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Microbiomes associated with avian malaria survival differ between susceptible Hawaiian honeycreepers and sympatric malaria-resistant introduced birds

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Abstract

Of the estimated 55 Hawaiian honeycreepers (subfamily Carduelinae) only 17 species remain, nine of which the International Union for Conservation of Nature considers endangered. Among the most pressing threats to honeycreeper survival is avian malaria, caused by the introduced blood parasite *Plasmodium relictum*, which is increasing in distribution in Hawai'i as a result of climate change. Preventing further honeycreeper decline will require innovative conservation strategies that confront malaria from multiple angles. Research on mammals has revealed strong connections between gut microbiome composition and malaria susceptibility, illuminating a potential novel approach to malaria control through the manipulation of gut microbiota. One honeycreeper species, Hawai'i 'amakihi (*Chlorodrepanis virens*), persists in areas of high malaria prevalence, indicating they have acquired some level of immunity. To investigate if avian host-specific microbes may be associated with malaria survival, we characterized cloacal microbiomes and malaria infection for 174 'amakihi and 172 malaria-resistant warbling white-eyes (*Zosterops japonicus*) from Hawai'i Island using 16S rRNA gene metabarcoding and quantitative polymerase chain reaction. Neither microbial alpha nor beta diversity covaried with infection, but 149 microbes showed positive associations with malaria survivors. Among these were *Escherichia* and *Lactobacillus* spp., which appear to mitigate malaria severity in mammalian hosts, revealing promising candidates for future probiotic research for augmenting malaria immunity in sensitive endangered species.

KEYWORDS
16S rRNA gene metabarcoding, conservation biology, Hawai'i 'amakihi (*Chlorodrepanis virens*), microbiota, *Plasmodium relictum*, probiotics

1 | INTRODUCTION

Anthropogenically introduced diseases to naïve ecosystems have been responsible for myriad local extinction events in wildlife populations and pose major concerns for the conservation of biodiversity worldwide (Rogalski et al., 2017). Increasing evidence indicates that microbiomes, the microbial communities residing on or within organisms, have strong influence over host health and fitness (Suzuki, 2017), and can shape various ecological and evolutionary processes such as host–parasite interactions (Waide & Schmidt, 2020), and disease susceptibility or severity (Broom & Kogut, 2018). Microbiome research is rapidly advancing with the improvement of sequencing capabilities such as 16S ribosomal RNA gene (16S rRNA) sequencing for bacterial identification, allowing for the analysis of microbial community composition from a wide range of sources, including faecal, anal or cloacal swab samples 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 results from studies of microbiota into conservation planning (Trevelline et al., 2019).

Island species can be particularly susceptible to introduced diseases, as they often lack the immunological adaptations that their mainland counterparts have evolved through historical exposure to pathogens (van Riper et al., 1986; Warner, 1968). Such species may benefit greatly from conservation strategies that manipulate microbiota to manage disease risks. Hawaiian honeycreepers (subfamily Carduelinae) have suffered devastating losses due to the introduction of *Plasmodium relictum*, a blood parasite that causes avian malaria (van Riper et al., 1986; Warner, 1968) which is effectively vectored by the non-native mosquito *Culex quinquefasciatus* (Fonseca et al., 2000). The substantial mortality of Hawaiian honeycreepers to avian malaria makes this group of native forest birds a prime example of an island taxon that may benefit from host-microbiome disease research. Of the 55 honeycreeper species that have been documented across the Hawaiian archipelago (Lerner et al., 2011; Pratt et al., 2009), only 17 remain, nine of which are considered endangered by the International Union for Conservation of Nature (IUCN, 2022). Most of the extant honeycreeper species can only persist in high-elevation forest refugia, where mosquito population dispersal and malarial sporozoite (the infectious life stage of *P. relictum*) development are limited by lower temperatures (LaPointe et al., 2010). As global temperatures rise due to climate change, avian malaria is expected to continue expanding into higher elevations, probably leading to multiple extinctions in the coming decades without intervention (Paxton et al., 2016, 2018). Protecting Hawaiian avifauna from extinction will require conservation strategies that combat avian malaria at multiple stages, including controlling the distribution of its mosquito vector, and facilitating honeycreeper persistence in environments with increased malaria prevalence (Paxton et al., 2018).

Recent experiments with mice have revealed strong connections 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, parasite burden and mortality rates after exposure to *Plasmodium* spp. (Martinez-Gómez et al., 2006; Morffy Smith et al., 2019; Stough et al., 2016; Villarino et al., 2016; Yilmaz et al., 2014). Additionally, caecal content transplants from the guts of mice that displayed malaria resistance to mice with sterilized guts resulted in less severe infection in the recipient mice than in those that received transplants from malaria-susceptible mice (Morffy Smith et al., 2019; Villarino et al., 2016). These encouraging relationships between malaria and the microbiome illuminate a novel approach to conservation of species sensitive to malaria infection, namely the development of antimalarial probiotics. However, although 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; Palinauskas et al., 2022; Videvall, Marzal, et al., 2021). Further, extrapolating insights gained from mammalian studies to avian systems may be challenging or inappropriate as current research indicates bird–microbe symbiosis dynamics are distinct from those of other vertebrates (Bodawatta et al., 2022). Nonetheless, the possibility of such relationships warrants further investigation.

In the present study, we used quantitative polymerase chain reaction (qPCR) to determine malaria infection status and 16S rRNA gene sequencing to characterize cloacal microbiomes of wild Hawai'i 'amakihi (*Chlorodrepanis virens*) and warbling white-eyes (*Zosterops japonicus*), sampled across Hawai'i Island. The nonendangered Hawai'i 'amakihi is one of only two honeycreeper species that persists at high and low elevations throughout its historical range, including some areas of high malaria prevalence (Camp, 2019; Woodworth et al., 2005). 'Amakihi display differential mortality rates from avian malaria infection across an elevational gradient, with low-elevation populations experiencing 17% mortality compared to 60% in high-elevation populations, indicating increased disease pressure at lower elevations is selecting for the evolution of immune strategies in these populations (Atkinson et al., 2013). Regardless, 'amakihi, like all honeycreepers, are highly susceptible to infection with avian malaria, and individuals that survive infection maintain chronic low levels of the parasite in their blood following exposure, even after all disease symptoms have resolved (Atkinson et al., 2001). In contrast, warbling white-eyes, like other bird species with evolutionary histories of exposure to avian malaria, probably are able to clear circulating parasites from their blood in most instances (Cellier-Holzem et al., 2010). Warbling white-eyes, which were introduced to Hawai'i in the 1920s (Guest, 1973), suffer little to no mortality from malaria infection (van Riper et al., 1986), but serve as important reservoirs for malaria in Hawaiian forests (McClure et al., 2020), and probably retain a suite of immunological strategies against malaria, to which they are exposed in their historical range. One such strategy may be harbouring a microbiome that can confer resistance or tolerance to malarial parasites.

In this study, we included both ‘amakihi and warbling white-eyes as these two species are ecologically similar and sympatric but differ in their resistance to malaria. White-eyes are abundant in all ‘amakihi habitats (McClure et al., 2020), and both species have a similar generalist diet, foraging mainly on nectar, arthropods and fruit (Mountainspring & Scott, 1985). Microbiomes of individuals are shaped by multiple factors, such as host taxonomy (Hird et al., 2015), geographical location (Klomp et al., 2008) and diet (Bodawatta et al., 2021; Bragg et al., 2020; Hammons et al., 2010), and therefore contrasting the microbiomes of these two species with similar habitat and diet allows us to differentiate environmental effects from variation in microbiome diversity associated with malaria infection. Our goal was to identify microbes that might be associated with avian malaria infection levels and host survival by contrasting within and between species and across sites. Specifically, we (i) examined if cloacal microbiome alpha diversity (within sample variation) or beta diversity (between sample variation) covaries with *P. relictum* infection, and (ii) evaluated whether specific bacterial taxa within the cloacal microbiome were associated with malaria survival by comparing uninfected and infected ‘amakihi and white-eyes. These questions have potentially significant conservation implications as elucidating a relationship between the microbiome and malaria induced mortality in Hawaiian honeycreepers is an essential first step in assessing the feasibility of mitigating the honeycreeper extinction crisis through the manipulation of gut microbiota.

2 | MATERIALS AND METHODS

2.1 | Study sites and sample collection

Hawai‘i ‘amakihi (N = 174) and warbling white-eye (N = 172) samples were collected from 16 locations and grouped into six regions across Hawai‘i Island (Figure 1) based on geographical proximity. To assess parasitaemia levels and cloacal microbiomes of focal species, blood from the brachial wing vein and cloacal swab samples were collected from birds passively captured in mist-nets between February and July in 2019 and 2020. This time period encompasses the peak breeding season of most Hawaiian forest birds and in the aseasonal climate of Hawai‘i coincides with peak flowering and fruit availability (Wolfe et al., 2017). Moreover, this sampling window does not overlap with peak malaria transmission, which occurs in autumn (Atkinson & Samuel, 2010), when we would be more likely to sample individuals undergoing active infections. Blood samples were stored in Queen's lysis buffer and cloacal swabs were stored dry in 2-ml tubes at −20°C. In a few instances a bird was recaptured and resampled in subsequent banding efforts, but after all sequence filtering steps were complete only one sample remained for each of these birds, and therefore only one sample per bird was used in analysis.

2.2 | Determining malaria infection status

DNA was extracted from blood samples using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol at the Pathogen and Microbiome Institute at Northern Arizona University. Samples were then evaluated for *Plasmodium relictum* (genetic lineage GRW4) parasitaemia via a qPCR assay with a hydrolysis probe (Videvall, Paxton, et al., 2021) and adapted from primers targeting the cytochrome B region (Zehtindjiev et al., 2008). Each sample was tested in duplicate and threshold cycle (C_t) values were averaged between the two runs (Paxton, 2022). Samples with an average C_t value greater than 38 were classified as uninfected because repeatability of *P. relictum* detection decreased substantially after cycle 38 (Navine et al., 2022a). Relative *P. relictum* parasitaemia intensity was calculated using the formula $1/(2^{[avgCt-25]})$. 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). Thus, uninfected white-eyes in our study could comprise individuals either naïve to *P. relictum* exposure or survivors of an infection prior to capture, whereas infected white-eyes were probably 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 parasitaemia following exposure (Atkinson et al., 2001). Consequently, uninfected ‘amakihi in our study were probably naïve to *P. relictum* exposure. Although a small number of birds with low-level infections (average C_t values >30) may have been in the early phase of acute infection, parasitaemia levels climb rapidly in the first few days following inoculation (Atkinson et al., 2001) and it is unlikely we caught many birds in this narrow window. Therefore, birds with low-level infections were assumed to be those that had recovered from infection prior to capture. We selected a C_t value of 30 as the cutoff for differentiating between low- and high-level infections based on changes in C_t values of experimentally infected ‘amakihi monitored throughout infection (Videvall, Paxton, et al., 2021).

2.3 | Cloacal microbiome amplicon sequencing

We used molecular methods similar to those used in contemporary microbiome studies at the Smithsonian Conservation Biology Institute's Center for Conservation Genomics (Bragg et al., 2020; Rao et al., 2020). Briefly, we isolated DNA from the cloacal swabs and from negative control swabs using the QIAamp PowerFecal Pro DNA Kit, then quantified extracted DNA concentrations using the Qubit dsDNA HS Assay Kit and Qubit 2.0 Fluorometer (Invitrogen). We prepared DNA libraries for 16S rRNA sequencing at the University of Hawai‘i at Hilo Core Genetics Facility by amplifying the V3–V5 regions of the 16S rRNA gene, ~570 bp, using the 16S Illumina Amplicon Protocol (Illumina) and universal primers 515F (GTGCCAGCMGCCGCGGTAA) and 939R (CTTGTGCGGGCCCCCGTCAATTC). We then performed index

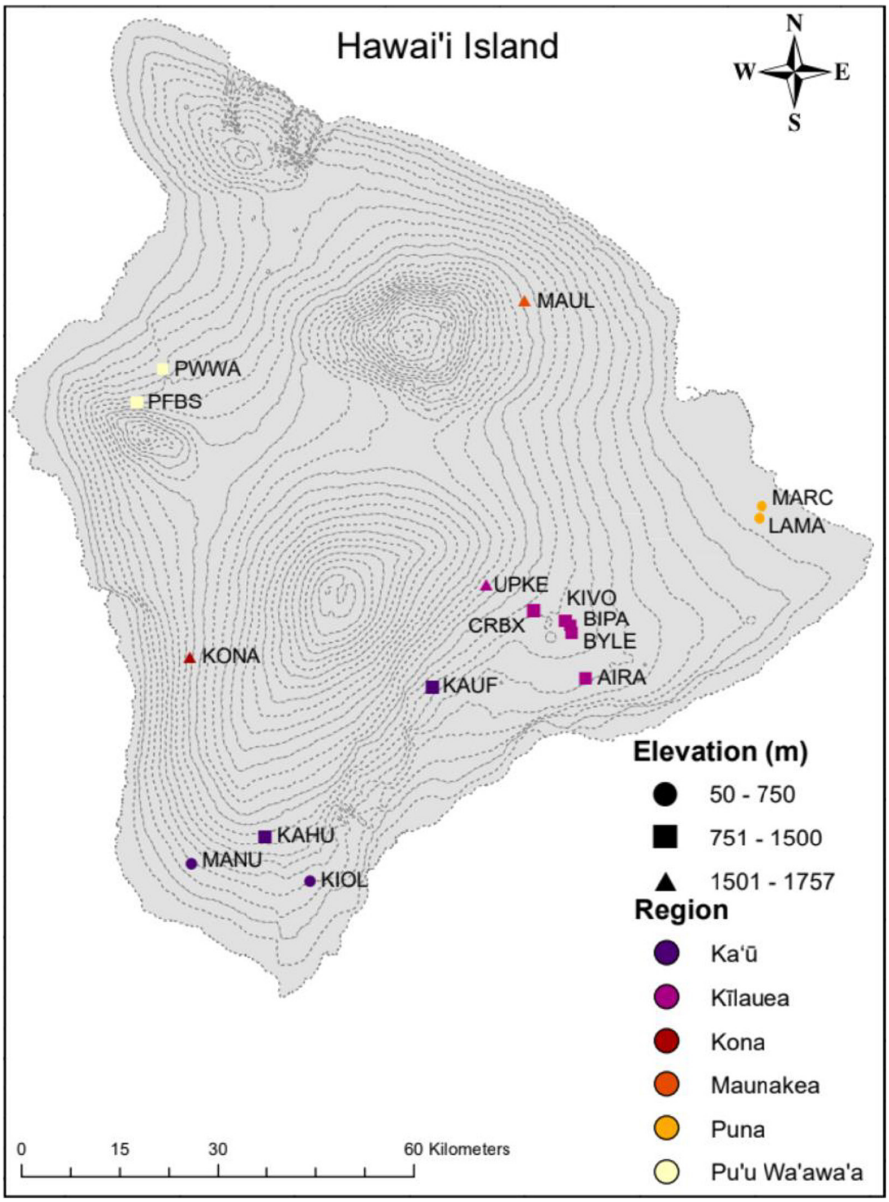

FIGURE 1 Topographic map of Hawai'i Island with 150-m elevation contours and sampling locations. The elevation of each site is indicated by point shape and assigned region is indicated by point colour.

PCR on the amplicons to attach custom i5 and i7 adaptors that provided each cloacal sample a unique sequence barcode identity. To remove off-target sequences, we used the BluePippin protocol (Sage Science) trained on a 500–640-bp fragment collection for size selection. We used Qubit to quantify the final pooled samples 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 version 3 with a 25% PhiX spike (Navine et al., 2022b).

2.4 | Bioinformatics and statistical analysis

We used FASTQC (Andrews, 2010) to quality screen the sequenced reads, TRIMMOMATIC (version 0.39; Bolger et al., 2014) to remove primer sequences, and DADA2 (version 2020.11; Callahan et al., 2016) to denoise and quality trim the reads in QIIME2 (version 2020.11; Bolyen et al., 2019). Amplicon sequence variants (ASVs) were taxonomically assigned using a naïve Bayes classifier trained on our data set using SILVA (version 138) full-length reference sequences (Quast et al., 2013). Using MAFFT (Katoh & Standley, 2013) and FASTTREE2 (Price et al., 2010) we generated a phylogenetic tree of the identified ASVs within QIIME2. Further processing and all statistical analyses were conducted in R (version 4.2.1; R Core Team, 2022). We used the R package DECONTAM (version 1.16.0; Davis et al., 2018) to filter out ASVs identified in the negative control samples. Reads identified as mitochondrial, chloroplastic or eukaryotic DNA, and reads that could not be assigned to the kingdom level, as well as ASVs with mean relative abundance $<10^{-5}$ were filtered from the data set using the package PHYLOSEQ (version 1.40.0; McMurdie & Holmes, 2013). We removed samples that had fewer than 500 reads remaining after filtering. Data visualizations were generated using the R package GGPLOT2 (version 3.3.6; Wickham, 2016).

To assess whether cloacal microbiome diversity covaries with malaria infection, we assessed both microbial alpha diversity, which

measures within-sample variation, and beta diversity, which assesses between-sample variation. We measured alpha diversity with integer-rounded Shannon diversity indices, which account for species richness and evenness (Shannon, 1948), using both rarefied and nonrarefied reads and found no differences in results. To avoid discarding reads unnecessarily, we proceeded with analyses using nonrarefied reads (McMurdie & Holmes, 2014). We assessed differences in Shannon diversity indices between host species ('amakihi, white-eye) and age (adult, juvenile, unknown), sampling years (2019, 2020), months (February–June) and regions (six regions identified in Figure 1), malaria infection status (infected, uninfected), and relative parasitaemia intensity (relative values between 0 and 1) using a negative binomial generalized linear model (nbGLM) to account for overdispersion in index values with the R package MASS (version 7.3-557; 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). We analysed Bray–Curtis dissimilarities with a permutational multivariate analysis of variance model (PERMANOVA) using the ADONIS function from the R package VEGAN (version 2.6-2; Oksanen et al., 2022), again comparing host species and age, sampling year, month and region, malaria infection status, and relative parasitaemia intensity.

To identify specific microbes that may be associated with malaria survival, we used PHYLOSEQ functions to visualize variation in the relative abundances of microbes at the phylum level between years, regions, host species and infection statuses. To identify any bacteria in 'amakihi microbiomes positively associated with disease pressure, we 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 (Paxton, 2022) while controlling for sampling year and month. 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.

We used DESEQ2 (version 1.36.0; Love et al., 2014) to test for differentially abundant ASVs between different groups while controlling for sampling year, month and region, again using the BH correction to generate *p*-adj. We made three specific comparisons. First, we compared 'amakihi with low-level malaria infection, that is individuals that survived acute malaria and are persistently infected, to uninfected 'amakihi, which were probably naïve to exposure (Atkinson et al., 2001). If certain gut microbes are conferring malaria protection, we would expect to find them in greater abundance in the survivors than in the naïve birds. Second, we compared uninfected white-eyes, all assumed to be capable of surviving malaria infection (van Riper et al., 1986), to uninfected 'amakihi. Because warbling white-eyes are resistant and able to clear the infection (Cellier-Holzem et al., 2010), we would expect to find a greater number of potentially beneficial microbes in warbling white-eyes than in uninfected 'amakihi. Among the ASVs uncovered in these two comparisons are microbes that possibly modulate malaria severity, and thus they are all candidates for further investigation, particularly ASVs that have been shown to provide malaria protection in other hosts. Last, we compared uninfected white-eyes to chronically infected 'amakihi. If gut microbes are part of the reason why chronically infected 'amakihi were able to survive, we may expect that the abundance of some of the candidate microbes identified in infected 'amakihi will be similar to the cloacal microbiomes of the malaria-resistant white-eyes.

To separate microbial communities associated with malaria infection survival from those experiencing acute disruptions associated with the active phase of infection (Ippolito et al., 2018), we excluded 'amakihi with high-level parasitaemia (average C_t values <30) and all infected white-eyes from the linear models and differential abundance analyses. We 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 of infection in mice (Mooney et al., 2015). Second, in 'amakihi that survive malaria, parasitaemia drops to chronic low levels within 30 days of infection (Atkinson et al., 2001, 2013).

3 | RESULTS

3.1 | Cloacal microbiome diversity does not covary with malaria infection

Of the 346 birds in this study, malaria infection was detected in 49 'amakihi (28%) and 39 white-eyes (23%; Table S1). Within the infected groups, nine 'amakihi and three white-eyes had high parasitaemia levels indicative of the acute phase of malaria. The other 40 infected 'amakihi were classified as malaria survivors with persistent low-level parasitaemia. Cloacal microbiome alpha diversity was strongly influenced by sampling year (nbGLM: $\chi^2[1] = 23.22$, $p < .001$; Figure 2b), but did not differ between months ($\chi^2[5] = 1.01$, $p = .962$), regions ($\chi^2[5] = 4.95$, $p = .422$; Figure 2a), bird species ($\chi^2[1] = 1.37$, $p = .242$; Figure 2a–c), ages of birds ($\chi^2[2] = 1.25$, $p = .536$) or infection status ($\chi^2[1] = 0.10$, $p = .757$; Figure 2c), nor did it covary with relative parasitaemia intensity ($\chi^2[1] = 0.09$, $p = .765$; Figure S1). The largest amount of variation in cloacal microbiome beta diversity was explained by the year the sample was collected (PERMANOVA: $R^2 = .12$, $F_{1,330} = 52.24$, $p = .001$; Figure S2b), followed by sampling region ($R^2 = .07$, $F_{5,330} = 6.50$, $p = .001$; Figure S2a), sampling month ($R^2 = .04$, $F_{5,330} = 3.53$, $p = .001$), bird species ($R^2 = .02$, $F_{1,330} = 7.66$, $p = .001$; Figure S2a–d) and age ($R^2 = .01$, $F_{2,330} = 1.50$, $p = .038$; Figure S2d). Beta diversity did not vary between infected and uninfected birds ($R^2 = .00$, $F_{1,330} = 1.36$, $p = .125$; Figure S2c), nor did it covary with relative infection intensity ($R^2 = .00$, $F_{1,330} = 0.91$, $p = .581$).

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

We identified 3751 ASVs in all cloacal microbiome samples. For both 'amakihi and white-eyes, the most abundant phyla were

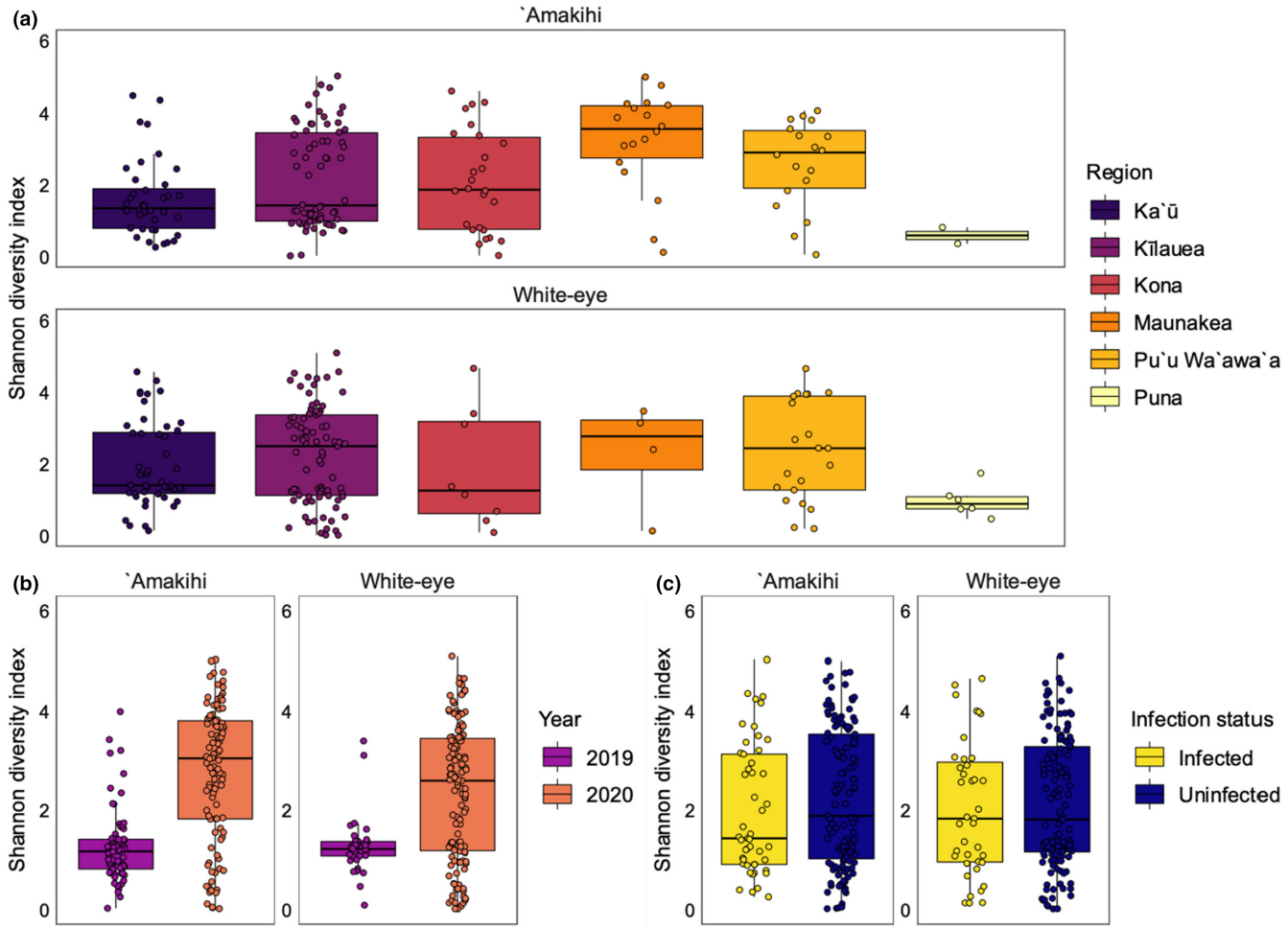

FIGURE 2 Shannon diversity indices of Hawai'i 'amakihi ($N = 174$) and warbling white-eye ($N = 172$) cloacal microbiomes sampled from (a) different regions on Hawai'i Island and categorized by (b) sampling year and (c) malaria infection status. Boxplots show the median and interquartile range for each population, and whiskers represent the 25th and 75th percentiles.

Proteobacteria (69% and 58%, respectively), Firmicutes (7% and 19%, respectively) and Actinobacteria (9% and 12%, respectively; Figure 3; Table S2). The most abundant genera differed between the two host species. The top genera in 'amakihi microbiomes were *Pseudomonas* (17%), *Cutibacterium* (4%), *Thermomonas* (2%) and *Blastomonas* (2%), while the top genera of white-eye samples were *Lactobacillus* (12%), *Pseudomonas* (11%), *Escherichia/Shigella* (7%; these genera are closely related and difficult to distinguish—Devanga Ragupathi et al., 2017—and thus were grouped together) and *Cutibacterium* (6%; Table S2).

Of the 3751 ASVs in our study, three of them, an alphaproteobacterium that could not be identified beyond the class level (LM: $R^2 = .07$, $F_{1,163} = 13.74$, p-adj = .026) and two *Pseudomonas* spp. ($R^2 = .08$, $F_{1,163} = 14.67$, p-adj = .018; $R^2 = .12$, $F_{1,163} = 23.63$, p-adj = .001; Table S3), increased in abundance with increasing malaria prevalence at sampling sites in 'amakihi microbiomes. Additionally, 354 ASVs were identified in our differential abundance analyses, with 149 being positively associated with birds likely to survive malaria infection (p-adj < .05; Figure 4; Tables S4–S6), and therefore candidate microbes for further exploration. Our comparison between chronically infected 'amakihi (malaria survivors) and uninfected 'amakihi (malaria naïve) revealed 107 differentially abundant ASVs, 66 of which were more abundant in the survivors (Figure 4a). In our first interspecific comparison, 128 ASVs were differentially abundant between uninfected white-eyes and uninfected 'amakihi, with 61 showing positive associations with white-eyes (Figure 4b). In the second comparison, 119 ASV abundances differed between uninfected white-eyes and infected 'amakihi, but only 22 of those ASVs were more abundant in the white-eyes (Figure 4c).

4 | DISCUSSION

Our study is among the first to assess correlates between malaria infection and microbiome composition in avian hosts sampled in a natural system. The volume of research supporting critical connections between microbiomes and malaria susceptibility and severity in mammals is steadily growing (Bamgbose et al., 2021); however, little comparable research has been conducted in other host taxa or in wild environments (Trevelline et al., 2019; but see Lutz et al., 2021;

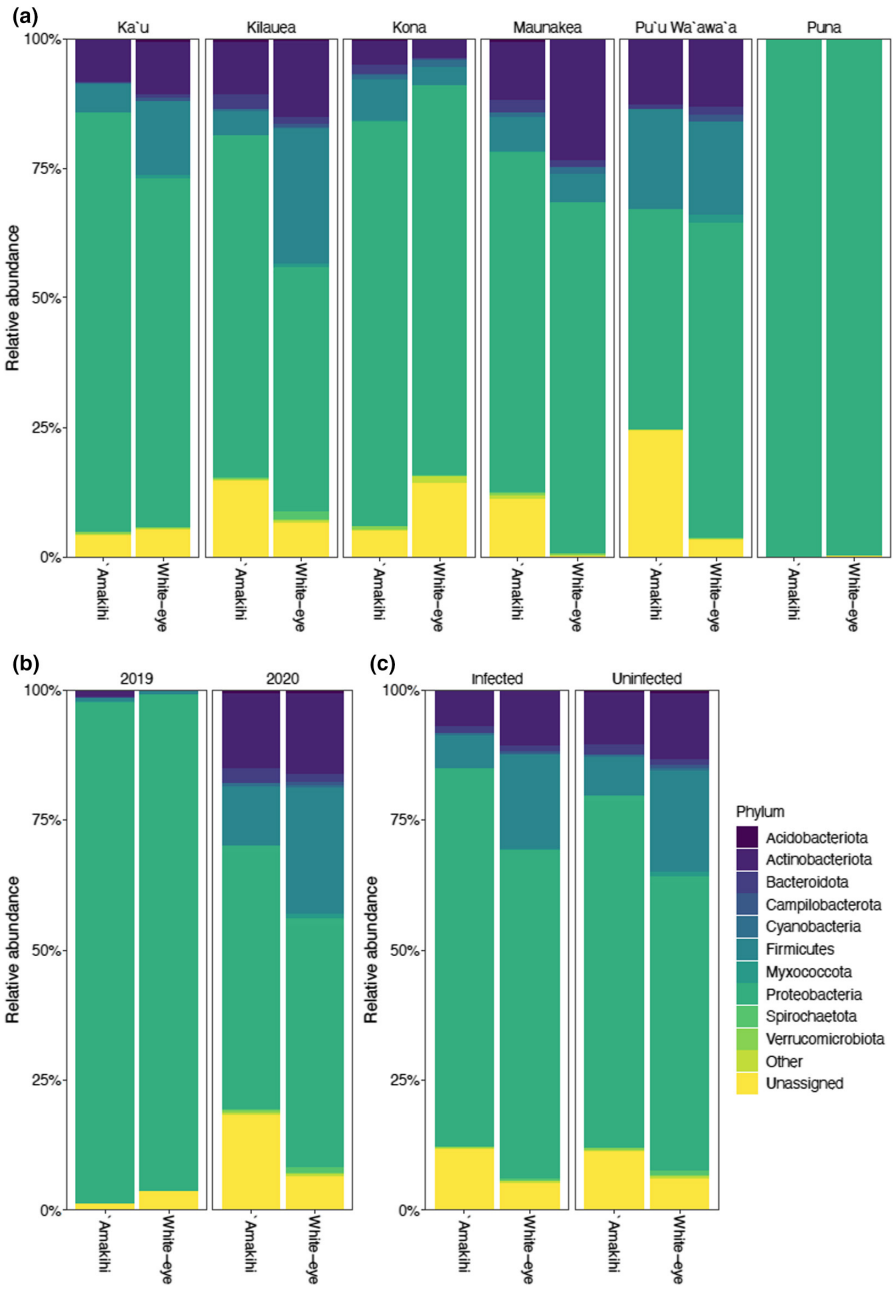

FIGURE 3 Relative abundances of the 10 most common phyla in the cloacal microbiomes of Hawai'i 'amakihi ($N = 174$) and warbling white-eye ($N = 172$) organized by (a) sampling region, (b) sampling year and (c) malaria infection status.

Palinauskas et al., 2022; Videvall, Marzal, et al., 2021). We found no difference in cloacal microbiome alpha diversity between Hawai'i 'amakihi and warbling white-eyes and only a small, yet significant, difference in beta diversity, which aligns with several studies that have reported few consistent differences across host species that are relatively close phylogenetically (Hird et al., 2014). Although microbiome diversity did not covary with malaria infection, we identified 149 bacterial ASVs that were more abundant in birds that probably possess some immunity to malaria. Some of these candidate microbes may contribute to malaria severity modulation in wild birds and are valuable targets for functional profiling aimed at uncovering a mechanism by which malaria immunity may be augmented in Hawaiian honeycreepers.

Environmental factors have been shown to strongly influence avian microbial communities (Bodawatta et al., 2022; Hird et al., 2014), and therefore finding significant levels of variation in the cloacal microbiomes of birds sampled at different sites that ranged from 50 to 1750 m in elevation and from heavily anthropogenically disturbed to nearly pristine native forest was expected. However, finding that microbiome diversity was highly variable between the 2 years, despite no methodological differences in sample collection and processing techniques, was unexpected. It is possible that microbiome differences between the 2 years may have been partially attributable to variable weather patterns altering food item availability in the months leading up to sampling (Bodawatta et al., 2021). For instance, mean and minimum monthly temperatures, as well as departure from normal mean and minimum monthly temperatures, in January to July (our sampling windows) varied between 2019 and 2020, with 2020 being generally warmer (National Oceanic and Atmospheric Administration,

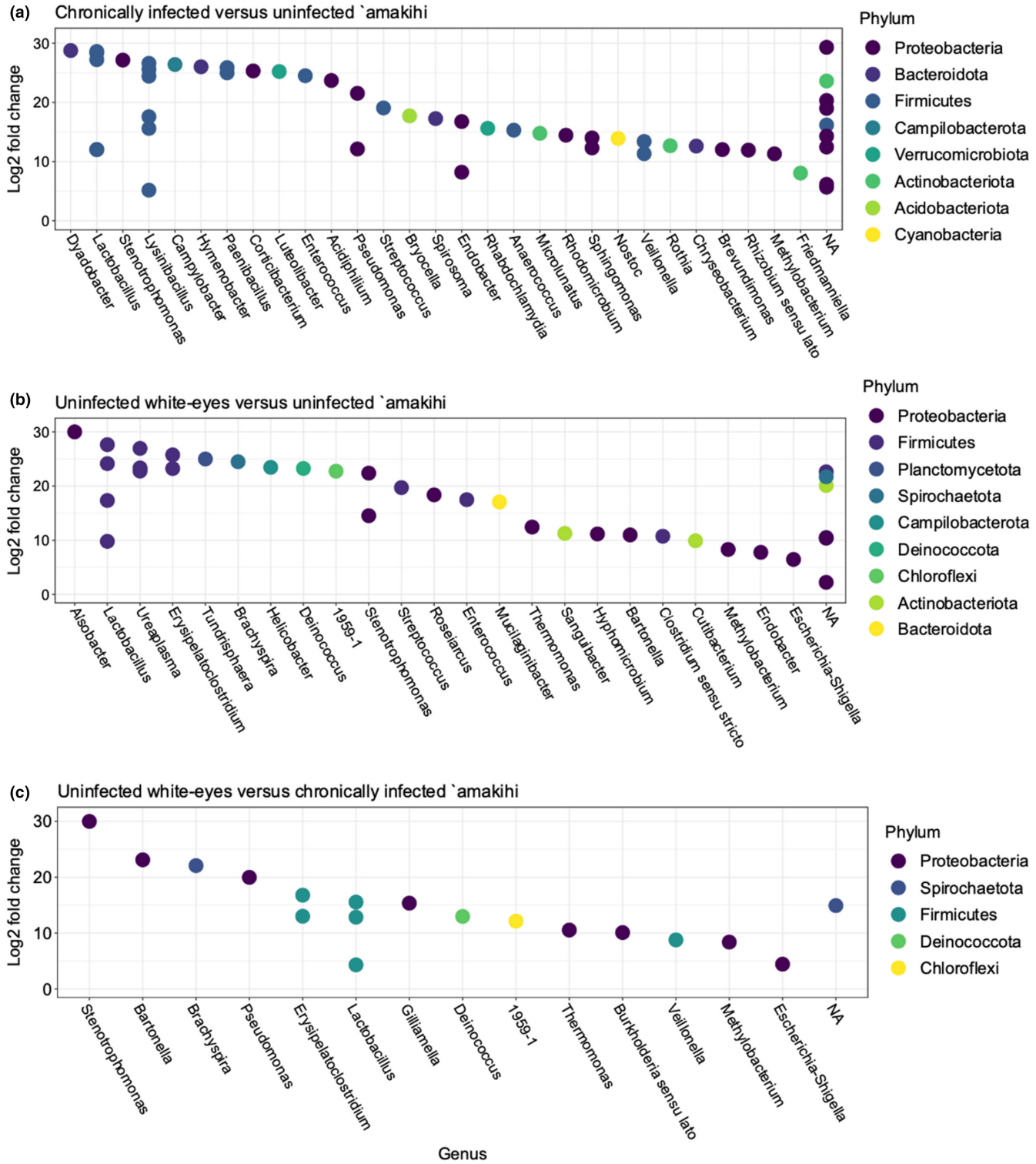

FIGURE 4 Log_2 fold increase of ASV abundance (p-adj < .05) in the cloacal microbiomes of (a) chronically malaria infected ($N = 40$) versus uninfected ($N = 125$) Hawai'i 'amakihi, (b) uninfected warbling white-eyes ($N = 133$) versus uninfected 'amakihi ($N = 125$) and (c) uninfected white-eyes ($N = 133$) versus chronically infected 'amakihi ($N = 40$). Points indicate ASVs more abundant in infected 'amakihi in (a) and in white-eyes in (b, c). NA indicates ASVs without taxonomic genus classification.

National Centers for Environmental Information, 2022). However, total monthly rainfall and deviation from normal rainfall did not vary between years in the months that were sampled. Climatic 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. Both Hawai'i 'amakihi and warbling white-eyes positive for *P. relictum*

showed higher abundance of Proteobacteria, which is congruent with gut microbiota imbalances seen in mammals with malaria (Bamgbose et al., 2021; Ippolito et al., 2018). Proteobacteria are frequently among the most abundant bacteria in bird gut microbiomes (Bodawatta et al., 2022), and their higher abundance in infected birds may be due to opportunistic expansion in compromised hosts (Shin et al., 2015). More informative are the patterns that we 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 disease severity in mammalian species (Bamgbose et al., 2021). In laboratory mice, experimental colonization of the gut with *Escherichia coli* O86:B7 stimulated the production of galactose-alpha-1,3-galactose (alpha-gal) antibodies by the host's immune system, enabling the rapid identification and suppression of alpha-gal-expressing *Plasmodium* during infection (Yilmaz et al., 2014). A recent study demonstrated that infection with *P. relictum* induces alpha-gal antibodies in bird sera (Palinauskas et al., 2022), indicating a similar mechanism of protection may extend to avian hosts. *Lactobacillus* spp. are common vertebrate symbionts with diverse functions and documented abilities to modulate host immune responses, and have been the focus of many probiotic studies, including those exploring malaria control strategies (Bamgbose et al., 2021). In a model mouse system, supplementation of yogurt spiked with *Lactobacillus* spp. significantly attenuated the severity of malaria in susceptible mice (Villarino et al., 2016), potentially by increasing nitric oxide concentrations in the host's blood, thereby inhibiting *Plasmodium* growth during the erythrocytic stage of infection (Martinez-Gómez et al., 2006). No connection between *Pseudomonas* spp. in host microbiomes and *Plasmodium* infection has yet been uncovered. However, the presence of *Pseudomonas putida* in vector mosquito midguts, where the bacteria can interact with parasites directly, has been implicated in blocking *Plasmodium* development (Bahia et al., 2014).

Lactobacillus and *Escherichia/Shigella* were among the top genera in white-eye microbiomes, accounting for ~12% and 7% ASV relative abundance, respectively, but both were relatively uncommon, <1% relative abundance, in ʻamakihi. Further, four *Lactobacillus* spp. and an *Escherichia/Shigella* sp. (as well as two ASVs in the same family, Enterobacteriaceae, that could not be identified to the genus level) were significantly more abundant in white-eyes than in ʻamakihi. Cross-reactive alpha-gal antibody production stimulated by these microbes may be working synergistically with white-eye genetics and behaviours to protect them from malaria mortality. Interestingly, one *Lactobacillus* sp. in particular was significantly more abundant in white-eyes and in ʻamakihi that survived malaria infection than in ʻamakihi naïve to *P. relictum* exposure. However, white-eyes and survivor ʻamakihi had similar abundances of this *Lactobacillus* sp. Taken together, these comparisons indicate that harbouring a greater abundance of these microbes in the gut microbiome may potentially be contributing to increased survival rates in ʻamakihi. *Pseudomonas* was the top genus in ʻamakihi microbiomes, accounting for ~17% ASV relative abundance, and two *Pseudomonas* spp. found in ʻamakihi microbiomes were positively associated with malaria prevalence at sampling sites. However, because malarial parasites circulate in the blood of avian hosts, it is unclear if *Pseudomonas* spp. can block *Plasmodium* development in birds as has been found in mosquito midguts (Bahia et al., 2014).

Although the number of avian microbiome studies is rapidly growing (Bodawatta et al., 2022), the current lack of characterized avian microbes in taxonomic databases poses barriers to the interpretation of the data generated by studies such as ours. Even at the phylum level, we were 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 (Morffy Smith et al., 2019; Stough et al., 2016; Villarino et al., 2016; Yilmaz et al., 2014); however, we used cloacal microbiome samples because they are substantially less invasive to collect and were logistically practical. Cloacal and faecal 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, caecum or colon in laboratory experiments may reveal stronger associations between commensal microbes and infection response. Field studies that concentrate efforts on resampling individuals over time or controlled laboratory experiments (such as proposed in Palinauskas et al., 2022) would facilitate tracking microbiome changes over the course of infection, and potentially also the sampling of microbiomes of birds that succumb to malaria, which was not possible for our study. We used cross-sectional comparisons between chronically infected survivors and uninfected, malaria-naïve ʻamakihi, as well as comparisons with a malaria-resistant species, to identify microbes that may be associated with malaria immunity. Thus, we are unable to distinguish if greater ASV abundances result from a selective pressure to harbour bacteria with protective effects, a proliferation as a result of the potential disruptive effects of chronic infection, or species-specific differences. Moreover, our pool of naïve ʻamakihi probably consisted of a mixture of birds that would either survive or die upon exposure; this heterogeneity may have weakened our ability to detect if beneficial microbes are in greater abundance in survivors. Although additional studies to support our findings are needed, we were able to detect promising associations between gut microbiomes and malaria parasites in both susceptible and resistant birds in a natural setting.

To conclude, we have shown that the malaria-resistant white-eye harbours microbes with established connections to malaria immunity in greater abundance than ʻamakihi. The candidate microbes we have outlined here warrant further investigation 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 (Bamgbose et al., 2021). *Pseudomonas*

spp. may also be valuable candidates for investigation as they appear to be naturally abundant in ʻamakihi microbiomes, may inhibit *P. relictum* development (Bahia et al., 2014), and could represent a unique and unexplored immune strategy evolving in ʻamakihi. Orally delivered prophylactic probiotics have been effective in mitigating disease experimentally with mice and poultry models (Broom & Kogut, 2018; Hodžić et al., 2020; Mateos-Hernández et al., 2020; Villarino et al., 2016) and have garnered scientific attention for their potential to lessen the burden of malaria on human health (Bamgbose et al., 2021; Hodžić et al., 2020; Ngwa & Pradel, 2015). They may also provide a useful strategy for the conservation of wildlife species on the brink of extinction. Targeted probiotics that could be administered in food supplementations to endangered Hawaiian honeycreepers in the wild and captive breeding programmes may be an effective and practical approach for bolstering malaria immunity in the face of increasing disease pressure in Hawaiʻi.

AUTHOR CONTRIBUTIONS

E.V., K.L.P., E.H.P., R.C.F. and A.K.N. conceived the experimental design. E.V., K.L.P., R.C.F., E.H.P. and P.J.H. were involved in funding acquisition. E.H.P. was responsible for supervising sample collection and A.K.N participated in sample collection. J.T.F. and N.M. supervised laboratory work and A.K.N. performed laboratory work. N.M. sequenced the samples. E.V. designed the bioinformatics pipeline and E.V. and K.L.P. supervised data analysis. A.K.N. analysed the data, generated data visualizations and wrote the original manuscript. All authors were involved in reviewing, editing and approving the final manuscript.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest for this article.

DATA AVAILABILITY STATEMENT

16S rRNA sequences have been deposited in the Sequence Read Archive at NCBI (PRJNA794822). Metadata and code scripts have been deposited with Dryad (https://doi.org/10.5061/dryad.g1jwstqsg). Malaria prevalence data are available at https://doi.org/10.5066/P9F519WO (Navine et al., 2022a, 2022b; Paxton, 2022).

BENEFIT-SHARING

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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