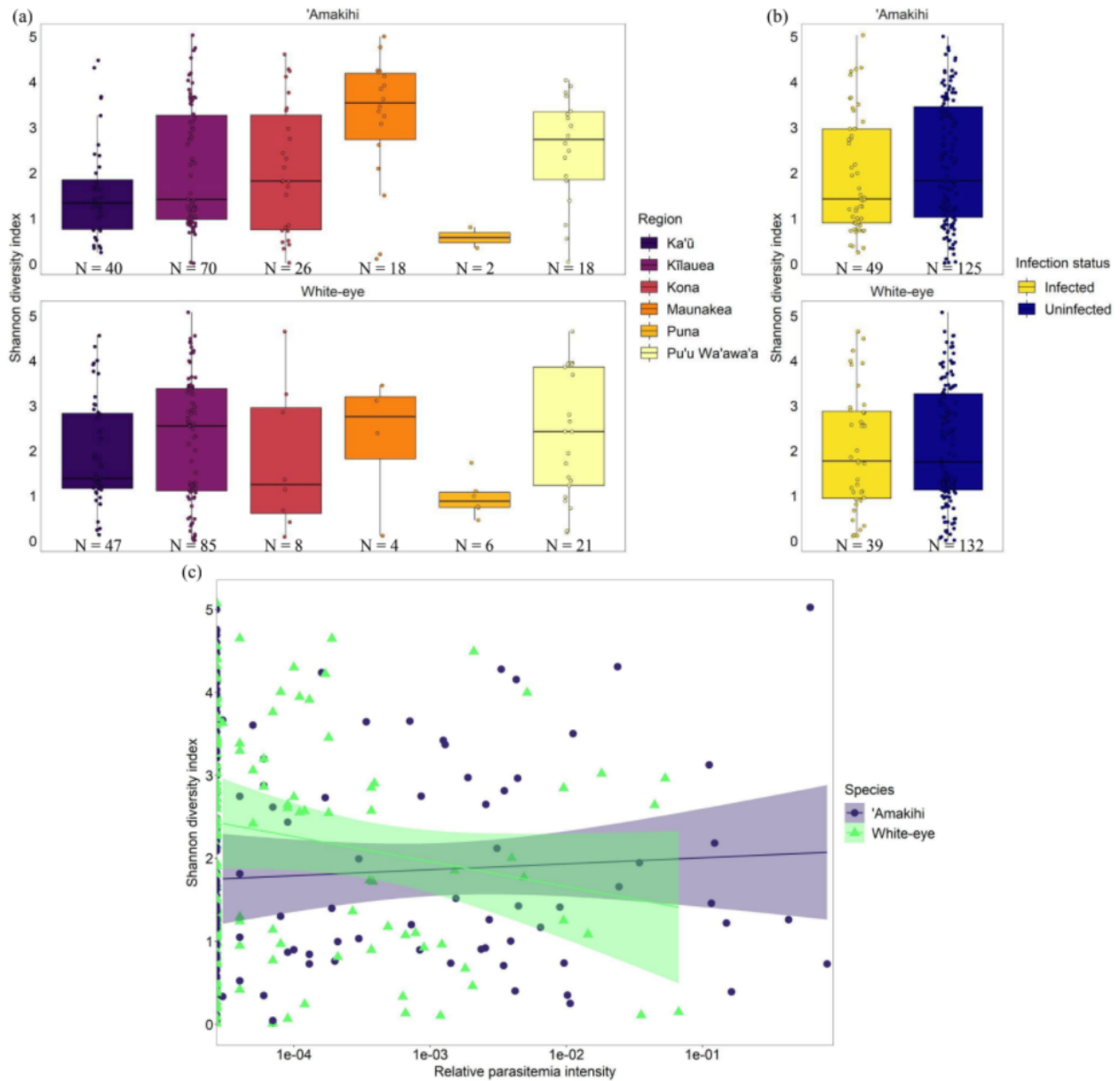


Figure 2. Shannon diversity indices of Hawai'i 'Amakihi (N = 174) and Warbling White-eye (N = 171) cloacal microbiomes (a) from different regions on Hawai'i Island, (b) categorized by *Plasmodium relictum* infection status, and (c) versus parasitemia intensity relative to the sampled population. In (a,b) boxplots show the median and interquartile range for each population, and whiskers represent the 25th and 75th percentile. In (c) curves represent linear regressions and shaded bands denote the 95% confidence interval.

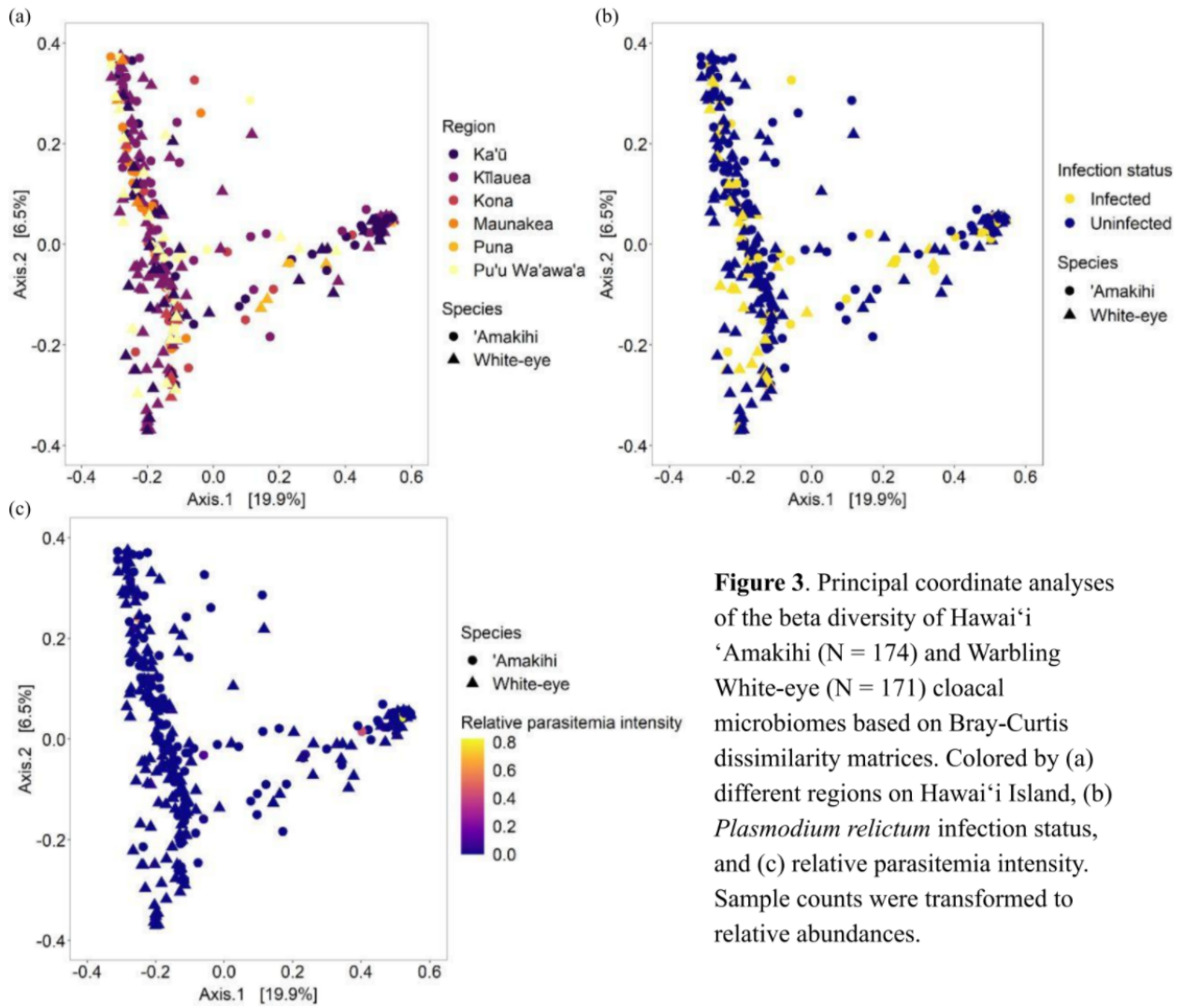


Figure 3. Principal coordinate analyses of the beta diversity of Hawai'i 'Amakihi (N = 174) and Warbling White-eye (N = 171) cloacal microbiomes based on Bray-Curtis dissimilarity matrices. Colored by (a) different regions on Hawai'i Island, (b) *Plasmodium relictum* infection status, and (c) relative parasitemia intensity. Sample counts were transformed to relative abundances.

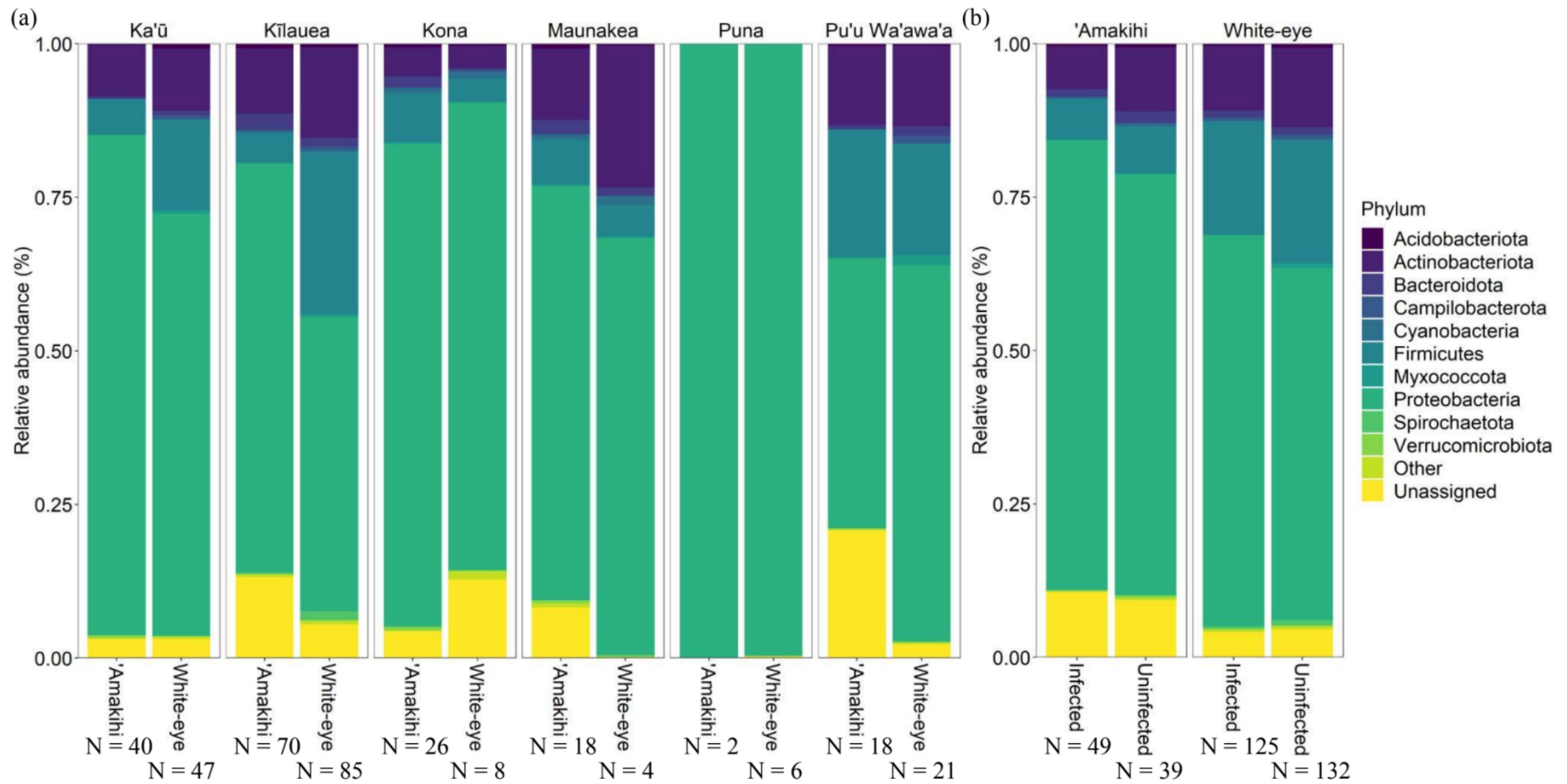


Figure 4. Relative abundances of the 10 most common phyla in the cloacal microbiomes of Hawai'i 'Amakihi (N = 174) and Warbling White-eyes (N = 171) organized by (a) region on Hawai'i Island and (b) *Plasmodium relictum* infection status.

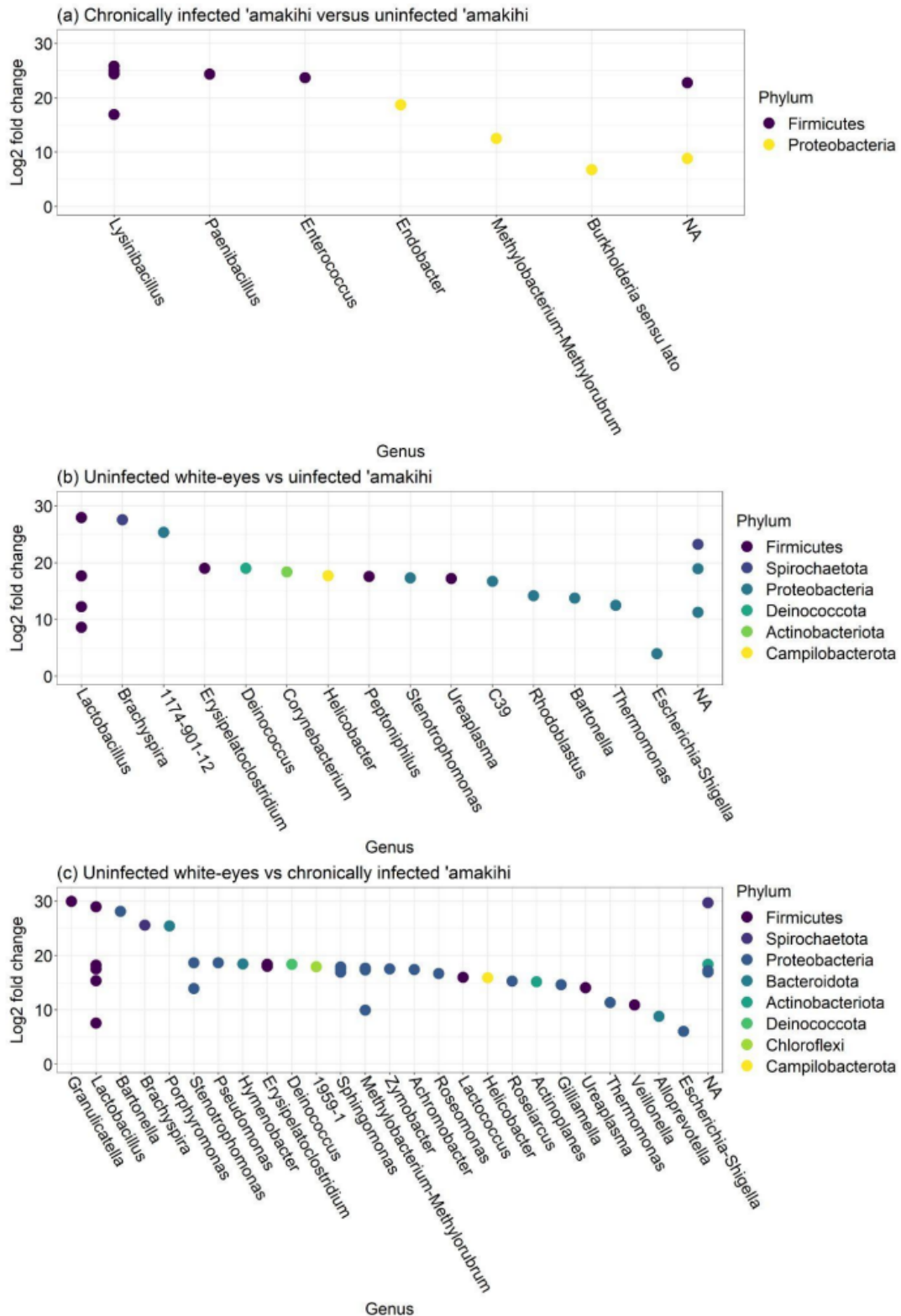


Figure 5. Log₂ fold increase of ASV abundance (p -adj < 0.05) in the cloacal microbiomes of (a) Hawai'i 'Amakihi chronically infected with avian malaria (N = 40) versus uninfected 'amakihi (N = 125), (b) uninfected Warbling White-eyes (N = 132) versus uninfected 'amakihi, and (c) uninfected white-eyes versus chronically infected 'amakihi from different regions of Hawai'i Island. NA indicates ASVs without taxonomic genus classification.

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