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INFLUENCES OF ANTI-*BD* BACTERIA ON AMPHIBIANS AND THEIR
MICROBIOMES

by

Emme L. Schmidt

A Thesis Submitted in Partial Fulfillment
Of the Requirements for the
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ABSTRACT

Influences of Anti-*Bd* Bacteria on Amphibians and their Microbiomes

Emme L. Schmidt

Director: Jake Kerby, Ph.D.

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been threatening amphibian populations across the globe. Recent work has discovered that bacteria isolated from certain populations of amphibians protect hosts from this pathogen. This project focuses on the anti-fungal mechanisms of *Serratia marcescens*, a species of bacteria isolated from a Costa Rican frog that dramatically inhibited *Bd* in culture. Wild-type and recombinant *S. marcescens* was introduced to the microbiomes of a live amphibian host, *Acris blanchardi* (Blanchard's Cricket Frog) and then challenged with *Bd* to examine the protective effects of this bacteria. The experiment includes recombinant *S. marcescens* with the genetic pathway responsible for the production of prodigiosin interrupted. This work observed significantly greater movement among frogs treated with wild-type *Sm* when compared to the other bacteria treatments. There were also significant differences in mean mass and snout-vent length (SVL) between the washed and unwashed treatments. In particular, mean SVL was greater in washed frogs compared to unwashed frogs of the wild-type *Sm* and no bacteria treatments. These efforts will aid in the future conservation of amphibian species worldwide.

KEYWORDS: *Batrachochytrium dendrobatidis*, Chytridiomycosis, *Serratia marcescens*, Genetic engineering

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CHAPTER ONE

Influences of Anti-*Bd* Bacteria on Amphibians

Introduction

Emerging infectious diseases are threatening populations of wildlife across the globe and are of great focus in conservation efforts (Rachowicz et al., 2005). Any species of plant or animal, including humans (*Homo sapiens*), can be affected by emerging infectious diseases. Mycoplasmal conjunctivitis arose in house finches (*Haemorhous mexicanus*) in the United States in the mid-1990s and *Mycoplasma gallisepticum* was isolated from affected birds. This was the first time *M. gallisepticum* had been associated with disease in wild birds (Williams et al., 2002). Caliciviruses causing rabbit hemorrhagic disease were spread via globalization from rabbitries in China to Europe, North Africa, Mexico, and eventually Australia and New Zealand. This marked the first time the rabbit (*Oryctolagus cuniculus*) populations of Europe were exposed to this particular virus and resulted in major population declines (Williams et al., 2002). The Hendra and Menangle viruses were described and classified as paramyxoviruses after emerging in Australia in the mid-1990s. The Hendra virus expanded its host range showing up in horses (*Equus caballus*) and humans. The Menangle virus first appeared in piglets in Australia. Infection with the virus in pigs (*Sus domesticus*) led to smaller litters and more stillbirths. The Menangle virus was also capable of infection in humans leading to flu-like symptoms (Williams et al., 2002). Mycoplasmal conjunctivitis in house

finches, hemorrhagic disease in European rabbit populations, and paramyxoviruses are all examples of emerging infectious diseases and how they can affect many species.

There are more than 31,000 species classified as threatened with extinction (International Union for Conservation of Nature [IUCN], 2020). This includes an estimated 41% of all amphibian species (IUCN, 2020). This puts amphibians at greater risk for extinction than birds (12%) or mammals (23%) (Stuart et al., 2004). There are several hypotheses regarding global amphibian population declines including habitat loss, land-use change, climate change, enigmatic decline, contaminants, and emerging infectious diseases. There was major deforestation and drainage of wetlands in the 1800s leading to rapid and large amphibian population declines in North America (Collins & Storfer, 2003). Changes to amphibian habitat occur today, but are not the sole reason for declines in recent decades. Global climate change, specifically, the average global rise in temperature and drier than normal environment in rainforests has led to negative impacts on the reproductive ability of amphibian species; therefore, creating population decline (Beebee & Griffiths, 2005). One study provided evidence to support the thermal mismatch hypothesis, which suggests amphibians from cooler climates are more susceptible to death from *Bd* infection under warmer conditions than amphibians from warm climates (Cohen et al., 2018). There are also many species of amphibians experiencing seemingly inexplicable declines, known as “enigmatic decline” (Stuart et al., 2004). Another likely contributor to population declines is the presence of contaminants in amphibian habitat. It has been demonstrated that maternal transfer of inorganic contaminants occurs among amphibians and negatively impacts the viability of offspring (Hopkins et al., 2006). Another study found that the commonly used pesticide,

Diuron was 100% lethal to bullfrog tadpoles (*Lithobates catesbeianus*) in concentrations of 40 mg a.i./L or greater (Moreira et al., 2019). There is also evidence of a link between neonicotinoid insecticide contaminants and immune suppression of animals leading to greater infectious disease vulnerability (Mason et al., 2013). Immune suppression is one way for emerging infectious diseases to occur. It allows for diseases to infect species that otherwise would not be afflicted. The emerging infectious disease may then lead to individual death and overall population decline.

There are many emerging infectious diseases contributing to amphibian declines specifically. The first is *ranavirus*, which infects many amphibians leading to organ death and hemorrhaging (Miller, Gray, & Storfer, 2011). *Ranavirus* has caused amphibian population declines in North America and Europe (Cunningham et al., 1996; Green, Converse & Schrader, 2002; Ariel et al., 2009). Chytridiomycosis is also of great concern to amphibian populations on a global scale. Chytridiomycosis is a fungal infection leading to death. Infection has been shown to be caused by two species of fungi, *Batrachochytrium salamandrivorans* (hereafter *Bsal*) and *Batrachochytrium dendrobatidis* (hereafter *Bd*). *Bsal* was identified as the cause of chytridiomycosis in fire salamanders (*Salamandra salamandra*), which were experiencing major population declines in the Netherlands (Martel et al., 2013). Salamanders exhibited symptoms of anorexia, apathy, ataxia, and signs of skin lesions as a result of infection. The salamanders ultimately died after seven days (Martel et al., 2013). The movement of *Bsal* into Europe has caused rapid and seemingly unrecoverable amphibian declines and as such represents a serious global threat to amphibians. *Bsal* has not yet established a presence in North America, but the United States Geological Service (USGS) National

Wildlife Health Center (NWHC) is working with the USGS Amphibian Research and Monitoring Initiative (ARMI) to further test for the presence of *Bsal* in the United States (United States Geological Service, 2016). *Bd* has a significant presence in North and Central America (Longcore, Pessier & Nichols, 1999). *Bd* is causing chytridiomycosis infection and significant amphibian population declines in over 200 species, threatening biodiversity on a global scale (Harris et al., 2009a; Miaud et al., 2016). There are several signs and symptoms indicative of *Bd* infection. First, mass can be measured and used as an indicator of infection. Decreased mass over time has been shown in amphibians infected with *Bd* (Harris et al., 2009b; Murone, DeMarchi & Venesky, 2016). Second, snout-vent length (SVL) has exhibited a negative relationship with infection in metamorphs (Kriger et al., 2006). Third, measures of lethargy, such as movement have been used in the methods of previous studies examining infection with *Bd* of amphibians (Voyles et al., 2007). Fast quantitative PCR is effective in diagnosing *Bd* infection by testing for the presence or absence of *Bd* zoospores from swabs taken from amphibians (Kerby et al., 2013).

There are a few host defenses against *Bd* that have been identified. Two key ones are antimicrobial peptides secreted by the host and microbiome defense. Select species of amphibians have immunity from *Bd* infection because they produce several skin peptides with antimicrobial properties allowing them to fight the pathogen and avoid infection (Woodhams et al., 2007a). The other mechanism of defense, the cutaneous microbiome relies on the presence of anti-fungal bacteria residing within the amphibian microbiome. Studies have shown that the presence of anti-fungal bacteria in the cutaneous microbiome of amphibians improves *Bd* infection outcomes (Harris et al., 2009a; Harris et al., 2009b;

Ibáñez et al., 2019). Emphasis on the amphibian microbiome is critical because microbiomes are important in many cases of disease across species. For example, the relative composition of human microbiomes significantly differs between healthy humans and those suffering from various disease states, such as inflammatory bowel disease, type 2 diabetes, and necrotizing enterocolitis (Johnson & Versalovic, 2012). Bat microbiomes have been shown to be influenced by many environmental factors and as such can serve as an indicator of the host's life history, physiology, and geography (Philips et al., 2012). Another example is the rainbow trout (*Oncorhynchus mykiss*) microbiome. One study found several bacterial species within the microbiome of the rainbow trout to inhibit aquatic fungal pathogens (Lowrey et al., 2015). Bacterial species capable of inhibiting fungal pathogens in amphibians have been isolated from amphibian microbiomes as well (Harris et al., 2006; Madison et al., 2017). It is important to have identified these anti-fungal bacteria resident to amphibian microbiomes because the microbiome can also influence the success of the animal's reintroduction to the wild. It has been suggested that non-resident bacteria introduced to microbiomes of animals do not persist in the animals' native environment and thus, does not perform its intended function so the animal dies after reintroduction (Redford et al., 2012). Both the previously mentioned examples of the rainbow trout microbiome and amphibian microbiome studies utilized bacterial species native to the hosts' microbiomes in an effort to eliminate the risk accompanied by non-resident bacteria (Lowrey et al., 2015; Harris et al., 2006; Madison et al., 2017). As such, this study utilizes a strain of *Serratia marcescens* isolated from amphibian populations in Costa Rica (Madison et al., 2017). One method for reducing the presence of transient bacteria on captured amphibians is via rinsing or washing. It is thought the

washing is simply effective for preventing the influence of environmental bacteria in results (Lauer et al., 2008; Kueneman et al., 2014). However, it may also play a role in increasing bacterial diversity of the microbiome (Madison et al., 2019). Thus, this study also looks at other ways the washed treatment may have influenced the frogs.

The microbiome can be manipulated to provide an additional tool for defense against *Bd* infection (Bletz et al., 2013). Bacteria that inhibits the growth of *Bd* must be used in order to do this. Previous studies have identified many species of bacteria capable of inhibiting *Bd* growth *in vitro* (Harris et al., 2006; Lauer et al., 2008; Madison et al., 2017). This study examines the effects on amphibians of one of these bacterial species *in vivo*. A couple of studies have shown inhibition of *Bd in vivo*, but did not investigate the specific bacterial pathways responsible for this inhibition (Harris et al., 2009a; Harris et al., 2009b).

Here, I used *Serratia marcescens*, the wild-type strain of which produces a known anti-fungal agent, prodigiosin (Okamoto et al., 1998). I also used a genetically altered strain of *S. marcescens* ($\Delta pigM-Sm$) that did not have the ability to produce prodigiosin to determine whether or not prodigiosin was responsible for inhibiting *Bd in vivo*. I hypothesized frogs treated with the wild-type *S. marcescens* and *Bd* would exhibit more movement and maintain mass than those treated with the $\Delta pigM-Sm$ and *Bd*.

Methods

I. Capture

Animals used in this experiment were wild caught *Acris blanchardi*. Metamorphs were captured with nets or gloved hands in Nebraska along the Missouri River Valley.

Metamorphs were used in this study based on natural availability. Collection of frogs was permitted under a Nebraska Game and Parks Commission Scientific and Educational Permit (#1006). After capture, each frog was put in an individual plastic bag placed in a cooler for transportation to the University of South Dakota. At intake, the mass and snout-vent length were measured of each frog. Mass and SVL were not significantly different between frogs prior to experimentation. The frogs were also swabbed for the presence of *Bd* as determined by qPCR. Half of the frogs were washed with 6 mL of Nanopure water using a 1 mL pipette.

II. Housing

Experimentation with the frogs was in accordance with an approved University of South Dakota IACUC protocol. All experimentation was done in a BSL-2 facility in the animal care facility located at the University of South Dakota. Each frog was kept in an individual plastic container, which had been sterilized prior to use via a four step process: (1) plastic containers and lids were first submerged in a 10% bleach solution for one minute; (2) rinsed with tap water; (3) wiped down with a 70% solution of ethanol; and (4) lastly, rinsed with Nanopure water. Every container held a bed of Coco Husk (Exo Terra, Rolf C. Hagen Corp., Mansfield, MA, USA), which was sterilized via autoclave (15 m, 121° C). The environment was kept at 20.5°-21° C and light/dark cycles set to represent the natural conditions of 42.5° N.

III. Treatments

Individual frogs were kept under 12 treatment conditions representing a 2x2x3 factorial design (table 1): live *Bd* or no-*Bd*; washed or unwashed; and no *S. marcescens* (hereafter *Sm*), wild-type *Sm*, or $\Delta pigM$ -*Sm*.

Table 1. The number of frogs kept under each treatment condition.

Treatments	No <i>Sm</i>	$\Delta pigM$-<i>Sm</i>	Wild-type <i>Sm</i>
Unwashed (no <i>Bd</i>)	9	7	9
Washed (no <i>Bd</i>)	9	7	8
Unwashed + <i>Bd</i>	9	7	10
Washed + <i>Bd</i>	8	8	8

The wild-type *Sm* strain used was previously isolated and characterized by our laboratory from the microbiome of a Costa Rican frog (Madison et al., 2017). The $\Delta pigM$ -*Sm* was developed in our laboratory by disrupting the prodigiosin producing gene (*pigM*) with the insertion of a kanamycin resistance cassette (Madison et al., 2017). Frogs were given 48 hours to acclimate before introduction of the treatments. Each treatment held at least 7 frogs. First, individuals were randomly selected to receive treatment of the control broth only (LB-Miller), wild-type *Sm* ($\sim 35 \times 10^6$ cells/mL), or $\Delta pigM$ -*Sm* ($\sim 12 \times 10^6$ cells/mL). This was done by placing each frog in a 50.0 mL tube containing 10.0 mL of its respective treatment for 30 minutes. Following this treatment, individuals were held for three days before the introduction of *Bd* to allow for the establishment of *Sm* to the microbiome. The frogs were then placed in treatment tubes containing either the control,

tryptone-gelatin-hydrolysate-lactose (TGhL) broth only, or *Bd* and TGhL ($\sim 1.125 \times 10^8$ zoospores/mL) broth for 30 minutes. Again, each frog was placed in a 50.0 mL tube with 10 mL of treatment. The washed treatment was applied at the time of intake prior to the 48 hour acclimation period.

IV. Monitoring

Each frog was sprayed three times with autoclaved spring water daily. Following the spray, the frog was observed for 20 seconds to record movement. Movement qualified as a change in position of the entire body of the frog. Frogs were fed with two crickets three times a week. For the duration of the experiment, all frogs were measured weekly for mass and SVL. They were also swabbed twice each week: (1) to determine presence of *Bd* and (2) for sequencing of the microbiome. Sterile rayon swabs were used to complete swab samples with three strokes ventral and three strokes dorsal on each frog (Becker et al., 2014). The swabs were then frozen at -20°C to preserve them for later analysis via qPCR. Frogs alive at the end of the experiment were euthanized via a topical application of Benzocaine (Orajel, Church and Dwight Canada Corp., Mississauga, Ontario, Canada). Frogs that died prior to the end of the experiment were massed and stored in a freezer at -20°C .

V. qPCR

qPCR was done in accordance with a previously developed protocol to determine presence or absence of *Bd* prior to experimental introduction and to confirm infection post-introduction (Kerby et al., 2013). The total reaction volume was 10 μ L with 3 μ L of template DNA, 900 nmol of forward primer, 900 nmol of reverse primer, and 250 nmol minor groove binder (MGB) probe, and 2X Taqman Fast Universal Master Mix (Applied Biosystems, Foster City, CA, USA). On each plate, there was a control of Nuclease-Free water (Thermo Fisher Scientific, Waltham, MA, USA) and a series of standards with concentrations ranging from 0.06-60 zoospore equivalents to create a standard curve to compare samples against. Plates were run on a Quant Studio 4 qPCR machine at an activation stage of 95 °C for 20 s, followed with 50 cycles of 95 °C for 3 s and 60 °C for 30 s (Kerby et al., 2013).

VI. Analysis

Statistical analyses were completed in RStudio version 1.1.383 (RStudio, Inc., Boston, MA, USA). Analysis of variance tests were performed to identify potential effects of the *Bd*, bacteria, and washed/unwashed treatments on the response variables of movement, mass, and SVL of the frogs. Movement data was summed over the first eight days of the experiment. Mean mass, change in mass, and mean SVL data was calculated using the values through the fourth week of the study. These time frames were selected due to greater instances of frog mortality beyond these periods. The treatments were specifically compared against the sum of movement, mean and change in mass, and mean

SVL data. *Post hoc* Tukey pairwise tests were then completed on any significant findings.

Results

I. Movement

There was a significant difference in the number of instances of movement between bacteria treatments ($F_{2,90}=4.361$, $p=0.016$). There was greater movement in frogs of the wild-type *Sm* treatment (mean=5.44, SD=1.73) compared to the other two treatments (Δ -*pigM Sm* mean=4.34, SD=1.64; no *Sm* mean=4.42, SD=1.72) (figure 1). No difference was found in movement among the *Bd* treatments ($F_{1,90}=0.004$, $p=0.96$) or the interaction of *Bd* and bacteria ($F_{2,90}=2.363$, $p=0.10$).

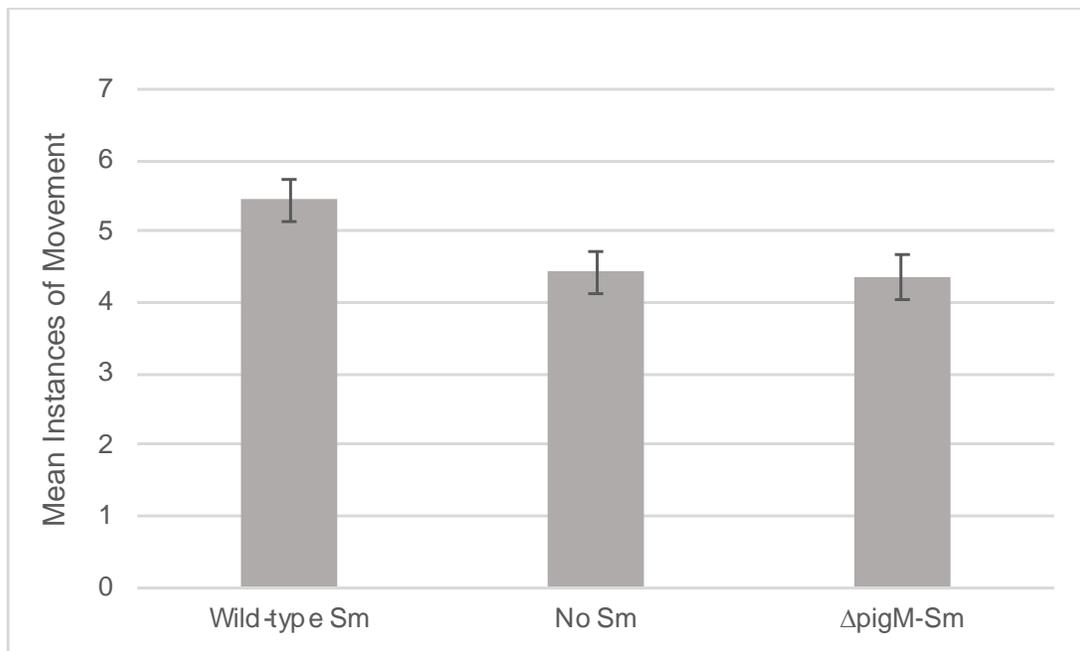


Figure 1. Mean number of times movement occurred over the sum of eight days between bacteria treatments with standard error. Frogs treated with wild-type *Sm* moved significantly more than frogs of the other bacteria treatments.

II. Mass

Washing produced a difference in mean mass ($F_{1,84}=8.71$, $p=0.004$). Washed frogs (mean=0.36 g, SD=0.11) had greater mean mass than frogs of the unwashed treatment (mean=0.29 g, SD=0.12) (figure 2). There were no relationships between mean mass and *Bd* ($F_{1,84}=0.136$, $p=0.71$), bacteria treatments (bacteria $F_{2,84}=1.472$, $p=0.24$), or their combinations (*Bd*:bacteria $F_{2,84}=0.451$, $p=0.64$; *Bd*:washed/unwashed $F_{1,84}=0.139$, $p=0.71$; Bacteria:washed/unwashed $F_{2,84}=2.786$, $p=0.07$; and *Bd*:bacteria:washed/unwashed $F_{2,84}=2.494$, $p=0.09$).

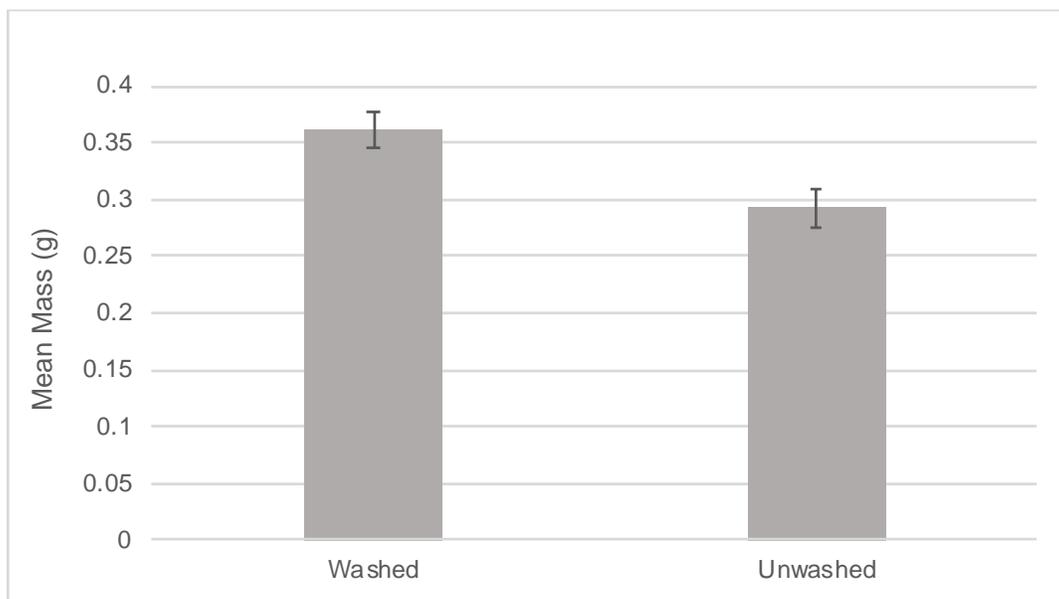


Figure 2. Mean mass over the first four weeks between frogs of the washed and unwashed treatments with standard error. Washed frogs had significantly greater mass than the unwashed frogs.

There was a small, yet significant decrease in mass for frogs ($p<0.05$) within all treatment groups from days 1 to 35, except for the no-*Sm* + *Bd*, unwashed treatment.

Differences in mass between treatments on the same days were not significant ($p>0.05$).

No significant relationships were found when comparing all treatments against change in mass (*Bd* $F_{1,89}=1.631$, $p=0.21$; bacteria $F_{2,89}=1.883$, $p=0.16$; *Bd*:bacteria $F_{2,89}=0.272$, $p=0.76$; washed/unwashed $F_{1,91}=1.510$, $p=0.22$; and *Bd*:washed/unwashed $F_{1,91}=0.102$, $p=0.75$).

III. Snout-Vent Length

SVL was significantly influenced by washing ($F_{1,84}=7.442$ and $p=0.008$). Washed frogs (mean=1.61 cm, SD=0.19) had greater mean SVLs than unwashed frogs (mean=1.51 cm, SD=0.19) (figure 3).

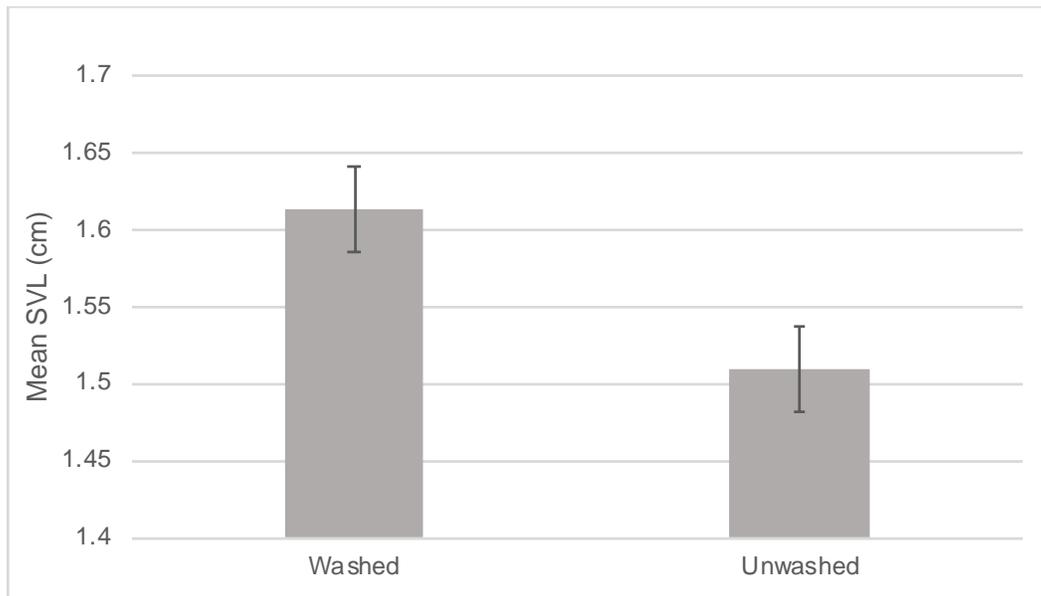


Figure 3. Mean SVL of washed and unwashed frogs with standard error. Washed frogs had a significantly greater SVL compared to the unwashed frogs.

Another significant difference existed in the mean SVLs between washed and unwashed frogs of the no *Sm* and wild-type *Sm* treatments ($F_{2,84}=5.420$, $p=0.006$). Washed frogs under the wild-type *Sm* and no *Sm* treatments had greater mean SVLs (wild-type *Sm*, mean=1.68 cm, SD=0.14; no *Sm*, mean=1.62 cm, SD=0.17) than their unwashed counterparts (wild-type *Sm*, mean=1.52 cm, SD=0.20; no *Sm*, mean=1.41 cm, SD=0.18) (figure 4). No such relationship was observed among the washed and unwashed frogs of the $\Delta pigM$ -*Sm* treatment. All other treatments and their respective relationships with mean SVL were insignificant (*Bd* $F_{1,84}=0.003$, $p=0.96$; bacteria $F_{2,84}=1.746$, $p=0.18$; *Bd*:bacteria $F_{2,84}=0.034$, $p=0.97$; *Bd*:washed/unwashed $F_{1,84}=0.357$, $p=0.55$; and *Bd*:bacteria:washed/unwashed $F_{2,84}=1.707$, $p=0.19$).

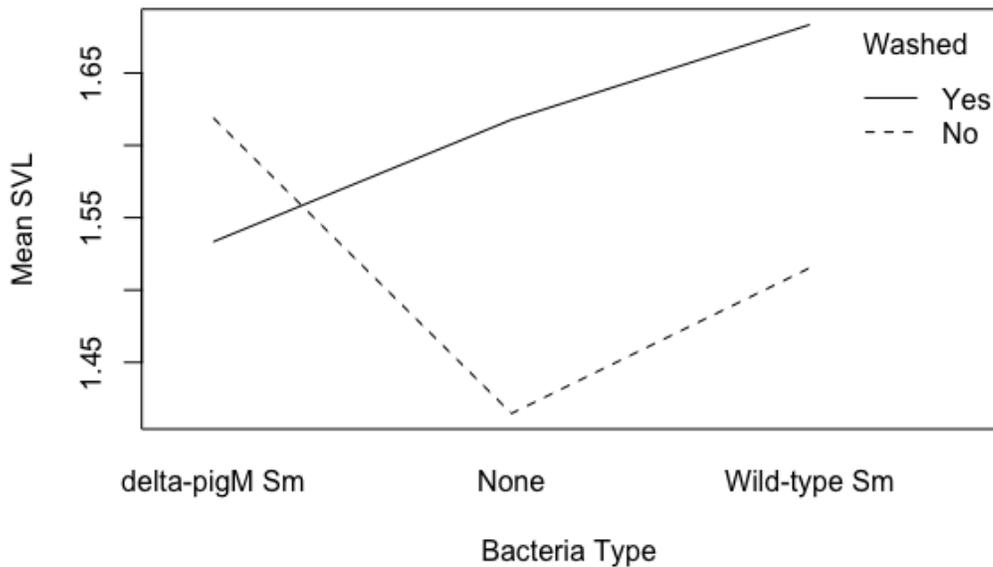


Figure 4. Interaction plot of the bacteria and washed/unwashed treatments for mean SVL. Washed frogs of the wild-type *Sm* and no bacteria treatments have significantly greater SVLs than the unwashed frogs of the same bacteria treatments. This difference was not observed between washed and unwashed frogs of the Δ -*pigM Sm* treatment.

Discussion

This is the first time recombinant *Sm* with an interrupted prodigiosin anti-fungal pathway has been used in an *in vivo* amphibian system. Previous studies have shown improvements in disease outcome with the introduction of anti-fungal bacteria species to the cutaneous microbiome of amphibians (Harris et al., 2009a; Harris et al., 2009b). We hypothesized the frogs treated with Δ *pigM-Sm* and *Bd* would produce less movement and experience more mass loss than the frogs treated with wild-type *Sm* and *Bd*. However, the most interesting difference found in this experiment is that average mass was greater in unwashed versus washed treatments. Washed frogs also had a higher average SVL than the unwashed frogs. This is of particular interest because in previous studies washing has served as an appropriate method for removing transient bacteria and debris from amphibians to prevent their influence on results, while having thought it affected other variables very little to not at all (Lauer et al., 2008; Kueneman et al., 2014; Walker et al., 2015). The reason for this relationship between washing and greater mass and SVL is unclear. Previous research suggests that washing does remove transient bacteria to ensure microbiome analysis is of primarily amphibian-associated bacteria (Culp, Falkinham, & Belden, 2007; Lauer et al., 2007). It is unknown whether washing may remove inhibitory factors to growth such as potentially harmful bacteria or environmental contaminants. It is also possible that washing removes some cutaneous *Bd* zoospores if they existed on the frog prior to experimentation. This discrepancy ultimately calls for further research on the use of washing as a method in amphibian research due to its unintended influences on other variables.

Another interesting relationship found was that between the interaction of the bacteria treatments with washed/unwashed treatments and the mean SVL variable. The washed frogs treated with wild-type *Sm* and no bacteria had much greater SVLs than their unwashed counterparts, but no significant difference was found between the washed and unwashed frogs of the $\Delta pigM$ -*Sm* treatment. This may further indicate the importance of the influence of washing even under the other treatments of this experiment. It is not clear why these differences exist. It may be that wild-type *Sm* was better able to establish a population within the microbiome after the wash. This establishment may have then led to an enhanced symbiotic relationship with the frog leading to the maintenance or gain of SVL over time. However, the observation that washed frogs with no introduced bacteria also experienced this difference in SVL does not support that wild-type *Sm* would have such an effect. Another possibility is that the introduction of $\Delta pigM$ -*Sm* within the microbiome had a unique influence within the microbiome and in its relationship with the frog host. Establishment of $\Delta pigM$ -*Sm* may not require the removal of transient bacteria or the removal of transient bacteria does not provide an advantage for $\Delta pigM$ -*Sm* to establish a population within the cutaneous microbiome. The introduction of $\Delta pigM$ -*Sm* appears to nullify the effects of the washed/unwashed treatment observed under the other bacteria treatments. Further research is required to determine why.

Mass as a measure can be used for indicating effects of *Bd* infection or lack thereof (Harris et al., 2009b). This experiment measured SVL to investigate any potential relationships with disease outcomes post-infection of *Bd*. Third, movement was measured in this experiment because decreased movement is indicative of infection (Carver, Bell, & Waldman, 2010). Frogs treated with wild-type *Sm* moved more than frogs under the

ΔpigM-Sm treatment. This result supports our hypothesis. It is most likely the wild-type *Sm* established a population within the cutaneous microbiomes of the frogs and developed a strong symbiotic relationship. This establishment would allow for the anti-fungal capabilities of the bacteria to take effect in the presence of *Bd*. This was anticipated since the wild-type strain retained its prodigiosin pathway. Frogs exposed to the *ΔpigM-Sm* instead of the wild-type *Sm* exhibited less movement. This was also anticipated because the lack of prodigiosin production in the recombinant strain reduced its anti-fungal capabilities likely leading to greater *Bd* infection in those frogs.

Identifying the role of the aforementioned significant relationships between treatments and measures in disease outcomes brings opportunities for further investigation into new prevention and treatment possibilities of chytridiomycosis, the disease caused by *Bd*.

Examining *Bd* in Blanchard's cricket frogs is important because they are declining across parts of their range and this could partially be due to *Bd*. Many species of amphibians are experiencing decline at least partially due to *Bd* infection, so this research is also important in helping combat global amphibian declines. To have the widest reach, it is also important to identify several effective bacteria species for this potential microbiome engineering treatment. Using bacteria native to the microbiome of the respective animal has also shown to be important in success of reintroduction to the wild, so identifying several anti-fungal bacteria is critical to developing this as a successful treatment of infection (Redford et al., 2012). Other studies used different species of bacteria shown to be effective in vivo against *Bd*, such as *Pseudomonas reactans* and *Janthinobacterium lividium* (Harris et al., 2009a; Harris et al., 2009b). This experiment examined the potential of *Sm* to be another one of these effective bacteria species. This also has

implications in disease outcomes of other wildlife, such as bat populations affected by white-nose syndrome, which is caused by a pathogenic fungus, *Pseudogymnoascus destructans* (Hoyt et al., 2015). In addition, bacterial microbiomes are known to defend plants from pathogens (Berendsen, Pieterse, & Bakker, 2012). As such, microbiomes of other animals and plants could be engineered to assist in defense against pathogens. It has even been suggested that microbiome engineering of this manner could be used to treat diseases in humans (Schwabe & Jobin 2013). Further research is required to determine the viability of microbiome engineering as a treatment of infection in wild animals. There are many obstacles to overcome, such as survival of animals with engineered microbiomes post-reintroduction. Investigation of unintended consequences, such as those seen with washing in this study, is required before this treatment can be widely used.

CHAPTER TWO

Influences of Anti-*Bd* Bacteria on Amphibian Microbiomes

Introduction

Microbiomes have shown great importance in the health of their hosts, yet many details of microbiome-host interaction remain unknown (Miles, Jonathan, & Handelsman, 2015; Cho & Blaser, 2012). The introduction of new bacteria to the microbiome has shown to influence host health when facing infection (Harris et al., 2009a). Although this interaction between bacteria and host health has been well studied, the changes in microbiome diversity due to a new bacteria species have not. Another concept that has yet to be studied in depth is the genetic recombination of bacteria species for the enhancement of a desired function (e.g., ability to fight fungal infection) of the microbiome. The methods for genetic engineering of this kind has been examined in bees (*Apis mellifera*) and mice (*Mus musculus*) (Leonard et al., 2018; Mimee et al., 2015). However, the use of these tools has an unknown influence on the diversity of the microbiome when introduced as treatment for host disease. This is an important area of study to make the introduction of recombinant bacteria to the microbiome a viable treatment option for host disease.

A major host disease of interest is chytridiomycosis, which is an infection of amphibians caused by the fungus, *Batrachochytrium dendrobatidis* (*Bd*) (Longcore, Pessier, and Nichols, 1999). *Bd* is causing serious amphibian population declines on a global scale (Vredenberg et al., 2010). Despite global declines, there are some amphibian

species that exhibit immunity to *Bd* infection (Whitfield et al., 2017). This immunity is likely due to some combination of unique major histocompatibility complexes (MHC) of the adaptive immune response, secretion of anti-microbial peptides, and resident bacteria of the cutaneous microbiome (Fu and Waldman, 2017; Bataille et al., 2015; Woodhams et al., 2007a; Woodhams et al., 2007b; Rebollar et al., 2016). The cutaneous microbiome holds the most potential for exploration and manipulation in efforts to combat *Bd* infection because of its proximity to the site of infection. Research thus far has led to the introduction of known anti-fungal bacteria native to the amphibian host microbiome (Lauer et al., 2008; Harris et al., 2006; Redford et al., 2012; Harris et al., 2009a; Harris et al., 2009b).

There are several known anti-*Bd* bacteria species (Woodhams et al., 2015). *Serratia* species are of particular interest in this study because of their antifungal properties. These include the production of chitinases and glucanases, the type-VI secretion system, and production of prodigiosin (Monreal & Reese, 1969; Trunk et al., 2018; Okamoto et al., 1998). *Serratia* is also of interest because it is commonly found in the microbiomes of Anura and Caudata (Woodhams et al., 2018). In this study, we focused on the prodigiosin pathway by introducing either a recombinant strain of *Serratia marcescens* with the pathway disabled ($\Delta pigM-Sm$) or a wild-type strain of *Sm*. We hypothesized that introduction of wild-type *Sm* versus $\Delta pigM-Sm$ would lead to significant differences in microbiome community structure relative to each strain and Blanchard's Cricket Frog (*Acris blanchardi*) response when challenged with *Bd*. We also hypothesized that these differences would be influenced by time, particularly the effects of time on microbiome diversity between treatments.

Methods

The same experimental subjects were used as in the previous chapter. Capture, housing, and treatments are summarized below, but see Chapter 1 for complete details.

I. Capture and Housing

Metamorph *A. blanchardi* were caught with gloved hands in Nebraska along the Missouri River Valley as described in Chapter 1. All frogs were housed in sterilized individual plastic containers under regulated conditions and temperatures per housing methods detailed in Chapter 1.

II. Growth Assays

Growth assays were completed to develop growth curves for each of the following strains of bacteria: wild-type *Sm* and $\Delta pigM$ -*Sm*. In addition, there were two broth only (LB-Miller) controls, one for each strain. Growth was carried out at 21°C. Concentration measurements of the bacteria growth for both strains were done every 24 hours with a spectrophotometer (OD=600). Samples were normalized for cell concentration as to have the same baseline.

Challenge assays were carried out using methods per Bell et al. (2013) to examine the effects of each strain of *Sm* on *Bd* growth. Varying concentrations of *Sm* supernatant (from centrifuged culture, 0.22 μ m filter-sterilized) were placed in 96 well plates. Starting growth values for beginning *Bd* concentration were normalized for all wells. *Bd* only dilution cultures were also created and examined to control for potential influence of

nutrient limitations. Growth of the challenge assays and *Bd* only dilution cultures was done at 21°C for 8 days.

III. Treatments

Individual frogs were kept under the 12 treatment conditions representing and 2x2x3 factorial design described in detail previously (Table 1, Chapter 1): live *Bd* (JEL 423), no-*Bd*; washed, unwashed; and no *Sm*, wild-type *Sm*, Δ *pigM-Sm*. All treatments had at least seven frogs up to ten. Treatment introduction and washing were completed as described in Chapter 1.

IV. DNA Extraction and Purification

Proteinase K digestion of all swab samples was done overnight at 56°C. DNA extraction was completed using the Qiagen (Hilden, Germany) DNeasy Blood and Tissue kit according to manufacturer's directions. Extracted DNA was then cleaned and concentrated using the ZR-9 Genomic DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA, USA).

V. qPCR

qPCR was done in accordance with a previously developed protocol to determine presence or absence of *Bd* and are detailed in Chapter 1 (Kerby et al., 2013). Samples were compared against controls lacking DNA and a standard curve of the *Bd* ribosomal internal transcribed spacer (ITS) gene. A sample was considered positive when two or

more of the three replicates indicated amplification and mean detection was at least 0.1 zoospore (9.8 copies) per previous research (Bloo et al., 2013).

VI. Library Preparation and Next-Generation Sequencing

All library preparation and sequencing was completed in WestCore at Black Hills State University (Spearfish, SD, USA) as is offered in Madison, Ouellette, Schmidt & Kerby (2019).

VII. Sequence Data and Statistical Analysis

Processing and analysis of raw sequencing data was done using Mothur v.1.39.5 (Schloss et al., 2009). The MiSeq standard operating procedure was followed starting with generating contigs from paired-end reads, clean-up steps (including screening, filtering and chimera removal using UCHIME), alignment to the Silva database [1.32], and generation of operational taxonomic units (OTUs) for statistical analysis (Kozich et al., 2013; corresponding webpage accessed 29 January 2018) according to Madison, Ouellette, Schmidt & Kerby (2019).

All statistical analyses were performed in R v.3.4.3. Linear models and repeated measures ANOVA were used in analysis of the *in vitro* experiments and measurements. Multiple treatment comparisons were made using estimated marginal means (EMMs) and Tukey's pairwise tests. This was done with the emmeans package (Madison et al., 2019)

Linear mixed-effects models were used to analyze the *in vivo* experimental results. Package lme4 was used to create the models of the relationship between the response variable and predictor variables: treatment, day, and individual (Madison et al.,

2019). A day-treatment interaction term was entered as a fixed effect. Individual frogs were included in the model as a random effect. The response variable used between models was of either inverse Simpson diversity, mass (g), *Bd* presence/absence or *Serratia* abundance. Visual inspection of residual plots in all models did not reveal any obvious deviations from normality or homoscedasticity. Model selection was carried out by likelihood ratio tests of the full model with the day-treatment interaction effect in question. Further *post hoc* testing with EMMs comparisons and Tukey's pairwise tests between treatments and between days was completed using the emmeans package (Madison et al., 2019).

All scripts and models used for the analysis of results presented in this paper have been deposited in GitHub (https://github.com/kvasir7/Acris_microbiome) and are publicly accessible. All 16S rRNA gene sequences have been deposited in the NCBI Sequence Read Archive (BioProject: PRJNA435631).

Results

As is presented in Madison, Ouellette, Schmidt & Kerby (2019), *Bd* growth was significantly reduced in the presence of wild-type *Sm in vitro* compared to the $\Delta pigM$ -*Sm* ($p=0.014$) and the *Bd* only control ($p<0.0001$). *In vivo*, the interaction between treatment and time significantly predicted frog mass, bacterial community inverse Simpson diversity, *Bd* presence/absence, and *Sm* abundances ($p<0.05$). The presence of zoospores was not significantly different between treatment groups at the same points in time ($p<0.05$). *Bd* was detected before the experimental introduction of *Bd*. This indicates some *Bd* prevalence in the environment where frogs were captured. Despite this finding,

Bd infection still qualitatively increased one week after exposure to *Bd*; this included individuals that were negative for *Bd*. *Bd* was absent from all remaining live frogs three weeks after initial experimental exposure.

I. Inverse Simpson Diversity

As is shown in Madison, Ouellette, Schmidt & Kerby (2019) there was a significant increase in diversity of the microbiome between day 1 and 29 in both washed and unwashed $\Delta pigM$ -*Sm* treatments ($p=0.033$ and $p<0.0001$, respectively) and in the washed control treatment ($p=0.001$). No significant change in inverse Simpson diversity was seen in the unwashed control or wild-type *Sm* treatments. It is also important to note that *Serratia* became a chief component (greater than 10.0% for at least one time point) of the microbiome after introduction of either strain (figure 5).

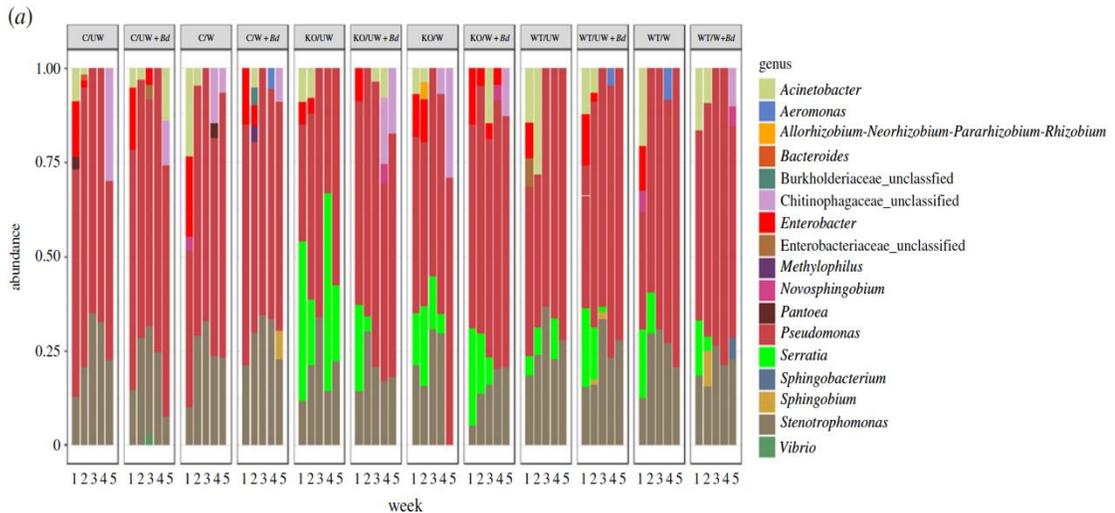


Figure 5. Bar graphs showing relative abundance (%) of bacterial genera comprising greater than 10% of the microbiome at a minimum of one time point.

II. Survival

Survival curve analysis from Madison, Ouellette, Schmidt & Kerby (2019) revealed a significant difference between bacteria treatments in the unwashed + *Bd* group (figure 6, $p(\text{global}) < 0.001$, d.f.=2). In the unwashed + *Bd* group, the ΔpigM -*Sm* treatment showed a significantly decreased likelihood of survival compared to the wild-type *Sm* treatment ($p = 0.00026$, d.f.=1). Significant differences were not observed in comparing the other bacteria treatments, wild-type *Sm* and no *Sm*, in the unwashed + *Bd* group ($p > 0.05$, d.f.=1). There were also no significant differences in survival observed between bacteria treatments in the washed + *Bd* group or in either of the no-*Bd* groups. A significantly higher survival rate was observed in the unwashed + *Bd* + no-*Sm* treatment when compared with the washed + *Bd* + no-*Sm* treatment ($p = 0.0071$, d.f.=1).

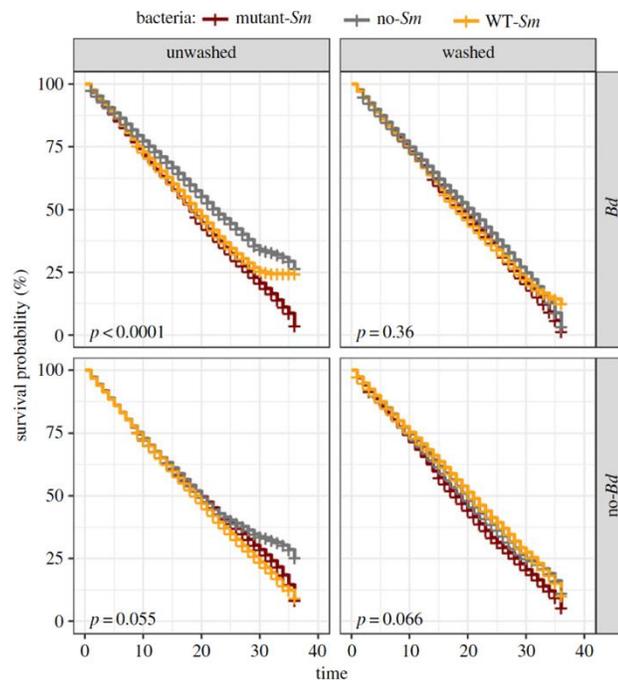


Figure 6. Survival plots with panels faceted by treatment conditions washed/unwashed and *Bd*/no-*Bd*. Curves in each sub-plot are color-coded by bacterial treatment: mutant (ΔpigM) *Sm*, wild-type *Sm* or no-*Sm* (given in figure legend). There is a significant difference between the curves of the *Bd*-unwashed sub-plot ($p < 0.001$).

Discussion

Using genetically engineered bacteria for fighting infection has yet to be thoroughly researched enough to allow for implementation. This experiment showed that inoculation of *A. blanchardi* with wild-type *Sm* or Δ *pigM-Sm* alters the microbiome diversity and survival of the host when challenged with *Bd*. The recombinant *Sm* was associated with greater microbiome diversity, but lower probability of host survival. This means the specific functions of a particular species of bacteria can have major effects on microbiome diversity, but not necessarily to the survival benefit of the host.

The low levels of *Bd* detected prior to experimental introduction of *Bd* JEL 423 is consistent with previous and current reports on *Bd* presence in the Midwestern United States (Steiner & Lehtinen, 2008). This was useful for this study as it was our goal to examine potential microbial therapies on amphibian populations that are not naïve to *Bd*, but potentially vulnerable to more virulent strains. The *Bd* strain JEL 423 used in this experiment is known for its high virulence (Hambalek, 2016); however, the individual frogs used in this experiment were only partially susceptible. It is possible environmental *Bd* exposure prior to capture or factors due to captivity influenced their susceptibility, but it remains poorly understood. It is also important to note the lack of *Bd* three weeks post-exposure. This is probably due to the death of infected frogs or the clearing of *Bd* by live frogs. It is possible that mortality among captured frogs could have been due to factors outside of *Bd* infection, such as pre-capture environmental factors or developmental stage. However, *Bd* was still a clear factor among treatment conditions for either having caused or was a significant factor in the observed differences in mortality between bacterial treatments.

Another interesting result was that bacterial community diversity of the host microbiome significantly increased over time in the $\Delta pigM-Sm$ and washed control treatment groups. The increase in diversity with $\Delta pigM-Sm$ may be due to the lack of the prodigiosin, since prodigiosin represents an inhibitory factor to bacterial growth. For instance, wild-type *Sm* with an intact prodigiosin pathway uses it to kill other bacteria species; therefore, reducing diversity. The increase of diversity in the washed control treatment may indicate that washing promotes microbiome diversity. The reasons for this are poorly understood but could be due to recolonization from the gut microbiome of the frog. This could occur since the gut microbiome is more stable in captivity than the cutaneous microbiome (Becker et al., 2014). Instead, washing may be responsible for removing a less adherent and dominant bacteria species from the microbiome allowing other bacteria to take its place. The removal of one species leaves a niche open for others. The availability of niches like this is important in the application of probiotics and therefore, may be important here as well (Zmora et al., 2018). Increased diversity of the microbiome does not necessarily mean increased odds of host survival when facing infection, however. Although $\Delta pigM-Sm$ inoculation was associated with increased diversity of the microbiome, it was also associated with decreased probability of survival when challenged with *Bd* compared to wild-type *Sm*. In addition to loss of anti-bacterial ability, the loss of prodigiosin in $\Delta pigM-Sm$ may also decrease survival in hosts, since prodigiosin is a known anti-*Bd* factor.

This study provides important information in showing the potential of genetic engineering and introduction of bacteria to host microbiomes for the treatment of disease. However, doing this in a predictive way is an important goal for implementation in wild

or laboratory settings. Another important feature to develop is the ability to up- and down-regulate protein or metabolite production of introduced bacteria. This would serve as an important safety feature to manage unintended effects on the host and the ecosystem (Smith, Van Elsas, & Van Veen, 1992). This methodology for the treatment of emerging infectious disease presents many challenges, yet has the potential to dramatically change treatment since there are few options. The results presented in this study, therefore, provide important insight into the use of genetically modified bacteria species in the management of host disease.

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