Fighting fungi with fungi: the mycobiome contribution to emerging disease in amphibians

Emerging infectious diseases caused by fungal taxa are increasing and are placing a substantial burden on economies and ecosystems worldwide. Of the emerging fungal diseases, chytridomycosis caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd) is causing a global amphibian extinction. The host frog does have come internal innate immunity, as well as additional resistance through cutaneous microbial communities, leading to the development of probiotic bacterial therapies with mixed results. Unknown is the role of fungi in the protection against Bd infection, and as such, we examined the overlapping roles of bacterial and fungal microbiota in pathogen defense with a combination of high-throughput sequencing and culturing of symbiotic fungi from poison arrow frogs (*Dendrobates* sp.). Our analyses revealed that abundance of cutaneous fungi contributed more to pathogen defense (~45%), than bacteria (~10%) and these differed from environmental microbiota. Further, we demonstrated that a fungal probiotic therapy did not induce an endocrine-immune reaction in contrast to bacterial probiotics that stressed amphibian hosts and suppressed antimicrobial peptide responses, limiting their long-term colonization potential. Our results suggest that probiotic strategies against amphibian fungal pathogens should refocus on host-associated and environmental fungi such as *Penicillium* and member of the families Chaetomiaceae and Lasiosphaeriaceae.

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Title: Fight Fungi with Fungi: The Mycobiome Contribution to Emerging Disease in Amphibians

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Running head: the fungal contribution to amphibian defense

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Abstract

Emerging infectious diseases caused by fungal taxa are increasing and are placing a substantial burden on economies and ecosystems worldwide. Of the emerging fungal diseases, chytridomycosis caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd) is causing a global amphibian extinction. The host frog does have come internal innate immunity, as well as additional resistance through cutaneous microbial communities, leading to the development of probiotic bacterial therapies with mixed results. Unknown is the role of fungi in the protection against Bd infection, and as such, we examined the overlapping roles of bacterial and fungal microbiota in pathogen defense with a combination of high-throughput sequencing and culturing of symbiotic fungi from poison arrow frogs (*Dendrobates* sp.). Our analyses revealed that abundance of cutaneous fungi contributed more to pathogen defense (~45%), than bacteria (~10%) and these differed from environmental microbiota. Further, we demonstrated that a fungal probiotic therapy did not induce an endocrine-immune reaction in contrast to bacterial probiotics that stressed amphibian hosts and suppressed antimicrobial peptide responses, limiting their long-term colonization potential. Our results suggest that probiotic strategies against amphibian fungal pathogens should refocus on host-associated and environmental fungi such as *Penicillium* and member of the families Chaetomiaceae and Lasiosphaeriaceae.

Introduction

Amphibians are participants in the sixth great mass extinction (Wake and Vredenburg 2008), and are globally at risk from a range of factors, not least of which are emerging fungal pathogens (Fisher et al. 2012). The chytridiomycete fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), induces a skin infection in amphibians that disrupts osmotic balance, leading to mortality (Voyles et al. 2009), and has been linked to the extinction of over 200 amphibian species. While chytridiomycosis often has a high mortality rate, species and populations can exhibit variable resistance to *Bd* infection and this differential susceptibility is due in part to skin microbiota (Woodhams et al. 2014).

The microbiome, or the microrganisms that live on and in organisms, can affect host health by influencing development, behavior, metabolism, and inflammation (Cho and Blaser 2012). In amphibians, the bacterial populations associated with the skin can provide protection against *Bd* infection through the production of anti-fungal metabolites, conferring resistance beyond the amphibian's own immune system (Bletz et al. 2013). While fungi have been known to produce anti-microbial compounds for some time (e.g., *Penicillin*, Strobel and Daisy 2003), their affects on amphibian health and interactions with host immune defense are not well known. In particular, studies of cutaneous fungal communities on amphibians infected with *Bd* are lagging, despite a growing literature on bacteria that are often suggested for probiotic applications (Rebollar et al. 2016, Woodhams et al. 2016).

We used a combination of high-throughput sequencing of bacterial and fungal communities and isolation of fungal taxa to test the hypothesis that Bd inhibitory taxa comprise a significantly greater proportion of the cutaneous microbiome compared to the bacteria on four species of poison arrow frogs (*Dendrobates* spp.). Further, we hypothesize that cutaneous fungal taxa supplement host defense against chytridiomycosis. To test these hypotheses we partnered with the New England Aquarium to examine the microbiota of captive poison arrow frogs including *Dendrobates auratus*, *D. leucomelas,* and two subspecies of *D. tinctorius* as well as their tank environment. In 2008, the Aquarium's *Dendrobates* collection experienced a *Bd* infection that eliminated populations of *D. auratus* and *D. tinctorius* while D. *leucomelas* individuals survived this exposure (Hirokawa et al. 2008).The controlled rearing conditions of these frogs on exhibit at the New England Aquarium, coupled with their divergent history of response to *Bd* infection, provides an excellent system to examine the relative contributions of bacterial and fungal skin communities on differential host defense.

Materials and Methods

Sample collection

Frogs were housed on exhibit at the New England Aquarium (Boston, MA, USA) and in its related holding facility 12 kilometers south in Quincy MA. All frog species on exhibit at the New England Aquarium were housed in a single tank. This 3 m^3 display had a glass public facing front and a solid fiberglass housing. It has an 80 L sump for water reserve that is pumped up to the exhibit. Water and bioifim samples were collected from the sump. The frogs at the Quincy holding facility were housed in species-specific pens. In 2015, holding cages were

plexiglass with a paper-towel floor with reserve water pool. In 2016, the holding cages were enhanced with soil, moss, and other plants. In these holding cages, biofilms were collected from the edge of the water pool. Approximately 75% of the frogs in collection were bred at the Quincy holding facility, with the remainder originating in other Association of Zoo and Aquarium accredited institutions.

Frogs were individually selected, rinsed with 15-mL of sterile water, and swabbed on the ventral surface. Swabs were placed in cryovials and stored on dry ice for transportation to the lab. All samples were kept at -80°C until DNA extraction. Water was collected in 15 mL sterile tubes, stored on ice, and filtered at University of Massachuetts, Boston. Biofilms were swabbed from the tanks and stored frozen in sterile cryovials.

DNA Extraction, PCR, and Sequencing

DNA was extracted from frog and tank biofilm swabs using the MoBio PowerSoil Total DNA Isolation kit (Carlsbad, CA, USA) following manufacturer's instructions. Water samples were extracted using the MoBio PowerWater Total DNA Isolation kit following manufacturer's instructions. All DNA extractions were verified by gel electrophoresis. Samples for bacterial community analysis were amplified in triplicate using the primer pair 515F and 806R (Caporaso et al. 2010) following previously published conditions (Caporaso et al. 2012). Primer constructs had Illumina adaptors and 12-bp GoLay barcodes. Proper product formation was verified with gel electrophoresis and samples were purified with the Qiagen QiaQuick Gel Purification Kit (Qiagen, Valencia, CA). Samples were quantified with a Qubit (Thermofisher) and pooled in equal masses for paired-end sequencing on the Ilumina MiSeq using V2 chemistry.

Fungal communities were amplified in triplicate with primers ITS1F and ITS2R (Walters et al. 2016) targeting the fungal internal transcribed spacer region (ITS). Primer constructs contained overhang sequences to allow downstream addition of dual Illumina indices and adapters. Proper product formation was verified with gel electrophoresis and samples were purified with the Qiagen QiaQuick Gel Purification kit. A second 8-cycle PCR was performed with the Illumina Nextera XT2 kit following the manufacturer's instructions to ligate dual indices and Illumina adaptors for each sample. Samples were then purified using a Qiagen PCR Purification kit, quantified with a Qubit, and pooled in equal mass for paired-end sequencing on the Illumina MiSeq using V2 chemistry.

Fungal isolation and Bd assays

To isolate fungi from the frog and the environment we swabbed the tank biofilm and the ventral surface of the frog in 2015 and plated the swabs on Potato Dextrose and Sabouraud Dextrose Agar. One millilter of water was spread on Potato Dextrose and Sabouraud Dextrose Agar as well. Plates were incubated at 25°C in the dark for 3 days and all distinct isolates were picked and isolated on Potato Dextrose Agar. Isolates were identified with sequencing at the Massachusetts General Hospital DNA Core Facility using the primer pair ITS1F and ITS4R (White et al. 1990).

To test the efficacy of isolates for inhibition of *Bd* zoospore growth we followed a protocol outlined previously (Woodhams et al. 2014). Briefly, all isolates were grown in 1% tryptone broth overnight in sterile centrifuge tubes. Following confirmation of growth, samples were centrifuged at 2,225 x *g* for 5 minutes to pellet the cells and the liquid was filtered through 0.22 µm filters. Isolate filtrates were kept at -20°C until inhibition assays. Two strains of *Bd* (JEL 197 and 423) were grown on 1% tryptone agar for 4-7 days to allow for the production of zoospores. Plates were flooded with 1% tryptone and the liquid was filtered through 0.45 µm filters. *Bd* zoospores were counted on a haemocytometer and diluted to 50 zoospores mL^{-1}. To assay the inhibition of *Bd* we inoculated 96-well plates with 50 µL *Bd* zoospores and 50 µL isolate filtrates. For negative controls we used heat killed Bd and wells containing no zoospores and for positive controls 50 µL of tryptone was added to zoospores. Growth was measured as changes in optical density at 480 nm at days 0, 3, 5, and 7. Differences in growth between isolates and controls was measured with a t-test in R (R core team 2012) using a Benjamini-Hochberg correction for multiple comparisons. To determine the phylogenetic relationship among fungal taxa we aligned ITS sequences with clustalW (Thompson et al. 2002) and constructed a phylogenetic tree based on maximum likelihood using RAxML (Stamatakis 2014) and visualized the tree with the Interactive Tree of Life (Letunic and Bork 2007). We tested confidence in tree topology with bootstrapping with 1000 restarts. To test for a phyogentic signal of inhibition or enhancement within our isolates we used a UniFrac significance test (Lozupone et al. 2006).

Quantitative PCR

Quantitative PCR (qPCR) was performed to assess the total abundance of the bacterial and fungal communities on all samples. DNA and standards were first quantified with a Qubit (Thermofisher). All samples were normalized to 3 ng μL^{-1} and serial dilutions of standards were prepared from purified PCR product of each gene. DNA from each sample was amplified in triplicate, along with standards and internal controls on a Strategene MX-3500P quantitative thermocycler (Stratagene, La Jolla, CA, USA). 16S rRNA and ITS genes were amplified in 25 µL reactions using 0.25 µL of each primer, 12.5 µL of Qiagen QuantiTect SYBR Green PCR Master Mix, 1 µL of DNA template, and 11 µL PCR grade water. Bacterial qPCR was performed with primers 357F and 515R (Biddle et al. 2008) following conditions described by Bowen et al. (2011). Fungal qPCR was performed with primers ITS1F and ITS2R with the same cycling conditions as the 16S rRNA gene. Proper product formation was verified with melt curves and gel electrophoresis. All standard curves possessed a high degree of linearity (>0.99 R^2) and PCR efficiency ranged from 95-101% for both bacterial and fungal qPCR. To assess differences in the abundance of 16S rRNA gene and ITS copy number we used a Welch's T-test or an ANOVA in R.

Sequence and Statistical analyses

Paired-end reads from 16S rRNA gene or ITS gene sequences were first joined with fastq-join (Aronesty 2011) and then quality filtered and demultiplexed in QIIME (version 1.91; Caporaso et al. 2010) following previously published guidelines (Bokulich et al. 2013). Fungal reads were further quality filtered using itsX to remove 5S and 18S fragments, which improves

fungal analyses (Bengtsson-Palme et al. 2013). Both bacterial and fungal sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity using uClust (Edgar 2010) against the GreenGenes (version 13.5) and UNITE (version 7.0) databases respectively. Following clustering, OTUs appearing only once (singeltons) and OTUs matching archaea, chloroplasts, and protists were removed from both datasets. Beta diversity was calculated using Bray-Curtis on OTU tables normalized to the lowest sampling depth (9,563 for bacteria and 6,753 for fungi). Beta diversity was visualized with a principal coordinates analysis. Significant differences in community composition were assessed with a permutational multivariate analysis of variance in QIIME (Anderson 2001).

To assess the percentage of bacterial communities that were inhibitory towards *Bd* we filtered our data set against a database of known Bd-inhibiting bacteria (Woodhams et al. 2015) in QIIME. We then filtered our fungal dataset against our Bd inhibiting/facilitating isolates. We visualized the results using a heatmap and dendrograms to assess similarity of anti-fungal communities. Dendrograms were calculated using the weighted pair group method and arithmetic mean (WPGMA) clustering. We tested for differences in percentages of inhibitory taxa between frogs and the environment using a Welch's T-test in R. To determine the interaction of inhibitory and enhancing bacterial and fungal taxa we constructed a bipartite OTU network following a previously described method (Bowen et al. 2013). We included sample types as their own nodes using the Fruchterman and Reingold (1991) algorithm for ease of visualization. Networks were visualized using the R package network (Butts et al. 2012).

Testing amphibian immune and stress response to bacteria and fungi

Midwife toads, *Alytes obstetricans* (n = 29), were raised in captivity from larvae at the Breeding Centre of Endangered Amphibians of the Guadarrama Mountains in Spain. Toad research conformed to the legal requirements of Consejerias de Medio Ambiente of Madrid. *Alytes obstetricans* were maintained on a 12:12h light cycle and fed *Acheta domesticus ad libitum.* After experimental treatments described below, toads were released into an outdoor mesocosm containing natural vegetation, a small pond, and pile of rocks for shelter. Frogs were photographed for individual identification upon recapture based on unique markings.

Midwife toads were randomly assigned to one of four treatments. Control toads (n = 12) were bathed in 20 ml sterile water for one hour. Toads treated with probiotics were bathed in 20 ml water containing 1 ml of either *Penicillium expansum* (n=9), *Janthinobacterium lividum* (isolate 77.5b1, 56 x 10^7 CFU, n = 4), or *Flavobacterium johnsoniae* (isolate 70c, 19 x 10^7 CFU, n=4) for one hour. The *P. expansum* was grown on Sabouraud Dextrose agar, while freshly growing bacteria were rinsed directly from 15mm Petri plates with R2A agar media supplemented with 1% tryptone.. Probiotic isolates were originally collected from wild *A. obstetricans* near Basel, Switzerland and chosen for this experiment based on their ability to inhibit *B. dendrobatidis* growth (Woodhams et al. 2014). Isolates used in this study were deposited in the Culture Collection of Switzerland (CCOS 423 & 433, http://www.ccos.ch/).

We assessed the stress response of frogs to probiotics using water-borne corticosterone release rates. Corticosterone is the primary amphibian stress hormone and water-borne corticosterone release rates are highly correlated with circulating corticosterone levels measured

from plasma (Gabor et al. 2013). An hour after removing the toads from probiotic treatments, they were placed in 40 ml of sterile water within a 100ml beaker for 1 hr to collect water-borne hormones. Frogs were carefully lifted out of the beaker and the remaining water sample was saved to assay corticosterone release rates. Water samples were immediately frozen at -20°C and the hormones were extracted from the thawed water using C18 solid phase extraction columns (SepPak Vac 3 cc/500 mg; Waters, Inc., Milford, MA, USA) with Tygon tubing (Saint Bobain formulation 2475) under vacuum pressure. After extraction the columns were immediately frozen at -20°C and sent to Texas State University where were eluted with methanol and then evaporated with nitrogen gas following Gabor et al. (2013). The residue was then resuspended in 5% ethanol and 95% EIA buffer (provided by Cayman Chemicals Inc. Ann Arbor, MI, USA) for a final re-suspension volume of 250 µL. Corticosterone release rates were measured in duplicate for all samples with an enzyme-immunoassay (EIA) kit (Cayman Chemicals Inc.) on a fluorescent plate reader (BioTek Powerwave XS). We examined the difference between treatment groups in the initial corticosterone stress (natural log transformed) using ANOVA with Tukey HSD pairwise comparisons (R Core Team 2012).

Following the hormone assay we released toads into the same outdoor mesocosm for four weeks after when we measured their skin peptide defense capacity. Peptide quantities recovered were compared among treatment groups. Peptides at a concentration of 500 µg ml^{-1} were tested for ability to inhibit the growth of *B. dendrobatidis*, *J. lividum*, and *F. johnsoniae*. The difference between treatment groups were compared using ANOVA with Tukey HSD pairwise comparisons (R Core Team 2012).

Results

Community composition and abundance

In the winter of 2015 and 2016 we sampled skin microbiomes from poison arrow frogs both from a mixed-species exhibit at the New England Aquarium (Boston, MA, USA), as well as from single species holding (Offsite Holding, Quincy MA, USA). High-throughput sequencing of the 16S rRNA gene and fungal intergenic transcribed spacer (ITS) region revealed distinct communities of microbes associated with host skin compared to the microbial communities found in the frog's environment (Fig. 1; PERMANOVA, $p<0.001$, $F_{(5,62)}=7.89$). Furthermore, we identified species-specific bacterial and fungal communities within the *Dendrobates* genus (Fig. 1; PERMANOVA, $p<0.01$, $F_{(4,45)}=6.98$) and this species specificity was maintained whether the frogs were reared in separate tanks at the Animal Care Facility, or in a shared tank on exhibit. We found lower bacterial abundance (Fig. S1; Welch's T-test, $p<0.01$, $T=12.43$) and higher fungal abundance (Fig. S1B, $p<0.01$, $T=7.89$) on frog skin compared to the frog environment. Surprisingly, there was no significant difference in fungal and bacterial abundance on frogs (ANOVA, $p=0.96$, $F=0.03$) or between species ($p=0.85$, $F=2.34$).

Fungal isolation and Bd challenge assays

We isolated 135 fungal strains, of which 58 were unique at 97% sequence identity, from the skin and environment of *Dendrobates* frogs (Table S1). The cultured isolates, when screened against the ITS amplicon sequences, accounted for 71% of the sequences present. Secondary

metabolites were collected from each isolate and tested for activity against zoospores from two strains of *Bd* (JEL 197 and 423). We found 32% (n=44) of isolates were capable of inhibiting at least one strain of *Bd* (Table S1). Surprisingly, 28% of isolates (n=39) were capable of facilitating the growth of at least one strain of *Bd*. Phylogenetic analysis of the fungal taxa (Table S1, Fig. S2) revealed diverse isolates primarily associated with phylum Ascomycota. A UniFrac significance test (Lozupone et al. 2006) indicated a significant phylogenetic signal (p<0.001) for both enhancement and inhibition of *Bd* growth, suggesting phylogenetic conservation of this trait.

Distrubution of Bd inhibiting/enhancing taxa

We assessed the community composition of *Bd*-inhibitory and *Bd*-facilitating taxa by screening our high throughput sequencing data against databases of known *Bd* inhibitory/facilitating bacteria (Woodhams et al. 2015) and fungi (Table S1). Our result revealed that a significantly higher percentage of the cutaneous bacterial and fungal communities were antifungal than was found in the frog's environment (Fig. 2A and 2B); however, there were significantly more anti-*Bd* fungi than anti-*Bd* bacteria (Welch's T-test, T=19.44, p<0.0001). In fact, approximately 45% of the fungal community on a given frog was capable of inhibiting *Bd* suggesting the fungal taxa present, rather then shear abundance most strongly influence *Bd* resistance. Further, the percentage of *Bd*-facilitating fungal taxa (Fig. 2C) was significantly lower on frog skin than the environment (Welch's T-test, T=12.90, p<0.01).

We next examined patterns of antifungal community structure among different *Dendrobates* species by comparing abundance of bacterial taxa that have demonstrated antifungal properties. Our results indicate species specific antifungal bacterial communities (PERMANOVA, p<0.001, $F_{(3,45)}$=12.34; Fig. 3A), which links the differential susceptibility among species of *Dendrobates* (Hirokawa et al. 2008) to their specific microbial communities (Fig. 1). All frogs were enriched in bacteria from the families Aeromonacaceae, Enterobacteriaceae, Pseudomonadaceae, and Xanthomonadaceae and from the genera *Cryseobacterium*, *Flavobacterium*, and *Comamonas* (Fig. 3A). *Dendrobates leucomelas*, the species that withstood a previous *Bd* infection (Hirokawa et al. 2008), was enriched in bacteria from the genus *Pseudomonas* suggesting this genus may play an important role in immune defense. Like the bacterial communities, the anti-*Bd* fungal communities displayed frog species specific inhibitory communities (p<0.003, F=4.667; Fig. 3B). All frog species had skin containing a large number of a highly divergent fungal taxon from the phylum Ascomycota, a taxon unknown at the kingdom level, and from the genus *Cladosporium* (Fig. 3). Compared to the other frog species, *D. leucomelas* was enriched in anti-fungal taxa from the phylum Ascomycota, in particular taxa from the families Chaetomiaceae, Lasiosphaeriaceae, and the genus *Pestalotiopsis*, suggesting an important role for these taxa in *Bd* defense.

To determine the interactions between *Bd*-enhancing and inhibiting taxa within the bacterial and fungal datasets we performed a bipartite network analysis (Fig. 4). Both bacterial and fungal networks indicated that frogs had a higher degree of network connectivity (mean=65.25 for bacteria, 38.75 for fungus) to Bd-inhibitory taxa than the environment (mean=32 for bacteria, 28 for fungus). Further, the number of Bd-inhibitory taxa unique to frog

skin (n=47 for bacteria , n=25 for fungi) was higher than those unique to the environment (n=0 for bacteria, n=11 for fungi) and was highest for *D. leucomelas* (n=4 for bacteria, n=7 for fungi). The presence of Bd-inhibitory bacteria and fungi present on frog skin and not in the environment suggests these taxa may be ideal candidates for use as probiotics in the treatment or prevention of chytridiomycosis. In particular, the fungal taxa from the family Chaetomiaceae and *Lasiosphaeriaceae* associated only with the *Bd* resistant *D. leucomales*, are of particular interest for future study.

Probiotic stress and immune tests on Midwife Toads (Alytes obstetricans)

To determine potential endocrine-immune interactions produced by applications of fungal or bacterial probiotics, we exposed a non-Bd resistant species, Midwife toads (*Alytes obstetricans*), to two bacterial strains (*Janthinobacterium lividum* and *Flavobacterium johnsoniae*) and a fungus closely related to an isolate from our data set, *Penicillium expansum* (Table S1). These probiotics were previously isolated from the target host species. Exposure to bacterial strains significantly increased corticosterone (stress hormone) release rates relative to *P. expansum* and control frogs (Fig. 5A; ANOVA, F=21.83, p<0.001). Exposure to *P. expansum* did not significantly decrease host antimicrobial peptide activity against *Bd* while exposure to both bacterial strains did (Fig. 5B; ANOVA, F=4.26, p=0.015).

Discussion

Host-associated microbial communities can have profound effects on host health and immune response (Cho and Blaser 2012). While microbes can directly modulate the host immune function through direct interactions with the host (i.e. inflammation), host associated taxa can provide additional immune defense against pathogens through the production of secondary metabolites. Our study sought to examine the role of host-associated bacterial and fungal communities in immune defense against chytridiomycosis in three species of poison arrow frogs (*Dendrobates* spp.). We observed species-specific bacterial and fungal communities associated with each frog that was distinct from their enclosure. Species-specific microbial communities have been observed at broad (Givens et al. 2015), narrow (Lee et al. 2011), and sub-species levels (Micallef et al. 2009). In addition our results suggest that the difference between the fungal communities of frogs and their environment is likely due to the high percentage of Bd-inhibitory taxa found on frog skin relative to their environment. The differences in microbiota observed among different frog species being held within the same exhibit as well as the consistency in community structure sampled in two different years suggest that these frogs can recruit and maintain specific taxa, including those that can provide protection against Bd. However, our study did not include field-caught frogs and the addition of wild forgs will help elucidate the strength of the pattern.

Much effort has been identifying bacteria capable of inhibiting Bd to be used as probiotics (Bletz et al. 2015, Woodhams et al. 2015, Walke and Belden 2016) and some bacteria have been shown to enhace the growth of Bd zoospores (Woodhams et al. 2015). Fungal taxa, in contrast, have been virtually ignored. Our study isolated 135 predominately uncultivated fungi, which possessed varying ability to inhibit (n=44) the growth of Bd zoospores. Further, the

percentage of inhibitory fungi was significantly higher than bacteria on frog skin suggesting fungi have the potential to be an important source of host defense against pathogens on amphibians and perhaps for other organisms (Dean et al. 2012). In addition to taxa with the ability to inhibit the growth of Bd zoospores, we identified several (n=39) taxa capable of enhancing the growth of Bd zoospores. While cooperation between pathogens and microbes is more common between closely related taxa (West and Buckling 2003, Griffin et al. 2004), our results may suggest that cooperation between fungal taxa may occur across greater phylogenetic distance indicating a lack of specificity in these interactions. Further, the presence of Bd inhibiting and enhancing fungal taxa suggests that the interactions between skin-associated taxa and potential probiotics is important for not only the establishment of potential probiotics (Becker et al. 2011, Kueng et al. 2014, Kueneman et al. 2016) but also for the immunological function of host-assocaited microbial communities.

Frogs from the *Dendrobates* population at the New England Aquarium have demonstrated differential susceptibility to Bd infection, with *D. leucomelas* having previously demonstrated the ability to clear itself of Bd infection (Hirokawa et al. 2008). Analysis of bacterial and fungal communities revealed species-specifc fungal and bacterial communities, suggesting the microbial communities may play an important role in host defense, in particular on *D. leucomelas*. Further, network analysis revealed groups of Bd-inhibitory bacteria and fungi found only on the skin of *D. leucomelas*, suggesting these may play an important role in the ability of *D. leucomealas* to rid itself of Bd infection. Bd inhibiting taxa from the genus *Pseudomonas* were enriched on the microbiome of *D. leucomelas* relative to other frogs. The genus *Pseudomonas*, a common bacterial genus across many biomes, is known to produce numerous extracellular and often antimicrobial metabolites (Holmström and Kjelleberg 1999) and its use as a probiotic has proven effective in plants (Picard and Bosco 2008), fish (El-Rhman et al. 2009), and shellfish (Hai and Fotedar 2009). In addition to bacteria, *D. leucomelas* was enriched in Bd inhibiting fungi from the families Chaetomiaceae, Lasiosphaeriaceae, and the genus *Pestalotiopsis*, suggesting an important role for these taxa in Bd defense. The persistence of Bd inhibiting taxa on amphibian skin, as well cosmopilitian distribution of many of these taxa across many biomes suggests these taxa would be ideal probiotic candidates for treating chytridiomycosis.

Our results have demonstrated a core role for amphibian-associated fungi in host defense against Bd infection. Further, probiotic application of *Penecillium expansum* to midwife toads did not significantly alter host immune or stress levels, while bacterial probiotics did. Applications of the probiotic bacterium *Janthinobacterium lividum* were protective against chytridiomycosis for several species of amphibians (Harris et al. 2009, Becker et al. 2009, Kuenman et al. 2016). Further, the viability of Bd zoospores was significantly reduced after exposure to mucus from frogs treated with the bacterium *Flavobacterium johnsoniae* and the fungus *P. expansum* (Woodhams et al. 2014). However, a recurrent problem with probiotic applications is the colonization resistance of hosts (Becker et al. 2011, Kueng et al. 2014), such that augmented bacteria fail to establish in the skin, particularly in the absence of an environmental reservoir for the probiotic bacteria (Kueneman et al. 2016). Additionally, Kueng et al. (2014) showed that some probiotic treatments may stress hosts or cause an immune

reaction in amphibians that prevents establishment of the probiotic. Our results suggest that fungal taxa such as *Penicillium*, that are common across amphibians and environments, may provide ideal candidates for amphibian probiotic therapy because these fungal probiotics may not induce host stress responses or repress the host's mucosal peptide response. Instead, they may, in addition to antimicrobial metabolite production, induce host defenses that target foreign fungi, including Bd.

Conclusions

We show species-specific bacterial and fungal communities associated with *Dendrobates* frogs that are distinct from their environment. The distinct microbiome (including the mycobiome) on host skin is, in part, due to host recruitment of potentially anti-microbial taxa that may help promote host health. Frog-associated bacterial communities possessed a significant portion of Bd-inhibitory taxa and the fungal communities were dominated by anti-*Bd* taxa, suggesting fungi may play a greater role in host protection than bacteria in amphibians. Our results suggest that host-associated fungi should be a greater focus of future efforts to develop probiotic therapies for the treatment of chytridiomycosis. When considering the host immune priming function provided by microbiota (Kurtz et al. 2007), and host resistance to bacterial colonization (Kueng et al. 2014), fungi may provide key probiotics needed for disease management in amphibians.

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Author contributions

PJK, JLB, MFT, and DCW designed the poison arrow frog experiment. PJK oversaw the students that performed all sequencing related activities. SF and PJK performed the qPCR, *Bd*

inhibition assays, and isolation of fungal cultures. SFB, CRG, JB, and DCW performed midwife toad experiments. PJK performed all sequence and statistical analyses. PJK, JLB, MFT, and DCW wrote the paper with contributions from SF, SFB, CRG, and JB.

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Figure legends

Figure 1- Principal coordinates analysis based on Bray-Curtis similarity for the bacterial (A) and fungal communities (B) from *Dendrobates* spp. frogs and their enclosure.

Figure 2- Percentage of bacterial (A) and fungal (B) communities that inhibit the growth of *Bd* and the percentage of fungal communities that facilitate the growth of *Bd* (C) from *Dendrobates* spp. frogs and their enclosure. Frogs have significantly higher antifungal taxa than their enclosure (Welch's t-test, $p<0.01$, $T=21.12$) and there is significantly more antifungal fungi than bacteria on frog skin ($p<0.01$, $T=29.01$). Boxes represent 25-75% quartiles, and the solid black line is the median value.

Figure 3- Heat maps of $\log_{10}$ abundance of antifungal bacterial taxa (A) and fungal taxa (B). Dendrograms are based on weighted pair group method and arithmetic mean (WPGMA) clustering. The highest level of taxonomic identification of each taxon is denoted by k=kingdom, p=phylum, o=order, f=family, and g=genus. DAU=*Dendrobates auratus*, DAZ= *D. tinctorius* "azureus", DTI= *D. tinctorius*, LEU=*D. leucomelas.*

Figure 4- Network analysis depicting the connectivity among sample types for anti-*Bd* bacterial (A) and fungal taxa (B). Different colors in (B) indicate the taxa's ability to either inhibit, enhance, or have no effect on *Bd* growth. In (A) "Frog only" taxa are found only in frogs while "all samples" are found in all sample types. *AU=Dendrobates auratus, AZ= D. tinctorius "azureus", T= D. tinctorius, L=D. leucomelas, W=water, B=Biofilm.*

Figure 5- Box plot of corticosterone release rates (A) and toad peptide capacity against Bd (B) in Midwife Toads measured after exposure to one fungal and two bacterial strains.

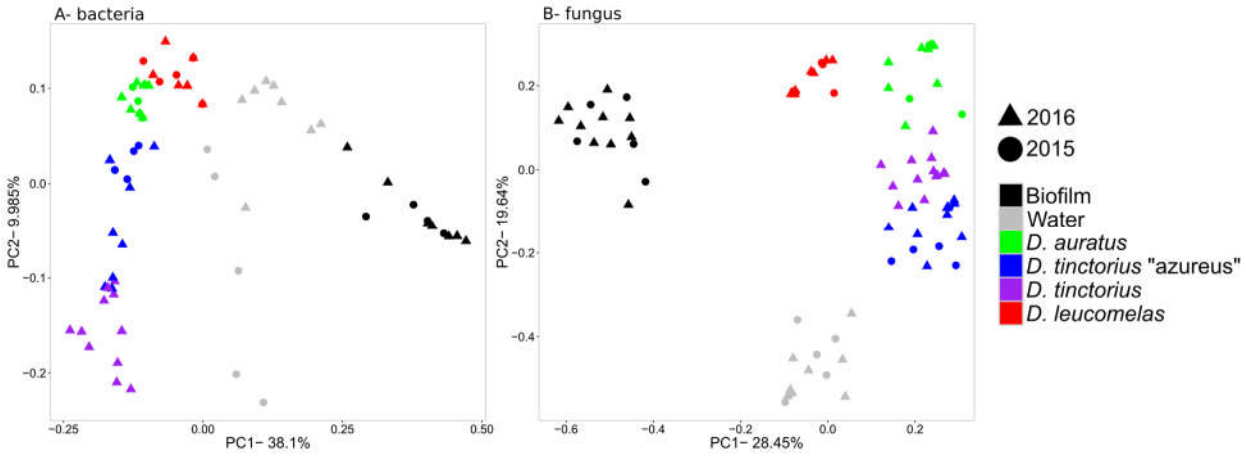
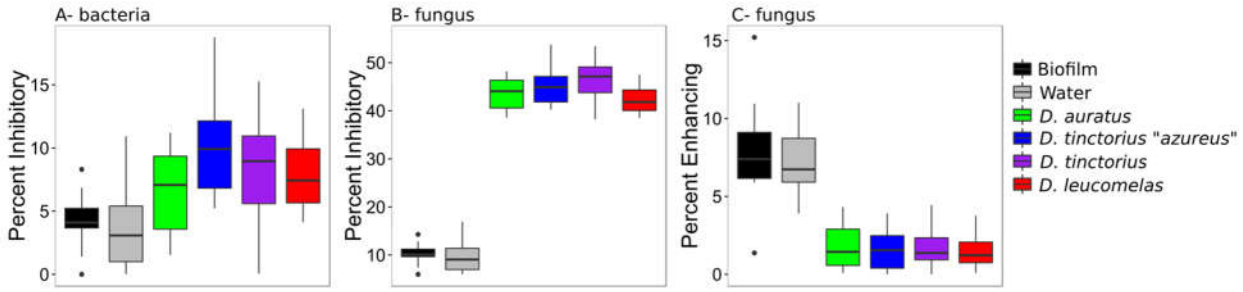


Figure 1.



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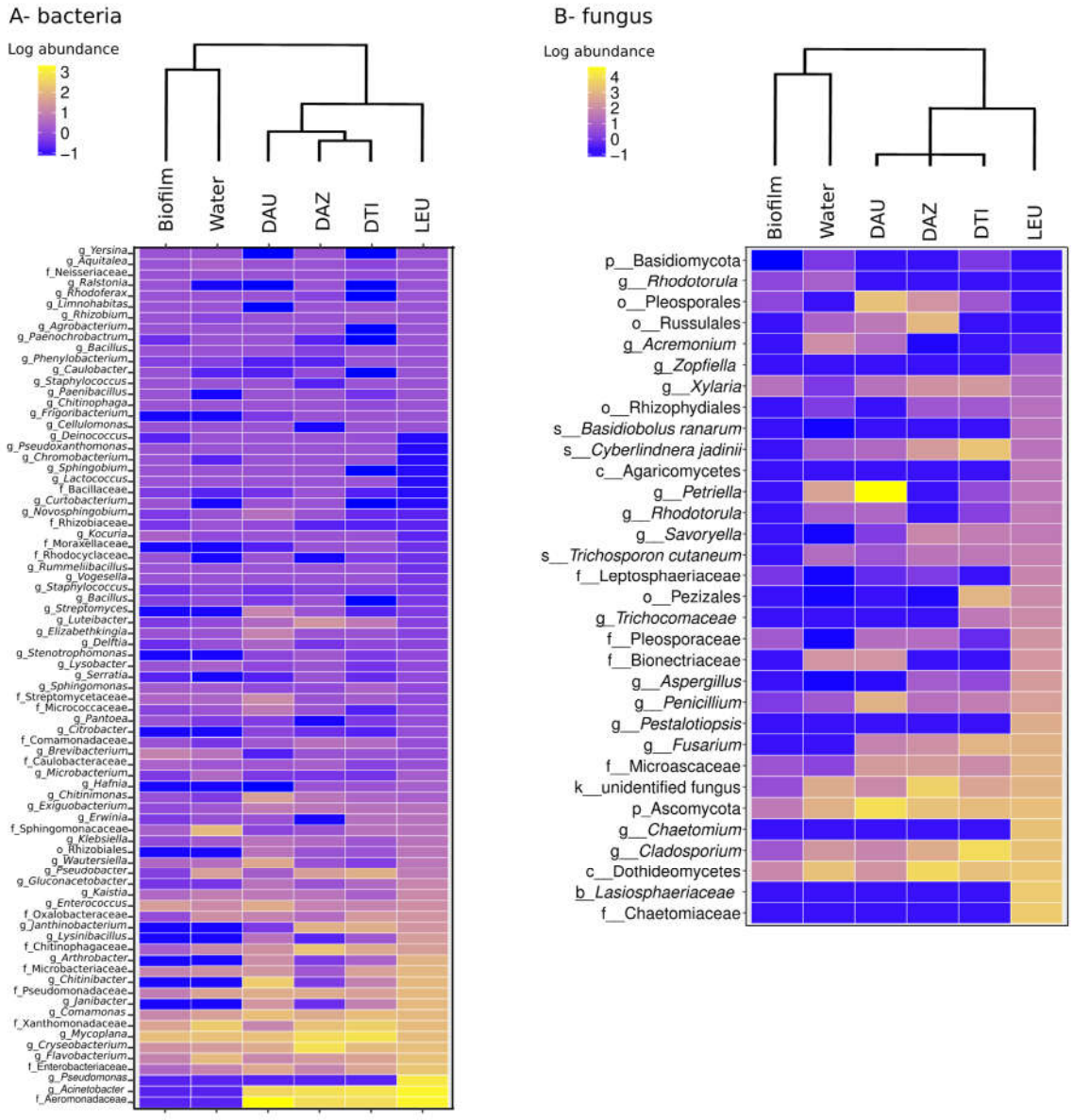

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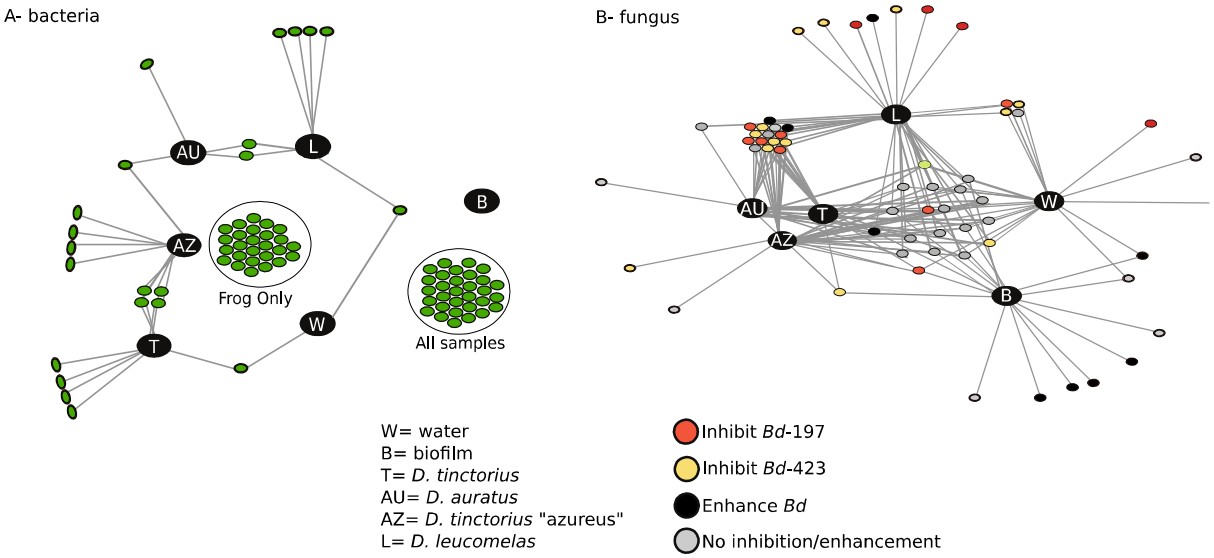

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612 Figure 4.

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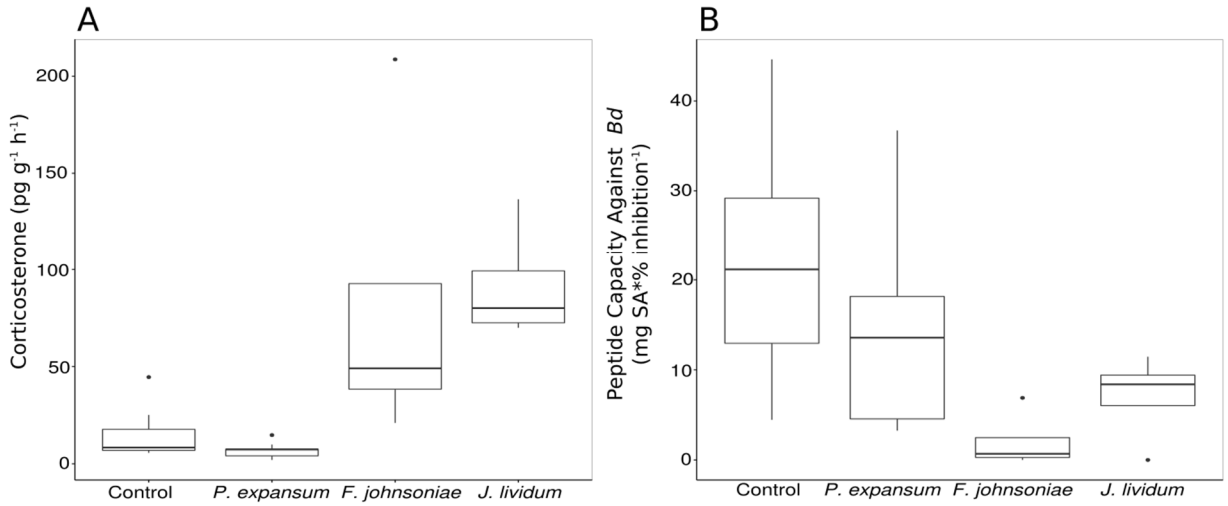

614 Figure 5.

615 **Supplementary Figures and Tables**

616 Figure S1- Quantitative PCR results comparing the abundance of bacteria (A) and fungus (B) in
617 *Dendrobates* spp. frogs and their enclosure. Boxes represent 25-75% quartiles and the solid
618 black line is the median value. Note the differing scales between (A) and (B).

619 Figure S2- Phylogenetic tree of distinct fungal isolates (97% sequence identitiy) based on
620 maximum likelihood. OTUs are colored by the ability to inhibit (blue), enhance (red), or have no
621 significant effect (black) on Bd growth. All branches colored red have bootstrap values >0.75,
622 green branches have bootstrap values >0.5, and black branches have bootstrap values <0.50.

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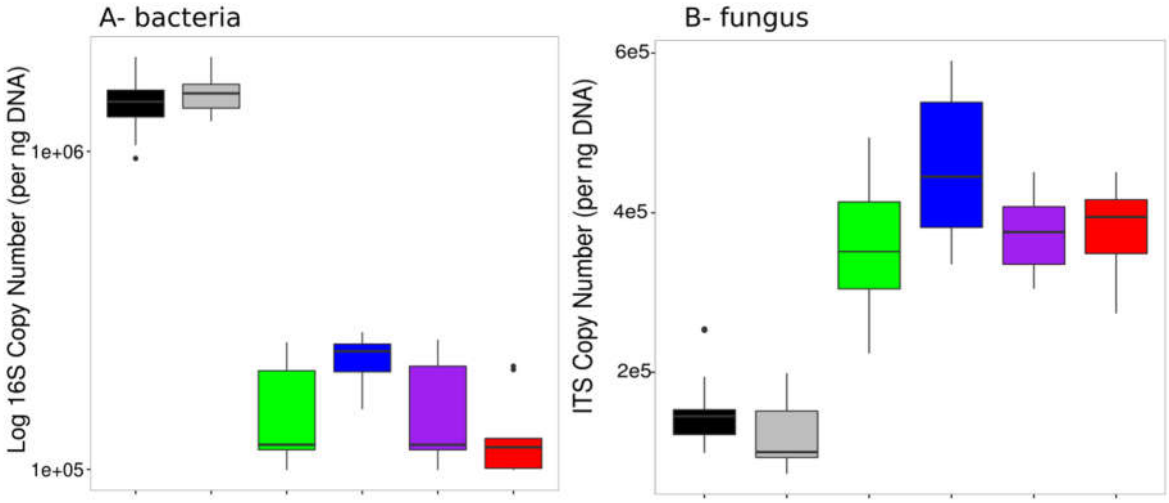

627 Figure S1.

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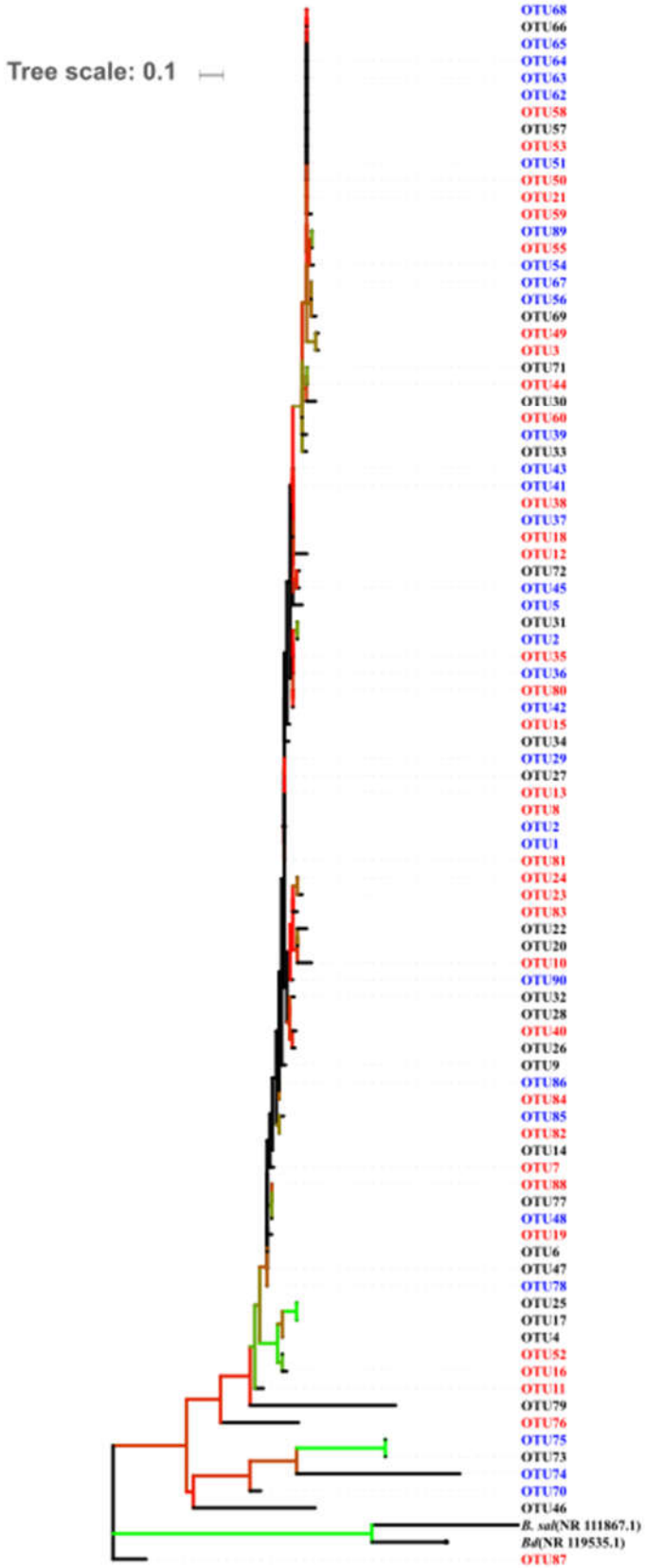
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OTU17
OTU4
OTU52
OTU16
OTU11
OTU79
OTU76
OTU75
OTU73
OTU74
OTU70
OTU46
B. sal(NR 111867.1)
Bd(NR 119535.1)
OTU87

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Figure S2.

Table S1- List of unique fungal isolates and whether or not they possess the ability to significantly (Benjamini Hochberg $p<0.05$) inhibit (<100%) or enhance (>100%) the growth of two strains of Bd, strains 197 and 423. Numbers in parentheses are standard error of the mean. Bolded taxa significantly inhibit at least one strain of Bd. The highest level of taxonomic classification, as determined by BLASTn is indicated. p= phylum, c=class, o=order, f=family, g=genus, s=species, and ns= not significant.

OTU #	Taxonomy	Inhib. 197	Inhib. 423
1	p_Ascomycota	49.32 (22.92)	66.99 (5.85)
2	p_Ascomycota	ns	49.57 (10.58)
3	c__Dothideomycetes	ns	172.84 (22.68)

3	c__Dothideomycetes	181.45 (2.61)	ns
4	c__Dothideomycetes	ns	147.35 (29.33)
5	c__Dothideomycetes	ns	143.02 (19.88)
6	c__Dothideomycetes	ns	ns
7	c__Dothideomycetes	171 (8.59)	154.87 (49.98)
8	c__Dothideomycetes	169.85 (7.83)	ns
9	c__Dothideomycetes	166.26 (23.05)	ns
10	c__Dothideomycetes	168.1 (4.95)	232.33 (19.46)
11	c__Dothideomycetes	165.99 (19.81)	145.12 (6.7)
12	c__Dothideomycetes	174.15 (16.71)	195.39 (5.18)
13	c__Dothideomycetes	140.3 (45.41)	141.51 (11.06)
14	c__Dothideomycetes	ns	158.63 (7.64)
15	c__Dothideomycetes	185 (15.18)	157.86 (2.53)
16	c__Dothideomycetes	186.62 (11.47)	165.49 (12.2)
17	c__Dothideomycetes	ns	142.02 (2.48)
18	c__Dothideomycetes	140.6 (40.07)	149.67 (15.84)
19	c__Dothideomycetes	161 (46.52)	152.5 (6.17)
20	c__Dothideomycetes	167.92 (11.98)	ns
21	c__Dothideomycetes	185.96 (3.07)	189.84 (3.08)
22	c__Dothideomycetes	ns	154.72 (7.59)
23	c__Dothideomycetes	ns	187.96 (8.07)
24	c__Dothideomycetes	140.99 (14.38)	166.61 (5.91)
25	c__Dothideomycetes	163.04 (7.98)	ns
26	c__Dothideomycetes	ns	187.85 (13.02)
27	c__Dothideomycetes	ns	ns
28	c__Dothideomycetes	ns	161.41 (10.54)
29	c__Dothideomycetes	1.78 (2.28)	14.94 (6.04)
30	c__Dothideomycetes	ns	ns
31	c__Dothideomycetes	143.23 (22.71)	ns

32	c__Dothideomycetes	211.57 (11.5)	ns
33	g__Cladosporium	ns	ns
34	g__Arthrographis	ns	139.53 (25.53)
35	o__Pleosporales	ns	181.82 (2.83)
36	o__Pleosporales	66.57 (7.8)	-1.77 (0.38)
37	f__Leptosphaeriaceae	53.74 (10.03)	74.2 (5.22)
38	f__Phaeosphaeriaceae	102.37 (9.2)	218.14 (16.98)
39	f__Pleosporaceae	18.78 (7.27)	-0.05 (0.33)
40	g__Curvularia	173.03 (11.82)	149.08 (57.17)
41	g__Aspergillus	64.21 (13.09)	58.21 (6.59)
42	g__Penicillium	17.71 (3.88)	21.8 (4.73)
43	s__Trichocomaceae sp	39.97 (6.37)	48.14 (12.47)
44	f__Teloschistaceae	ns	184.71 (18.47)
45	o__Pezizales	57.49 (10.44)	59.3 (9.16)
46	s__Helvella sp	137.91 (26.38)	ns
47	s__Plectania milleri	ns	ns
48	s__Cyberlindnera jadinii	19.03 (7.66)	46.85 (9.42)
49	c__Sordariomycetes	ns	146.54 (14.42)
50	o__Hypocreales	183.13 (6.32)	ns
51	f__Bionectriaceae	81.4 (44.96)	10.33 (10.76)
52	f__Hypocreaceae	156.38 (10.55)	143.6 (37.45)
53	s__Trichoderma spirale	161.67 (44.03)	163.16 (43.33)
54	s__Acremonium sp	15.54 (5.21)	75.61 (7.69)
55	g__Sarocladium	139.34 (32.12)	146.8 (15.26)
56	g__Fusarium	72.4 (8.33)	36.83 (13.08)
57	s__Nectriaceae sp	ns	ns
58	s__Volutella consors	151.4 (3.5)	171.24 (27.13)
59	f__Ophiocordycipitaceae	168.38 (11.05)	151.43 (34.1)
60	s__Purpureocillium lilacinum	ns	150.86 (32.07)

62	g__Savoryella	28.49 (9.79)	122.17 (13.55)
63	f__Microascaceae	61.15 (13.72)	-28.22 (26.7)
64	f__Chaetomiaceae	31.62 (28.96)	42.53 (16.08)
65	g__Chaetomium	59.86 (6.9)	44.87 (15.71)
66	s__Lasiosphaeriaceae sp	ns	ns
67	s__Lasiosphaeriaceae sp	31.17 (14.94)	55.61 (12.91)
68	s__Zopfiella sp	33.68 (1.43)	51.29 (4.99)
69	s__Zopfiella sp	ns	ns
70	g__Pestalotiopsis	ns	72.39 (9.25)
71	s__Microdochium sp	ns	ns
72	g__Xylaria	81.99 (6.66)	282.61 (54.06)
73	p__Basidiomycota	68.46 (7.29)	130.6 (4.66)
74	p__Basidiomycota	21.04 (0.34)	167.32 (15.56)
75	c__Agaricomycetes	67.56 (7.02)	ns
76	o__Agaricales	ns	162.87 (17.88)
77	f__Phanerochaetaceae	ns	ns
78	o__Russulales	64.62 (32.78)	ns
79	g__Lactarius	ns	ns
80	g__Stereum	134.58 (12.45)	308.51 (22.9)
81	s__Sebacinales Group B sp	144.13 (15.26)	ns
82	c__Microbotryomycetes	144.91 (3.9)	139.33 (38.43)
83	g__Rhodotorula	219.74 (57.49)	ns
84	s__Rhodotorula mucilaginosa	ns	188.14 (53.86)
85	g__Rhodotorula	ns	44.1 (45.79)
86	s__Trichosporon cutaneum	ns	77.8 (13.56)
87	o__Rhizophydiales	164.27 (11.81)	164.59 (5.6)
88	s__Rozellomycota sp	137.79 (25.27)	175.55 (24.48)
89	k__unidentified fungus	ns	-0.14 (0.89)
90	s__Basidiobolus ranarum	37.17 (21.83)	24.3 (6.72)

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