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ARTICLE

Disease Ecology

The interplay of fungal and bacterial microbiomes on rainforest frogs following a disease outbreak

Donald T. McKnight[1] | **Roger Huerlimann**[1,2] | **Deborah S. Bower**[1,3] | **Lin Schwarzkopf**[1] | **Ross A. Alford**[1] | **Kyall R. Zenger**[1]

[1]College of Science and Engineering, James Cook University, Townsville, Queensland, Australia

[2]Marine Climate Change Unit, Okinawa Institute of Science and Technology, Onnason, Okinawa, Japan

[3]School of Environmental and Rural Science, University of New England, Armidale, New South Wales, Australia

Correspondence
Donald T. McKnight
Email: donald.mcknight@my.jcu.edu.au

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Abstract

Emerging infectious diseases are a serious threat to wildlife populations, and there is growing evidence that host microbiomes play important roles in infection dynamics, possibly even mitigating diseases. Nevertheless, most research on this topic has focused only on bacterial microbiomes, while fungal microbiomes have been largely neglected. To help fill this gap in our knowledge, we examined both the bacterial and fungal microbiomes of four sympatric Australian frog species, which had different population-level responses to the emergence of chytridiomycosis, a widespread disease caused by the fungal pathogen *Batrachochytrium dendrobatidis (Bd)*. We sequenced 16,884 fungal amplicon sequence variants (ASVs) and 41,774 bacterial ASVs. Bacterial communities had higher richness and were less variable within frog species than were fungal communities. Nevertheless, both communities were correlated for both ASV richness and beta diversity (i.e., frogs with similar bacterial richness and community composition tended to also have similar fungal richness and community composition). This suggests that either one microbial community was having a large impact on the other or that they were both being driven by similar environmental factors. For both microbial taxa, we found little evidence of associations between *Bd* (prevalence or intensity) and either individuals' ASVs or beta diversity. However, there was mixed evidence of associations between richness (both bacterial and fungal) and *Bd*, with high richness potentially providing a protective effect. Surprisingly, the relative abundance of bacteria that have previously been shown to inhibit *Bd* was also positively associated with *Bd* infection intensity, suggesting that a high relative abundance of those bacteria provides poor protection against infection.

KEYWORDS

16S, amphibians, Anura, bacteria, *Batrachochytrium dendrobatidis*, chytridiomycosis, disease, fungi, ITS, metabarcoding, microbiome

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INTRODUCTION

Emerging infectious diseases threaten many wildlife populations, and conservation efforts will benefit from understanding the factors that cause some populations to experience epizootic outbreaks while allowing others to coexist with pathogens in an enzootic state (Daszak et al., 2000; Smith et al., 2006). It is well established that microbiomes play important roles in the ecology and biology of multicellular organisms, including mitigating diseases (Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Mao-Jones et al., 2010; Mattoso et al., 2011). Therefore, investigations of the relationships between pathogens and host microbiomes can help in understanding the emergence of diseases and managing the threats they pose (e.g., via bioaugmentation using probiotics).

The amphibian fungal disease chytridiomycosis is an ideal candidate for understanding the interactions of microbiomes with emerging infectious diseases. Chytridiomycosis is caused primarily by the pathogen *Batrachochytrium dendrobatidis* and has caused declines or extinctions in over 500 species of amphibians worldwide (Berger et al., 1998; Lips et al., 2006; Scheele et al., 2019; Skerratt et al., 2007). However, not all amphibian species are susceptible to chytridiomycosis, and some species and populations that underwent initial declines have transitioned to an enzootic state and are persisting or increasing, despite the continued presence of the pathogen (McKnight et al., 2017; Scheele et al., 2017). The reasons for these differences among species and populations are not entirely clear, but variations in host microbiomes may play a key role.

Most research on interactions between host microbiomes and disease has focused on bacterial microbiomes, and studies have shown promising results, particularly with regard to *Bd* mitigation (Rebollar et al., 2020). Some bacteria from amphibians' skin are capable of inhibiting the growth of *Bd* in vitro (Becker & Harris, 2010; Bell et al., 2013; Harris et al., 2006; Lauer et al., 2007, 2008), and supplementing amphibians' microbiomes with inhibitory bacteria can increase survival in laboratory trials (Becker et al., 2009; Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Muletz et al., 2012). Additionally, the composition of frogs' bacterial communities and host population persistence are often correlated (K. A. Bates et al., 2018; Bell et al., 2018; Burkart et al., 2017; Catenazzi et al., 2018; Flechas et al., 2012; Jani et al., 2017; Kueneman et al., 2016; Lam et al., 2010; Woodhams et al., 2007).

Despite this wealth of research on bacterial microbiomes, much less is known about fungal microbiomes (Rebollar et al., 2020). Within vertebrates, fungal microbiome studies are largely restricted to humans (Findley et al., 2013; Hoffmann et al., 2013; Huffnagle & Noverr, 2013; Wargo & Hogan, 2006), domesticated animals (Chermprapai et al., 2019; Kittelmann et al., 2013), and laboratory rodents (Scupham et al., 2006). Relatively few studies have examined fungal microbiomes of vertebrate wildlife (Allender et al., 2018; Chen et al., 2018; Harrison et al., 2021; Kearns et al., 2017; Kueneman et al., 2016, 2017; Medina et al., 2019). Further, while these studies provide valuable starting points, they have often had limitations, in that they were either conducted in captivity (Chen et al., 2018; Kearns et al., 2017), which alters microbiomes (Becker et al., 2014; Loudon, Woodhams, et al., 2014), or used 18S primers (Kueneman et al., 2016, 2017), which provide less information about fungal microbiomes than do internal transcribe sequences (ITS) primers (Schoch et al., 2012).

This dearth of research on vertebrate fungal microbiomes is particularly surprising given that fungal diseases are among the most widespread and virulent emerging infectious diseases in wildlife (Fisher et al., 2012). Fungal microbiomes may have important interactions with both the host and bacterial microbiomes and play key, but currently overlooked, roles in the infection dynamics of these diseases. Indeed, one of the few studies to examine fungal microbiomes and *Bd* found that captive poison dart frogs (*Dendrobates* spp.) harbored both fungi that inhibited the growth of *Bd* and fungi that enhanced its growth (Kearns et al., 2017). More research is needed to understand the relationships between bacterial and fungal microbiomes in vertebrates and whether those relationships influence infection dynamics and affect host responses to disease outbreaks.

The frog fauna of the Wet Tropics in Queensland, Australia provides a useful study system for examining these issues. This fauna includes four sympatric, stream-dwelling species of frogs that responded differently to a *Bd* outbreak in the late 1980s and the early 1990s. During the outbreak, the Australian lace-lid frog (*Litoria dayi*) was extirpated from upland sites (>300–400 m in elevation) but persisted in the warmer low-elevation sites (McDonald & Alford, 1999). Its populations have never recovered, and it continues to be restricted to sites below 300–400 m in elevation (Bell et al., 2020; McKnight et al., 2017, 2020). The waterfall frog (*Litoria nannotis*) experienced the same pattern of declines (Laurance et al., 1996; McDonald & Alford, 1999; Richards et al., 1993), but, starting in the early 2000s, it began recolonizing upland sites, and stable, breeding populations are now present at many (but not all) locations from which it had been extirpated (Bell et al., 2020; McKnight et al., 2017; McKnight, Lal, et al., 2019). The green-eyed treefrog (*Litoria serrata* [formerly *genimaculata*]) went through a similar pattern of declines and recoveries, but it never fully disappeared from

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upland locations, and has shown the strongest recovery (Bell et al., 2020; McDonald & Alford, 1999; Richards & Alford, 2005). Finally, the stony creek frog (*Litoria wilcoxii*) never noticeably declined at any elevation.

Our study had three related overarching goals: (1) to compare the attributes of fungal and bacterial microbiomes to further our understanding of their interactions and potential importance for their amphibian hosts, (2) to examine potential associations between fungal and bacterial microbiomes and historical, species-level patterns of declines and recovery from *Bd* (e.g., comparing alpha diversity among species with different histories of declines and recoveries), and (3) to examine the potential associations of fungal and bacterial microbiomes with *Bd* infections at the individual level (e.g., testing for correlations between individuals' alpha diversity and *Bd* load).

MATERIALS AND METHODS

Study sites and sampling

We sampled frogs from three national parks in the Wet Tropics of Queensland, Australia: Paluma Range National Park, Kirrama Range National Park, and Tully Gorge National Park (the lower section of Kirrama is now part of Girramay National Park, but we will refer to the entire area simply as Kirrama). At Paluma and Kirrama, upland and lowland sites were sampled, but at Tully, only a single lowland site was available. We defined lowland as <400 m in elevation, because that is the threshold above which declines and disappearances occurred in this area (McDonald & Alford, 1999). To reduce habitat effects within sites, when possible, we sampled all species from the same stretch of stream, and we sampled the upland and lowland portions of the same stream. To minimize potential effects of season and weather, at each sampling location, we sampled all individuals of all species within a single night (with a few exceptions at the Paluma lowlands), and we sampled all locations over an 11-night period. To quantify any contaminants present from sampling or laboratory methods, on each night of sampling, we collected a blank swab, and treated them as samples during extraction, amplification, and sequencing (McKnight, Huerlimann, et al., 2019b; Salter et al., 2014). We sampled a total of 169 frogs (Table 1).

We located frogs by spotlighting along streams at night. Each frog was captured and handled with new nitrile gloves, rinsed with sterile water to remove

TABLE 1 Summary of sample data

Species	Park	Elevation (m)	N	N *Bd*+
Litoria dayi	Kirrama	Lowland (288)	10[a]	8[a]
L. dayi	Tully	Lowland (213)	10	5
Litoria nannotis	Paluma	Lowland (304)	10	7
L. nannotis	Paluma	Upland (571)	10	8
L. nannotis	Kirrama	Lowland (288)	11	3
L. nannotis	Kirrama	Upland (720)	10[a]	7[a]
L. nannotis	Tully	Lowland (213)	8	5
Litoria serrata	Paluma	Lowland (351)	10	5
L. serrata	Paluma	Upland (679)	19[b]	10[b]
L. serrata	Kirrama	Lowland (291)	10	6
L. serrata	Kirrama	Upland (650)	16	9
Litoria wilcoxii	Paluma	Lowland (345)	9[c]	5
L. wilcoxii	Paluma	Upland (781)	10	4
L. wilcoxii	Kirrama	Lowland (272)	10	6
L. wilcoxii	Kirrama	Upland (594)	7	2
L. wilcoxii	Tully	Lowland (213)	9	4

Note: Elevation (m) is the mean elevation for a given species at a given site. N = total number of individuals sequenced. N *Bd*+ = the number of *Bd*-positive (infected) individuals based on qPCR.

[a]1 frog was not sequenced for fungi.

[b]17 frogs (9 *Bd*+) were sequenced for both fungi and bacteria, 1 (*Bd*+) was only sequenced for fungi, and 1 (*Bd*+) was only sequenced for bacteria.

[c]Two frogs were not sequenced for bacteria.

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transient bacteria (Lauer et al., 2007), and swabbed with a single, sterile, rayon-tipped swab (Medical Wire, MW113), using a total of 25 strokes: five on the stomach, five on the underside of each thigh, and five on the underside of each rear foot. After swabbing, swabs were immediately placed on dry ice, where they remained until they were transferred to a −80°C freezer. We released all frogs at their collection sites shortly after capture. For consistency, the same researcher (Donald T. McKnight) swabbed all frogs.

Throughout this study, we refer to *L. wilcoxii*; however, there is a wide zone of hybridization between *L. wilcoxii* and the morphologically indistinguishable *Litoria jungguy* (the two species were recently split), and either species or hybrids could have been present at some of our sites (Donnellan & Mahony, 2004). Neither species declined during chytridiomycosis outbreaks, and given that they do not differ noticeably in ecology or morphology, we will treat them as if they were a single species for the purposes of our study.

Laboratory methods

We extracted fungal and bacterial DNA from the samples using a modified version of the CTAB protocol (Doyle & Doyle, 1987). It was modified by adding a bead-beating step to lyse fungal cells and a lysozyme incubation step to lyse gram-positive bacteria. We also used an overnight proteinase K digestion (see detailed protocol in Appendix S1). The DNA from each extraction was used for *Bd* qPCR, bacterial sequencing, and fungal sequencing.

The *Bd* load on each sample was quantified using triplicate qPCR following the standard protocol for *Bd* (Boyle et al., 2004). We only scored a frog as *Bd*+ if all three replicates were positive. Standards containing the DNA of 70, 7, 0.7, and 0.07 *Bd* zoospore equivalents were used to construct a standard curve and calculate zoospore equivalents in each sample. The qPCRs were performed by a commercial laboratory (Cesar, Melbourne, Australia).

We prepared samples for sequencing following the Illumina 16S Metagenomics Sequencing Library Preparation guide (Illumina, 2017), with a few modifications. We amplified the bacterial DNA using the S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) primer pair recommended by Illumina, and we amplified the ITS2 region of the fungal genome using the ITS3_KY02 (5′-GATGAA GAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) primer pair (Toju et al., 2012). Separate PCRs and subsequent workflows were used for fungi and bacteria.

For each set of primers (bacterial and fungal), we used KAPA HiFi DNA Polymerase and triplicate 10 μl PCRs to amplify the DNA. We visually inspected for amplification with gel electrophoresis and pooled the triplicates for each sample. We cleaned the pooled samples with Sera-mag SpeedBeads followed by a 40 μl indexing PCR. We used gel electrophoresis to check the results for consistent amplification, cleaned the samples again, and quantified the DNA with a QuantiFluor. We standardized the DNA concentrations and pooled samples into a single fungal and single bacterial library. We sequenced each library on separate runs of an Illumina MiSeq (Reagent Kit v3 600 cycles PE, Illumina, San Diego, CA). We included 20% and 10% of PhiX in the fungal and bacterial sequencing runs, respectively.

Bioinformatics and quality control

Both bacterial and fungal data sets were processed using DADA2 (v1.14.1; Callahan et al., 2016) in R (v3.6.3; R Core Team, 2017). For bacteria, the DADA2 tutorial was followed (https://benjjneb.github.io/dada2/tutorial.html) with standard parameters except for truncLen = c (270, 230), trimLeft = c(20, 21), and maxEE = c(3, 5). The Silva database (v138.1; updated 10 March 2021; Quast et al., 2013) was used both for assignTaxonomy and addSpecies in DADA2. For fungi, the reads were processed using the DADA2 ITS Pipeline Workflow 1.8 (https://benjjneb.github.io/dada2/ITS_workflow.html) with default parameters except for maxEE = c (3, 4). Trimming was done using cutadapt (v3.4; Martin, 2011) as recommended by the workflow. Taxonomic assignment was done using the UNITE database (sh_general_release_dynamic_10.05.2021.fasta; Abarenkov et al., 2021).

Inhibitory bacteria

To identify bacteria that were putatively inhibitory to *Bd* in our samples (hereafter "inhibitory amplicon sequence variants [ASVs]"), we used a BLAST search to identify ASVs in our data set that were listed as inhibitory toward *Bd* in an antifungal isolates database (Woodhams et al., 2015). We filtered the results for 100% query coverage and percent shared identity ≥97%. The antifungal isolates database was compiled using studies from around the world, including studies on some of our study species at our study sites. Therefore, we also examined a subset of only ASVs that matched isolates that originated in Queensland, AU, and a subset of only ASVs that matched isolates that originated on one of our study species. Those subsets produced similar patterns that do not alter our

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conclusions and are presented in Appendix S2: Sections 5.3, 7.7, 8.3, and 9.3.

Based on these BLAST results, we calculated the relative abundance of putatively inhibitory bacteria as the proportion of reads per sample that belonged to inhibitory ASVs. We also examined the relative abundance of all bacteria in the genus *Pseudomonas*, because members of this genus are often highly inhibitory toward *Bd* and are candidates for probiotic treatments (Harris et al., 2006; Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Lam et al., 2010; Rebollar et al., 2016).

Fungi with and without *Bd*

Because *Bd* is an invading pathogen that often dominated the fungal communities, we usually removed it from the fungal community prior to normalizing the fungal data or applying statistical tests. However, in some cases, we included *Bd* so that its effects could be analyzed, and we could compare the communities with and without *Bd*. Note that several ASVs were assigned to *Bd*. These were summed into a single ASV for all analyses that included *Bd* as part of the community.

Statistical analyses and presentation: Overview

We ran analyses on four data sets: all bacteria, putatively inhibitory bacteria, fungi with *Bd* included in the community, and fungi without *Bd* included. For some tests, we were interested in comparing means among species or between fungi and bacteria. These tests involved an initial comparison of all groups (e.g., species) followed by standard post hoc tests of pairwise comparisons that controlled the type 1 error rate. In other cases, we were interested in associations between variables (e.g., richness and evenness), and we wanted to know both whether there was a general overarching effect across all species and whether there were differing patterns among species. Therefore, for these tests, we first ran mixed-effects models, with frog species as a random effect, to look for overarching patterns. If the results were significant in those models, we ran separate fixed-effects models for each species. To control the type 1 error rate, for each set of tests, we applied a sequential Bonferroni correction (Holm, 1979) to the four species-specific models. We have presented these corrected *p* values throughout (adjusted with p.adjust in R; v4.0.3; R Core Team, 2017), and original *p* values are available in Appendix S2.

Although rarefaction curves suggested that sufficient read depth had been achieved, read depth and richness were strongly, linearly correlated for both bacteria and fungi, and strong correlations persisted even after rarefying the richness (Appendix S2: Section 3). Therefore, we used the full richness (not rarefied) throughout and included read depth as a covariate. Read depth was also included in models for evenness and beta diversity. Because read depths were much higher than any other values in the models, in all models except for generalized linear models, we rescaled the read depth for each sample by subtracting the mean read depth and dividing by the standard deviation (calculated separately for fungi and bacteria). For generalized linear models, the natural log of read depth was included as an offset.

All mixed-effects models were run using the lme4 package (v1.1-26; Bates et al., 2015), and significance for all general and generalized linear models was assessed using the ANOVA function in the car package (v3.0-10; Fox & Weisberg, 2011) with a type II sum of squares. Model assumptions were assessed with Q–Q plots and residual plots via the performance package (Lüdecke et al., 2021). To achieve an acceptable model fit, it was necessary to $\log_{10}$-transform richness and convert evenness to dominance (1-evenness) followed by a $\log_{10}$ transformation (dominance is the inverse of evenness).

Because of the large number of tests involved, we have presented model information concisely, and we have put results for many of the post hoc results in figures and tables, rather than the text. Similarly, we have only presented *p* values in the manuscript. Full information on all statistical models and outputs is available in Appendix S2.

Statistical analyses: Family level and ASVs

All analyses discussed were conducted at the ASV level unless otherwise specified. ASVs provide a very fine level of resolution, but often result in a given microbial species being split into several ASVs. Therefore, for thoroughness, we also ran most analyses at the family level (excluding analyses that would be redundant [e.g., relative abundance of inhibitory bacteria]). Results at the family level were highly similar to the results at the ASV level. Therefore, we generally will not discuss them, but they are available in Appendix S3.

Additionally, the data in this paper were previously clustered into operational taxonomic units (OTUs) and analyzed as part of a thesis (McKnight, 2019). These analyses generally showed the same patterns, providing confidence that the results are not artifacts of the chosen bioinformatics pipeline.

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Statistical analyses: Alpha diversity

We examined alpha diversity (richness and evenness) both within and between taxa. We calculated richness as the number of unique ASVs per individual, and we calculated evenness via Pielou's formula, where 1 = a totally even community, and 0 = a totally dominant community.

First, we compared alpha diversity among groups within taxa. For fungal richness, bacterial richness, fungal evenness, and bacterial evenness, we used linear models to look for differences among frog species, among parks, and between elevations. We performed post hoc comparisons using Tukey's test via the emmeans package in R (v1.5.4; Lenth, 2018).

Second, we used linear mixed-effects models to compare mean fungal richness or evenness (with and without *Bd*) with mean bacterial richness or evenness. Initial models included individual frog ID nested in species (as random effects); subsequent species-specific models only included individual ID as a random effect. All models included park and elevation as fixed effects, except for *L. dayi* models, which only included park because *L. dayi* was only found at lowland sites.

Third, we looked for correlations between fungal richness and evenness (with and without *Bd*), bacterial richness and evenness, fungal and bacterial richness, and fungal and bacterial evenness (fungi with and without *Bd*). We ran mixed-effects models with species as a random effect as well as species-specific fixed-effects models.

Statistical analyses: Beta diversity

We examined beta diversity by calculating the Bray–Curtis dissimilarities (which incorporate abundance) and the Jaccard dissimilarities (presence/absence) among individual frogs for both fungal communities (without *Bd*) and bacterial communities. Following the recommendations in McKnight, Huerlimann, et al. (2019a), we normalized samples to proportions (i.e., total sum normalization) prior to calculating the Bray–Curtis dissimilarities. For fungi, we normalized after removing *Bd*. For both beta diversity metrics, we used a Mantel test to look for correlations between bacterial and fungal (without *Bd*) beta diversity (permutations were constrained to within each species, park, and elevation combination).

We used PERMANOVAs via the adonis2 function in the vegan package (v2.5-7; Oksanen et al., 2017) to look for beta diversity differences among species, parks, and elevations. For each taxon, we constructed an initial model with all three factors and no constraints on permutations; then, we made pairwise comparisons for each pair of species with permutations constrained within each park and elevation combination. For these post hoc tests, we used the sequential Bonferroni corrections to control the type 1 error rates (applied separately for each taxon). We used 5000 permutations for each test and performed these analyses on both fungi and bacteria using both the Bray–Curtis and the Jaccard dissimilarities (i.e., four sets of comparisons). Additionally, we used the same set of PERMANOVAs to examine the fungal community with *Bd* included. These results were broadly similar to the results for with *Bd* and are available in Appendix S2: Sections 10.7 and 10.8.

Statistical analyses: Inhibitory bacteria

To compare the relative abundance of putatively inhibitory bacteria among species, we ran a negative binomial generalized linear model using the glm.nb function in R, with the number of inhibitory reads per sample as the response variable, species, park, and elevation as predictors, and the natural log of the total number of reads per sample as an offset. We performed post hoc tests with the emmeans package.

We also examined the relationship between the relative abundance of inhibitory bacteria and total bacterial richness. We ran a linear mixed-effects model with species as the random effect, richness as the response variable, and the relative abundance of inhibitory reads (proportion of reads), park, elevation, and read depth as the fixed effects. Subsequently, we ran species-specific linear models with the same structure but excluding species. Finally, we ran PERMANOVAs using the Bray–Curtis dissimilarities and the Jaccard dissimilarities following the same model structure as before (data were normalized after reducing the data to just the inhibitory bacteria).

Statistical analyses: Associations with *Bd*

We used several methods to look for associations between *Bd* and the bacterial and fungal communities. First, we looked for associations between *Bd* and fungal richness, bacterial richness, and the relative abundance of inhibitory bacteria. We did this in two ways. First, we used binomial models to examine *Bd* prevalence (i.e., comparing infected and uninfected individuals). Second, we used linear models to examine *Bd* intensity (i.e., the $\log_{10}$ of the qPCR results [only using infected individuals], calculated without adding a pseudocount to the data). For both types of test, we first ran mixed-effects models (with species as a random effect) and then ran

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species-specific fixed-effects models if the mixed-effects model was significant. In all cases, *Bd* (prevalence or intensity) was the response, and park, elevation, read depth, and either richness or inhibitory relative abundance were the predictors. Second, we used PERMANOVAs to look for associations between beta diversity (Bray–Curtis or Jaccard) and *Bd*. We tested *Bd* prevalence and intensity as before. For each test, permutations (5000) were constrained within unique combinations of species, park, and elevation. We ran these PERMANOVAs on all bacteria, inhibitory bacteria, fungi with *Bd*, and fungi without *Bd*.

Finally, for both fungi and bacteria, we looked for associations between individual ASVs and *Bd*. We used DESeq2 (Love et al., 2014) to look for ASVs that were differentially abundant between *Bd*+ and *Bd*− individuals. We did this separately for each species and included elevation and park as factors. Additionally, we ran models comparing the relative abundance of each ASV with the infection intensity of *Bd* (based on qPCR results) for infected individuals (separate tests for each frog species). For these models, not all ASVs had the same data distribution; therefore, for each ASV, we used AICs to compare model fit for the linear, negative binomial, Poisson, zero-inflated negative binomial, and zero-inflated Poisson models, and we only assessed the significance of the model with the best fit. The number of reads for the ASV in question was the response variable, and total read depth was included as a covariate (linear models) or offset (following natural log transformation; generalized linear models). For all analyses, only ASVs that were present in at least 20% of the individuals in a test were included. Within each frog species, we controlled the type 1 error rate to a false discovery rate (FDR) of 0.01 (applied separately for fungi and bacteria).

RESULTS

Sequencing

We attempted to extract, amplify, and sequence fungal and bacterial DNA from 169 frogs (Table 1). However, one fungal sample could not be sequenced, and after quality control filtering, two additional fungal samples were removed due to low read depths (<5000 reads after chimera removal). Similarly, two bacterial samples were removed due to low read depths (<5000 reads), and one was removed because it had over twice the reads of any other sample, as well as a very high richness and a rarefaction curve with an atypical slope. We did not consider that sample to be trustworthy and removed it to avoid biasing the data. We decided to remove these six samples prior to any analyses. For the remaining frog samples, sequencing produced a total of 6.49 million fungal reads and 6.87 million bacterial reads. Following all quality control filtering steps and application of microDecon to remove contaminant reads (McKnight, Huerlimann, et al., 2019b), 3.28 million fungal reads (mean = 19,761 per sample) and 3.81 million bacteria reads (mean = 22,975 per sample) were retained (details on filtering steps and microDecon are available in the archived data).

Taxonomic results

The fungal data produced 16,884 ASVs, representing 13 phyla, 50 classes, 160 orders, 417 families, and 1,110 genera. Most ASVs were in the phyla Ascomycota (9665 ASVs) and Basidiomycota (4527 ASVs), while all other phyla were poorly represented, ranging from one ASV (Blastocladiomycota, Calcarisporiellomycota, and Monoblepharomycota) to 89 ASVs (Rozellomycota). Fungal relative abundance followed a largely similar taxonomic pattern. Ascomycota (51.0% of reads) was the most abundant phylum, followed by Chytridiomycota (24.7%) and Basidiomycota (21.3%). The remaining phyla ranged from 0.0003% of reads (Blastocladiomycota) to 2.1% (Basidiobolomycota). Within Chytridiomycota, 98.8% of reads belonged to *Bd*. The remaining ASVs within Chytridiomycota were assigned to nine classes, seven orders, 12 families, and 14 genera (many could not be assigned at a particular taxonomic level; details in Appendix S2: Sections 4.6 and 4.7).

The bacterial data produced 41,774 ASVs, representing 48 phyla, 115 classes, 284 orders, 419 families, and 1,177 genera. Most ASVs were in the phylum Proteobacteria (10,943 ASVs), followed by Bacteroidota (5078 ASVs), Planctomycetota (4435 ASVs), and Actinobacteriota (3368 ASVs). Bacterial relative abundance followed a largely similar taxonomic pattern. Proteobacteria (46.3% of reads) was the most abundant phylum, followed by Bacteroidota (16.8%), Cyanobacteria (12.8%), and Actinobacteriota (9.7%).

For both taxa, not all ASVs could be assigned at every taxonomic level. This was particularly pronounced for fungi, where 14.1% of ASVs could not be assigned below the level of kingdom (compared with 0.5% for bacteria). Also, for both taxa, most ASVs were rare and often only occurred on one individual. Stacked barplots for each taxon (including a Chytridiomycota subset) are presented in Appendix S2: Section 4.

Alpha diversity (richness and evenness)

Fungal richness differed significantly among frog species ($p < 0.001$) and parks ($p = 0.005$), but not elevations

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($p = 0.692$). Bacterial richness also differed significantly among species ($p < 0.001$) and parks ($p < 0.001$), as well as elevations ($p = 0.021$). For both taxa, *L. dayi* had the lowest richness (particularly pronounced for bacteria), but not all pairwise comparisons were significant (Figure 1). However, when fungal and bacterial richness were combined (total richness), *L. dayi* had significantly lower richness than any other frog species (summed read depth was used as a covariate; Appendix S2: Section 5.1.3). These patterns persisted at the family level, and *L. dayi* had significantly lower bacterial family richness than any other frog species (Appendix S3: Section 3).

There was a significant positive correlation between bacterial and fungal richness in both the full model and the models for each frog species (all $p < 0.010$; Figure 2; Table 2). However, bacterial richness was significantly higher than fungal richness in both the full model and species-specific models (i.e., individual frogs tended to have more unique bacterial ASVs than unique fungal ASVs; all $p < 0.001$; Figure 1; Table 2).

Fungal evenness differed significantly among frog species both when *Bd* was included as part of the community ($p < 0.001$) and when *Bd* was excluded ($p = 0.008$). Fungal evenness did not differ significantly among parks or elevations in either case (all $p > 0.292$). For bacteria, evenness differed among species ($p < 0.001$), parks ($p = 0.008$), and elevations ($p = 0.039$).

Unlike richness, evenness patterns among frog species differed between fungi and bacteria. *L. dayi* had the highest fungal evenness of any frog species (with *Bd* excluded from the community), but the lowest bacterial evenness (Figure 1). For fungi (without *Bd*), *L. dayi* had significantly higher evenness than *L. nannotis* ($p = 0.012$), but no other comparisons were significant. For bacteria, *L. dayi* and *L. serrata* had significantly lower evenness than *L. nannotis* ($p = 0.005$ and 0.002, respectively), and no other comparisons were significant. When *Bd* was included in the fungal community, evenness was greatly reduced, and no comparisons with *L. dayi* were significant, but *L. nannotis* had significantly lower fungal evenness than *L. serrata* ($p = 0.016$) or *L. wilcoxii* ($p < 0.001$). In further contrast to richness patterns, bacterial evenness and fungal evenness were not significantly correlated regardless of whether *Bd* was included or excluded ($p = 0.451$ and 0.695, respectively; Figure 2; Table 2).

When *Bd* was included as part of the community, there was no significant difference in evenness between bacteria and fungi in the full model ($p = 0.514$; Figure 1; Table 2). However, when *Bd* was excluded from the fungal community, fungi had significantly higher evenness than bacteria in the full model ($p < 0.001$) and the model for all species (all $p < 0.012$) except *L. nannotis*

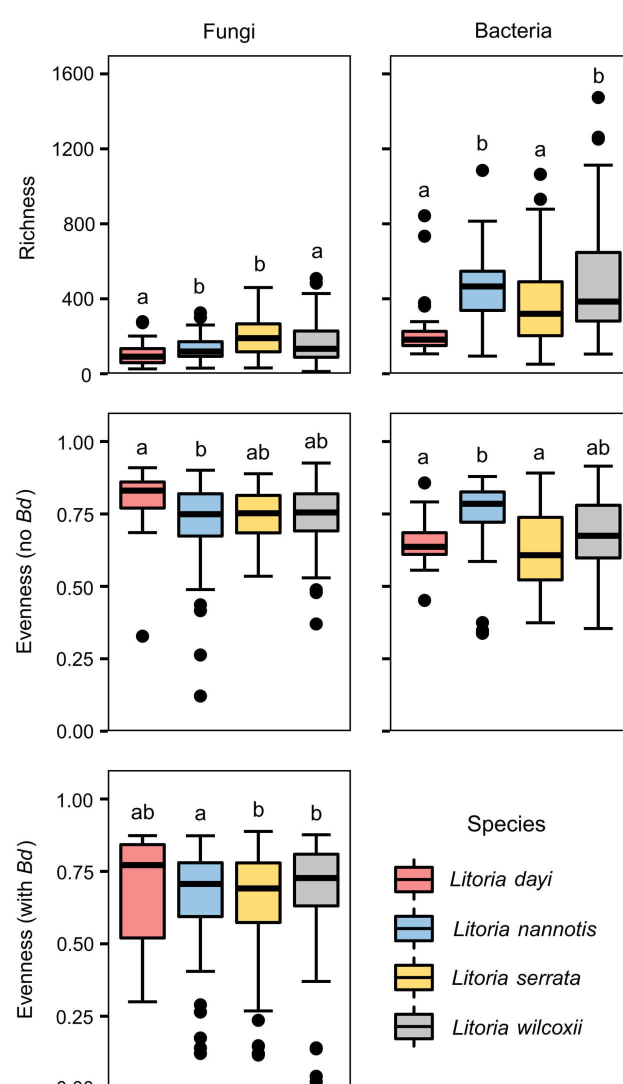

FIGURE 1 Boxplots of microbiome alpha diversity values for each frog species (across all parks and elevations without compensating for read depth). Evenness (no *Bd*) = for fungi, evenness was calculated after removing *Batrachochytrium dendrobatidis* (*Bd*) from the community. Evenness (with *Bd*) = *Bd* was included as part of the community. Boxplots were made using the default formula in ggplot2 (v3.3.3). Lowercase letters indicate significant differences within plots (based on models that accounted for additional factors)

($p = 0.391$; Figure 1; Table 2). Indeed, while not significant, *L. nannotis* had the opposite trend of all other species (i.e., higher evenness in bacteria than in fungi).

For fungi (with *Bd* included), there was a significant positive association between richness and evenness in the full model and the model for each species (all $p < 0.008$). When *Bd* was excluded, however, there was a significant positive association in the full model ($p < 0.001$), but *L. nannotis* was the only species with a significant trend ($p = 0.009$). In contrast, bacterial richness and evenness

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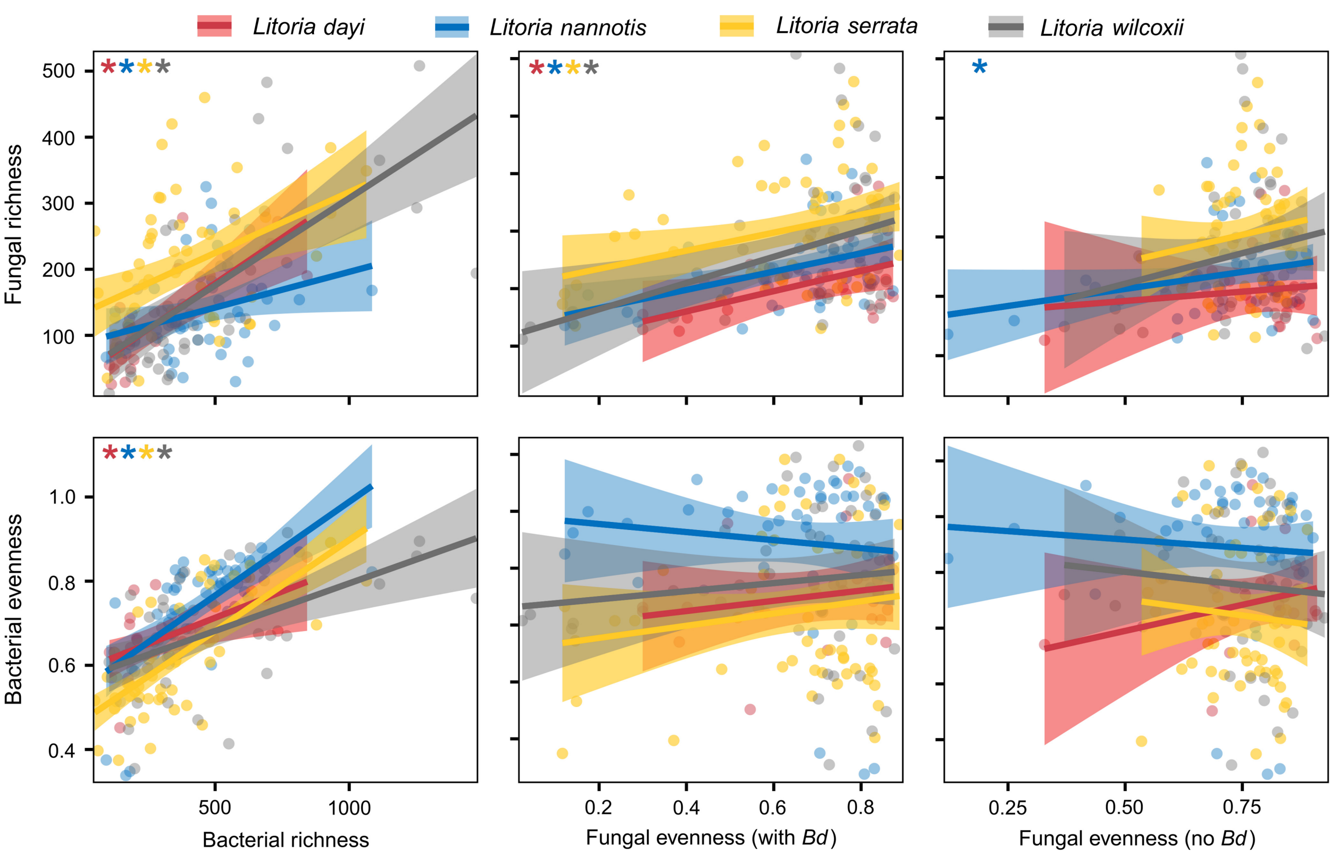

FIGURE 2 Linear regressions of microbiome alpha diversity values split by frog species. Evenness (with *Bd*) = for fungi, evenness was calculated with *Batrachochytrium dendrobatidis* (*Bd*) as part of the community. Evenness (no *Bd*) = for fungi, evenness was calculated after removing *Bd* from the community. Shading represents 95% confidence intervals for each species. Regressions are simple linear regressions that do not compensate for additional factors. Asterisks indicate significant correlations (based on models including read depth, parks, and elevations)

showed a strong positive correlation in the full model ($p < 0.001$) and the model for each species (all $p < 0.001$; Figure 2; Table 2).

Beta diversity

Beta diversity patterns of fungal (without *Bd*) and bacterial communities were positively correlated based on both the Bray–Curtis dissimilarities (Mantel's $r = 0.121$, $p < 0.001$) and the Jaccard dissimilarities (Mantel's $r = 0.300$, $p < 0.001$; i.e., a pair of frogs with similar fungal microbiomes tended to also have similar bacterial microbiomes). Both communities had high dissimilarities, but fungal dissimilarities were generally higher than bacterial dissimilarities, indicating less stability of the fungal microbiomes across individuals (Appendix S2: Section 10.12). Indeed, for bacteria, there were 18 pairwise comparisons with a dissimilarity of one (i.e., no ASVs in common; always between individuals from different species), whereas for fungi, there were 1,425 pairwise comparisons with a dissimilarity of one (10.8% of all comparisons), and comparisons with a dissimilarity of one were present within all four species (347 comparisons in total). A general pattern of higher fungal dissimilarities persisted at the family level, but it was less pronounced (Appendix S3: Section 8.12).

For both bacteria and fungi, PERMANOVAs based on either the Bray–Curtis or the Jaccard dissimilarities showed significant differences among species, among parks, and between elevations (all $p < 0.001$); however, patterns were somewhat less obvious for fungi than for bacteria (Figure 3). Further, in post hoc comparisons, for bacteria, all pairwise comparisons were significant for species (all $p < 0.002$) and parks (all $p < 0.004$), whereas for fungi, p values were higher, and the *L. dayi* × *L. serrata* and Kirrama × Tully Gorge comparisons (both based on the Bray–Curtis) were not significant ($p = 0.102$ and 0.079, respectively). All other comparisons were significant for fungi (all $p < 0.040$). Patterns for inhibitory bacteria and fungi with *Bd* were similar (Appendix S2: Section 10), as were tests at the family level (with a reduction in the

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TABLE 2 Statistical results (p values) for alpha diversity and relative abundance of inhibitory bacterial (Inhib. relative abund.) analyses, including correlations within a taxon, correlations and comparisons between taxa, correlations with *Batrachochytrium dendrobatidis* (*Bd*) infection intensity, and comparisons between infected and infected individuals (*Bd* prevalence)

Analyses	Full	*Litoria dayi*	*Litoria nannotis*	*Litoria serrata*	*Litoria wilcoxii*	Dir.
Comparisons of means						
Richness: Bacteria versus fungi	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	B > F*
Evenness: Bacteria versus fungi (with *Bd*)	0.514	—	—	—	—	
Evenness: Bacteria versus fungi (no *Bd*)	<0.001*	<0.001*	0.391	<0.001*	0.011*	F > B*
Bd prevalence: Bacterial richness	0.330	—	—	—	—	
Bd prevalence: Fungal richness	0.032*	1.000	1.000	0.006*	0.136	+ > −*
Bd prevalence: Inhib. relative abund.	0.137	—	—	—	—	
Correlations						
Richness: Bacteria × fungi	<0.001*	<0.001*	0.010*	0.010*	<0.001*	+*
Evenness: Bacteria × fungi (with *Bd*)	0.451	—	—	—	—	
Evenness: Bacteria × fungi (no *Bd*)	0.695	—	—	—	—	
Bacteria: Richness × evenness	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	+*
Bacteria: Richness × Inhib. relative abund.	<0.001*	0.007*	0.072	<0.001*	0.001*	−*
Fungi (with *Bd*): Richness × evenness	<0.001*	0.007*	<0.001*	<0.001*	0.007*	+*
Fungi (no *Bd*): Richness × evenness	<0.001*	0.592	0.009*	0.096	0.592	+*
Bd intensity × Bacterial richness	0.502	—	—	—	—	
Bd intensity × Fungal richness	0.155	—	—	—	—	
Bd intensity × Inhib. relative abund.	0.004*	0.013*	0.899	0.899	0.288	+*

Note: The "Full" column shows results for mixed-effects models with frog species included as a random effect, and subsequent columns show fixed-effects models on each species. A sequential Bonferroni correction (via the p.adjust function in R) was used to control the type 1 error rate for the four fixed-effects models in each row (adjusted p values are shown). "Dir." shows the direction of significance ("B" = bacteria, "F" = fungi, "+ > −" = infected greater than uninfected, "+" = positive association, "−" = negative association). An asterisk indicates statistical significance following a sequential Bonferroni correction, and "—" indicates that a test was not conducted due to a lack of significance in the "Full" model. Detailed model outputs are available in Appendix S2.

number of significant post hoc tests, particularly for fungi; Appendix S3: Section 8).

Inhibitory bacteria

A BLAST search aligning our data to a database of bacteria known to inhibit *Bd* (Woodhams et al., 2015) resulted in a subset of 1,735 putatively inhibitory ASVs. Within this subset, all species, parks, and elevations were strongly dominated by Proteobacteria at the phylum level and Gammaproteobacteria at the class level (both generally > 75% of reads). Burkholderiales was generally the order with the highest mean relative abundance (proportion of reads within the inhibitory community), but Pseudomonadales was also consistently abundant (sometimes with a higher relative abundance than Burkholderiales). Differences among frog species became pronounced at the family level. Alcaligenaceae was generally the family with the highest relative abundance for *L. dayi*, *L. serrata*, and *L. wilcoxii* (but its relative abundance varied among them), and Comamonadaceae was relatively uncommon. In contrast, for *L. nannotis*, Alcaligenaceae was very rare, and Comamonadaceae dominated. Pseudomonadaceae was the second most common family for all species. Stacked barplots are presented in Appendix S2: Section 4.2.

The total relative abundance of inhibitory bacteria (i.e., the proportion of all bacterial reads that were assigned to inhibitory ASVs) differed significantly among species ($p < 0.001$) but not parks ($p = 0.196$), or elevations ($p = 0.055$). *L. dayi* had the highest relative abundance of inhibitory bacteria (Figure 4), but the only statistically significant difference was that *L. nannotis* was significantly lower than all other species (all $p < 0.004$). The low relative abundance of

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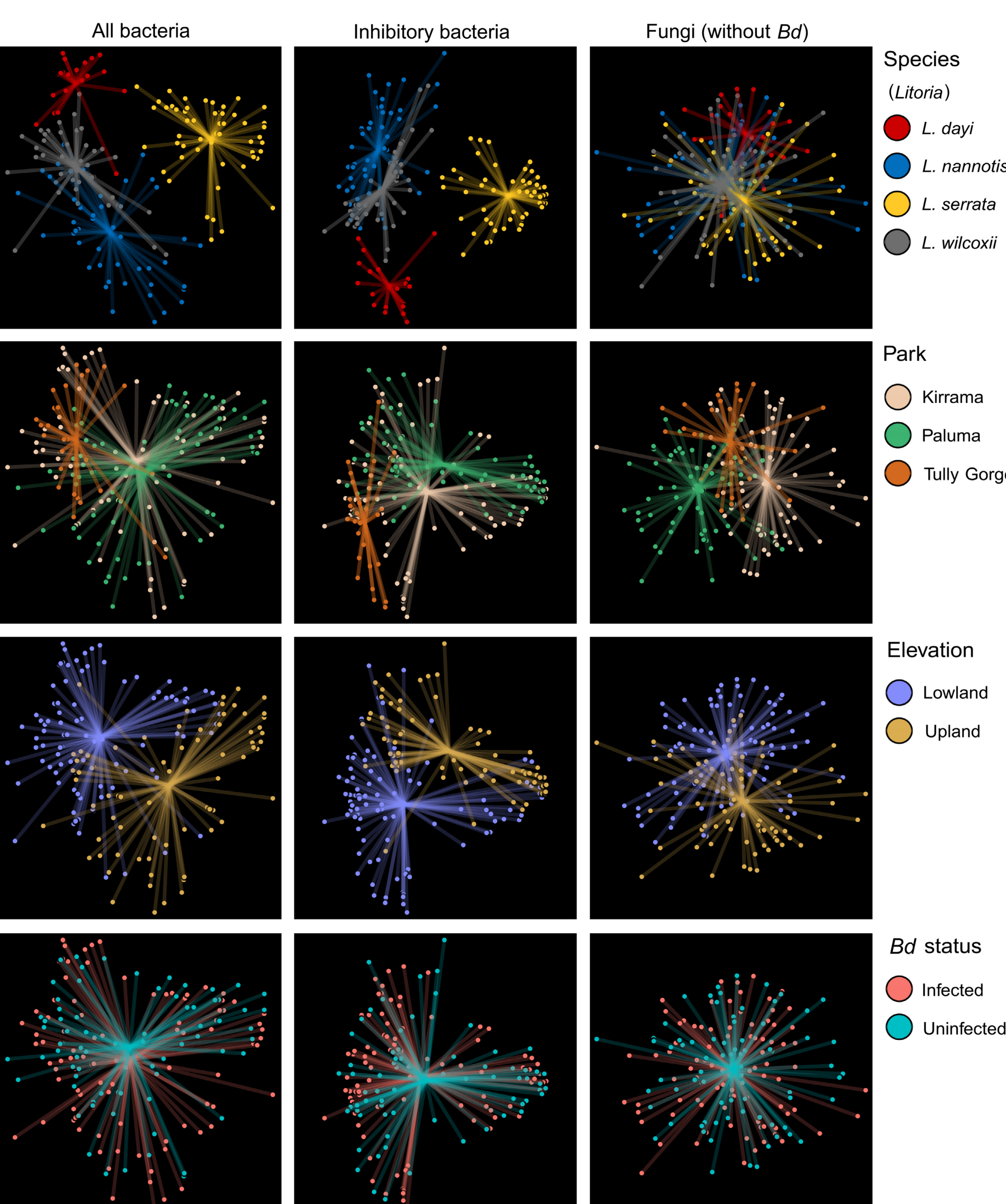

FIGURE 3 nMDS ordination plots based on the Bray–Curtis dissimilarities for all bacteria, inhibitory bacteria, and fungi without *Batrachochytrium dendrobatidis* included as part of the community (normalization took place after removing *Bd*). Each column shows the same four ordinations, but the colors and centroids are based on different factors. For all plots, $k = 4$. Stress = 0.145 (all bacteria), 0.126 (inhibitory bacteria), and 0.191 (fungi). Because axis values are arbitrary in nMDS plots, they are not shown, but within each plot, the difference between the minimum and the maximum is the same for the *x*-axis and the *y*-axis (i.e., they are scaled symmetrically). Plots based on the Jaccard dissimilarities and plots for fungi with *Bd* are available in Appendix S2: Section 10, and plots at the family level are available in Appendix S3: Section 8

inhibitory bacteria in *L. nannotis* and difference between *L. dayi* and *L. wilcoxii* became more pronounced using only inhibitory bacteria isolated in QLD or isolated from one of the study species (Appendix S2: Section 5.3). There was a significant negative association between total bacterial richness and the relative abundance of inhibitory bacteria in the full model ($p < 0.001$) and all species-specific models (all $p < 0.008$), except for *L. nannotis* which was nearly signfiant ($p = 0.072$; Table 2).

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A total of 132 ASVs were assigned to the genus *Pseudomonas*. Most reads for *Pseudomonas* ASVs could not be identified at the species level, but 10 species were identified, the most abundant of which was *Pseudomonas koreensis*, though its relative abundance varied greatly (Appendix S2: Section 4.3). Most of the *Pseudomonas* ASVs (121) matched sequences in the Woodhams et al. (2015) inhibitory database. The remaining 11 ASVs were uncommon (the sum of all 11 = 0.01%–3.22% of all reads per individual). All individuals had at least one *Pseudomonas* ASV except for four *L. nannotis* and one *L. serrata*. The relative abundance of *Pseudomonas* ASVs differed significantly among species ($p < 0.001$) but not among parks ($p = 0.577$) or elevations ($p = 0.623$). *L. dayi* generally had the highest relative abundance of *Pseudomonas* spp., followed by *L. wilcoxii* (Figure 4). Both frog species had significantly higher levels of *Pseudomonas* spp. than *L. nannotis* or *L. serrata* (both $p < 0.014$) but were not significantly different from each other ($p = 0.800$).

Another highly inhibitory genus, *Janthinobacterium* (Bletz et al., 2013; Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Muletz et al., 2012), was only represented by five ASVs and was only present in eight samples (one *L. nannotis*, five *L. serrata*, and two *L. wilcoxii*). It was always rare, comprising only 0.03%–0.32% of reads per sample.

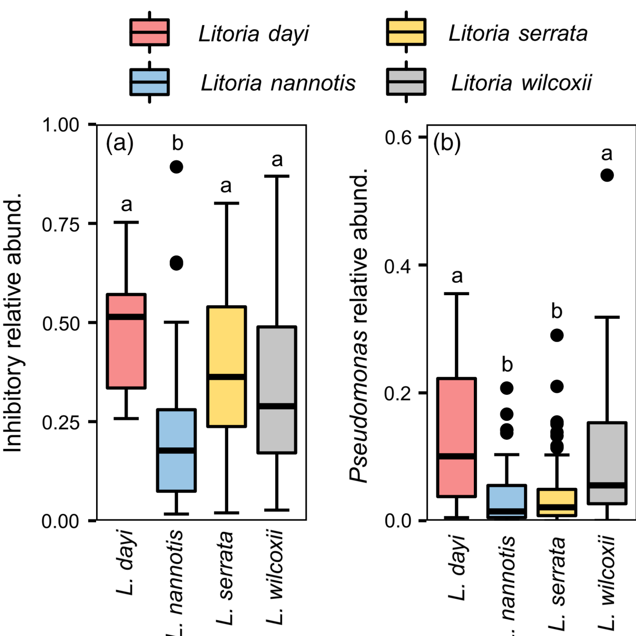

FIGURE 4 Boxplots split by frog species showing (a) the relative abundance (proportion of reads) of inhibitory bacteria and (b) relative abundance (proportion of reads) of bacteria in the genus *Pseudomonas*. Boxplots were made using the default formula in ggplot2 (v3.3.3). Lowercase letters indicate significant differences within plots (based on models including parks and elevations)

Associations with *Bd*

For *Bd* prevalence (*Bd*+ individuals compared with *Bd*− individuals), there were no significant effects of bacterial richness ($p = 0.330$) or the relative abundance of inhibitory bacteria ($p = 0.137$), but there was a significant difference among *Bd*+ and *Bd*− individuals for fungal richness ($p = 0.032$; Figure 5). However, the fungal result is likely an artifact from samples with high *Bd* loads having few reads available for other fungal ASVs and should be viewed with caution (Appendix S2: Section 8.2.3.1).

For *Bd* intensity models (based on qPCR results and only using infected individuals), associations with richness were not significant for bacteria ($p = 0.502$) or fungi ($p = 0.155$). However, unexpectedly, there was a significant positive relationship between the relative abundance of inhibitory bacteria and *Bd* intensity in the full model ($p = 0.004$) and the model specifically for *L. dayi* ($p = 0.013$). The other species had positive slopes but were not statistically significant (all $p > 0.288$; Figure 5).

PERMANOVAs testing associations between community composition and either *Bd* prevalence or intensity found no significant associations for bacterial (full community or inhibitory community) or fungal communities (without *Bd*) using either the Bray–Curtis or the Jaccard dissimilarities (all $p > 0.073$; Figure 3). When *Bd* was included as part of the fungal community, however, the dominating effect of *Bd* altered the pattern, and tests became significant for both prevalence and intensity using either dissimilarity (all $p < 0.001$; Appendix S2: Section 10).

There was little evidence of associations between *Bd* and any individual fungal or bacterial ASV. DESeq only identified one fungal ASV and seven bacterial ASVs that differed between infected and uninfected frogs (FDR = 0.01), none of which were significant in more than one frog species. Four of the seven bacterial ASVs did match sequences in the antifungal isolates database (Woodhams et al., 2015), but three of those four showed an increased abundance with *Bd*.

Models comparing the relative abundance of ASVs with infection intensity (within infected individuals) found significant associations in 18 fungal ASVs (FDR = 0.01). Interestingly, a disproportionate number of the significant fungal ASVs (15) were significant in *L. dayi*. Only three fungal ASVs were significant in more than one species (always *L. dayi* and *L. wilcoxii*) and two of the three had a positive association with *Bd* in *L. dayi*, and negative association in *L. wilcoxii*.

For bacteria, 66 ASVs were significantly associated with *Bd* infection intensity. Twenty-five were significant in *L. dayi*, 15 in *L. nannotis*, 10 in *L. serrata*, and 20 in *L. wilcoxii*. Four ASVs were significant in two species,

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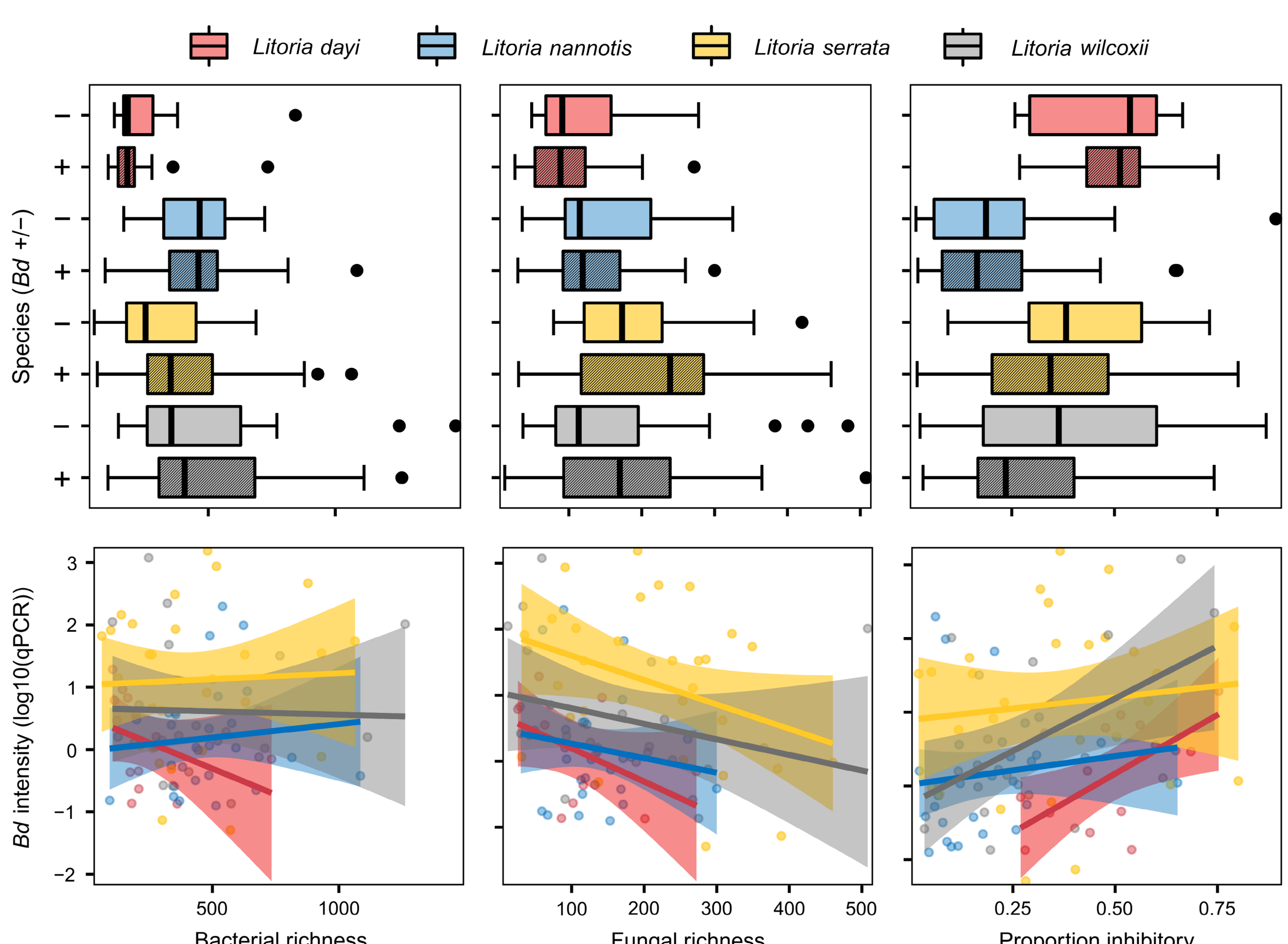

FIGURE 5 Comparisons between *Batrachochytrium dendrobatidis* (*Bd*) prevalence (row 1) and intensity (row 2) based on qPCR results ($\log_{10}$-transformed without a pseudocount). Row 1 compares infected (+; shaded) and uninfected (−; unshaded) individuals for each species. Row 2 shows linear regressions based on infection intensity results (only *Bd*+ individuals are included, so some points in row 1 are not present in row 2). Boxplots were made using the default formula in ggplot2 (v3.3.3). Shading indicates 95% confidence intervals for each species. Regressions are simple linear regressions that do not compensate for additional factors used in the statistical models. The only species-specific significant results were *L. serrata* for fungal richness × *Bd* prevalence, and *L. dayi* for proportion inhibitory × *Bd* intensity

but two of those four were positively associated with *Bd* in one species and negatively associated in the other. Nineteen of the significant ASVs matched sequences in the antifungal isolates database (two were among the ASVs that were significant in two frog species), but only 10 of their association with *Bd* were negative, while 11 were positive.

DISCUSSION

Fungal and bacterial microbiomes

Our study detected rich fungal and bacterial microbiomes on rainforest frogs. In contrast to microbiome research that used the 18S region (Kueneman et al., 2016, 2017), we found that frog skin contained a large number of fungal ASVs. However, the microbial richness on individual frogs was generally higher for bacteria across all four frog species. This is consistent with studies on humans and ruminants (Kittelmann et al., 2012; Qin et al., 2010). Similarly, a study looking at a wide range of animal hosts found that Shannon diversity was higher for bacteria than for fungi in 47 out of 49 species (Harrison et al., 2021).

Consistent with one of the few previous studies on frog fungal microbiomes (Medina et al., 2019), Ascomycota and Basidiomycota were the most common phyla (excluding Chytridiomycota), but at lower taxonomic levels, there was little evidence of dominance (excluding *Bd*), and (with the exception of *L. nannotis*) frogs generally had higher fungal evenness than bacterial evenness. Bacterial richness and evenness showed strong positive correlations in all frog species, whereas for fungi

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(without *Bd*), there was a general positive trend, but *L. dayi* was the only significant species in the species-specific models.

Fungal communities also tended to be more variable (higher beta diversity) than bacterial communities, with fewer consistent patterns among species and parks. This is evidenced by the high dissimilarities for fungi compared with bacteria (Appendix S2: Section 10.12) and the reduced clarity of patterns in some ordination plots (Figure 3). As with the richness patterns, this result is consistent with results from humans and ruminants, which showed more variation among samples and less stability for fungal communities than for bacterial communities (Kittelmann et al., 2013; Underhill & Iliev, 2014).

Despite differences in richness and variability, there were some similarities between fungal and bacterial communities. Within both communities, there were significant beta diversity (Bray–Curtis and Jaccard) differences among species and parks, and the Mantel tests found significant associations between the dissimilarities of both communities (even after constraining permutations within each combination of species, park, and elevation). Further, fungal richness and bacterial richness were strongly, positively correlated for all frog species, and for both taxa, *L. dayi* had the lowest richness of any of the frog species. These results agree with a previous study on amphibians (Medina et al., 2019); however, they partially differ with a study on human skin, which found that fungal and bacterial communities were associated by region (e.g., different areas of the feet clustered together), but there was no general association between the richness of the two communities across body sites, suggesting that different processes were driving the communities' alpha diversity (Findley et al., 2013). Likewise, Harrison et al. (2021) did not find correlations between fungal and bacterial alpha diversity using a data set from 49 animal species, but they did find that both fungi and bacteria were strongly associated with host phylogeny.

Associations between fungal and bacterial communities can occur when the microbiomes are not independent and one is influencing the other (Wargo & Hogan, 2006), a process that is well established in mycorrhizal systems, where the fungal community often dictates the bacterial community (Bonfante & Anca, 2009). Conversely, in boreal toads, bacteria inhibited fungi and strongly influenced the fungal community (Kueneman et al., 2016). While these intertaxon effects could explain the correlation we observed between beta diversity values, they do not provide a satisfactory explanation for the strong correlations between the richness (alpha diversity) of both communities. An alternative (but non-mutually exclusive) explanation is that both communities were being influenced by the same processes. Thus, a frog that is suitable for hosting a rich assemblage of bacteria may also be suitable for hosting a rich assemblage of fungi. Indeed, Harrison et al. (2021) found evidence of co-occurrence between fungi and bacteria and associations between host phylogeny and microbiomes across a range of species, suggesting that aspects of the hosts were simultaneously selecting specific fungi and bacteria. This explanation is reasonable given that, on a particular frog, both communities will be exposed to the same antimicrobial peptides and experience the same environments and climate.

Associations with *Bd*: Richness and inhibitory bacteria

We found mixed results regarding associations between *Bd* and richness. Looking at the host species level, *L. dayi*, the species that has never recovered from the *Bd* outbreak, had the lowest bacterial and fungal richness (at both the ASV and family levels). Further, consistent with a previous culture-based study in this region (Bell et al., 2018), the uplands (where recoveries have taken place) had higher bacterial richness than did the lowlands. Multiple studies of the interactions between bacterial microbiomes and pathogens suggested that high levels of richness can provide a protective effect against diseases (Dillon et al., 2005; Eisenhauer et al., 2013; Fraune et al., 2015; Harrison et al., 2019; Matos et al., 2005). Additionally, laboratory trials found that multispecies cultures are more effective at inhibiting *Bd* than are single isolates (Antwis & Harrison, 2018; Piovia-Scott et al., 2017), and in some cases, coculturing bacteria greatly increased metabolite production (Jousset et al., 2014) and resulted in synergistic inhibitory effects (Loudon, Holland, et al., 2014). Further, field-based studies in both North America (Jani et al., 2017) and Europe (Bates et al., 2018) found that populations that have shifted to an enzootic state and are coexisting with *Bd* have high bacterial diversity compared with populations that are still experiencing epidemics.

Our study builds on these results by providing a large, sequence-based comparison of Australian species and showing that the species that has yet to recover has lower richness than species that either have recovered or never declined. However, some other studies have failed to find significant associations between alpha diversity (not always explicitly richness) and *Bd* (Becker et al., 2015; Belden et al., 2015; Jani & Briggs, 2014; Rebollar et al., 2016), and neither our comparisons of infected and uninfected individuals nor our tests of associations between *Bd* infection intensity and richness were

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significant for bacteria, and while there was a positive association between fungal richness and *Bd* intensity, that result is likely an artifact caused by high *Bd* loads restricting the reads available for other ASVs during sequencing (Appendix S2: Section 8.2.3.1). Thus, our species-level patterns are consistent with historical patterns of declines and recoveries, but the patterns break down at finer scales.

Surprisingly, the relative abundance of putatively inhibitory bacteria was the opposite of what we predicted based on patterns of species declines and recoveries. The relative abundance of inhibitory bacteria was highest in *L. dayi* and lowest in *L. nannotis*, despite the fact that *L. dayi* populations have not recovered from the *Bd* outbreak, whereas *L. nannotis* populations have largely recovered (Bell et al., 2020; McKnight et al., 2017). Indeed, a mean of 48.3% of *L. dayi* bacterial reads (SD = 0.153) matched isolates in the antifungal isolates database (Woodhams et al., 2015). Similarly, *L. dayi* had the highest relative abundance of *Pseudomonas* spp., a genus that is often highly inhibitory toward *Bd* (Harris et al., 2006; Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Lam et al., 2010; Rebollar et al., 2016). Further, there were significant positive associations between *Bd* infection intensity and the relative abundance of inhibitory bacteria both in our mixed-effects model with species as a random factor, and in our model that just examined *L. dayi*.

These results suggest that simply having a high relative abundance of inhibitory bacteria is insufficient for population persistence or recovery when faced with a *Bd* outbreak. Indeed, the positive associations between *Bd* and the relative abundance of inhibitory bacteria suggest that either *Bd* creates conditions in which those bacteria thrive, or some aspect of the frogs' microhabitats or even skin results in conditions that are favorable for both inhibitory bacteria and *Bd*. Alternatively, the general negative relationship between richness and relative abundance of inhibitory bacterial ASVs may indicate that inhibitory bacteria also reduce the richness of the bacterial microbiome, which could, in turn, put frogs at greater risk of *Bd* infection. Finally, it should be acknowledged that it is possible that there is a survivorship bias in which *L. dayi* with low relative abundance of inhibitory bacteria quickly die out and were therefore unavailable for us to sample. Similarly, perhaps within species, only individuals with high levels of inhibitory bacteria can survive high *Bd* loads, resulting in the positive associations between *Bd* and inhibitory bacteria. These explanations are, however, clearly speculative, and future research should prioritize disentangling these causes and effects.

In contrast to our results, some other studies have found significant associations between inhibitory bacteria and infection status (Kueneman et al., 2016) or population persistence (Bell et al., 2018; Burkart et al., 2017; Catenazzi et al., 2018; Flechas et al., 2012; Kueneman et al., 2016; Lam et al., 2010; Woodhams et al., 2007). Also, it should be acknowledged that we were only able to measure relative abundance of inhibitory bacteria rather than total abundance. Thus, if *L. dayi* harbors fewer bacteria per unit area than the other frog species harbor, it would have few inhibitory bacteria (in terms of actual abundance) even though inhibitory bacteria constitute a large portion of its microbiome. There is, however, no a priori reason to expect *L. dayi* to have low total bacterial loads.

One potential weakness of our result is that our definition of inhibitory bacteria was necessarily restricted to ASVs with a 97% similarity to bacteria that had been identified as inhibitory in previous studies. Thus, frogs in our study may have possessed inhibitory bacteria that were not identified as such because they were not cultured in previous studies, and ASVs that matched known inhibitory bacteria may have been from non-inhibitory strains. However, the dominant members of amphibian bacterial microbiomes are culturable (Walke et al., 2015), and the inhibitory bacterial database we used (Woodhams et al., 2015) was constructed from the results of multiple studies, including studies that examined some of the same species of frogs and study sites that we used in this study (Bell, 2012; Bell et al., 2013). Further, the general patterns persisted even if we only looked at inhibitory bacteria that matched isolates from our region (Queensland) or study species, thus minimizing the possibility of large numbers of missing ASVs or large mismatches between the strains we sequenced and the strains in the database (Appendix S2: Sections 5.3, 7.7, 8.3, and 9.3). Also, large portions of our communities were inhibitory, and our results agreed with a previous culture-based study in our system (Bell et al., 2018). Additionally, unidentified inhibitory bacteria would not explain the high relative abundance of *Pseudomonas* spp. on *L. dayi*, and we would have expected any individual unidentified inhibitory ASVs that had a strong effect on *Bd* to be detected in our differential abundance tests. Therefore, we do not think that unidentified or misidentified inhibitory bacteria are likely to have substantially influenced our results.

Associations with *Bd*: ASVs and beta diversity

Differential abundance tests and linear models found little consistent evidence of associations between individual ASVs of either taxon and *Bd*. Few ASVs were significantly

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associated with *Bd*, and the few that were significant were usually only significant in one frog species or had inconsistent patterns between species. Similarly, for the bacterial ASVs that were significantly associated with *Bd* and matched sequences in the antifungal isolates database (Woodhams et al., 2015), roughly half of them showed positive associations, which is the opposite of what we would expect from an inhibitory effect, but is interesting given the significant associations between the relative abundance of all inhibitory bacteria and *Bd*. This lack of compelling patterns was surprising because many fungi (Kearns et al., 2017) and bacteria (Woodhams et al., 2015) either enhance or reduce the growth of *Bd*. However, many of those studies were performed on isolated laboratory cultures, and effects can vary in more complex cultures (Antwis & Harrison, 2018). Thus, it may be difficult to isolate the effects of individual bacteria or fungi within a complex community.

PERMANOVAs also failed to find significant associations between *Bd* and the beta diversity of either taxon, with the exception of tests where *Bd* was included as part of the fungal community. Significant patterns when *Bd* was included were unsurprising because, when *Bd* was present, it often dominated the fungal microbiome and was frequently the most common fungal ASV, comprising up to 99.2% of fungal reads on a frog. Thus, including it as part of the community reduced species evenness (Figure 1) and had a homogenizing effect on the fungal communities, which reduced differences among species and resulted in *Bd* explaining much of the variation in fungal communities. When this dominating effect is considered alongside the lack of significant patterns when *Bd* was excluded from the communities, the results suggest that although *Bd* dominated the fungal community in sheer numbers (resulting in a strong influence on the Bray–Curtis dissimilarities), the rest of the community was not strongly influenced by its presence or relative abundance, nor did the underlying community composition affect its ability to invade hosts.

The dominating effect of *Bd* on fungal communities and lack of underlying effects of fungal beta diversity echo the results of Medina et al. (2019), but the lack of associations between *Bd* and bacterial beta diversity (using either all bacteria or just inhibitory bacteria) is more surprising because several studies have found evidence that bacterial community composition is important in *Bd* infection dynamics. In Panamanian golden frogs (*Atelopus zeteki*), for example, individuals that were able to clear *Bd* infections had significantly different communities (beta diversity) from individuals that did not clear infections, but the alpha diversity did not differ between the two groups (Becker et al., 2015). Similarly, a comparison between susceptible and non-susceptible frog species in South America found that the community structures differed between each group, but alpha diversity patterns did not match the pattern of susceptibility (Rebollar et al., 2016). Further, in Sierra Nevada Mountain yellow-legged frogs (*Rana sierrae*), the structure of the microbial communities, but not the alpha diversity, correlated with *Bd* (Jani & Briggs, 2014). Some studies have also found differences between the community structure of populations that are coexisting with *Bd* and populations that are experiencing epizootics (Bates et al., 2018; Jani et al., 2017). It should be noted that we did observe significant differences in beta diversity among species for both fungi and bacteria; therefore, we have not falsified the possibility that these species-level differences are driving the historical patterns of declines and recoveries (e.g., *L. dayi* may have a microbiome that is more susceptible to invasion by *Bd*), but we were unable to actually test that possibility at the beta diversity level.

Differences among studies

As noted throughout, studies of *Bd* and microbiomes reveal a range of different patterns, and studies have reported both negative and positive associations between *Bd* and alpha diversity, beta diversity, differential abundance of specific bacteria, and inhibitory bacteria. There are several possible explanations for these discrepancies among studies. First, many different methodologies have been employed, including field-based surveys, laboratory infection trials, culture-dependent approaches, and next-generation sequencing of entire communities. Therefore, some differences may be methodological artifacts. Second, as our study demonstrates, microbiomes and their interactions with their hosts and environment are exquisitely complex, and we have only scratched the surface of that complexity. Amphibian microbiomes are strongly affected by factors such as habitat, season, and study site (Bird et al., 2018; Bletz et al., 2017; Longo et al., 2015; Longo & Zamudio, 2016; Medina et al., 2017). Thus, the effects of microbiomes on *Bd* are likely determined by a complex series of interactions involving characteristics of the habitats, hosts, and the microbiomes. Different populations or species may be persisting or recovering via different mechanisms, and in some populations, ASV richness may be very important, while in others, having a high abundance of inhibitory bacteria may be more important. Additionally, there are other possible explanations for population recoveries, such as changes in habitat, behavior, or immune system function, which may be interacting with microbiomes or acting independently (McKnight et al., 2017; Scheele et al., 2015, 2017). Indeed, a study of the population genetics of *L. dayi* found

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evidence that it may currently be in the process of adapting to *Bd* (McKnight et al., 2020), while a similar study of *L. nannotis* and *L. serrata* did not find evidence of ongoing adaptation (McKnight, Lal, et al., 2019).

Another possibility, and additional difficulty in interpreting microbiome studies, is the entanglement of cause and effect. Microbiomes can coevolve with hosts, resulting in increased benefits for the host (Ford & King, 2016). Additionally, several studies have suggested that infection by *Bd* can shift host microbiomes (Jani et al., 2017; Jani & Briggs, 2014; Longo & Zamudio, 2016). This makes it difficult to determine which features of the microbiome played a causal role in the patterns of declines and recoveries and which features were caused by the outbreak. The positive correlation that we observed between inhibitory bacteria and *Bd* infection intensity, for example, could be caused by *Bd* creating an environment on the frogs in which those bacteria thrive. More laboratory trials are needed to elucidate this further. It would also be invaluable for researchers to collect and archive samples from sites that are not currently infected but are likely to become infected (e.g., Papua New Guinea) so that cause and effect can be disentangled in the future (Bower et al., 2017, 2020).

CONCLUSION AND MANAGEMENT IMPLICATIONS

Our study is among the first to sequence both the fungal and bacterial microbiomes of amphibians. We showed that the four species tested had rich fungal microbiomes and their fungal microbiomes differed among species. We also found that fungal and bacterial microbiomes were positively correlated for both richness and beta diversity. We did not, however, find strong evidence of associations between the fungal microbiome and *Bd* infections. Because so few studies have examined this topic, it is, however, difficult to make broad generalizations and more studies of amphibian fungal microbiomes are needed.

We found some suggestive evidence that microbiome richness may have played a role in historical patterns of declines and recoveries, but a lack of finer-scale associations makes it difficult to draw firm conclusions. It would be useful to examine other species that also show limited or no recovery (e.g., *Litoria rheocola*; Bell et al., 2020; McKnight et al., 2017) to see whether they also have low richness.

Importantly, patterns of relative abundance of inhibitory bacteria were the opposite from our predictions if those bacteria were having a protective effect against *Bd*. *L. dayi* has not recovered from the outbreak despite having the highest relative abundance of inhibitory bacteria generally and *Pseudomonas* spp. specifically. Further, across species and specifically within *L. dayi*, highly infected individuals tended to have the highest relative abundance of inhibitory bacteria. Therefore, at least for this species, simply having a high relative abundance of inhibitory bacteria is not sufficient for population recovery. This has important conservation implications. In laboratory trials, seeding amphibians with inhibitory bacteria (probiotics) often reduces mortality and allows hosts to clear infections or reduce infection intensity (Becker et al., 2009; Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Muletz et al., 2012). As a result, bioaugmentation is widely considered a promising strategy for mitigating *Bd* in wild populations and assisting population recoveries (Bletz et al., 2013; Woodhams et al., 2011, 2012; Yasumiba et al., 2016), but our results suggest that this strategy may be unlikely to succeed. We found few significant associations between *Bd* and individual ASVs, and the associations that were present usually varied by frog species. Further, for *L. dayi*, 48.3% of their bacterial microbiomes, on average, already consisted of inhibitory bacteria, making it unlikely that adding a few more inhibitory bacteria will make a substantial difference. Further, if richness is having a protective effect, a better approach may be to use a diverse consortium of bacteria, with the goal of increasing the richness of the inhibitory community, rather than its abundance (Antwis & Harrison, 2018; Loudon, Holland, et al., 2014; Piovia-Scott et al., 2017); it may, however, be difficult to get a diverse assemblage to establish on hosts. More work is clearly needed to disentangle these complex relationships and design optimal strategies for facilitating species recoveries, and bioaugmentation strategies may need to be species-specific.

AUTHOR CONTRIBUTIONS

Donald T. McKnight and Deborah S. Bower collected the samples. Donald T. McKnight and Roger Huerlimann performed the laboratory work and bioinformatics. Roger Huerlimann, Deborah S. Bower, Lin Schwarzkopf, Ross A. Alford, and Kyall R. Zenger supervised the project, including providing input and advice for the design of the project and analysis of the data. Donald T. McKnight led the analyses and writing. All authors edited, read, and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (McKnight et al., 2022) are available from Dryad: https://doi.org/10.5061/dryad.tht76hf16.

ORCID

Donald T. McKnight https://orcid.org/0000-0001-8543-098X
Roger Huerlimann https://orcid.org/0000-0002-6020-334X
Deborah S. Bower https://orcid.org/0000-0003-0188-3290
Lin Schwarzkopf https://orcid.org/0000-0002-1009-670X
Ross A. Alford https://orcid.org/0000-0002-3010-6768
Kyall R. Zenger https://orcid.org/0000-0001-6817-8786

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