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Comparative analysis of anti-Bd bacteria from six Malagasy frog species of Ranomafana National Park

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Comparative analysis of anti-Bd bacteria from six Malagasy frog species of Ranomafana National Park

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Abstract

As Malagasy amphibians are facing an impending extinction crisis from the lethal skin fungus *Batrachochytrium dendrobatidis* (Bd), it has become imperative to proactively mitigate the threat. Bd sporangia develop in the skin of infected amphibians and cause the skin to thicken, leading to ionic imbalance and eventual heart failure. It has been shown that certain bacterial species are able to inhibit Bd growth on amphibians by producing antifungal metabolites. Community-based probiotics are one approach used to combat chytridomycosis by inoculating an environment with Bd-inhibitory bacteria so that many amphibian species are treated at once. With this method, it is important to minimize effects on non-target organisms by selecting anti-Bd bacteria that occur on the amphibians' skins with the goal of augmenting bacterial abundance. The purpose of this study was to determine which bacteria from an amphibian community at Ranomafana National Park, Madagascar, are capable of inhibiting Bd. To identify anti-Bd bacterial isolates, inhibition assays of each isolate against Bd were conducted. Changes in optical density (492nm) of isolates' culture filtrates with Bd were compared to controls. After finding which bacteria were positive for inhibition, relationships between the anti-Bd bacterial families, genera, and species and the relative abundances found on each frog species were assessed. Overall, Bd inhibitory isolates were found on every species. Several bacterial isolates were able to inhibit Bd as found in previous studies. However, only two isolates of *Sphingobacterium multivorum* were identified to inhibit Bd at the species level. Based on this study, I recommend that more research is conducted with greater sample sizes to identify a possible probiotic species that is more commonly found than *S. multivorum*.

Introduction

Amphibian declines have been documented in almost every corner of the globe for the past 25 years (Beebee and Griffiths, 2005). In fact, amphibians are arguably the vertebrates most susceptible to extinction with 32.5% of species threatened (Kilpatrick et al., 2010). Reasons for this decline include land use change, contaminants, and climate change (Collins, 2010). Another major cause of amphibian declines is infectious disease such as chytridiomycosis, which is recently negatively impacting large numbers of amphibians (Kilpatrick et al., 2010).

Batrachochytrium dendrobatidis (Bd), the causal agent of chytridiomycosis, is a fungal pathogen greatly affecting amphibian populations by causing large-scale population declines worldwide. For example, 92.5% of 'critically endangered' species are undergoing dramatic declines that could be attributed to Bd (Kilpatrick et al., 2010). This fungus has been found worldwide except for a few locations such as some parts of Madagascar (Vrendenburg et al., 2012). Bd is thought to be an emerging pathogen as it was first identified in 1999 (Longcore et al., 1999). However, recent genomic studies demonstrate that Bd arose approximately 1,000 to 10,000 years ago (Rosenblum et al., 2013). This evolutionary time period is sufficient to allow quite variable strains as seen around the world, including one pathogenic strain, global pandemic lineage (GPL), responsible for amphibian population decline.

The introduction of a virulent strain to naïve amphibian populations is controversial as there is no clear answer to its origin in terms of location or host

species. Some findings support the hypothesis that Bd originated in Africa and was spread by the host species *Xenopus laevis* as this frog was traded internationally in the mid 20th century for use as a pregnancy test (Rosenblum et al., 2010). However, others hypothesize that the North American bullfrog, *Rana catesbeiana*, was the original host based on the high allelic variance of Bd isolates on this host species (Rosenblum et al., 2010). Based on the rate of virulent Bd spread worldwide, there is no obvious answer to the question of its origin.

The lifecycle of Bd has two major stages: a swimming zoospore and a fixed sporangium (Bletz et al., 2013). Bd infects its host by zoospores colonizing the amphibian's skin. During Bd's lifecycle, the zoospores form sporangia in the deeper layers of keratinized skin (Fisher et al., 2009). The sporangia disrupt the vital ion transport mechanisms of the amphibian's skin, causing eventual cardiac arrest (Campbell et al., 2012). Bd virulence and growth rate are quite variable depending on the strain or environmental factors such as temperature. Cooler temperatures are associated with faster Bd zoospore production rates, while warmer temperatures allow for the sporangium to mature at faster rates (Kilpatrick et al., 2010).

Some amphibian populations persist with Bd because they can counteract chytridiomycosis through a variety of defense strategies. First, amphibians are able to produce antimicrobial peptides that inhibit potentially harmful microorganisms (Harris et al., 2009). Another line of defense against Bd are commensal bacteria on the frog's skin that produce antifungal metabolites. By introducing or increasing

such probiotic bacteria onto infected frogs (bioaugmentation), the infection rate of Bd can decrease (Harris et al., 2009; Becker and Harris, 2010).

By implementing probiotic bioaugmentation of anti-Bd bacteria to amphibian skin, Bd-naïve locations, such as areas of Madagascar, can be proactively protected against the disease (Bletz et al., 2015). Thus, the purpose of my study is to test bacteria obtained from Malagasy frogs for Bd inhibition in order to obtain bacterial strains that can one day be used as probiotics that can protect amphibians of Madagascar, where there is an estimated 465 endemic amphibian species (Fisher et al., 2009)

Based on previous research, I expect to find several anti-Bd bacterial isolates. However, I expect to find a different community of anti-Bd bacterial species on each frog species due to the different array of anti-microbial peptides produced by each frog species (Woodhams et al., 2006). These peptides likely control which bacterial species can live on frog skin. I expect that a limited number of anti-Bd bacterial species are shared among several frog species based on previous research in North America (Bell et al., 2013), and these species would be potential community probiotics.

Materials and Methods

Sample Collection

Six Malagasy amphibian species from Ranomafana National Park were studied in this analysis: *Blommersia blommersae, Boophis elenae, Boophis guibei, Gephyromantis tschenki, Guibemantis liber,* and *Mantidactylus majori*. Of these species, *B. elenae* and *G. tschenki* are considered "data deficient" as a conservation status, with the rest of the species being of "least concern" (AmphibiaWeb, 2015). Sample sizes at the level of amphibian species were low, with the highest being *B. guibei* (n=6 individuals) and the lowest being *B. elenae* (n=3).

Based on the amount of bacterial samples processed from the Malagasy frogs, 255 bacterial samples were studied. Molly Bletz and Reid Harris collected bacterial swab samples by collecting frogs at night using a different pair of gloves for each frog handled. Next, the skin of the ventral side of each frog was swabbed, and the swab was frozen in glycerol solution until bacterial isolation in the lab.

This strategy of sampling frogs from one location allows for the possibility of discovering a community-wide probiotic if a consistent anti-Bd isolate is found among several frog species. For example, if anti-Bd bacteria species 1 and 2 are primarily associated with frog species A, anti-Bd bacteria species 1 and 3 with frog species B, and anti-Bd bacteria species 1 and 4 with frog species C, then bacteria species 1 would be a good candidate for a community-based probiotic as it is found across multiple frog species, thus reducing potential secondary effects such as creating major differences in bacterial community of some frog species. This

probiotic could be inoculated to amphibians by introducing the bacteria to an environmental source, such as a pond, so that many amphibians can be bioaugmented in a relatively short amount of time (Muletz et al., 2012; Bletz et al., 2013). Studying the relationships between species of amphibians and anti-Bd bacterial isolates will be an important factor of this method of experimentation.

Inhibition Assay Preparation

An inhibition assay is a method of finding anti-Bd bacteria by challenging Bd zoospore growth while in the presence of the bacterium's metabolites and comparing Bd growth to several controls that are described below. One percent tryptone broth cultures of each bacterium were made from the original bacterial isolate plates from the frog skin swabs and were allowed to grow in the presence of Bd, so as the bacterium was more likely to produce anti-Bd metabolites, if capable. To standardize the amount of bacteria used in each inhibition assay, cultures of all bacterial species will be assayed after three days of growth.

Bd was prepared for use in inhibition assays by using strain JEL in 1% tryptone broth culture. New plates were prepared by pipetting 1mL of Bd liquid culture onto 1% tryptone plates and swirling to ensure the plate was covered evenly and then incubating the plates for 3 days at 21°C. The controls and Bd used to grow with the bacteria were also be prepared as liquid cultures and incubated at 21°C with agitation for 3 days in preparation for the inhibition assays.

Bd for inhibition assays was obtained by flooding the Bd plates with 5mL of tryptone and allowing them to sit for 10 minutes with periodic agitation. The liquid

was removed and filtered through an autoclaved coffee filter into a new falcon tube. Zoospores passed through the filter and were counted twice using a haemocytometer, and this average initial concentration was used to create the final Bd concentration of $2x10^6$ zoospores in 15mL of tryptone solution. To prepare the heat killed Bd for the "heat-killed" control, 500µL of quantified Bd was put into a 1.5mL microcentrifuge tube and heated at 60°C for 60 minutes. The bacterial and Bd solutions were places into new 1.5mL microcentrifuge tubes and centrifuged at 10,000 rpm for five minutes to create bacterial cell free supernatants (CFSs) or Bd cell free supernatants, which were controls grown without the presence of bacteria. Using a needle attached to a 3mL syringe, I collected 1mL of supernatant. The supernatant was then filtered through a 0.22µm syringe filter into a sterile microtube to ensure supernatants were cell-free. This experimental design is based on Bell et al. (2013).

Inhibition Assay Experimental Design

The experimental samples included 50µL of Bd zoospore solution and 50µL of bacterial CFS. A nutrient depleted control samples had 50µL of Bd zoospore solution and 50µL of sterile water (Figure 1). The nutrient depleted control served as the basis of comparison of growth for Bd as it controls for differing levels of tryptone nutrients after depletion by bacterial in broth culture. This nutrient depleted control is important since the Bd zoospores that were added to the 96 wells in the inhibition assay were in wells that were nutrient depleted by bacterial growth to various extents depending on how much each bacterial strain grew while

in pure culture. Bd and bacteria use the same nutrients to some degree, so it is important to distinguish between the effects of nutrient depletion and the effects of anti-Bd metabolites secreted by bacteria on Bd growth. The positive control contained 50 µL of Bd zoospore solution with 50µL of Bd CFS to show growth attributed to Bd zoospores alone (Figure 1). The positive control shows growth of Bd zoospores without the effects of bacterial presence. The heat-killed Bd controls contained 50µL of heat-killed Bd zoospores, as described above, and 50µL of Bd CFS (Figure 1). This control was a measure of total mortality of Bd. Finally, the negative control contained 50µL of 1% tryptone medium and 50µL of positive extract (Figure 1). The negative control focused on possible contamination presence, as no growth should be seen from the lack of Bd zoospores.

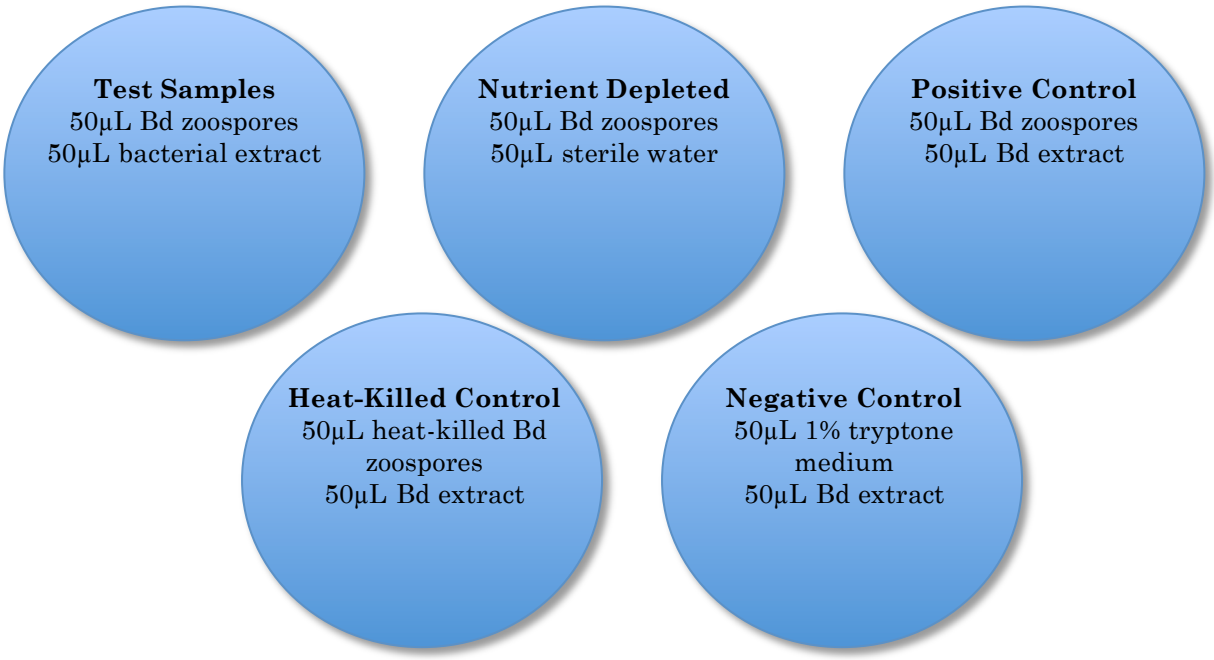


Figure 1. Inhibition assay set up for each control and test samples that will be in 96 well plates.

The optical density (OD at 492 nm) of all samples was determined on days 0, 4, 7, and 10 after the start of the inhibition assay. OD is a measure of Bd growth, as samples with larger Bd population densities will have higher OD readings. Samples were incubated at 21°C. during this time. Each sample had 3 replicates, and growth curves were created for each isolate's average rate of growth and compared to the nutrient depleted control. As mentioned above, the nutrient depleted control samples were used as a benchmark to determine which isolates had anti-Bd properties.

Genetic Analysis

The final analysis of this experiment is to analyze the relationships between species of amphibians and families of bacteria by completing polymerase chain reactions (PCR) in order to sequence a portion of the 16S rRNA gene of the anti-Bd bacterial isolates. These sequences are used as a way to identify each strain by comparing each isolate's sequence to the NCBI BLAST database.

Statistical Analysis

A bacterial isolate was classified as Bd-inhibiting if the mean proportion of inhibition of growth for the three replicates, compared to the nutrient depleted control, was greater than or equal to 0.85. This threshold was assigned in order to be consistent with Molly Bletz's larger data set analysis. By comparing inhibition to the nutrient depleted control's growth, the inhibition proportion accounts for varying amounts of tryptone nutrients being depleted by either bacterial isolates or Bd zoospores. Due to small sample sizes, several nonparametric statistical tests

were used. Wilcoxon rank sum test was used to compare values of inhibition, and Fisher's exact test was used to compare proportions of inhibitory isolates compared to all isolates found. Standard boxplots formed from the 25^{th}, 50^{th}, and 75^{th} quartiles, along with tails created by adding and subtracting the interquartile range multiplied by 1.5, were constructed to depict variation in inhibition among the samples. ANOVA was used to compare average inhibition scores between bacterial families and genera.

Results

Amphibian Level

Inhibition of Bd was estimated by calculating the median inhibition score across all of the bacterial strains on all individuals for each amphibian species. This value was around 0.6 with low variability. The Wilcoxon rank sum test indicated that only the *B. blommersae* and *G. liber* species pair differed in inhibition values (W=1296; p = 0.006) (Figure 2).

Median inhibition values were calculated for each individual frog within a species with boxplots depicting the variation in values for each frog (Figure 3). For *B. blommersae*, a significant difference was found between J4-010 and J4-013 (W=20, p-value=0.025), J4-011 and J4-013 (W=6, p-value=0.029), J4-012 and J4-013 (W=4, p-value=0.005), and J4-013 and J4-040 (W=76, p-value=0.046) using Wilcoxon rank sum test (Figure 3). For *B. guibei,* the only statistical difference in median inhibition values was between J4-021 and J4-024 (W=40, p-value=0.036) (Figure 3). For *G. liber,* the only statistical difference in inhibition values was between J4-030 and J4-032 (W=91, p-value=0.047) (Figure 3). There were no statistical differences in inhibition values for *B. elenae, G. tschenki*, and *M. majori* (Figure 3). To understand how many inhibitory isolates were found on each species that made up the inhibition scores, proportions of inhibitory isolates to total isolates were calculated.

When comparing the proportion of inhibitory isolates found across amphibian species, a different pattern is seen. There is no statistically significant difference

between species using pair-wise Fisher's exact test with all p-values greater than 0.05 (Figure 4). In all species every individual frog had at least one inhibitory isolate with the exception of *G. tschenki* and *M. majori,* which had two out of four frogs and two out of six frogs without at least one inhibitory isolate, respectively (Figure 4).

Across individual frogs, there was variation among proportions of inhibitory isolates (Figure 5). However, the level of variation changes among species (Figure 5). Of all six frog species, only two species exhibited a statistically significant difference in proportion of inhibitory isolates among individuals. Only J4-010 and J4-012 of *B. blommersae* and A3-032 and A3-034 of *M. majori* showed a difference in proportion of inhibitory isolates within their respective species using Fisher's exact test with p-value of 0.0498 and 0.0455, respectively (Figure 5). Low sample sizes of frogs within each species may have contributed to the lack of significant differences in proportion of inhibitory isolates. Overall, inhibitory isolates made up the minority of the isolates found in cutaneous cultures from each frog, except for two frogs, as they had less than 50% inhibitory isolates (Figure 5).

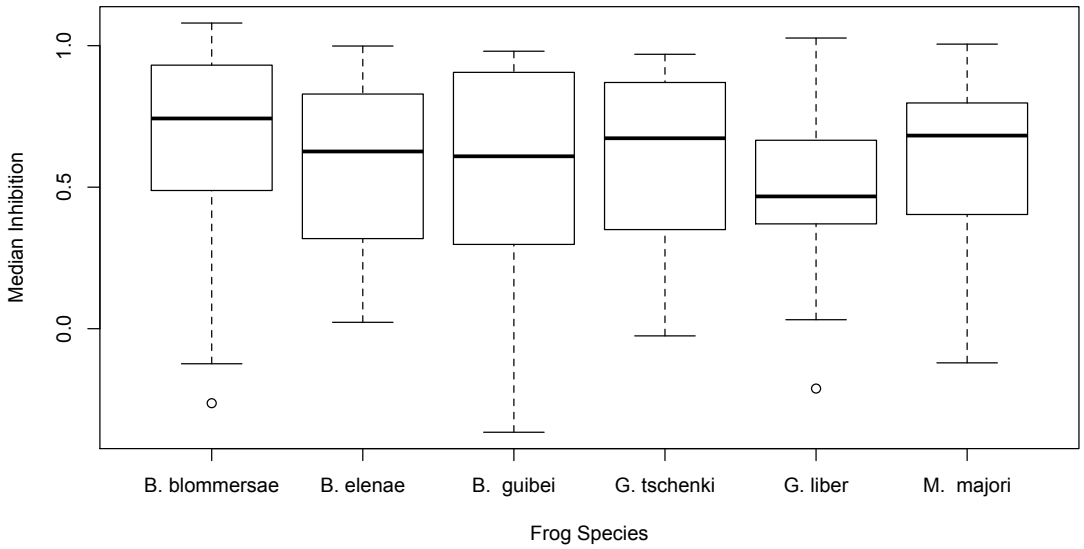


Figure 2. Median inhibition for each frog species. Each boxplot represents the median of inhibition across all frogs sampled of each species in addition to 25%, 75% quartiles and outliers. Species are arranged by alphabetical order. A high inhibition represents a better ability of inhibiting Bd growth. The only statistical difference in inhibition values is between *B. blommersae* and *G. liber* using Wilcoxon rank sum test.

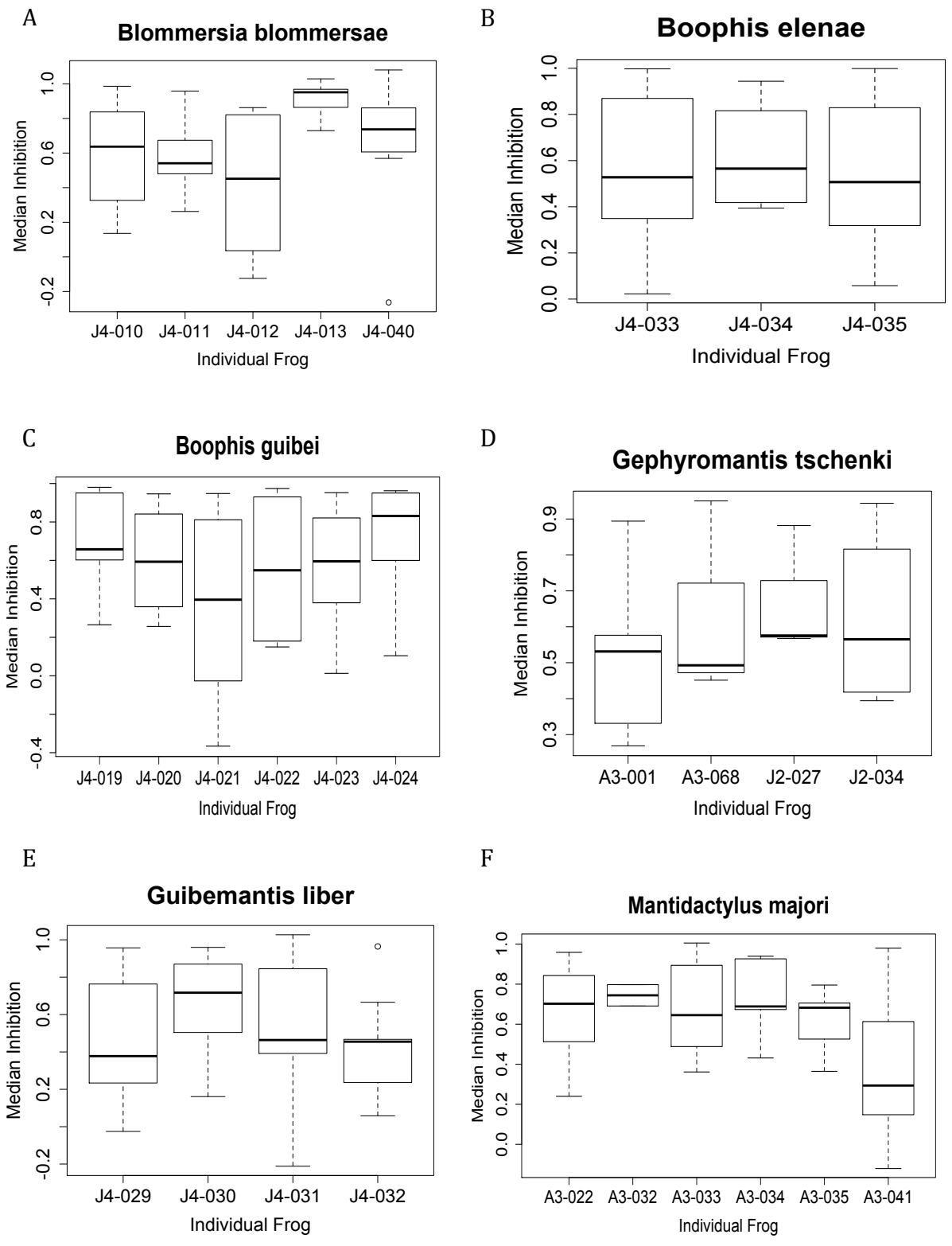


Figure 3 Median inhibitions for each individual frog. Each boxplot represents the median of inhibition across all frogs sampled of each species. A high inhibition

represents a better ability of inhibiting Bd growth. Statistical differences in inhibition values between *B. blommersae* frogs, graph A, using Wilcoxon rank sum test are between J4-010 and J4-013, J4-011 and J4-013, J4-012 and J4-013, and J4-013 and J4-040. For *B. guibei,* graph C, the only statistical difference in inhibition values is between J4-021 and J4-024. For *G. liber,* graph E, the only statistical difference in inhibition values is between J4-030 and J4-032. There are no statistical differences in inhibition values for *B. elenae* (graph B), *G. tschenki* (graph D), and *M. majori* (graph F).

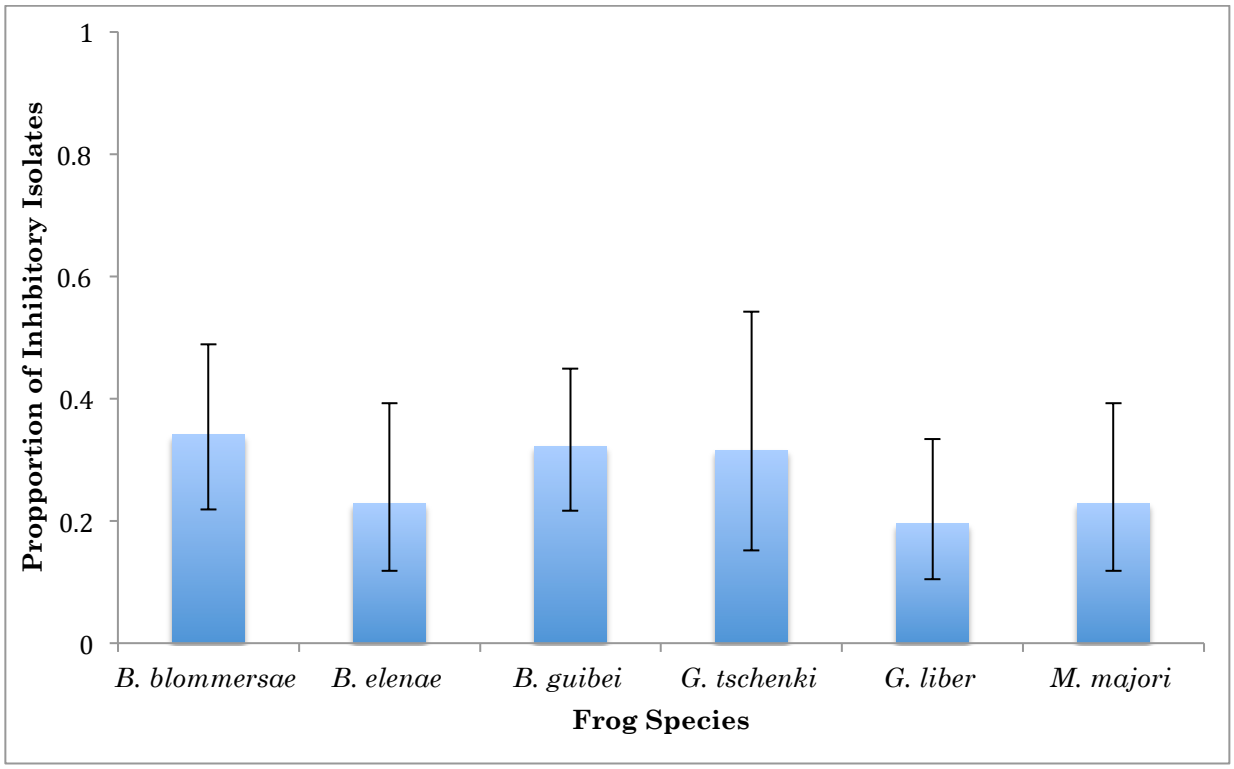


Figure 4. Proportion of inhibitory isolates by frog species. Each bar represents the proportion of inhibitory isolates compared to total number of isolates found on each frog species. The error bars represent the 95% confidence interval. No statistically significant difference between any species was seen using Fisher's exact test.

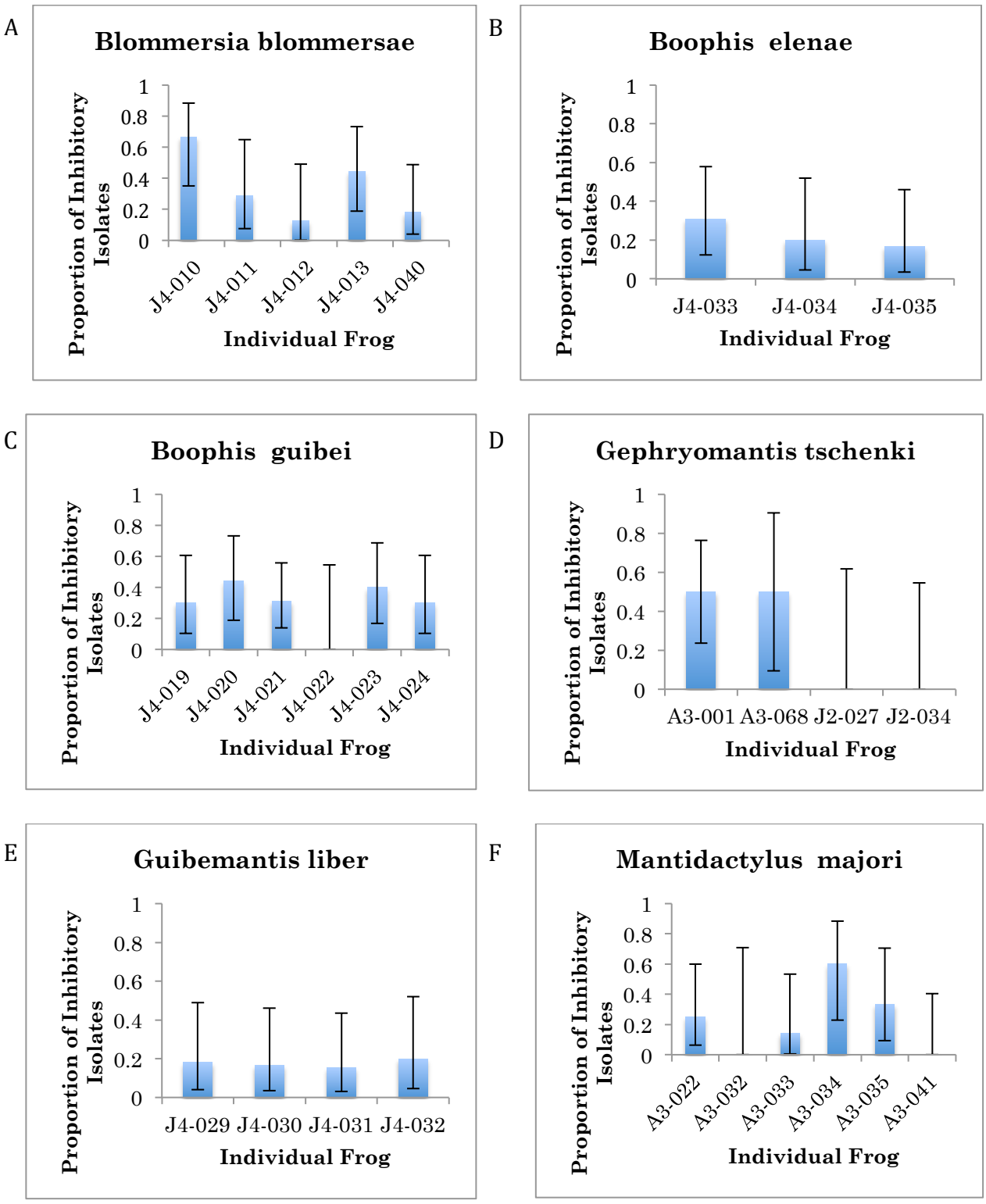


Figure 5. Proportion of inhibitory isolates by individual frog. Each bar represents the proportion of inhibitory isolates compared to the total number of isolates found on each individual frog. For *B. blommersae,* graph A, the only statistically different proportions are between J4-010 and J4-012 using Fisher's exact test. For *M.*

majori, graph F, the only statistically significant different proportions are between A3-032 and A3-034 using Fisher's exact test. For *B. elenae*, *B. guibei*, *G. tschenki*, and *G. liber,* graphs B, C, D, and E respectively, there are no statistically significant differences between proportions of inhibitory isolates using Fisher's exact test.

Bacterial Level

Average inhibition scores were calculated for each identified bacterial family, genus, and species to identify groups of anti-Bd bacteria to help guide options for a community probiotic. Of the families identified, three had average inhibition scores over the threshold of 0.85 when comparing more than one isolate, noted by a § in Figure 7. I have also identified the five most commonly identified bacterial families, those with the five highest relative abundances across amphibian species, noted by an * (Figure 6, Figure 7). *Xanthomonadaceae, Caulobacteraceae,* and *Sphingobacteriaceae* were identified as families having an average inhibition score above 0.85, and their relative abundances were 4.7%, 4.0%, and 2.5% respectively (Figure 7). The five most commonly found bacterial families, in order, are *Microbacteriaceae* (26.9%)*, Micrococcaceae* (11.3%)*, Brevibacteriaceae* (9.8%)*, Streptomycetaceae* (5.7%)*,* and *Xanthomonadaceae* (4.7%) (Figure 6, Figure 7). Of these families, only *Xanthomonadaceae* had an average inhibition over 0.85 (Figure 7). These families differed in average inhibition scores ($F= 6.67$, $df=6, 139$, p-value=3.21E-6).

Of the genera identified, two had average inhibition scores over the threshold of 0.85 when comparing more than one isolate, noted by a § (Figure 9). I have also identified the five most commonly identified bacteria, those with the five highest

relative abundances across amphibian species, noted by an * (Figure 8, Figure 9). *Sphingobacterium* (from the *Sphingobacteriaceae* family) and *Stenotrophomonas* (from the *Xanthomonadaceae* family) were identified as genera having an average inhibition score above 0.85, and their relative abundances were 1.6% and 2.4% respectively (Figure 9). The five most commonly found bacterial genera, in order, are *Microbacterium* (16.8%), *Brevibacterium* (9.8%), *Kocuria* (8.5%), *Sphingomonas* (3.9%), and *Curtobacterium* (2.9%) (Figure 8, Figure 9). Of these genera, none had an average inhibition over 0.85 (Figure 9). These genera differed in average inhibition scores ($F= 3.80$, $df=6,97$, p-value=0.002).

Of the species identified, one had an average inhibition score over the threshold of 0.85 when comparing more than one isolate, noted by a § (Figure 10). I have also identified the five most commonly found bacterial species, those with the five highest relative abundances across amphibian species, noted by an * (Figure 10). *Sphingobacterium multivorum* was identified as a bacterial species having an average inhibition score above 0.85 and had a relative abundance of 0.78% (Figure 10). The five most commonly identified bacterial species, in order, are *Brevibacterium aureum* (7.1%), *Microbacterium chocolatum* (3.9%), *Kocuria palustris* (2.4%), *Acinetobacter rhizospaerae* (1.2%), and *Luteibacter rhizovicinus* (1.2%), as seen in Figure 10. Of these species, none had an average inhibition over 0.85 (Figure 10). The species did not differ in inhibition scores ($F= 2.06$, $df=5$, p-value=0.09) probably due to large standard error bars for some species and low sample size in others.

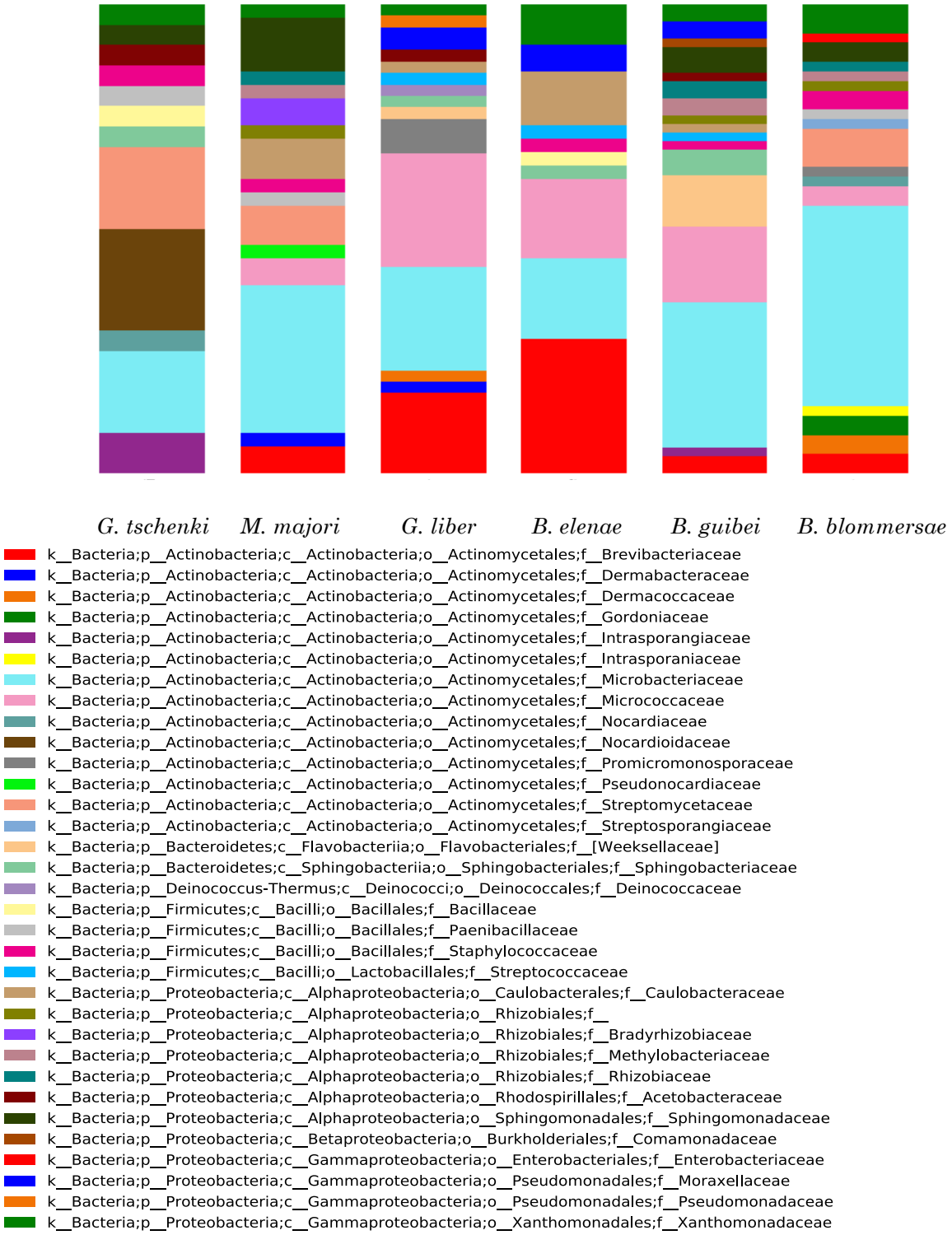


Figure 6. Bacterial community structure on the skins of six amphibian species. Bacteria were identified to the family level.

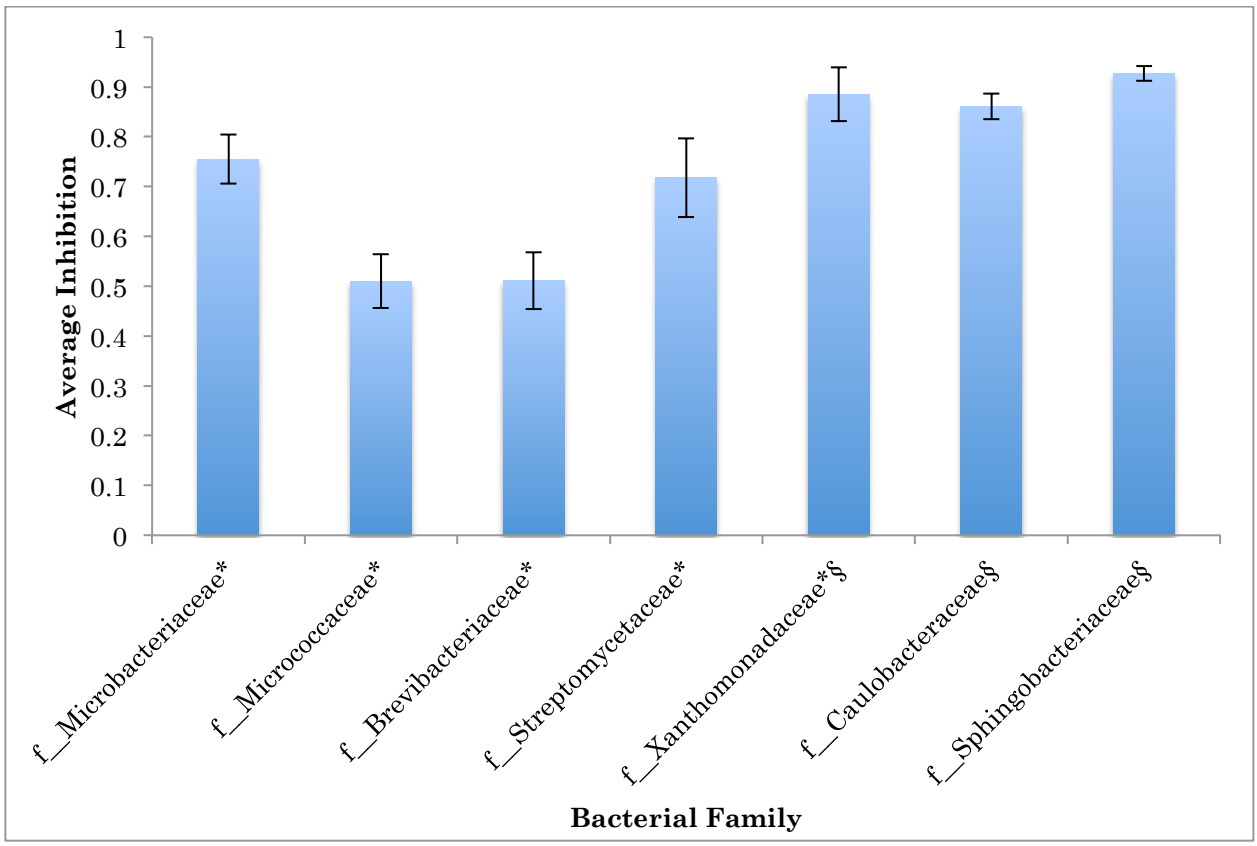
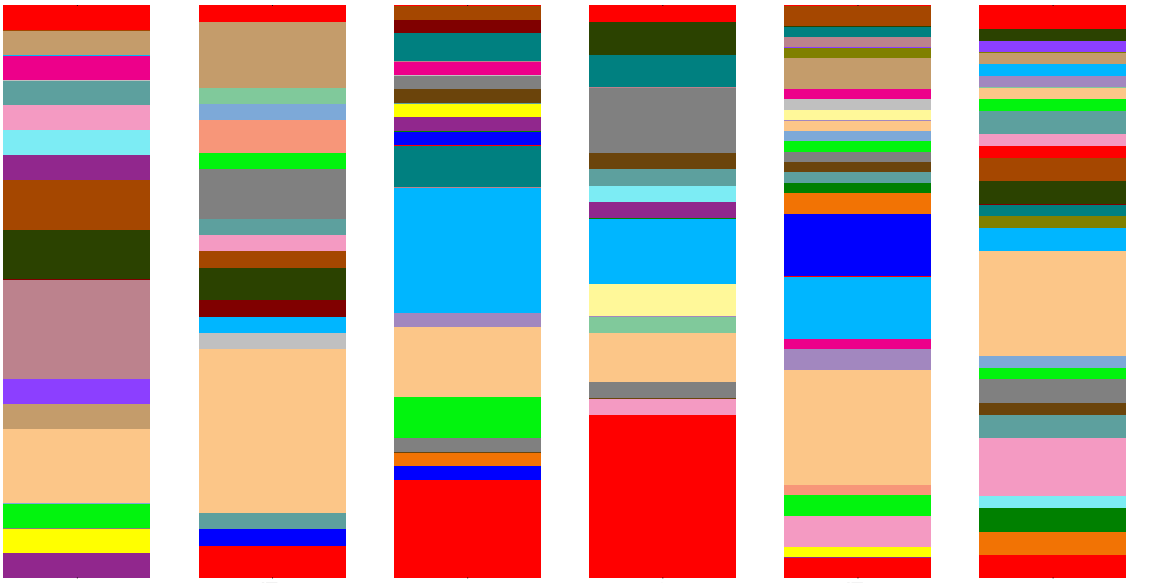


Figure 7. Average inhibition for each bacterial family identified. Each bar represents the average inhibition for a bacterial family. Depicted families represent the five most commonly found families (noted with *) and families with an average inhibition greater than 0.85 with more than one isolate (noted with §). Relative abundance of each family decreases from left to right. Error bars represent standard error for each family. Families were statistically different using ANOVA.



G. tschenki *M. majori* *G. liber* *B. elenae* *B. guibei* *B. blommersae*

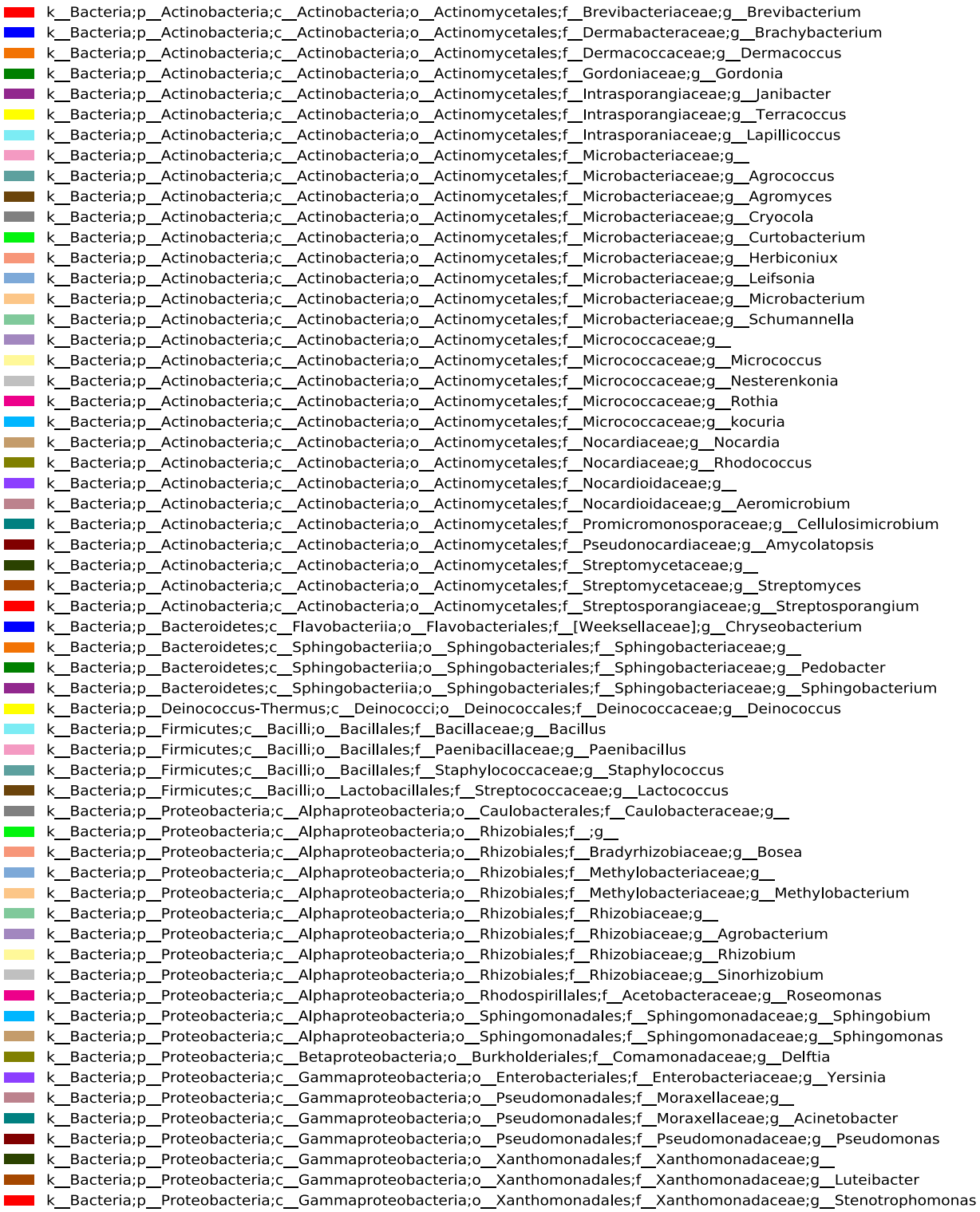


Figure 8. Bacterial community structure on the skins of six amphibian species. Bacteria were identified to the genus level.

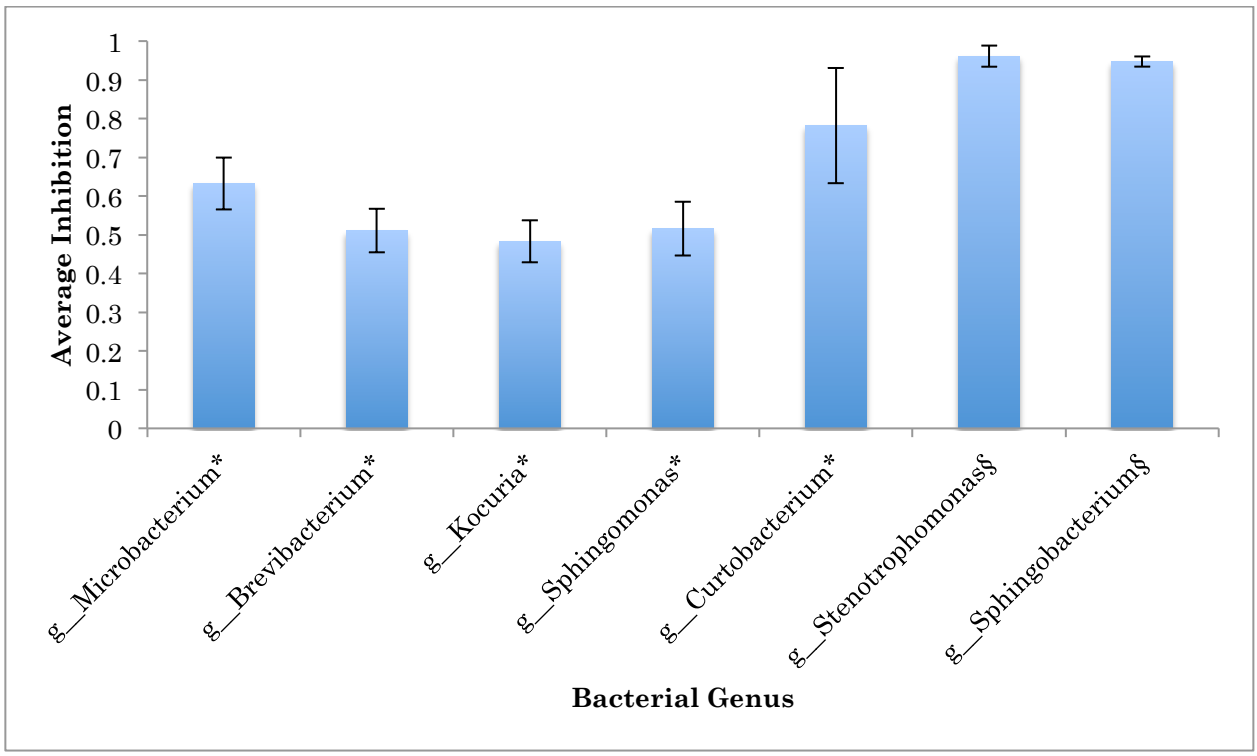


Figure 9. Average inhibition for each bacterial genus identified. Each bar represents the average inhibition for a bacterial genus. Depicted genera represent the five most commonly found genera (noted with *) and genera with an average inhibition greater than 0.85 with more than one isolate (noted with §). Relative abundance of each genus decreases from left to right. Error bars represent standard error for each genus. Genera were statistically different using ANOVA.

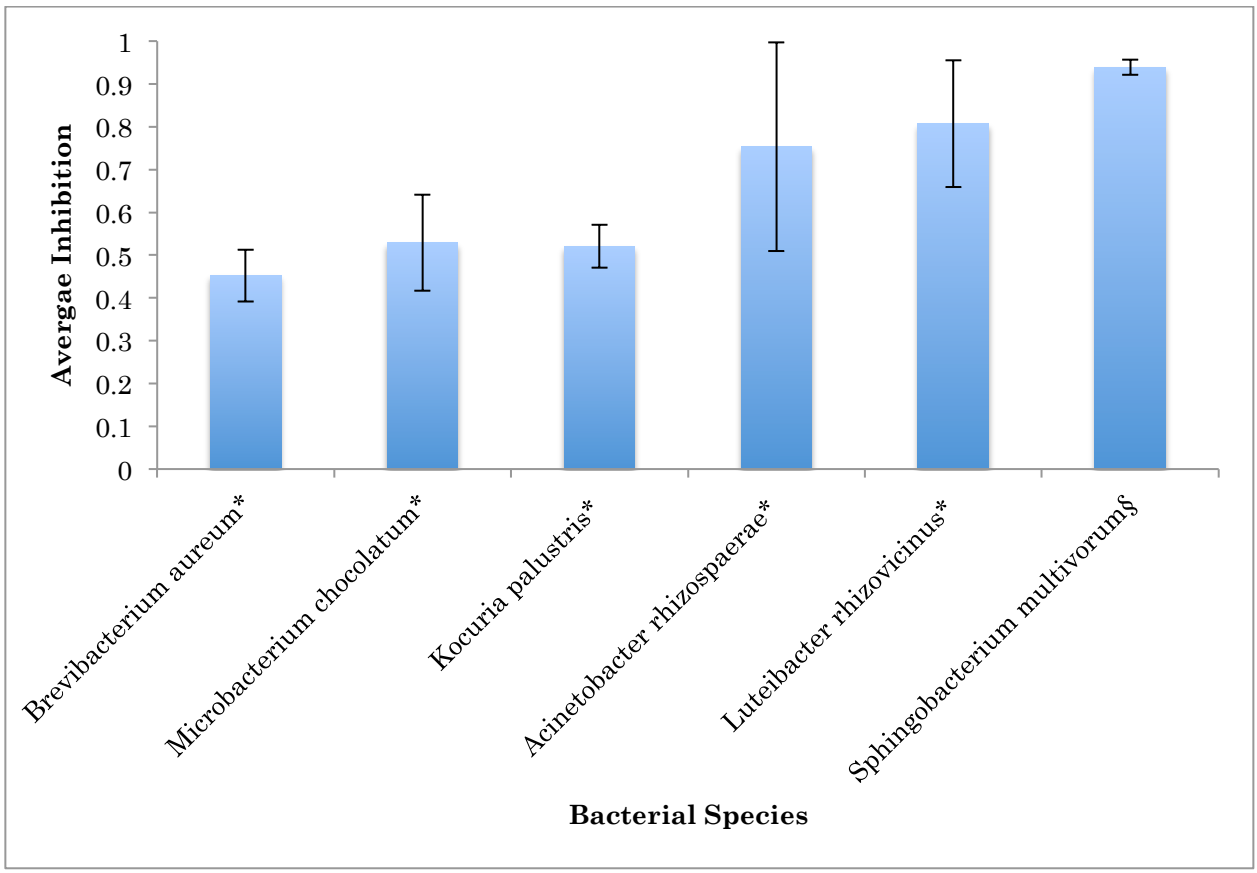


Figure 10. Average inhibition for each bacterial species identified. Each bar represents the average inhibition for a bacterial species. Depicted species represent the five most commonly found species (noted with *) and species with an average inhibition greater than 0.85 with more than one isolate (noted with §). Relative abundance of each species decreases from left to right. Error bars represent standard error for each species. Species were not statistically different using ANOVA.

Discussion

With this study, I tested for relationships between amphibian and bacterial species within the same community. Using the community probiotic approach, a Bd inhibitory bacterial species found commonly among amphibian species would be a great candidate for future approaches to amphibian protection (Bletz, 2013). Such a wide distribution would reduce the chances of non-target effects of bacterial introduction to the environment since many of the amphibian species already have the isolate as a member of its normal microbiota (Bletz, 2013). Part of the selection process is to analyze the current inhibition abilities of isolates for each frog species found at the site while studying variation across individual frogs within a species.

The overall median degree of inhibition across species was relatively constant and lower than 0.85, which means based on microbial protection alone that at least half of the individuals are at risk of Bd infection. However, as yet it is not known how the 0.85 threshold relates to individual frog survival when infected with Bd. However, this result shows that all species studied should be targeted for probiotic protection to the same degree. Each species also had inhibitory bacterial species making up the minority of total isolates since all proportions of inhibitory isolates were below 0.5 (Figure 4). However, each species studied had at least one inhibitory isolate found in its normal microbiota (Figure 4). When studying the proportion of inhibitory isolates on the individual frogs, there is not much variation between frogs of the same species (Figure 5).

For bacteria, only a few families, genera, and one species could be identified as inhibitory of Bd. Only *Xanthomonadaceae* was identified as a bacterial family whose average inhibition scores of its isolates exceeded the 0.85 threshold, while also being commonly found on the frogs, one of the five highest relative abundances across amphibian species (Figure 7). In fact, this family was found on all six species of frogs studied (Figure 6). No genera were identified as being both one of the most commonly found genera, those with the five highest relative abundances across amphibian species, and having an average inhibition score over 0.85. However, the inhibitory genus *Sphingobacterium* was found on three of the six frog species, *G. tschenki, G. liber,* and *B. elenae,* and the genus *Stenotrophomonas* was found on four of the six frog species, *G. tschenki, M. majori, B. elenae,* and *B. guibei* (Figure 8). Only one bacterial species, *S. multivorum*, was identified as having an average inhibition score over 0.85. This species only had two isolates, out of 255 isolates in the study, based on culture-dependent genetic analysis. Although this was the only species that met the 0.85 threshold of average inhibition scores, other species identified did meet this qualification but only one isolate was identified. Because of low relative abundance of the inhibitory isolate *S. multivorum*, I cannot recommend it as a future probiotic focused on the species studied until culture independent abundance can confirm its spread across the amphibian species studied. Using culture media to encourage *S. multivorum* growth, rather than 1% tryptone, in the future may also give a more accurate relative abundance that is found on the amphibians. As *S. multivorum* was the only bacterial species found that was

inhibitory, I cannot recommend other possible probiotics on the species level based on the outcomes of this study.

Variation among some isolates of the same bacterial species (i.e. *A. rhizospaerae*) contributed to a high p-value for the ANOVA test, but does indicate different strains of the species with widely varying Bd inhibition abilities. Such variation in inhibition traits for some species could harm community probiotic approaches as high variation within certain bacterial species may indicate a high mutation rate. When depending on a probiotic approach to defend against Bd, such high mutation rates may hinder defense against Bd, or may even help the fungus to grow in some cases. As such, researchers should understand the bacterium of choice in detail, such as the variation in inhibition between strains of the species, before adding it to the community in case of future issues.

The vulnerability level of the amphibian species studied was "least concern", or "data deficient", suggesting that they have a large population size with large genetic diversity (AmphibiaWeb, 2015). Such diversity in genetics could lead in a diversity of antimicrobial peptides produced by the host amphibians. As such, cutaneous microbiota should be diverse within species to reflect the diversity of antimicrobial peptides produced as a result of genetic variation among the species. In addition to antimicrobial peptides, amphibian habitat and life history could also contribute to the diversity in skin microbiota. However, more work in the field of diversity needs to be conducted so that researchers can use probiotics in a safe manner. Such future work could include assaying different species genetic

diversity, studying antimicrobial peptide diversity and its effects on bacterial Bd inhibition, and microbial diversity in relation to habitat and life history in general. By identifying certain bacterial families, genera, and species that are capable of Bd inhibition this study serves as a stepping stone for researchers to find a probiotic that is suitable for the Ranomafana area of Madagascar while also cautioning the amount of variation possible for inhibition within bacterial species

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