PROBIOTIC APPLICATION OF SYMBIOTIC BACTERIA ISOLATED FROM WESTERN BAT SPECIES ONTO CAPTIVE AND FIELD BATS TO PREVENT WHITE-NOSE SYNDROME

by

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ABSTRACT

Wildlife diseases can have drastic consequences for species at an unprecedented rate. Without proper intervention, diseases can threaten population viability and result in species extinction. The delicate balance of an ecosystem is associated with the niche of an individual species and can quickly become unstable upon the introduction of an invasive disease. Managing these diseases is often quite challenging and typically requires immediate action to prevent further ecological loss. The psychrophilic fungus, *Pseudogymnoascus destructans*, *Pd*, has devastated populations of several North American bat species. White-nose syndrome (WNS) is a deadly disease that indirectly causes hibernating bat mortality by growing on the cutaneous surfaces of the bat and causing more frequent arousal periods. In addition to physiological imbalances caused by the degradation of wing tissue, the decreased torpor length and associated increased metabolic rate will burn precious fat stores, cause rapid dehydration, and lead to bat mortality. Reducing the severity of the *Pd* infection could result in lower mortality rates. Experiments on captive bats and free-flying wild bats were conducted to alter native wing microflora found on *Myotis yumanensis*, using a prophylactic topical probiotic cocktail. The probiotic contains *Pseudomonas* bacterial species that were isolated from wings of healthy British Columbia bats and were found to inhibit *Pd*. The four main objectives of this study were: (1) to develop a technique of applying the anti-*Pd* microbes to Myotis bats roosting in buildings and/or bat boxes; (2) to test whether an microbiome enhanced with anti-*Pd* microbes can be sustained on bat wings; (3) to replicate captive trial tests and procedures in a field trial setting; and (4) to quantify the ability of the probiotic to inhibit *Pd* germination/growth on live bat skin. Two captive bat trials at the B.C. Wildlife Park and one lab hibernation trial were conducted. I developed an effective two-step application process of misting a roosting substrate with water, followed by powdered clay containing freeze-dried probiotic cells. Quantitative PCR analyses confirmed successful long term adherence of the probiotic cells to bat boxes, and successful transfer of these microbes to bats' wings. The probiotic was field-trialed at summer maternity roosts in the Greater Vancouver area, successfully applying probiotic at building and bat box roosts, and through swab sampling of captures of free-flying bats, determined the probiotic was successfully transferred. In a laboratory

experiment, I placed probiotic bacteria and *Pd* onto separated bat patagia, with the goal of adapting and refining tissue explant chamber technology to keep bat skin cells partially alive on which to test the interaction of *Pd* and probiotic cells. This probiotic tool provides one potential prevention measure for reducing the spread, or at minimum the severity, of WNS in the Pacific Northwest of North America.

Key-words: bats, *Pseudogymnoascus destructans*, probiotic*, Pseudomonas,* white-nose syndrome (WNS), disease management, captive trial, field trial, prophylaxis, bats, *Myotis yumanensis*

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Glossary of terms

ANOVA Analysis of Variance
BCWP BC Wildlife Park
BSC Biosafety Cabinet
CFU Colony Forming Units
DAPG Diacetylphloroglucinol
DNA Deoxyribonucleic Acid

EPFU Big brown bat (*Eptesicus fuscus*)

EWL Evaporative Water Loss

GLM General Linear Model

LB Lysogeny Broth

MYLU Little brown bat (*Myotis lucifugus*)

MYYU Yuma myotis (*Myotis yumanensis)*

NA Nutrient Agar

OD Optical Density

PBS Phosphate Buffer Saline

PCR Polymerase Chain Reaction

Pd *Pseudogymnoascus destructans*

PIT Passive Integrated Transponder

qPCR Quantitative Polymerase Chain Reaction

RNA Ribonucleic Acid

SDA Sabouraud Dextrose Agar

TMR Torpor Metabolic Rate

TRU Thompson Rivers University

WCS Wildlife Conservation Society (Of Canada)

WNS White-Nose Syndrome

Chapter 1: INTRODUCTION

Chiroptera is the second largest mammalian order with over 1,400 species found worldwide excluding the arctic poles (Simmons and Cirranello 2021). One hundred and nine of these species are considered vulnerable, eighty-three are currently endangered, and twenty-one are critically endangered (BCI 2021). New bat species are continuously being discovered and 242 bat species are data deficient with an unknown conservation status (BCI 2021). British Columbia is home to 17 of the 19 bat species found in Canada (Naughton 2012).

All bat species in Canada are insectivores that feed on arthropods (Harvey, et al. 2011; Nagorsen and Brigham 1995). As such, when insects are not present, starting in fall, bats must either migrate or hibernate until insect prey are again abundant. Most bat species in Canada are hibernating species, with only 3 that are considered migrating species: *Lasiurus borealis*, *L. cinereus*, and *Lasionycteris novtivagans* (Harvey, et al. 2011; Nagorsen and Brigham 1995). Hibernating species generally roost within mines, caves, rock crevices, and in some areas some species will hibernate in trees and buildings (Naughton 2012). Hibernation is thought to occur shortly after mating swarms in September-October or later (i.e. *Myotis. lucifugus*, *Eptesicus fuscus*, *M. yumanensis*, *M. californicus*, *Euderma maculatum*, *M. septentrionalis*, *M. evotis*, *Antrozous pallidus*, *M. thysanodes*, *M. volans*, *M. ciliolabrum*, *M. leibii*, *L. noctivagans*, *Parastrellus hesperus*, *Corynorhinus townsendii*). Bats may undergo torpor to conserve energy and reduce water loss when their ambient temperature is less than their thermoneutral zone (Herreid and Schmidt-Nielsen 1966). Torpor decreases their body temperature to match their ambient temperature and lower their metabolic rate (Geiser 2004). Basic bodily functions such as breathing and heart rate are slowed immensely and their immune system is suppressed throughout torpor, making them vulnerable to infection (Bouma et al., 2010; Geiser 2004; Moore et al., 2011). Durations of torpor are intermittent and often delayed by brief arousals that vary among species, in which body temperature and metabolic rate reach normothermic levels briefly before bats re-enter torpor. These arousals are the main contributor of stored energy usage when in hibernation (Thomas et al. 1990). Feeding, drinking, mating, grooming, and replenishing electrolytes have all

been documented in between bouts of torpor (Boyles et al. 2006; Wilcox et al. 2014). After emergence from hibernation, which typically occurs between March-May the following spring, females will typically return to previously used maternity roosts whether it be a bat box, building, or tree roost. Males will instead form smaller colonies or roost by themselves (Naughton 2012).

Temperate bats choose where to roost based on many factors such as thermoregulation, reproduction status, risk, coloniality, and roost quality (Boyles 2007; kerth et al. 2008; Lausen and Barclay 2006; Racey et al. 1973). Bats often choose roost based on their thermoneutral zone, in which they do not have to regulate their body temperature to match their roost (Boyles 2007). This will eliminate unneeded energy expenditure in colder temperatures and dehydration from warmer areas (Genoud et al. 1990). Furthermore, bats undergoing gestation or lactation will choose roosts that do not require torpor, to avoid negative effects such as parturition and reduced milk supply (Racey 1973; Studier and O'Farrell, 1972). Once maternity colonies are formed in the summer, individual bats must decide how to balance risks such as parasitism and disease risk in larger maternity colonies, with the benefits of coloniality such as social thermoregulation, predator avoidance, and maintenance of social relationships (Altrignham 2011; Bartonička and Gaisler 2007; Kerth 2008; Reckardt and Kerth 2007; Russo et al. 2017; Webber et al. 2018; Willis and Brigham 2007).

Roost switching is common to balance these factors and is different between artificial and natural roosts (Whitaker 1998). Man made structures such as bridges, and buildings often mimic natural roosts quite well, and provide better thermal stability, warmer microclimates, and greater protection from predators than natural roosts (Lausen and Barclay 2006; Rueegger 2016; Russo and Ancillotto 2015). These conditions promote reproductive success, better body condition, faster juvenile development, and inevitably less roost switching (Allen et al. 2010; Godhino et al. 2015; Lausen and Barclay 2006; Webber et al. 2016; Zahn 1999).

Species such as, but not limited to, *C. townsendii*, *E. fuscus*, and more commonly found, *M. lucifugus*, and *M. yumanensis* are all found in buildings throughout

the summer season (Nagorsen 1995). In BC, most bats raise a single young each summer, although some migratory species can give birth to up to 4 pups. Most species give birth in June each year and pups will depend on their mother's milk for roughly 6 weeks until they learn to fly and hunt on their own. Many maternity colonies break up in late summer, and it is thought that most bats will arrive at hibernation areas by October, and bats mate somewhere either en-route to hibernacula, or at the hibernacula. It is now known that some species of bats in B.C. will continue to mate during winter and in spring (C. Lausen, pers. comm.)

Because bats consume thousands of insect prey nightly in the summer, they are important environmentally and economically; the value of insectivorous bats in the United States is estimated to be roughly 22.9 billion USD a year (range $3.7 - $53 billion, Boyles et al. 2011). Humans have a significant impact on bat populations world-wide, through roost disturbance, habitat loss, direct mortality such as for food consumption and persecution, and indirect mortality including exposure to chemicals like pesticides (Berthinussen et al. 2014). In North America, deforestation, and developments such as roads, buildings, and windfarms contribute to habitat fragmentation. Such expansions can result in roost destruction, loss of traditional land used for migration and insect feeding and forced movements into uninhabited areas. Roads are particularly bad because they restrict foraging access for bats and directly contribute to fragmentation and in some cases, casualties (Kerth and Melber 2009; Lesinski et al. 2011). Bat casualties are often seen from wind turbines, in which hundreds of thousands of bat deaths each year from barotrauma (Baerwald et al. 2008; Smallwood 2013), due to migratory movements and unnatural attraction towards the turbines (Cryan and Barclay 2009). North American bat species face many cumulative conservation threats, however the biggest threat in the last decade, which can likely lead to the extinction of several bat species, is White-nose syndrome (WNS).

White-nose syndrome is a fatal bat disease that is a direct result of infection from the fungal pathogen *Pseudogymnoascus destructans*. The fungus was first discovered in a cave near Albany, New York in 2006 (Blehert et al. 2009; Frick et al. 2010). Since

then it has caused an unknown number of bat mortalities, having been estimated at more than 6 million bat deaths in 2012 (USFW 2012). Mortality rates as high as 90-100% have been reported (Drees et al. 2017; Frick et al. 2010; Frick et al. 2015; Leopardi et al. 2015). *Pd* is currently detected in 39 states and seven Canadian provinces (USFW 2020) and has not been officially documented in British Columbia yet. However, bat hibernacula in British Columbia (and the Pacific Northwest in its entirety) are largely unknown and un-surveyed (Weller et al. 2018). Therefore it is reasonable to think WNS has already reached British Columbia and we simply have not detected it yet, especially considering the cases found in Washington State came from Kentucky (Thapa at al. 2021).

Differential rate of mortality caused by WNS is due to many factors such as species, hibernacula microclimates, and individual bat interactions associated with cluster size (Lorch et al. 2011; Wilder et al. 2011). Certain species are more affected than others, particularly *M. lucifugus*), *Perimyotis subflavus*, *M. sodalis*, and more recently, *M. septentrionalis* (Frick et al. 2015). The origin of *Pd* is speculated to be from Europe (Drees et al. 2017; Leopardi et al. 2015). Eurasian bats have not suffered the same population declines or mass mortalities as seen in North American bats. This is likely due to WNS being an invasive novel fungus that North American bat species have never encountered or adapted to, unlike bats from Eurasia thought to have co-evolved with this fungus (Leopardi et al. 2015). Furthermore, there is genomic evidence that Eurasian bats may have co-evolved resistance or tolerance to WNS infection in the past (Hoyt et al. 2016; Leopardi et al. 2015).

Bats have suffered high mortality rates because of *Pd's* persistence, growth conditions, mechanism of infection, and difficulty to remove from hibernacula. *Pd* is a psychrophilic saprotroph that grows optimally between 12.5-15.8^{o}C in high relative humidity conditions of 81.5% (Marroquin et al. 2017; Verant et al. 2012). It may persist in caves for centuries and may reinfect bats that use the same area for hibernation (Reynolds et al. 2015). Bats who survive until emergence will groom off WNS hyphae and resume their normal life cycle until the next hibernation period with a normal body

mass and some wing tissue injuries (Fuller et al. 2020; Reichard and Kunz 2009). However, survival of bats after hibernation within infected caves has been largely dependant on body weight and greater fat reserves (Cheng et al. 2019; Jonasson and Willis 2011) with physiological disturbance and starvation being the main mechanism of mortality (Storm and Boyles 2011; Cryan et al. 2013; Warneke et al. 2013; Verant et al. 2014). *E. fuscus* can have similar hibernation conditions to vulnerable species, however it has exhibited resistance presumably due to its skin microbiota (Frank et al. 2014; Lemieux-Labonté et al. 2020). Bat species exposed to re-occurring infection from the same hibernacula either reach 100% infection or have a lesser mortality rate, possibly due to behavioural or skin microbiota adaptions that previous bats did not have (Frick et al. 2017; Hoyt et al. 2015; Langwig et al. 2017).

An augmented skin microbiota that provides resistance to *Pd* infection has been a promising method of controlling WNS spread. Many ex-vivo laboratory trials have demonstrated antagonistic interactions from certain bacteria due to the release of 2,4-Diacetylphloroglucinol antibiotic metabolite (Delany et al. 2000; Bangera and Thomashow 1999). *Pseudomonas* isolates are commonly used in research studies because of its natural abundance within environments, its successful history as an anti-fungal agent in agriculture, and detected presence within some wild bat wing microbiota (Cheng et al. 2016; Lemieux-Labonté et al. 2017; Hoyt et al. 2015).

Several studies have used bacteria from the *P. fluorescens* species complex for anti-*Pd* experiments, both in the lab and field (Cheng et al. 2016; Lemieux-Labonté et al., 2017; Hoyt et al. 2015; Hoyt et al. 2019). Bacteria within the *Pseudomonas* genus are characterized by their rod shape (0.5-1.0 µm diameter, 1.5-5.0 µm in length) and gram negative morphology. They are aerobic with an optimum growth temperature between 4-42^{o}C and are commonly found within soils (Bossis et al. 2000; Chakravarty and Gregory 2015). *P. fluorescens* is a species complex that was previously noted as an individual species but is instead composed of a wide range of ~52 diverse species that form their own phylogenetic group and comprise the same core proteome (Garrido-Sanz et al. 2017; Mulet et al. 2010; Nikolaidis et al. 2020). This classification was due to

the advantages of 16s rRNA genotyping in the late 20th century that has resulted in many bacteria to be re-classified from the *Pseudomonas* genus (Anzai et al. 2000). In this thesis, we use a probiotic cocktail of synergistic *P. fluorescens* bacteria species that display anti-*Pd* properties. All four species belong with the *P. fluorescens* species complex, *P. azotoformans* and *P. synxantha* strains A and B, and *P. antarctica* (Nikolaidis et al. 2020). *P. azotoformans* exhibits antifungal properties and have previously been used as a biocontrol agent against cucumber *Colletotrichum orbiculare* (Sang et al. 2014). It was isolated from an adult *C. townsendii* caught from a maternity roost in Deroche, British Columbia. *P. synxantha* is a bacteria species commonly found within the rhizosphere of plants and exhibits nematocidal and antifungal properties (Wechter et al. 2002)(Janakiev et al 2019). Strain A was isolated from a *M. yumanensis* at a mine hibernaculum near Salmo, British Columbia. Strain B was isolated from an adult female *E. fuscus* caught from a mine near Salmo, British Columbia. *P. antarctica* was first isolated by Gundlapalli et al. in 2004 in Wright Valley, Antarctica. It is a psychrophilic aerobic species that grows optimally between 4-30^{o}C. It is the most exotic species in our cocktail and was isolated from a female juvenile *M. evotis* at a mine hibernaculum near Nelway, British Columbia.

Very few studies have conducted field trials that implement anti-*Pd* bacteria into wild bat populations due to the inherit difficulty of treatment, especially in western bat species, and risks of upsetting the natural balance of skin microbiota (Cheng et al. 2016; Hoyt et al. 2019). Researchers have considered creative solutions such as raising bat hibernacula temperatures to reduce torpor and introducing UV lights to prevent *Pd* growth however these are largely dependent on knowledge of hibernacula (Boyles and Willis 2010; Palmer et al. 2018;). Studies have also considered and/or tested vaccines, antifungal drugs, chitosan, other fungi, propolis, orange oil and other volatile organic compounds to fight WNS (Boire et al. 2016; Chaturvedi et al. 2011; Cornelison et al. 2014a; Cornelison et al. 2014b; Ghosh et al. 2017; Hoyt et al. 2015; Kulhanek 2016; Lemieux-Labonté et al., 2017; Micalizzi and Smith 2020; Robbins et al. 2011; Rocke et al. 2019; Zhang et al. 2015).

There are currently no methods available to prevent the spread of WNS, especially in western bat species. There has been no official documentation of WNS in British Columbia (WNS 2019), therefore a proactive approach to preventing infection is still possible. Once infected, it would be difficult to treat bats in British Columbia due to hibernacula being largely unknown, likely remote, and all known hibernacula contain few bats and few species (Fletcher et al. 2020; Weller et al. 2018). Fletcher et al. (2020) recommends that treatment measures should be considered that do not require knowledge of bat hibernacula in western North America. This study proposes the first bioactive treatment of WNS for western bat species.

This project's goal was to test a newly developed anti-*Pd* prophylaxis (Forsythe et al. 2021) to prevent western bat mortality due to WNS. The first objective was to test the probiotic in a captive setting to confirm that a sustained changed in wing microbiota can be achieved. I hypothesized that if I introduce the probiotic bacteria onto bat wings, then probiotic bacterial species will be detected on their wings for some period of time following application. I wanted to determine how long the probiotic can be detected on captive and wild bat wings following inoculation. The second objective is to develop an application method that is safe, replicable, easy to do, and inexpensive for widespread use in the Pacific Northwest. If a universal application method was developed, then it could be transitioned into field testing and widespread use. Since probiotic cells cannot be inoculated directly, a carrier agent needs to be utilized, specifically one that does not harm bats and replicates a substance found in the wild. The third objective is to successfully apply probiotic onto wild bat roosts and monitor levels of detected probiotic on their wings. If I were to replicate the methodology from the captive trial into a field trial on wild bats, then there should be a detectable concentration of probiotic on their wings. This is especially important because widespread use is dependant on successful field trial results. Finally, the fourth objective involves describing and quantifying probiotic and *Pd* interactions on live bat skin. If I inoculate *Pd* onto wing tissue from the captive trials, then I should see some antagonistic interactions from the probiotic bacteria concentrations on their wings. This can be further examined using explant

chambers, in which wing tissue is inoculated with *Pd* and the probiotic bacteria at the same time and monitored for abundance using qPCR.

Here I present, in chronological order, the research that was conducted during the testing and field pilot implementation of the probiotic. The first chapter describes the captive trials, with the preliminary findings which facilitated field implementation; in the second chapter I present the field trial application of the prophylaxis; and in the third chapter I describe a pilot ex-vivo *Pd*-challenge experiment on severed bat wings that were inoculated with the probiotic.

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Chapter 2: Testing the Efficacy of a Topical Probiotic on Captive Bats to Prevent White-Nose Syndrome

INTRODUCTION

White-nose syndrome (WNS) is one of the deadliest wildlife diseases in recorded history and is causing devastating consequences for bat populations in North America (Frick et al. 2010). White-nose syndrome is caused by *Pseudogymnoascus destructans* (*Pd*) and has resulted in greater than 90% mortality rates in some bat roosts (Frick et al. 2015; Lorch et al. 2011). It was introduced to the east coast of North America in 2006 and has been spreading ever since (Frick et al. 2010, Lorch et al. 2016), including a giant leap of the fungus into Washington state (USFWS 2019), the only western state where WNS has been found to date.

Pseudogymnoascus destructans is a psychrophilic fungus that grows optimally between 12.5-15.8°C and 70-81.5% relative humidity and causes mortality of hibernating bats (Johnson et al. 2014; Marroquin et al. 2017; Verant et al. 2012; Warnecke et al. 2012). Hibernating bats have arousals periods for several physiological reasons, however, once infected with *Pd*, a bat will arouse much more than usual, experience higher rates of evaporative water loss (EWL), and blood electrolyte disruption (Jonasson and Willis 2012, McGuire et al. 2017, Reeder et al. 2012, Verant et al. 2014, Warnecke et al. 2013). These physiological imbalances will eventually kill the bat because of greater energy expenditure, thus burning fat stores built up from the fall season. Bats will arouse from hibernation due to WNS disturbance and attempts to groom the fungus off of their bodies (Reeder et al. 2012). Mechanically, the fungal hyphae will penetrate the connective tissue of the cutaneous sections of the bat and cause “cup-like” epidermal erosions on the wings, among small tears and patches of rough skin. The hyphae will noticeably invade hair follicles, sebaceous and apocrine glands and regional connective tissues; while producing a noticeable opaque white texture among the cutaneous sections of the muzzle, wings and tail regions (Meteyer et al. 2009).

If the bat has survived until spring, it will move to summer roosts where continued grooming, and a warm body temperature above that which *Pd* can survive, will reduce *Pd* spore loads to nearly zero for the duration of the reproductive period if they do not return to the winter roosts. The *Pd* fungus has been known to persist in caves because of the continuous lower temperature and high humidity (Hoyt et al. 2015a).

For the most vulnerable bat species, such as *Myotis septentrionalis* and *M. lucifugus*, once a winter colony has been exposed to *Pd* there is reduced chance of survival; however, some populations, especially of less vulnerable species, have shown less infection intensity after surviving WNS (Langwig et al. 2017). Hypotheses for why some bats survive WNS included gene selection pertaining to immune response and wound healing, skin microbiota, and behavioural adaptions (Frick et al. 2017; Hazim et al. 2018; Langwig et al. 2017; Lemieux-Labonté et al. (2017). However, cumulative mortality risk models suggest that even populations of less susceptible bat species may follow a stabilized negative growth rate after a major mortality event (Maslo et al. 2017), leading to a false sense of security in the fate of some bat species.

WNS along with other fungal diseases such as chytridiomycosis (*Batrachochytrium dendrobatidis*) and ophidiomycosis (*Ophidiomyces ophiodiicola*), which has caused rapid decline in amphibian and snake species, respectively, are threatening wildlife in unprecedented ways, and urgency surrounds the management of these diseases (Becker et al. 2015, Lorch et al. 2015). Reducing the mortality of bats from WNS could have important benefits for the ecosystem and economy. It is especially important to consider the economic benefits bats provide. They control some insect populations and reduce the use of pesticides in North American agriculture and forestry industries; without the ecosystem services provided by bats, it is estimated that the US could see an economic loss of 3.7-53 billion dollars a year (Boyles et al. 2011).

A vaccine showing promising results has recently been conducted *in vivo* and is awaiting field testing (Rocke et al. 2019). However, administering a vaccine can be difficult on free ranging or wild animals. Administering requires consistent human intervention as it would require vaccination of all individuals recruited into a population

each year, and depending on its efficacy, could require repeated vaccinations of the same individual. It can be expensive and could cause repeated disturbance to bats (Langwig et al. 2015).

The microflora present on an animal's skin plays a vital role in the health of the individual. Healthy bacteria have been proven to provide increased protection from pathogen colonization by increased processing of skin proteins and overall inhibition of invasive transient microorganisms (Clay 2014; Grice and Segre 2011). Probiotics offer advantages over chemical antifungal treatments, being safer and longer lasting (Thomas and Willis 1998, TUFTS University 2011). Traditionally, fungicides have been used to combat diseases in agriculture (e.g., Knight et al. 1997). Broad use of fungicides can be dangerous to native microbiomes and expensive (Thomas and Willis 1998, TUFTS University 2011). However, bacteria belonging to the *P. fluorescens* species complex have been increasingly considered for biocontrol of pests in agriculture because of its production of bioactive metabolites and rapid growth within the rhizosphere (Prasad et al. 2018). Other examples of probiotics assisting with fungal diseases include clearing chytridiomycosis infection through bacterial control (Becker et al. 2015); defending crustacean embryos with symbiotic marine bacteria (Gil-Turnes et al. 1989); and protecting wasp larvae from fungal infection (Kaltenpoth et al. 2004).

Anti-*Pd* bacteria have shown to be a promising choice for protecting bats from WNS mortality (Hamm et al. 2017, Hoyt et al. 2015b, Hoyt et al. 2019). Utilizing bacteria found on native bat species can prevent complications and assist with anti-*Pd* colonization into bat wing microbiomes (Bletz et al. 2013). Further, there is potential for probiotics to coevolve with the pathogen and provide long lasting solutions not seen in chemical fungicides (Thomas et al 1998).

Studies using microbials to inhibit *Pd* include *Trichoderma polysporum* fungi (Zhang et al. 2015), and *Rhodococcus rhodochrous* to inhibit *Pd* growth when it is induced with urea (Cornelison et al. 2014). Three other studies have tested the *Pseudomonas fluorescens* species complex for its anti-*Pd* activity (Cheng et al. 2017; Hoyt et al. 2015b; Hoyt et al. 2019). Utilizing naturally occurring *Pseudomonas* bacteria

to inhibit *Pd* as a biocontrol agent was first proposed by Hoyt et al. (2015b). Cheng et al. (2016) produced mixed results, in which bacteria from the *P. fluorescens* species complex were applied in large doses to bats which were hibernated in a laboratory setting. Hoyt et al. (2019), applied the same probiotic in a controlled field trial at a mine hibernaculum, reporting obvious survival benefits from application of the probiotic -- five-fold survival of the probiotic-treated group compared to the control group.

We have developed a prophylaxis composed of *Pseudomonas* bacteria naturally found on bats' wings in British Columbia, Canada. Candidate isolates were identified based on *Pd* inhibition, and combinations of isolates were previously tested for synergistic activity ex-vivo (Appendix A; unpublished data). Four isolates were selected for the final probiotic cocktail based on their mix of partial and full inhibition, and additive inhibition against *Pd*: *P. azotoformans*, *P. synxantha* strains A and B, and *P. antarctica*.

The premise of our WNS prophylaxis approach is that through summer application of probiotic on wings, bats arrive at winter hibernacula with enhanced wing microbiomes containing anti-*Pd* probiotic bacteria. This may be able to delay or prevent *Pd* growth for a critical portion of the winter.

Here, we test this probiotic cocktail in varying dosages, on captive free-flying bats. Our goals were to develop a passive method of application that could also be used in field applications; confirm that a sustained change in wing microbiome could be achieved, in which probiotic bacteria would become incorporated into the wing microbiome; compare temperature and probiotic cell counts on an empty bat box; and test the health of captive bats in 'treatment' and 'control' groups to ensure the probiotic caused no ill effects.

I describe two captive trials on captive *M. yumanensis* bats free-flying in large open air enclosures during summer months – one pilot trial in 2018 and a comprehensive trial in 2019. The latter trial expanded into fall and early winter to include a laboratory hibernation experiment of captive bats. The hibernation experiment

examined the response of our probiotic bacteria on hibernating bat in a controlled environmental chamber.

METHODS

Captive Bat Enclosures

Two large bat enclosures were built at the BC Wildlife Park (BCWP) in Kamloops, located away from the public and enclosed in an area only accessible by employees. Each of the two enclosures were covered by a large carport to provide shelter from sun and weather; however, bats were still exposed to environmental fluctuations including changes in temperature, humidity, and wind because the ends of the carport was left open and the enclosures' outer walls were constructed of wire mesh. A solid middle wall divided each enclosure in half, forming two flight chambers of equal area and design (each chamber 3.05m long x 1.37m wide x 3.05m high). The two enclosures were approximately 30 metres from each other. Some of the mesh walls were strategically covered with corrugated plastic board to reduce wind turbulence which could result in air exchange between sides, and to ensure that bat feeding and water dishes were not upset by strong gusts of wind. All human entry was through a vestibule on one end of each flight chamber, and these vestibules were on opposite ends of the chambers to further reduce the chance of contamination. Vestibules had a door on each side – one for entry/exit from the vestibule to the outdoors, and the other door which entered into the flight chamber. Vestibules' outside doors were hinged solid wood with spring hinges for automatic closure, and the inside doors were zippered vinyl to ensure bats could not escape from the chamber. A Tyvek suit, boot covers, shower caps, and disposable gloves were donned while standing in the vestibule so that all entry in and out of the chamber reduced chance of cross-contamination. Each day, the 'control' bats were tended to first and then the 'treatment' bats to reduce chance of accidental introducing probiotic to the control side. There was a large Rubbermaid storage bin outside each vestibule which held items specific to each enclosure group. Items that did move between sides were sterilized with 70% ethanol before reuse.

Each flight chamber had a single chamber bat house (with hinged front door to allow access to bats) mounted on the middle dividing wall for bats to roost in. On each end (opposite ends of each flight chamber), there was a watering dish, and a food station. For details on bat care see Appendix B.

Only one enclosure had been built at the time of the 2018 pilot captive trial, and one flight chamber was used for the Treatment bats and the other for the Control bats. In 2019, we used two enclosures, one enclosure housed two groups of Treatment bats (Treatment 1, Treatment 2), each in their own flight chamber (Figure 2.1). And the other enclosure housed the Control bats, all on one side in one flight chamber. The second flight chamber of this enclosure was not used. A HOBO microclimate data logger (Onset Corporation, SN 20340415 for treatment 1 and SN 20340414 for treatment 2) was installed into each treatment chamber to measure temperature and relative humidity. Each logger had two probes: one was placed ¾ of the way into the left side of the box and the other probe was attached to the wall of the same enclosure within a cut-out yogurt container to shield it.

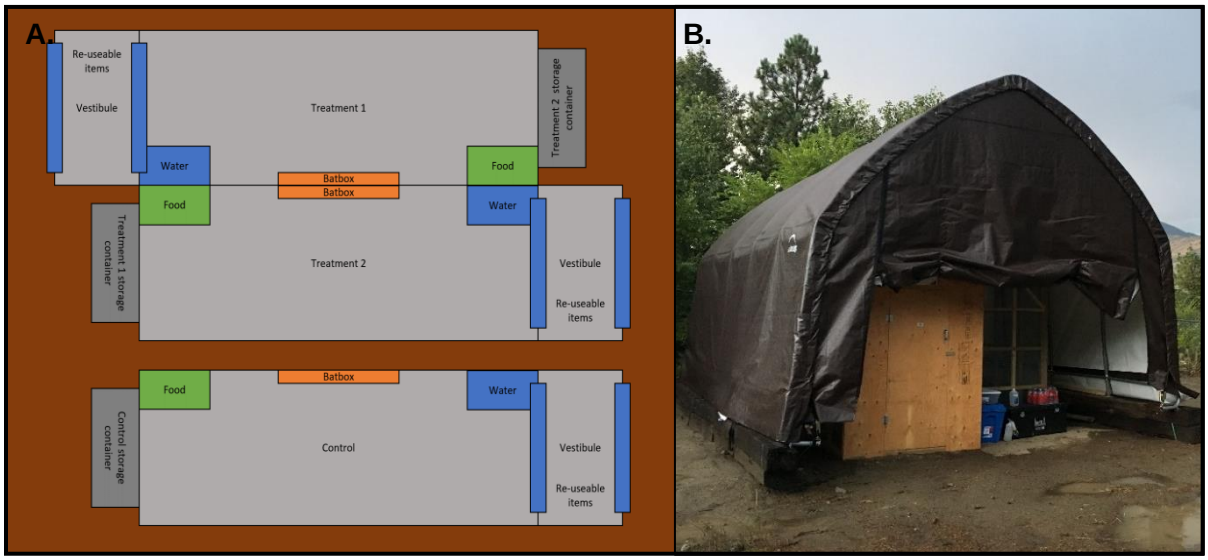

Figure 2.1. A. Floor plan of control and treatment enclosures. The control enclosure was approximately 30m away from the treatment enclosure. B. Outside view of treatment enclosure.

Animal Collection and Handling

M. yumanensis bats were captured from Vernon, Chase, Greater Vancouver, or Creston, BC, under the permit issued to C. Lausen, WCS Canada, 287882. Each bat used in the trial had a colored or numbered forearm band on its wing to differentiate them from one another. They were taught to self-feed on mealworms and self-drink out of a water dishes. In 2018, 11 adult *M. yumanensis* were taken captive (10 males, 1 female), and in 2019, 20 adult *M. yumanensis* were taken captive (19 males, 1 females).

Probiotic Composition

In 2018, two synergistic strains, which were *Pseudomonas* strains that exhibited full inhibition of *Pd* growth *in vitro* were used in our pilot probiotic. Both isolates were sourced from a *Eptesicus fuscus* female adult bat from a mine hibernaculum near Salmo, BC. One of these strains were identified as *P. synxantha* strain B and was used in our 2019 experiment probiotic. Full inhibition isolates are characterized by their ability to fully inhibit *Pd*, which is contrast to a partial inhibitor, that only suppresses hyphal growth.

In 2019, four synergistic *Pseudomonas* strains were included into a final probiotic: *Pseudomonas synxantha* strains A (full inhibitor) and B (full inhibitor)*, P. azotoformans* (full inhibitor)*,* and *P. antarctica* (partial inhibitor). Each isolate was previously sourced from bats in BC. *Pseudomonas synxantha* strain A was isolated from a *M. yumanensis* at a mine hibernaculum near Salmo, British Columbia; strain B was isolated from an adult female *E. fuscus* captured free-flying at a mine near Salmo, BC (as told above); *P. azotoformans* was isolated from an adult *Corynorhinus townsendii* caught from a maternity roost in Deroche, British Columbia; and *P. antarctica* was sourced from a female juvenile *M. evotis* at from a mine hibernaculum near Nelway, BC.

Probiotic strains were made in large quantities and pelleted and lyophilized using a benchtop freeze-dryer (Labconco). All water was extracted from cell pellets after 3

hours at -50°C. Viability and inhibition of probiotic cocktail was confirmed after the freeze-drying process. Dry pellets were ground into a fine powder using a sterile pestle.

In 2018, probiotic was applied directly to bats' wings in sterile water using a pipette and in 2019, probiotic was instead applied by freeze drying bacteria and combining it with sterile potter's clay. Two hundred and fifty million Colony Forming Units (CFU) of each bacterium were used as a cumulative dose of about 1 billion cells for each dosage. Each dose was applied with sterile water onto each bat house to reactivate the freeze-dried bacteria.

Development of Probiotic Application

An application method for the four probiotic bacteria underwent different revisions throughout the 2019 trial. The first method incorporated a combination of our bacteria directly with water before application. 1 billion lyophilized cells were combined with 275mL of sterile water and sprayed onto the Treatment 1 bat house on April 26th. Bats were removed from their bat house and placed into sterile cotton bags before removing their bat house from the treatment chamber. The bat house was put flat onto the ground and the door was opened to expose the roosting chamber. A hand pump sprayer was used to apply the probiotic-water solution evenly onto the bat house chamber. Afterwards, the bat house sat overnight until it was dry and re-established within their treatment enclosure. A different sterile bat house was used as a substitute.

Water and clay were added separately for the second application method and were used in a two-step process. Bats were removed from their bat box and placed into cloth bags during the inoculation. The bat box was removed and sprayed with a minimal amount of sterile water evenly over the entire surface. The probiotic was prepared in sterile potter's clay and spread evenly over the bat box within the enclosure. After ten minutes the bat box was placed into the same location previously and bats were placed back into the box. Bacteria amounts were consistent for repeated doses, however different amounts of clay and water were utilized for our Treatment 1 bat house in order to establish an ideal consistency for repeated applications: 125mL water and 10g of clay

on May 12th and 24th, 50mL water and 30g clay on June 24th, and 29th. The Treatment 2 bat house received the same later dosage on June 15. Finally, two dosages of 1 billion cells were applied to our treatment 2 captive bat boxes on September 30th and October 7th before acclimation began on November 3rd for the hibernation experiment. The method of applying water and then powered clay became the final application method used, although there was further work done on how to create a sprayer to put clay powder up into bat box chambers in the field where chambers are vertical and cannot be accessed via a door as they were in the single chamber bat boxes used in the enclosures (see Chapter 3 methods).

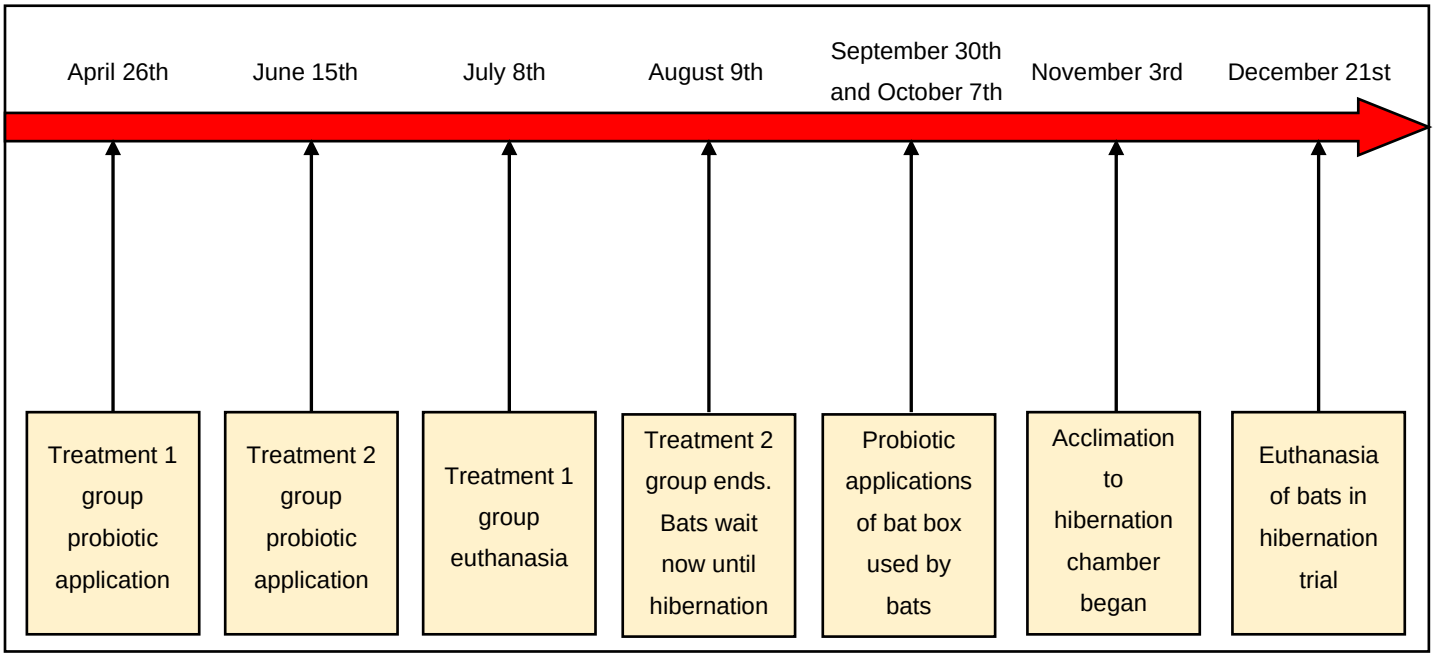

Figure 2.2. Timeline of probiotic application and euthanasia of Treatment 1, 2 and Hibernation groups. **Bats remained in outdoor enclosure throughout September and October.*

Four-chambered Bat Box

In 2019, a four-chambered bat box (Chamber 1 directly above landing platform; Chamber 4 was furthest from the landing platform) was treated with probiotic-laden clay, just as were the single-chamber bat boxes within each enclosure's flight chamber. However, this box was mounted on a pole in direct sunlight, as would a typical field-

based bat box for wild bats. The bat box was hung 3m off the ground and faced the sunset (west) starting May 17th, 2019. A HOBO microclimate datalogger (Onset Corporation, SN 20577875 for chamber 1, SN 20577881 for chamber 2, SN 20577876 for chamber 3, and SN 20569683 for chamber 4) was placed into each chamber of the bat house to measure temperature and relative humidity (chamber 4's sensor recorded temperature only). This box's entrance was screened off to prevent it from being used by wild bats in the area; probiotic concentrations and internal microclimate were measured throughout the summer.

The loggers were strategically placed for different experiments: initially at a depth of 15.25cm from May 17th-July 14th for the first experiment, and 30.5cm into the box from July 14th until the end of the trial for the second experiment. Each chamber was inoculated with a 'dose' of probiotic clay powder and a clay paste on May 23rd, both of which were 15.25cm deep into the bat box (Figure 2.3). On July 20th a second powder treatment was inoculated deeper within each bat box chamber at 30.5cm. Each treatment was separated and swabbed individually by chamber each week until the end of August.

Figure 2.3. A. Four-chamber bat box hanging off the ground and away from our captive bat enclosures. B. Clay paste application (left), powder application (right) within the four-chamber bat box.

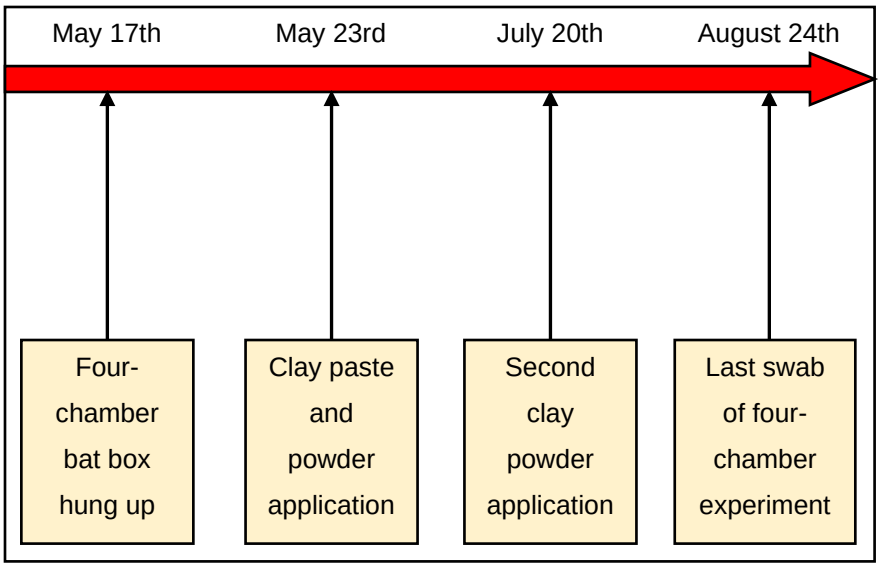

Figure 2.4. Timeline of four-chamber bat box experiment.

Sampling of Microbes from Wings and Roost Substrates

Treatment 1 group of captive bats were swabbed with sterile polyester tips from April 28th until July 8th. The second treatment group was swabbed from June 15th until August 24th. Finally, the hibernation trial bats were swabbed from November 16th to December 21st. Swabs were performed once a week to prevent bat stress and interference with treated bats microflora. Each bat was individually swabbed by rolling the polyester tip evenly onto the bat's wings.

Swabbing protocol evolved as the experiment proceeded and this meant that Treatment 1 bats were swabbed differently throughout the trial. Wing area was mapped by measuring left and right arm length (see Appendix C). On May 26th and June 1st, the ventral and dorsal side were swabbed together. One swab constituted both wings, wing tip to armpit along the wing membrane, on one side of the bat (ventral or dorsal). One swab for both wings, wingtip to armpit on the dorsal side, were used permanently from June 8th until the end of the trial.

Bats from the hibernation trial were removed from the cooler every 7-10 days to offer water and food, if needed, and to perform swab sampling. Bats were warmed with

heat pads for approximately 5 minutes before food and water were offered. A polyester swab was rolled along the top portion of one wing from tip to armpit. Left and right wings were alternated when swabbing to prevent accidental rubbing off of probiotic from previous swabbing. We swabbed along the myotis finger bones. Each week a different fingerbone was used. polyester swab tips were broken off into Eppendorf tubes containing 1mL of sterile water and were immediately transported to the lab.

Processing of Swab Samples

Using sterile polyester-tipped swabs, bats and roost substrates were periodically sampled to quantify presence of each probiotic bacterial strain using qPCR. Custom probes were designed specific to each bacterial strain. Upon swabbing of bat wings or roosting substrates, swabs were immediately placed into Eppendorf tubes, refrigerated, and shipped for qPCR analysis at McMaster University. In 2018, the wing area for each bat was measured using photography and subsequent calculation of area (SketchAndCalc 2021). Subsequent analyses showed no significant difference among wing areas, and thus for 2019, mean swabbing areas were used to calibrate all cell concentration for wings depending on swab location (/cm^2; see Appendix C for measurements and dates used).

An additional test of viability on our samples was introduced on June 8th until the end of the trial. This test required the swab sample to be split: each Eppendorf vial containing a swab was vortexed on medium-high for 20 seconds and the tips were removed from the water inside the tube. The remaining 1mL was divided into a separate Eppendorf containing 1mL of LB broth, and the remaining water was frozen. The LB broth containing half our sampled cells were placed onto a shaker at 25^{o}C on medium for 12 hours. Afterwards the broth was frozen and transported to McMaster University alongside our original frozen water samples for qPCR analysis. The surface area was measured of each wing section to account for differences in inoculated area.

Collaborative researchers at McMaster University used the following protocol for qPCR analysis. Species-specific gyrB primers (modified from Yamamoto et al. 2000)

were used to quantify cell concentrations in each swab compared to background levels. Swabs tips were washed with PBS in sterile tubes and vortexed. This suspension was then sonicated for 30 seconds in a sterile glass vial. Standards were created through serial dilution of cells (10^5, 10^4, 10^3, and 10^2) which were prepared identically to wing swab samples. All reactions were carried out in triplicate containing: 10µl of 2X NEB Luna qPCR (SYBR green dye), 0.5µL of each primer at 10µM, and 2µL of sonicated cells. Reactions were run on a BioRad CFX96 machine (Mississauga, Ontario). Initial denaturation was performed at 95°C for 2 minutes, followed by 40 cycles of additional denaturation at 95°C for 30 seconds and annealing/extension for 30 seconds at 60°C. Using CFX Manager software (V3.0) a standard curve was created using the Cycle Threshold (Cq) values from samples of known concentration of cells. Fitting the Cq values from samples of unknown concentration allow for us to extrapolate the number of cells present in each swab. These swabs were corrected for the average wing area. Standard error was calculated for all means. See appendix G for sample sizes and details on mean and error calculations. Unfortunately, due to unreliability with the *P. antarctica* probe, only some of the samples managed to be salvaged and presented appropriately.

Hibernation Trial

Three *M. yumanensis* bats from our previous captive trial were acclimated into the hibernation chamber gradually over 5 days, followed by another decrease of 1ºC 14 days later (see Appendix D for environmental chamber conditions). A modified glass-door wine fridge of approximate dimensions was used as a hibernation chamber: width of 59.4cm, height of 85.6cm and depth of 62.5cm (Figure 2.5). The fridge was maintained at a temperature of 4.5-8ºC throughout the trial, using a digital thermostat controller which was accessible on the outside of the fridge. Internal humidity was measured and controlled to range between 90-100% humidity by using a terrarium humidifier (Zoo Med Reptile Fogger) ; humidity control was automated using a humidity controller (Inkbird) to replicate *M. yumanensis* hibernating environmental conditions. A reptile humidifier was used to achieve high humidity and an electronic humidity sensor

powered the humidifier when humidity dropped below 90%. Temperature and humidity were monitored using a HOBO microclimate monitor (SN 20577876) and conditions were modified upon observation.

Mesh screen lined the fridge to allow bats to move around in the chamber. A cloth roosting pouch, a tin food dish, and a small water dish (with marbles and sponge to prevent drowning) were placed into the hibernation chamber. A wide-angle infrared camera (Model number: GXLFPW7WE58LHJS2111A) was placed into the chamber which enabled monitoring of the bats without disturbance. Video footage was automated and could be accessed via a smartphone app (iCookyCam). The glass door of the fridge was covered with a thick blanket to keep out light, and the room housing the fridge was kept dark. An oxygen tank was connected by an external hose which reached into the chamber; oxygen was monitored using an oxygen sensor and air levels were manually maintained between 19-24% oxygen using an external air tank. An oxygen monitor was installed inside the chamber. Once every ~10 days the door of the fridge was opened, bats were weighed, warmed, swab-sampled and then offered food (mealworms) and water.

Figure 2.5. A modified beverage cooler was converted into an environmental chamber for the bat hibernation experiment. The temperature and humidity were controlled by a thermostat and reptile humidifier, respectively. Humidity was monitored with a sensor to

retain >90% relative humidity. A wireless camera was installed to view the bats and record activity. The roost pouch and food were removed shortly after acclimation.

Necropsies and Histology

All captive bats were ultimately euthanized (see figure 2.2 for dates). Necropsies and wing tissue histology (n = 9 bats in 2018; n = 13 bats in 2019) were conducted by Dr. Glenna McGregor (BC Animal Health Lab, Abbotsford, BC examined skin of wings, ears, nose and tail looked for the following potential pathologies: acanthosis, bacterial epidermal, bacterial invasion, dermal inflammation, epidermal inflammation, hemorrhage, hyperkeratosis, hyphae, mites, monocytes, overall inflammation, rabies, segs (mature neutrophiles), ulceration and yeast).

RESULTS

Swab Sample Results - 2018 Pilot

From wing samples taken at the start of the 2018 pilot captive trial, we detected large quantities of the probiotic strains on bats from the treatment side of the enclosure (Figure 2.6B). The probiotic abundance on samples taken from the control side of the enclosure was low at first, but increased over time, increasing to levels similar to that of treated bats (Figure 2.6A), providing conclusive evidence of cross-contamination, likely through probiotic travelling in the air between sides of the enclosure during spraying of probiotic on treatment bats. As such, the wing concentrations from the 2018 captive bat trial were not used in further analyses, and are presented here (Figure 2.6) only to provide context for the necropsies and histology examination. Refinement of methods from the 2018 trial informed the 2019 captive trial, including the building of a separate enclosure 30 metres away for the Control bats to eliminate the problem of cross-contamination.

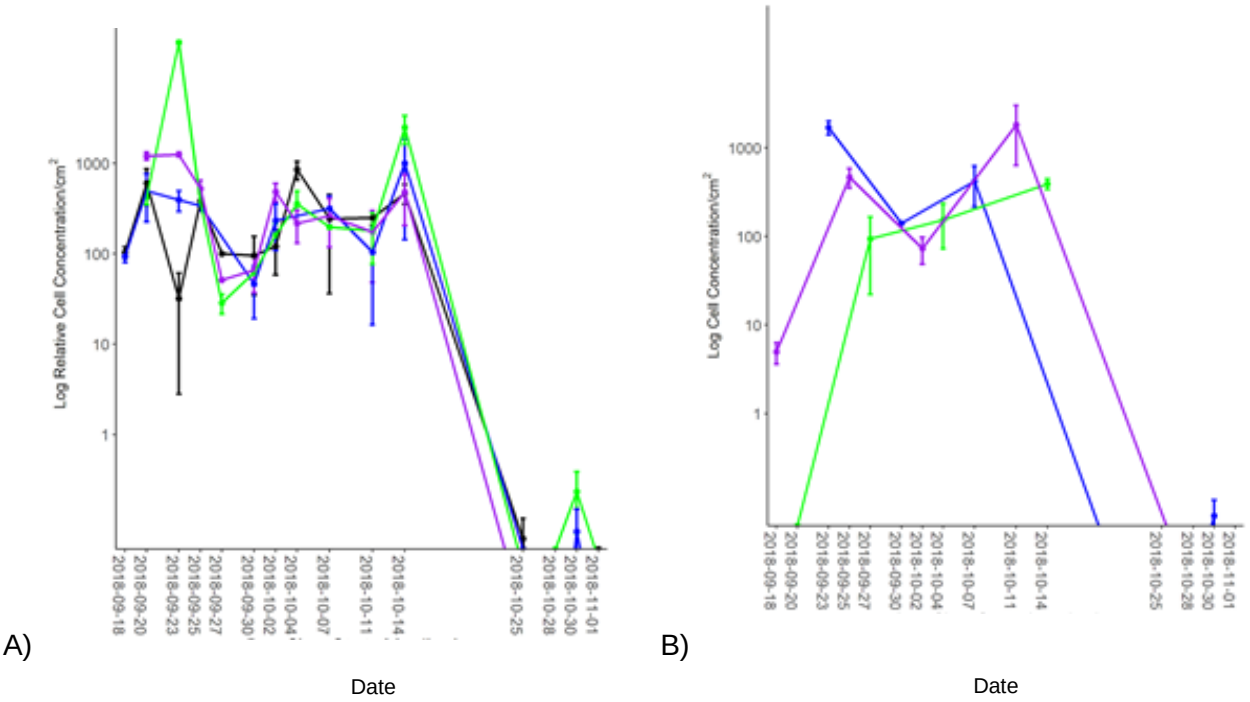

Figure 2.6. A) The number of probiotic cells detected on treated bats was determined using qPCR of wing swabs, relative to pre-exposure concentrations, corrected by wing area. B) The number of probiotic cells detected on control bats using qPCR of wing swabs, corrected by wing area. Each colour is one individual captive bat. Figures prepared by Adrian Forsythe.

2019 Captive Summer Trial

Baseline and control swabs

Concentrations of each probiotic bacteria were quantified on every bat when they were first introduced into captivity on April 21st (Figure 2.7). Background levels of all three probiotic bacteria were detectable from their wings and averaged to find a mean. There were 4.99 ± 1.21 cells/cm^2 (31 replicates) of *P. synxantha* B, 88.4 ± 19.9 cells/cm^2 (n=30 replicates) of *P. synxantha* A, and 1.62 ± 0.37 cells/cm^2 (n=21 replicates) of *P. azotoformans*. Probiotic bacteria was found on the control bat box throughout the captive trial however it was very low relative to the treatment bat boxes (Figure 2.8, 2.10 and 2.12).

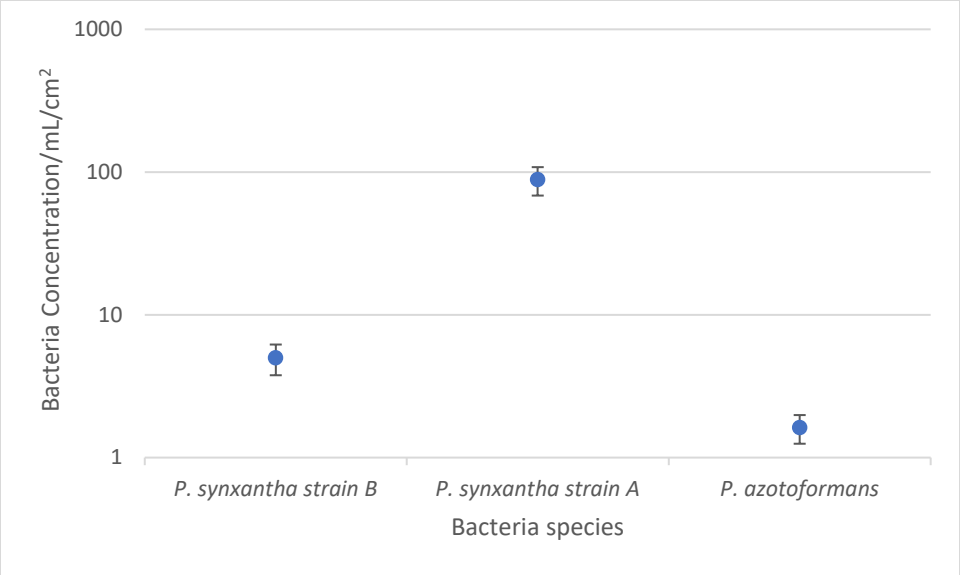

Figure 2.7. Number of probiotic cells present on captive bats on April 21st, before probiotic application began. Note the logarithmic scale.

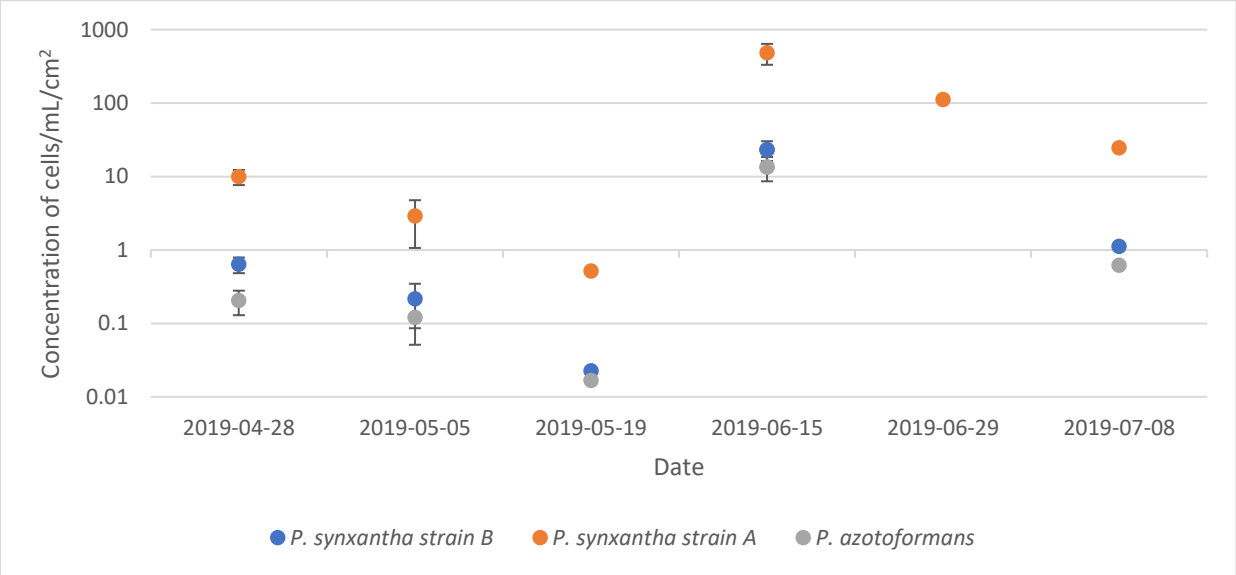

Figure 2.8. Number of probiotic cells present on the control bat box throughout the trial. Note the logarithmic scale.

Broth Results

The broth results were dropped from our analysis because of too many confounding variables and randomness of the data. Factors that lowered the reliability of our results included: competing microbes growing in the broth, only incubating the samples for 12 hours, and inability to reliably split a suspension 50/50 in terms of cell count. Results for the broth test are found in Appendix E.

Treatment 1

Concentrations were measured on bat wings from May 26th until June 29th. Concentrations of each bacteria followed a similar trend and *P. synxantha* A was significantly higher throughout the trial than *P. azotoformans* and *P. synxantha* B (Figure 2.9) per cm^2 of bat wings. *P. synxantha* A ranged between 5.95 ± 3.77 x10^2 to ± 4.88 ± 2.57 x10^5 cells/cm^2 throughout the trial and was consistently at least ~15 times higher in concentration if we do not consider the similar concentrations of *P. synxantha* A and *P. synxantha* B on June 1st. Concentrations of the other 3 bacteria ranged from 19.1 ± 12.4 cells/cm^2 (*P. azotoformans* strain B on June 1st) to 1.74 ± .904x10^4 cells/cm^2 (*P. synxantha* B on May 26th).

Bat box concentrations (figure 2.10) show a similar pattern seen in the bat swabs, in which *P. synxantha* A was much higher than the other two probiotic bacteria in quantity. *P. synxantha* A cell concentration ranged between 1.95 ± ,144 x10^4 cells/cm^2 to 3.95 ± .197 x10^4 cells/cm^2 throughout the trial period. The other three bacteria ranged from 563 ± 32.5 cells/cm^2 (*P. azotoformans* on June 1st) to 2.08 ± .12 x10^3 cells/cm^2 (*P. synxantha* B on June 8th). The concentration of *P. synxantha* A was consistently at least ~18.5 times higher than the other two bacteria concentrations on each swabbing date.

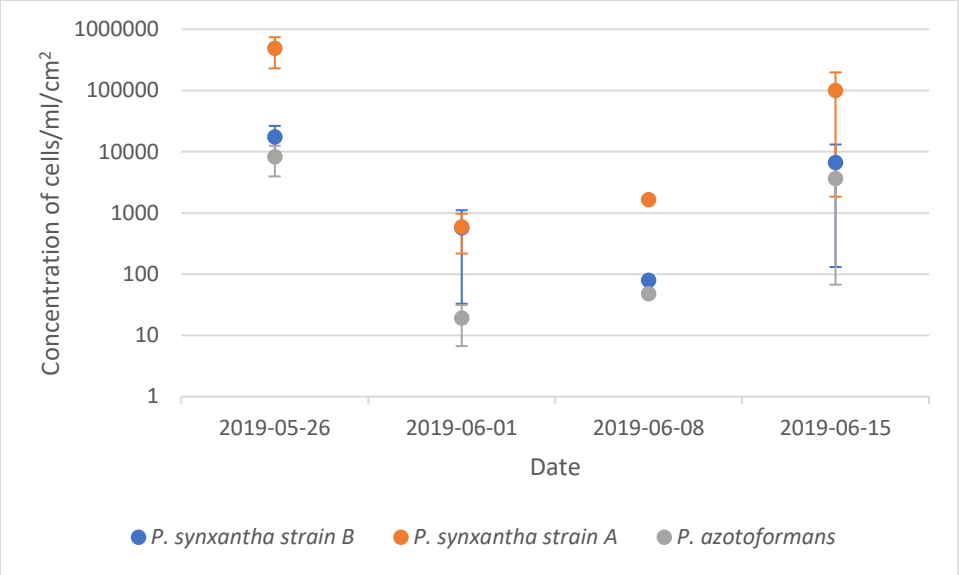

Figure 2.9. Starting quantities of probiotic cells detected on treated bats in the Treatment 1 group using qPCR for each probiotic bacteria species. Concentrations are corrected by wing area to represent cm^2. Note the logarithmic scale.

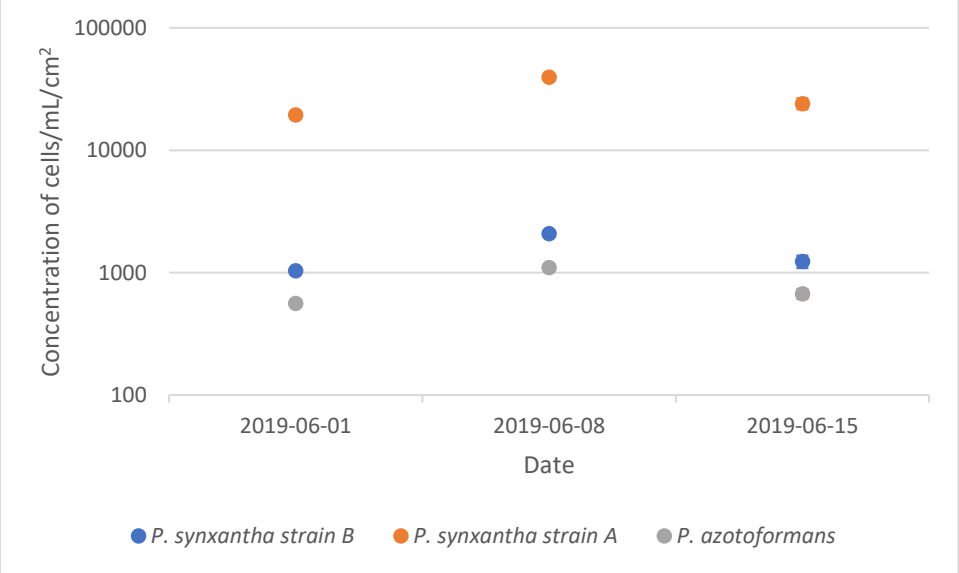

Figure 2.10. Concentrations of probiotic cells detected within the bat box in the Treatment 1 group using qPCR for each probiotic bacteria species. Concentrations are corrected by wing area to represent cm^2. Note the logarithmic scale.

Enclosure and bat box temperatures were compared between Treatment 1 and Treatment 2 groups for any significant differences. The temperature of each enclosure and bat box within treatment group 1 and 2 were measured from May 12th until August 7th (n=41,601 for each location and treatment group). There were no significant differences between enclosure temperatures (19.1 ± 0.03 ^{o}C treatment 1; 19.00 ± 0.03 ^{o}C treatment 2; P>0.05), however, bat box temperatures were significantly different (19.6 ± 0.03 ^{o}C treatment 1; 19.4 ± 0.03 ^{o}C treatment 2; P<0.05). The significant difference is very small with a mean difference (T1-T2) of 0.190 ± .001 ^{o}C.

Treatment 2

Concentrations were calculated based on area of the skin in cm^2 and a mean was found between all bat wing swabs. Bat swabs taken from the Treatment 2 group have a detectable concentration of each species throughout the trial from June 29th until August 14th (Figures 2.11). *P. synxantha* A was overall the most abundant among each probiotic species, however relative concentrations between the probiotic species are mixed. *P. synxantha* A had a range of 13.6 ± 8.63 cells/cm^2 to 667 ± 0 cells/cm^2 and was only significantly higher than the other 3 bacteria on July 13th and August 9th (at least ~19 times higher). The other 3 bacteria ranged between 12.7 ± 0.86 cells/cm^2 (*P. antarctica* on August 9th) and 1.36 ± 1.35 x10^3 cells/cm^2 (*P. azotoformans* on August 14th)

Treatment 2 bat box swabs showed an abundant probiotic concentration throughout the trial since application on June 13th (Figure 2.12). *P. synxantha* A ranged between 1.68 ± .81 x10^4 cells/cm^2 to 3.99 ± 2.07 x10^4 cells/cm^2 and was consistently at least ~18 times higher than the other bacteria species. Each of the other three probiotic species ranged between 91.2 ± 42.5 cells/cm^2 (*P. antarctica* on August 18th) and 2.17 ± 1.14 x10^3 (*P. synxantha* B on July 27th) throughout the swabbing period.

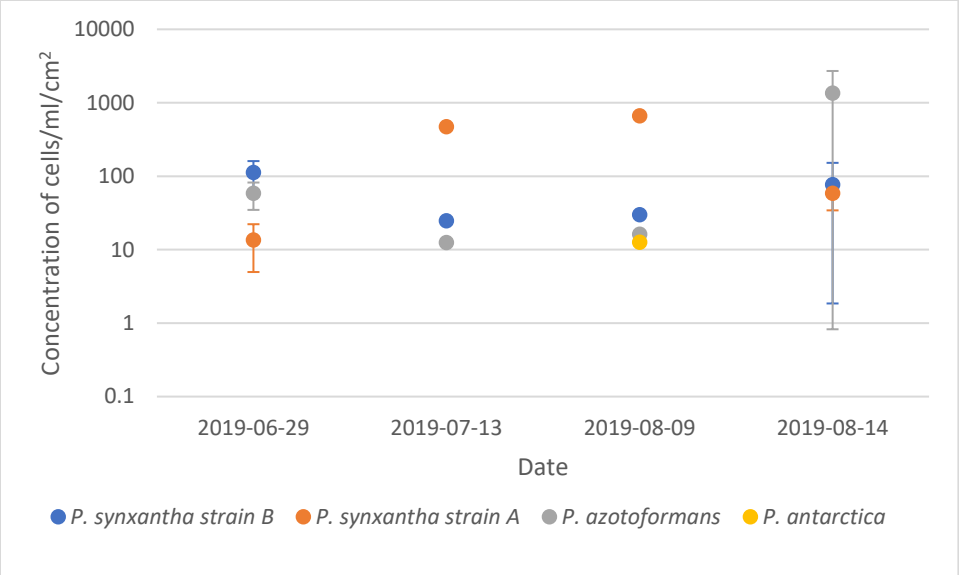

Figure 2.11. Starting quantities of probiotic cells detected on treated bats within the Treatment 2 group using qPCR for each probiotic bacteria species that was frozen in H_2O. Note the logarithmic scales.

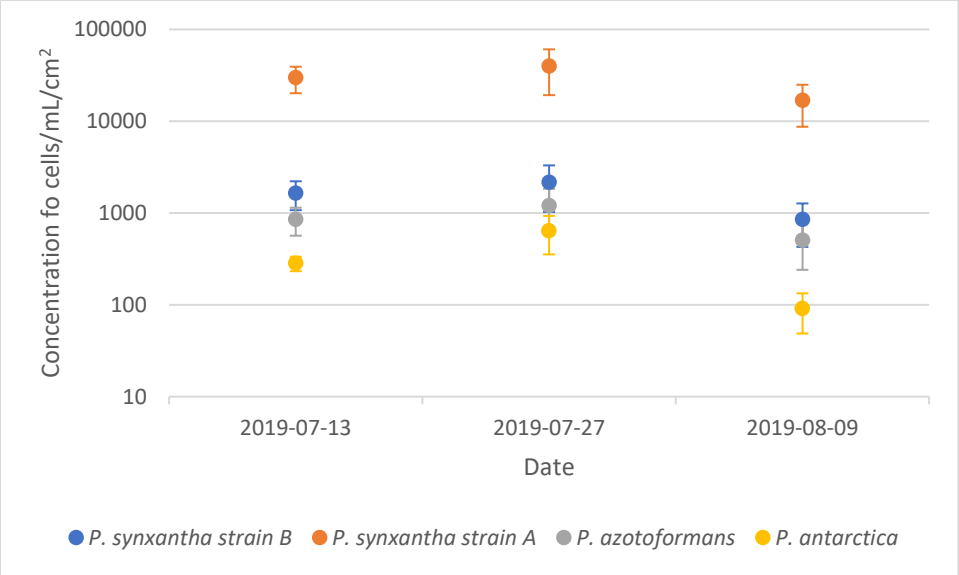

Figure 2.12. Starting quantities of probiotic cells detected on the bat box within the Treatment 2 group using qPCR for each probiotic bacteria species that was frozen in H_2O. Note the logarithmic scales.

Four-chamber Bat box

The probiotic bacteria were still detectable using qPCR when exposed to summer heat within the four-chamber bat box three months after original application, from July 20th until August 24th (Figure 2.13). The results from the paste application were excluded due to small amounts of detectable bacteria found immediately after application. All chambers were combined to form a mean of detectable bacteria found in the four-chamber bat box across all chambers for each date swabbed. This allowed us to look for general trends across all samples of our data.

P. synxantha A was significantly more abundant than *P. synxantha* B, *P. antarctica*, and *P. azotorformans*, which all had similar quantities throughout the trial. Results are similar to the bat box results for treatment 1 and treatment 2. In which *P. synxantha* A is approximately 18.5 times higher than other individual probiotic bacteria species across all sampling dates except for June 9th, in which it is only 4.4 times higher. *P. synxantha* A concentration ranged from 9.73 ± 1.88 x10^{3} to 1.58 ± .860 x10^{5} cells/cm^{2} throughout the trial, whereas the other 3 bacteria ranged from 23.9 ± 7.26 cells/cm^{2} (*P. antarctica* on August 24th) to 1.49 ± 1.14 x10^{4} (*P. antarctica* on August 9th).

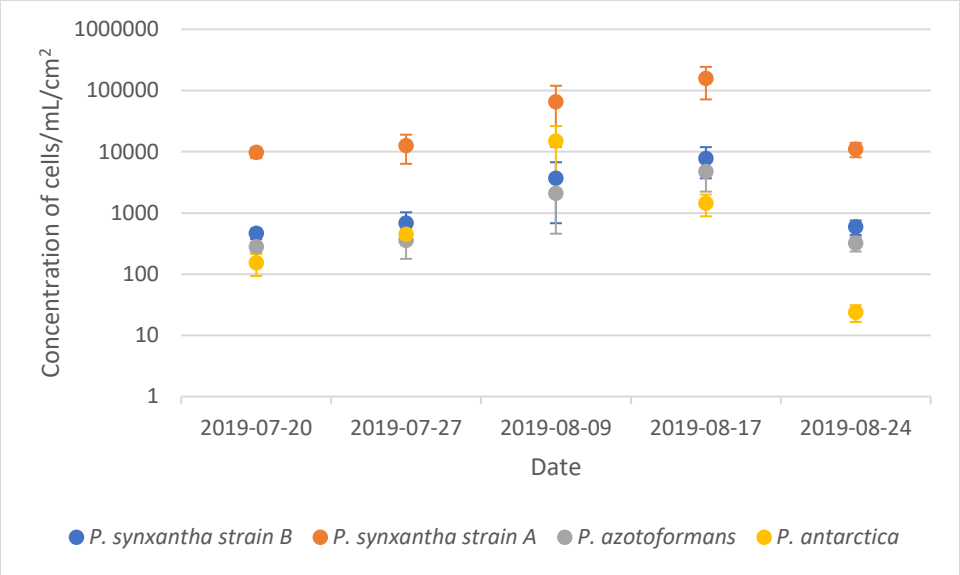

Figure 2.13. Concentration of probiotic cells detected in combined chambers of the four-chamber bat box using qPCR for each probiotic bacteria species frozen in H_2O. Note the logarithmic scales.

Daily temperatures within each chamber averaged between 15.78ºC-33.75ºC across the entire monitoring period (Figure 2.14). Chamber 4 (front chamber) exhibited the highest average daily temperature from the end of July and into August, however none of the chambers average values were significantly different throughout the trial period (P=0.456; Table 2.1). Chamber 1 had a statistically significantly higher maximum daily temperature when compared to chambers 2, 3 and 4 (P<0.001) (Figure 2.15) throughout the trial period. The maximum temperatures seen in chambers 1-4 were 57.4ºC, 49.7ºC, 49.5ºC, and 51.8ºC, and the minimum daily temperatures were 5.5ºC, 6.3ºC, 6.5ºC, and 6.4ºC, respectively. Chamber 1 had a significantly higher maximum temperature than other chambers (P<0.05; Table 2.2). We saw no significant differences in average daily relative humidity or minimum temperatures among chambers (P>0.5) (results not presented).

Individual chambers differed significantly in probiotic concentrations (table 2.3). Chamber 1 was the highest, followed by chamber 2, 3 and then 4 (P<0.05). When

bacteria concentrations are compared, *P. synxantha A* was the only significant probiotic bacteria (P<0.05), whereas *P. azotoformans, P. synxantha* B, *P. antarctica* are not significantly different from each other (P<0.05).

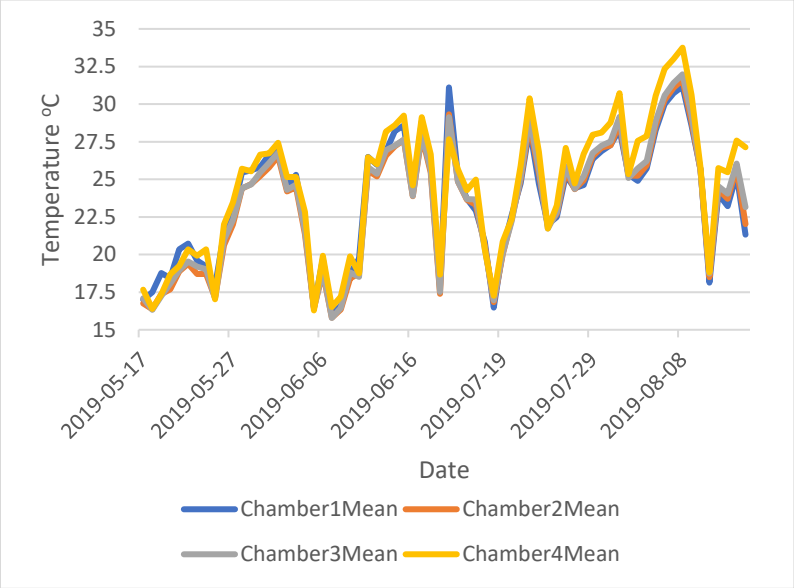

Figure 2.14. Average daily temperature for each chamber of the four-chamber bat box between 2019-05-17 and 2019-08-15.

Table 2.1. One Way Analysis Of Variance and Post Hoc test between each chamber of the four-chamber bat box comparing average daily temperature. Means that do not share a letter are signficantly different.

Analysis of Variance for Average Daily Temperature

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	46.41	15.47	0.87	0.456
Error	268	4756.41	17.75		
Total	271	4802.81			

Factor	N	Mean	StDev	95% CI
Chamber1Mean	68	23.638	4.01	(22.633, 24.644)
Chamber2Mean	68	23.345	4.138	(22.339, 24.351)
Chamber3Mean	68	23.525	4.185	(22.519, 24.531)
Chamber4Mean	68	24.426	4.503	(23.420, 24.431)

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Chamber4Mean	68	24.426	A
Chamber1Mean	68	23.638	A
Chamber3Mean	68	23.525	A
Chamber2Mean	68	23.345	A

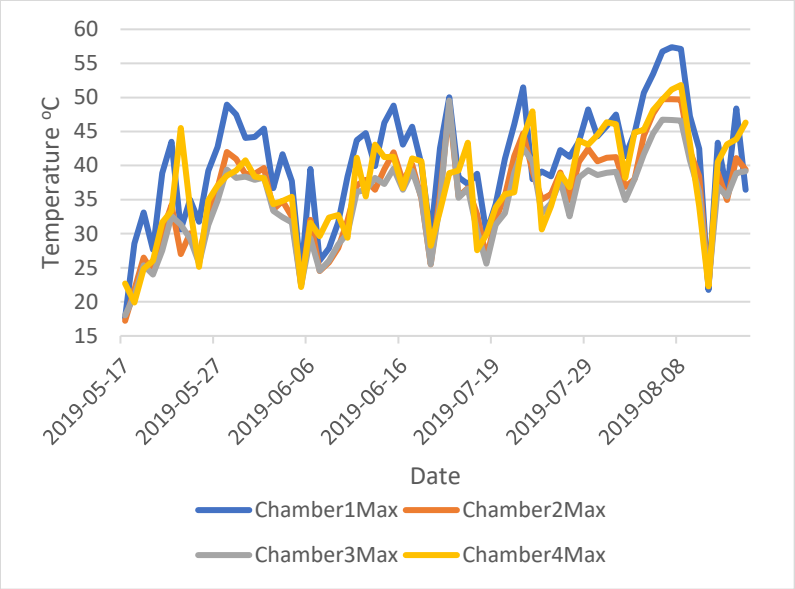

Figure 2.15. Average daily maximum temperature for each chamber of the four-chamber bat box between 2019-05-17 and 2019-08-15.

Table 2.2. One Way Analysis Of Variance and Post Hoc test between each chamber of the four-chamber bat box comparing daily maximum temperatures. Means that do not share a letter indicate statistically signifcant differences.

Analysis of Variance for Maximum Daily Temperature

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	1349	449.71	8.28	0.000
Error	268	14557	54.32		
Total	271	15906			

Factor	N	Mean	StDev	95% CI
Chamber1Mean	68	23.638	4.01	(22.633, 24.644)
Chamber2Mean	68	23.345	4.138	(22.339, 24.351)
Chamber3Mean	68	23.525	4.185	(22.519, 24.531)
Chamber4Mean	68	24.426	4.503	(23.420, 25.431)

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping	
Chamber1Mean	68	24.426	A	
Chamber4Mean	68	23.638		B
Chamber2Mean	68	23.525		B
Chamber3Mean	68	23.345		B

Table 2.3. General Linear Model for details of frozen water samples on chamber, bacteria species, and interactions between the two. Means that do not share a letter indicate statistically signifcant differences.

Analysis of Variance for Frozen Water Samples

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chamber	3	28.723	9.5743	15.06	0.000
Bacteria Species	3	79.773	26.591	41.84	0.000
Chamber*Bacteria Species	9	1.142	0.1269	0.2	0.994
Error	171	108.683	0.6356		
Total	186	218.205			

Tukey Method and 95% Confidence

Chamber	N	Mean	Grouping
1	47	4.23486	A
2	46	3.64363	B
3	47	3.40292	B, C
4	47	3.18837	C

Bacteria Species	N	Mean	Grouping
P. synxantha A	48	4.72339	A
P. synxantha B	48	3.44183	B
P. azotoformans	48	3.18439	B
P. antarctica	43	3.12017	B

Bat Box Probiotic Comparison

P. synxantha A has a higher ratio of cell abundance compared to other probiotic bacteria found within each bat box (Table 2.4). When the ratio of *P. synxantha* to the average value of other probiotic bacteria is tested, an increase in concentration of *P. synxantha* is seen over time. Treatment 1 bat box *P. synxantha* A concentration increased by 4% from June 6th to June 15th, Treatment 2 bat box increased by 9.1%

from July 13th to August 9th, and the four-chamber bat box increased by 8.3% from July 20th to August 24th.

Table 2.4. Ratio of *P. synxantha* strain A to all other probiotic bacteria found within the bat box, from first swabbing date to last. A higher ratio signifies greater abundance of *P. synxantha* A to other probiotic bacteria.

Treatment 1 Bat Box	(*P. synxantha* strain A)/((*P. synxantha* B + *P. azotoformans*/2))
2019-06-01 2019-06-15	24.3 25.3
Treatment 2 Bat Box	(*P. synxantha* strain A)/((P*. synxantha* B + *P. azotoformans*+*P. antacrtica*/3))
2019-07-13 2019-08-09	32.0 34.9
Four-chamber Bat Box	(*P. synxantha* strain A)/((*P. synxantha* B + *P. azotoformans*+*P. antacrtica*/3))
2019-07-20 2019-08-24	32.6 35.3

Hibernation Captive Trial

Primers specific to each of the probiotic species were used to quantify the abundance of probiotic present on wing tissue throughout the hibernation trial. Bats were not exposed to probiotic bacteria since the application dates of September 30th and October 7th. Results suggest that each of the probiotic species stabilized in concentration throughout the testing period from November 16th until December 21st (Figure 2.16). However, *P. synxantha* largely dominated with a higher concentration and was ~18 times higher than the other individual bacteria concentrations except for the last swab on December 21st. Detectable concentrations of each bacteria significantly dipped in concentration on December 18th but returned to previous levels on December 21st, in which *P. antarctica* reached 6.95 ± 3.9 x10^9 cells/cm^2 and trumped *P. synxantha* A by 141,919-fold. Each bacteria species significantly increased in concentration from their original starting quantities from November 16th, however *P. antarctica* increased

4.66x10^6 fold higher in comparison to the other bacteria which increased ~14 fold from their starting quantities, although this could be an outlier in the data (Table 2.5). This increase suggests that the probiotic bacteria were proliferating on the captive bat wings during hibernation when exposed to the high humidity and low temperature conditions. Captive myotis body weights slowly decreased throughout the trial and can be attributed due to their disturbance from weekly swabbing and artificial hibernating conditions. Bats were largely torpor throughout the hibernating conditions but were removed once they reached a critical body mass of ~4.0g.

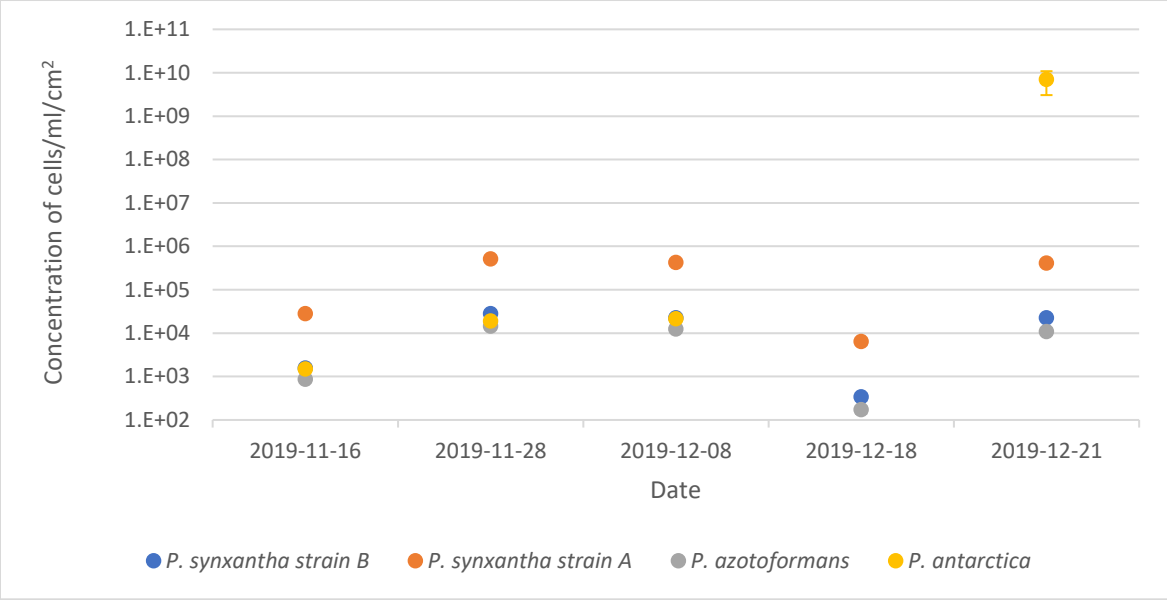

Figure 2.16. Concentration of probiotic cells detected on treated bats using qPCR for each probiotic bacteria species after being placed in sterile water and immediately frozen. Cell concentrations are corrected by wing area to represent cm^2. Note the logarithmic scale.

Table 2.5. Concentrations of probiotic detected on bats in hibernation chamber, at the start and end of the 35 day experiment. Concentrations (adjusted by wing area, cm^2) were determined using qPCR. N=3 bats. Note: the final value of *P. antarctica* may be an outlier.

	P. synxantha B	***P. synxantha A***	***P. azotoformans***	***P. antarctica***
Start concentration (/cm²)	1.56 ± .186 $x10^3$ (.691 - 2.32 $x10^3$)	2.79 ± ,337 $x10^4$ (1.24 - 4.1 $x10^4$)	870 ± 103 (.395 - 1.3 $x10^3$)	1.49 ± .096$x10^3$ (1.21 - 1.81 $x10^3$)
End concentration (/cm²)	2.24 ± .148 $x10^4$ (1.88 - 2.82 $x10^4$)	4.11 ± .275$x10^5$ (3.27 - 4.96 $x10^5$)	1.08 ± .081 $x10^4$ (8.72 - 1.37 $x10^4$)	6.95 ± 3.9 $x10^9$ (8.35$x10^1$ -2.02$x10^{10}$)
Fold increase	**14.4**	**14.7**	**12.4**	**4.66x10⁶**

Necropsies and Histology

Fall Trial

Necropsies were performed by Dr. Glenna McGregor, BC Animal Health Lab. A summary of microscopic test scores from the fall captive trial (Appendix F) reveals that most of the bats that remained in captivity until the end of the trial period showed overall high scores (negative health), ranging from 9 - 94 (mean = 46; n = 6). Bats that died prior to the end of the trial and were thus only in captivity for less than 1 month, showed overall lower scores (better health), ranging from 1 – 13 (mean = 5.3; n = 3), and showing overall less inflammation. In general, G. McGregor commented that the inflammation was higher in all bats that were examined in contrast to what she would typically see in wild bats; this included more acanthosis (thickening of the skin), potentially due to repeated swabbing. Bats that were euthanized at the end of the trial were examined for neutrophils in the lung and spleen and it was found that there was a large number of these indicative of a systemic inflammatory response (G. McGregor, pers. comm.), but also commonly associated with a response of hibernators when entering deep bouts of torpor (Bouma et al. 2010).

Summer Captive Trial

Necropsies performed on the control group were compared to the Treatment 1 group. Wings, ears, tails, noses, lungs, spleens were examined for a lengthy number of histology criteria (Appendix F) such as inflammation, neutrophil and monocyte count, acanthosis and hyperkeratosis. Results were similar to the fall trial histology examination; however, the control bats more closely resembled the treatment bats based on criteria and no significant differences were found between the two groups ($P>0.05$; not shown here) when a General Linear Model is used to compare histology criteria (Appendix F). The only significant outlier was dermal inflammation in the tail ($P=0.045$), in which the control had more inflammation than our treatment group, which is a negligible finding and is likely due to handling.

DISCUSSION

We saw no detrimental effects from the prophylaxis application in two separate captive trials and the probiotic can easily be transported and implemented using the clay substrate with freeze dried cells application method that we developed. Probiotic bacteria were persistent and presumably viable on captive bats and bat boxes months after application, particularly *P. synxantha* A, including when exposed to high summer heat in the 40-50^{o}C range, and substantially increased in viability and growth when exposed to environmental conditions in the hibernation trial. *P. synxantha* A abundance was significantly higher in treatments 1 and 2 and the four-chamber bat box, whereas *P. antarctica* was significantly higher at the end of the hibernation experiment.

Solutions are urgently needed to prevent WNS infection from inevitably causing bat mortalities in Western Canada. *Pd* infection has not been officially documented in British Columbia (WNS 2019), however, bat mortalities due to WNS are already evident in Washington state (WDFW 2021). *M*. *septentrionalis* is threatened and other bat populations could collapse without human intervention (Frick et al. 2015). British Columbia has the largest number of bat species in Canada, and thus stands to lose significant biodiversity if WNS affects many western species. Few studies have tested

WNS treatment methods on live bats, and fewer have investigated use of bacteria antagonistic against *Pd* (Cheng et al. 2016; Hoyt et al. 2019). This study is the first one to test an anti-*Pd* probiotic cocktail on captive bats in summer on free-flying bats.

I describe the cellular concentration of bacteria on wings in relation to length of time swabbing – in summer (captive enclosures roosting in bat boxes) and winter (hibernation chamber) conditions. There is a clear pattern in probiotic bacteria numbers that I describe. I also describe the relationship between roost microclimate temperatures in the four-chamber bat box in relation to changes in bacterial concentrations on roosting substrates. We determined that the final probiotic cocktail is safe to use, having found no negative effects on the health of bats associated with its application.

Treatment 1 probiotic bat swab concentration decreased by more than half from the May 26th to June 15th samples. By June 15th, bacteria concentrations were 6.62 ± 6.5 x10^{3} cells/cm^{2} for *P. synxantha* B (62% lower), 1.0 ± .98 x10^{5} cells/cm^{2} for *P. synxantha* A (79.5% lower), and 3.66 ± 3.59 x10^{3} cells/cm^{2} for *P. azotoformans* (55.6% lower). The treatment 2 probiotic bat swab concentrations changed in concentration from June 29th to August 14th with final concentrations of 77.0 ± 75.2 cells/cm^{2} for *P. synxantha* B (31% lower), 58.4 ± 24.1 cells/cm^{2} for *P. synxantha* A (4.29x higher), and 1.36 ± 1.35 x10^{3} cells/cm^{2} for *P. azotoformans* (23.2x higher). The standard error is quite large for some values which is because of the large variability seen in the qPCR triplicate values of each swab. The larger values in treatment 1 bats are likely because of the multiple treatments and revisions of probiotic application they underwent on April 26th, May 12th, and May 24th. Therefore, it is likely that treatment 1 bats had a background concentration of probiotic from their previous treatments leading into May 26th, when their probiotic concentrations were highest. Treatment 2 bats were only exposed to one probiotic application on June 15th and have a relatively lower concentration. These results encourage the use of multiple treatments to increase probiotic bacteria concentration on bat wings. Overview on experimental design and justification can be found in Appendix G.

Concentration of probiotic species are comparable between the treatment 1 and 2's bat box and the four chamber bat box, and each species appears to follow a similar trend of relative concentrations. *P. synxantha* A consistently hovers between $1x10^5$ to $1x10^6$ cells/cm^2 in the treatment 1 and 2 and four-chamber bat box. *P. synxantha* A has nearly always remained approximately 18 times higher in concentration than the other species (~18.5 fold for treatment 1, ~18 fold for treatment 2, and ~18.5 fold for the four chamber bat box). This contrasts with *P. antarctica*, which appears to have the lowest abundance in bat boxes and on bat wings relative to the other probiotic bacteria except for the hibernation trial. Arguably this pattern suggests that *P. synxantha* A is either proliferating within the bat boxes and on the bat wings, or is dying off at a slower rate than the other three bacteria. A clear pattern is evident from the data that *P. synxantha* A is outnumbering each of the other three probiotic bacteria, which all show similar values. This idea is further promoted by table 2.4 when we see an increase of *P. synxantha* A's ratio to other bacteria from start to finish. It is difficult to determine the specific reason or variables for this difference, but it may be due to differences in generation time and/or resilience of environmental factors such as temperature, moisture, and pH.

There are many factors that can influence the growth and proliferation of bacterial strains on bat skin, however, they are largely unknown, particularly for this study. Weather, nutrients, humidity, where bats were sourced, grooming behaviour, and other competing microbes are all covariates that could influence the results. The microbiota likely varied significantly between our treatment bats and interacted in unknown ways with our probiotic treatment. Bats were captured from different locations and previous research has shown that microbiota can vary between populations significantly (Avena et al. 2016; Lemieux-Labonté et al. 2016; Winter et al. 2017). Further study is warranted to examine what constitutes ideal growing conditions of the probiotic bacteria on bat wings. For example, what conditions promote anti-*Pd* antifungal compounds to be secreted when *Pd* is present within the environment? Clay has been shown to promote biofilm in *Pseudomonas* bacteria (Alimova, et al. 2009). As such, the use of clay to adhere the microbes on the roosting surfaces of the bat boxes

might additionally act as nutrient supplementation. This might in fact be essential to the production of secondary metabolites and thus the efficacy of this treatment (Duffy and Défago 1999). Testing the proliferation and persistence of the probiotic on bat box substrates in absence of clay, would be needed to determine this.

The four-chamber bat box had a larger abundance of *P. synxantha* A than the other probiotic bacteria. When the chamber data is pooled (figure 2.13), there is an obvious trend of *P. synxantha* A being significantly larger than the other 3 probiotic bacteria as seen in treatment 1 and 2 bat boxes. This result is replicated when each chamber is looked at individually and *P. synxantha* A is still the highest (Table 2.3). However bacteria concentrations significantly ($P<0.001$) differed between individual chambers of the four-chamber bat box and each bacteria species (Table 2.3). Concentrations of all probiotic bacteria remained stable from July 20th until the end of the trial on August 24th. Each chamber significantly differed from at least one other chamber, with the largest difference being between the deepest one, chamber 1, and the outside one, chamber 4. Variables such as humidity, average daily temperature, and minimum daily temperatures were all similar ($P>0.05$) however chamber 1 had significantly hotter maximum daily temperatures when compared to chamber 2, 3 and 4 throughout the entire trial ($P<0.001$). Interestingly, chamber 1 had a significantly higher concentration of probiotic bacteria than chambers 2,3 and 4 ($P<0.001$). Based on the humidity and temperature variables we tested, bacteria proliferation and viability increased when maximum temperatures increased within the bat box. Furthermore, the lowest maximum temperatures seen were in chamber 4, although not a significantly different temperature in comparison between chambers; this chamber had a significantly smaller number of bacteria. The only correlation that is evident from the data, based on the variables we tested, is that the higher maximum temperature may have encouraged probiotic bacteria growth based on the significantly higher abundance of probiotic bacteria, whereas a lower maximum temperature decreased abundance of probiotic bacteria. In the future, a controlled laboratory experiment that tested different temperatures may give clearer results when correlating temperature to bacteria growth.

There are many variables that may have interfered with these results such as weather, UV light, and overall sun exposure to each of the chambers.

Histology scores between Treatment 1 and the control group were not significantly different. I can therefore conclude that there are no negative effects from the probiotic application that hindered the treatment bats when compared to the control. Although both groups had what was thought to be more inflammation than wild bats, captivity is likely to explain this (G. McGregor, pers. comm.). Additionally, bats from the pilot trial had increased numbers of neutrophils in the lungs and spleen, but these bats were euthanized shortly after cold fall temperatures set in (November, when bats were using long bouts of torpor and likely preparing for hibernation). Leukocytes are less commonly found in the blood when bats are torpid; instead, immune system cells will move into organs to support torpor once bats transition into hibernation (Bouma et al. 2010).

Because the higher wing cell concentrations occur shortly after bat box inoculation (Figure 2.9), in field use with wild bats, we would recommend applying the probiotic late in the summer season, close to the time when bats leave maternity roosts for their hibernacula. However, there are other considerations that just be made such as frequency and timing of bat visits to inoculated roosts (roost switching) and thus it might actually be better to inoculate well before the end of summer (see next chapter), with optional reinoculation of roosts as opportunity allows. Even if wing concentrations at the end of summer are low when bats leave their maternity roosts, results of our hibernation experiment are encouraging, showing at least a 12.4-14.7 fold increase (Table 2.5) in probiotic cells within ~ one month of hibernation for *P. azotoformans, P. synxantha* A and B, and as such, even small concentrations of probiotic on bats entering into hibernation may be enough to protect bats from *Pd* growth. Unsurprisingly, *P. antarctica* grew to extremely significant concentrations by the end of the trial and was 4.66×10^6 times higher with a final concentration of $6.95 \pm 3.9 \times 10^9$ within a single month. However this large increase may be because of the final value being an outlier. Concentration of *P. antarctica* outnumbers the other 3 probiotic bacteria significantly and future studies

may consider utilizing only *P. antarctica* for probiotic applications that occur in late fall before bats leave for hibernation because of its psychrophilic characteristics. We did not perform a histological examination of the hibernation bats however, they did not differ in behaviour throughout the experiment.

In conclusion, the probiotic we have developed is safe to use on bats, can be successfully transferred to bats' via their roosting substrates, and presents promise of preventing WNS disease in hibernating bats. *P. synxantha* A and *P. antarctica* are promising probiotic bacteria because of their distinguishably high concentrations in summer and hibernation experiments. Future researchers should consider testing only *P. synxantha* A and *P. antarctica* together in higher concentrations, specifically in a large comprehensive field trial.

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Chapter 3: Field Application of *Pseudomonas fluorescens* Species Complex Bacteria onto Bat Boxes in the Greater Vancouver Area

INTRODUCTION

White-nose syndrome (WNS) is a fungal disease that can kill hibernating bats (Lorch et al. 2011; Frick et al. 2010). Mortality rates of up to 90-100% result from a cascade of physiological events and wing damage stemming from dehydration, electrolyte depletion, and starvation (Cryan et al. 2012; Turner at al. 2011; Verant et al. 2014; Frick et al. 2015; Warnecke et al. 2013). White-nose syndrome is caused by the fungal pathogen *Pseudogymnoascus destructans* (*Pd*) and is touted as one of the most catastrophic wildlife diseases in recorded history (Drees et al. 2017; Frick et al. 2010; Frick et al. 2015; Leopardi et al. 2015). Three species are highly vulnerable to WNS, including M*yotis lucifugus*, *Perimyotis subflavus*, and *M. septentrionalis*, all listed as endangered in Canada (Committee on the Status of Endangered Wildlife in Canada, 2013). The first infection occurred near New York Albany in 2006, presumably transferred from Eurasia (Blehert et al. 2009; Frick et al. 2010; Puechmaille et al. 2011a; Puechmaille et al. 2011b). *Pd* has been documented in seven Canadian provinces and 39 American states and west of the Rockies Mountains, is found only in one state so far, Washington state (WNS 2019), although *Pd* has been detected in California. *Pd* is predicted to persist in cave hibernacula for up to a century after it is introduced due to its ability to act as a saprotroph and expand once introduced (Frick et al. 2017; Lindner et al. 2011; Lorch et al. 2013a; Lorch et al. 2013b; Reynolds and Barton 2014; Reynolds et al. 2015). Spores, when airborne, spread easily and are found on surfaces of infected bat hibernacula each hibernation cycle (Langwig et al. 2015a; Kokurewicz et al. 2016; Hoyt et al. 2019; Zhelyazkova et al. 2020). Bats returning to hibernation are rapidly re-infected each year until the infection rate reaches near 100% after 2-3 years (Frick et al., 2017). However, this infection rate can be reduced if spore loads in the environment are kept low (Hoyt et al. 2020). Strategies to reduce growth of *Pd* on hibernating bats can reduce spore loads on individual bats, reducing likelihood of mortality, and reduce

overall environmental loads and thus reduce population-level infection rate in a hibernaculum.

Locations of bat hibernacula in the Pacific Northwest and Western North America are mostly unknown (Weller et al. 2018). This challenges most mitigation methods relying on knowledge of bat hibernacula (Fletcher et al. 2020; Weller et al. 2018). However, bat boxes used by maternity colonies present an alternative treatment site to roost hibernacula. Used annually by large numbers of adult females typically showing high fidelity to roosts (AK Department of Fish and Game 2021; www.batwatch.ca; www.bcbats.ca), maternity roosts are increasingly being identified by government outreach initiatives (e.g., AK Department of Fish and Game 2021) and community bat programs (e.g., www.batwatch.ca; www.bcbats.ca).Therefore a WNS mitigation method that targets maternity colonies could be highly impactful at a local population level to reducing mortality caused by WNS.

With the goal of preventing *Pd* infection through application of a summer prophylaxis, rather than treating WNS in winter, we conducted a field trial on maternity colonies in the greater Vancouver area of Western Canada in August of 2019. Roosting substrates of maternity colonies were sprayed with a probiotic of anti-*Pd* bacteria (see Chapter 1) that were naturally sourced from wild bats wings. We selected four study sites, each with a large mixed colony of *Myotis yumanensis* and *M. lucifugus.* The roosts of two of these colonies were treated with probiotic, while there was no application of probiotic at the other two sites, leaving them as controls for comparison. Bats were studied at all four sites to establish baseline ecological and behavioural data and identify main roosts used during the pup-rearing season (June – August; Rensel 2021).

Bats and roosting substrates were inoculated with probiotic and then opportunistically swabbed for analysis of probiotic cell concentrations using quantitative PCR (qPCR). In order to track individual bats over time, including post-hibernation return (survival) rates, bats were tracked using Passive Integrated Transponder (PIT)-tags and arm bands in collaboration with the University of British Columbia Okanagan

(Rensel 2021). Although we performed the pilot probiotic application late in the season, we still managed to compare treatment bats to controls in their separate locations prior to bats leaving for hibernation.

The probiotic cocktail we developed and field test here is comprised of 4 anti-*Pd* bacteria species belonging to the *Pseudomonas fluorescens* species complex: *P. azotoformans, P. antarctica,* and two strains of *P. synxantha.* These bacteria demonstrated inhibition of *Pd* growth *in vitro* and were isolated from *Eptesicus fuscus*, *M. yumanensis, Corynorhinus townsendii*, and *M. evotis*. Many species of bacteria within the *P. fluorescens* species complex are known for secreting antimicrobial secondary metabolites, and numerous studies have now shown that several strains of bacteria from the *P. fluorescens* species complex inhibit *Pd* growth both *in vitro* and *in vivo* in lab settings (Cheng et al. 2016; Lemieux-Labonté et al., 2017; Hoyt et al. 2015) and recently in a controlled hibernation field trial (Hoyt et al. 2019). Hoyt et al. (2019) demonstrated that certain bacteria from the *P. fluorescens* species complex reduce the mortality rate of WNS. Here I describe the first study of bacteria from the *Pseudomonas fluorescens* species complex to be used as a prophylaxis and applied at maternity roosts, in advance of hibernation. We used autoclaved clay powder mixed with freeze-dried probiotic to dilute the probiotic and distribute it within bat boxes. Clay is inert and resembles similar substrates found in natural crevice roosts used by bats such as rock crevices. The premise of our approach is that bats coat their wings and bodies with probiotic clay as they roost against the inoculated surfaces of their bat box or building roosts. My objectives were to inoculate maternity roost substrates with the four-strain probiotic cocktail, targeting human-built structures in summer, and subsequently monitor the presence, concentration and viability of probiotic bacteria on treated roosting substrates and wings of bats that have roosted on these substrates. Additionally, I aimed to examine longevity of persistence on roosting surfaces and wings between seasons, and monitor return rates (survival) of bats post-hibernation following exposure to the probiotic cocktail. To date, I have been able to test roosting surfaces, but due to COVID-19 field restrictions, no swab-sampling of bat wings has occurred. Additionally, because *Pd* has not yet been detected in the study area and PIT tag surveillance is not

possible at all four field sites, comparison of return/survival rates among sites is not yet possible.

METHODS

Applicator design and probiotic preparation

Four different species of bacteria were included in the probiotic mixture: *Pseudomonas azotoformans* isolated from a *C. townsendii, P. synxantha* A isolated from a *M. yumanensis, P. antarctica* isolated from a *M. evotis,* and *P. synxantha* B isolated from a *E. fuscus*. These bacterial species inhibit the growth of *Pd* and are a mix of partial and full inhibitors (see Chapter 2). Bacteria were individually grown in 100mLof lysogeny broth (LB) and incubated at 30^{o}C, shaking at ~150rpm over night. Culture's were then centrifuged at 5000 rpm for 10 min and rinsed with Phosphate Buffer Saline (PBS) three times. The cells were mixed with 1g of sterile clay powder and freeze-dried in a cryo-lyophilizer for a minimum of 2 hours. Viability and bacteria counts were tested by diluting and spread-plating and serial diluting 0.01g of freeze-dried suspension onto LB agar , incubating at 25^{o}C for 24hrs and counting the Colony Forming Units (CFU) the next day. The *synxantha* A species final yield was $2.54x10^{9}$ cells per gram of freeze-dried suspension, the *P. synxantha* B species final yield was $1.02x10^{12}$ cells per gram of freeze-dried suspension, the *P. antarctica* species was $9.82x10^{10}$ cells per gram of freeze-dried suspension, and the *P. azotoformans* species final yield was $6.64x10^{11}$ cells per gram of freeze-dried suspension. Each dosage was composed of 250 million cells of each species that was mixed with 30g of clay for treatment of an individual bat box chamber. Clay was added and vortexed on medium speed for 20 seconds in small increments of 5g to promote homogeneity in each dosage. The final product was 40 doses of probiotic containing 1 billion freeze-dried probiotic per dose per bat box chamber.

The probiotic-containing clay powder then needed to be propelled up into the bat box chambers. I devised a propellant technique using a can of emzone compressed air attached to a 50ml centrifuge tube containing the probiotic-clay mixture. I drilled a small

hole into each end of the centrifuge tube; using a silicone seal, I attached the thin 1.5mm diameter tube that is supplied with the compressed air can to one end of the centrifuge tube, and a large 10mm diameter rubber straw to the other end which was the centrifuge tube's lid. The rubber straw was 30cm long and flexible for directing the spray up into each chamber of the bat box. I loaded the probiotic-clay powder into the centrifuge tube by opening and closing the screw cap lid that was now attached to the rubber straw. During deployment, powdered clay placed in the tube was propelled out of the straw into each chamber of the bat box by depressing the nozzle of the spray can. A light mist of water (~50ml of sterile de-ionized water per bat box chamber) was sprayed into the box using a manual pump garden wand sprayer (with a misting wand with a nozzle small enough in diameter to reach into each chamber) beforehand to enable the propelled powder to adhere to the wood and assisted with activating the freeze-dried bacteria. In subsequent field deployments, we substituted a manual action tire pump in place of the aerosol spray can. This reduces cost and is quicker, because the aerosol air cans can become very cold, requiring a resting period before continuing to spray, and they are non-refillable.

Field Application

In 2018 and 2019, 464 bats were individually marked with either PIT-tags (Biomark, ID, USA; 8 mm) or metal arm bands (Porzana, UK; 2.4 or 2.7 mm diameter, split-ring lipped). Bats were captured and marked at three study sites in the lower mainland of British Columbia (Figure 3.1): 220 bats were banded at Colony Farm Regional Park, 213 bats were banded and 493 were PIT-tagged at Stave Lake BC Hydro (of which 15 received both PIT-tags and bands), and 235 bats were banded and 337 were PIT-tagged at Alice Lake Provincial Park (of which16 bats received both PIT-tags and bands. Each band and/or PIT tag enabled individual identification of bats in subsequent monitoring. PIT tagged bats, when entering/exiting a roost equipped with a PIT tag reader of probiotic levels and diversification of controls vs treatment.

Probiotic was applied to roosting surfaces at two study locations: Colony Farm Regional Park and Stave Lake BC Hydro. On August 7th, 2019, I inoculated the four

main bat boxes used by bats at Colony Farm park (numbered 1-4 from left to right of the park). I used a standard dose one billion cells (250 million cells of each of the four probiotic species) per chamber: each bat box had 7 chambers, for a total of 28 doses of probiotic-clay deployed at this site. Three bat boxes were sprayed at night when bats left to forage and one bat box was sprayed during daylight hours when no bats were present.

At Stave Lake BC Hydro, probiotic-clay powder was sprayed into each chamber of three four–chambered bat boxes, and on the outside of the roost entrance to a building (Stave Lodge) on August 8th, 2019. Bats were not present in the bat boxes during the day when probiotic was applied, but were present inside the lodge roost.

Figure 3.1. Map of field study sites in the Greater Vancouver region in southwestern British Columbia. Treatment sites were Colony Farm Regional Park and Stave Lake BC Hydro. Control location was Alice Lake. Each location has multiple roosts used by a mix of *M. yumanensis* and *M. lucifugus.*

Roost Substrate and Wing Swab Samples

To track the presence of each probiotic bacteria, bats' wings were swabbed with a sterile polyester swab is lightly dragged along the edge of both bat's arm bones on the dorsal surface (Appendix H). Each polyester swab tip was stored in a sterile Eppendorf tube, and kept frozen prior to processing. Swabs were washed with 0.5ml PBS solution and vortexed to dislodge cells picked up in the swab. The PBS suspension was then sonicated in a sonicating water bath for 30 seconds in a sterile glass vial. Similarly, for swab sampling of roost substrates, a polyester swab with a long handle was dragged across a roost surface along a predetermined line or within a predetermined area. Swabbing occurred at the entrances of each inoculated bat box chamber and the boiler room entrance. Swabbing areas were kept consistent between bats and between roost surfaces; therefore the measured starting quantities are only comparable relative to their own groups. Bat swabs were divided based on mean wing area seen in the captive trial of 19.7cm due to measurement of field trial bats not taken at the time. The boiler room entrance and bat box swabs were not measured for swabbing area.

Baseline and control swabs of bats were taken before the bat box probiotic inoculations. Adult females from each site were swab-sampled for baseline concentrations of naturally-occurring bacteria matching the probiotic strains constituting the probiotic cocktail: one *M. yumanensis/lucifugus* bat from Colony Farm on July 11th; one *M. yumanensis/lucifugus* bat from Stave Lake on July 29th. An individual *M. yumanensis/lucifugus* bat was swabbed from Alice Lake on July 24th, July 31st, and August 14th as a control. This latter sample from the Control site was one week after application of the probiotic at the treatment sites. All samples were qPCR-analyzed with the custom probes for 3 of the 4 bacteria making up the probiotic. These baseline levels formed the zero baseline when measuring the starting quantity of the treatment bat swabs.

Treatment of bat boxes occurred on August 7th for Colony Farms and August 8th for Stave Lake. There were three bat boxes in total at Stave Lake, two of which attached to a lodge and designated as "#1" and "#2", and a third bat box known as

“Hayward Bat Box”. Four bat boxes were inoculated at Colony Farms. Bat boxes were monitored manually and through PIT tag readers for bat activity in the following weeks after probiotic inoculation. Bats were not seen roosting in any bat boxes at Colony Farms when inspected on August 13th and a lack of a PIT tag reader at this site precluded knowing if bats were present on other days. An adult female *M. lucifugus* and a juvenile female *M. yumanensis/lucifugus* species were captured in the Colony Farm site using mist nets and swabbed for qPCR analysis of the probiotic species. It could not be confirmed that they had been using the probiotic-inoculated bat box roosts. There were no bats detected in roost boxes from Colony Farms throughout the rest of the trial, and this was unsurprising given that this colony leaves these roosts by mid-August each year (J. Saremba, Burke Mountain Naturalists, pers. comm.).

Each chamber of the three inoculated bat boxes and the boiler room roost entrance were swabbed on August 22nd, 14 days following application. Swabs were attached to a wood dowel and rubbed against a pre-determined and consistent bat box surface area vertically in each chamber. Relative quantity of each of the probiotic bacteria in each swab was measured using qPCR. On August 27th, 19 days after probiotic application at roosts, 33 bats were swabbed at the Stave Lake study site. Bats were captured using a harp trap from the Hayward Lake bat box.

On March 3rd, 2020, overwinter survivability of the probiotic species was tested by swab-sampling previously inoculated roosting surfaces. We sampled bat boxes 1-4 from Colony Farms three times (n=12 swabs), 2 swabs were frozen in water and the third swab was placed in broth and allowed to grow in an incubator for 12 hours before being frozen. Box 1 from Stave Lake was sampled three times (n=3), 2 swabs were frozen in water and the third swab was placed in broth and allowed to grow in an incubator for 12 hours before being frozen.

qPCR Analysis

All swab samples were processed using a relative-quantity multiplex probe-based qPCR assay. Species-specific gyrB primers (modified from Yamamoto et al.

2000) were used to quantify cell concentrations in swabs for each probiotic isolate. We designed strain-specific qPCR probes, with a 5' fluorophore tag and 3' quencher, for use in multiplex reaction, measuring the relative combination of 3 of the 4 probiotic species in each reaction. Species measured with qPCR were *P. azotoformans*, *P. synxantha* A and B. Standards were created through serial dilution of cells suspension (10^5, 10^4, 10^3, and 10^2) from each probiotic strain and all reactions were carried out in triplicate. We then created a standard curve using the Cycle Threshold (Cq) values from cell standards of known concentration. Fitting the Cq values from samples of unknown concentration enable extrapolation of the number of cells present in each swab. Standard error is presented for all means.

RESULTS

Summer Swab Samples – Bats and Roosts

Three strains of the probiotic bacteria were detected on both bats from Colony Farms, with *P. synxantha* strain A 18.7-36.2 times higher in concentration than the other two bacteria when both swab concentrations are combined. *P. synxantha* strain A concentration was 2.36 ± .602 x10^3 cells/cm^2 (1.28 - 3.36 x10^3, n= 3 replicates) on a juvenile and 3.87 ± 3.18x10^4 cells/cm^2 (5.47 - 102 x10^3, n= 3 replicates) and on an adult female; *P. synxantha* strain B was 136 ± 36.0 cells/cm^2 (70.6-195, n= 3 replicates) and 2.06 ± 1.67 x10^3 cells/cm^2 (295-5429, n= 3 replicates); *P. azotoformans* was 67.8 ± 18 cells/cm^2 (37.1-99.3, n=3 replicates) and 1.06 ± .865 x10^3 cells/cm^2 (.153-2.79 x10^3, n=3 explicates), respectively. Unfortunately due to problems with the 4th PCR probe, presence of *P. antarctica* could not be tested. High variance between replicates likely reflects variability occurring from sampling during qPCR.

Three bat boxes (Lodge Bat Box #1, Lodge Bat Box #2, and Hayward Lake Box) and the boiler room roost entrance Stave Lodge were swabbed at Stave Lake BC Hydro on August 22nd for detection of probiotic 22 days after the roosts were first inoculated. All but one of the Lodge boxes had detectable amounts of probiotic bacteria within each chamber; Lodge Bat Box 1 inexplicably had no detectable amount of probiotic in any of the chambers. At the other roosts, *P. synxantha* strain A was the most abundant

bacteria (10-12.5 times more; Figures 3.2 and 3.3) within each chamber of both bat boxes and at the boiler room roost entrance (Figure 3.4). Lodge Bat Box 2 and 3 each varied in the amount of probiotic among chambers; the former had the greatest abundance of probiotic bacteria within its third chamber, followed by the first, fourth, and second and each probiotic bacteria species was detected in measurable quantities. Hayward Bat Box had a greater number of probiotic bacteria present within each chamber compared to Lodge Bat Box 2. These results might reflect the randomness of the application process given that not all clay powder sticks when sprayed into the bat box chambers, and the spraying consistency across the substrate is largely uncontrolled which means that depending on where the swab sample is taken, probiotic concentrations can vary widely. Interestingly, bacteria in Hayward Bat Box was detected highest in the outer chamber 4, and progressively less bacteria found in each chamber, with chamber 1 having the least (Figure 3.3). Bats typically roost in chamber 1 as that chamber is the first one they enter from the landing platform. Bats can move progressively from one chamber to the next moving outward, and therefore all bats will pass through chamber 1, each picking up probiotic clay, and depending on the number of bats in the box, they may not proceed past chamber 1.

At the entrance of the boiler room roost entrance, two swabs were taken (Figure 3.4) and each had a detectable number of bacteria: *P. synxantha* A had a marginally greater number of cells compared to *P. synxantha* B and *P. azotoformans*.

At the control site, Alice Lake Provincial Park, 3 bats were captured (one per night on 24 July, 31 July and 14 August, 2019). No detectable probiotic bacteria were found on their wings through qPCR analysis. The two baseline swabs from each bat at Colony Farms and Stave Lake on July 11th and July 29th had no detectable probiotic.

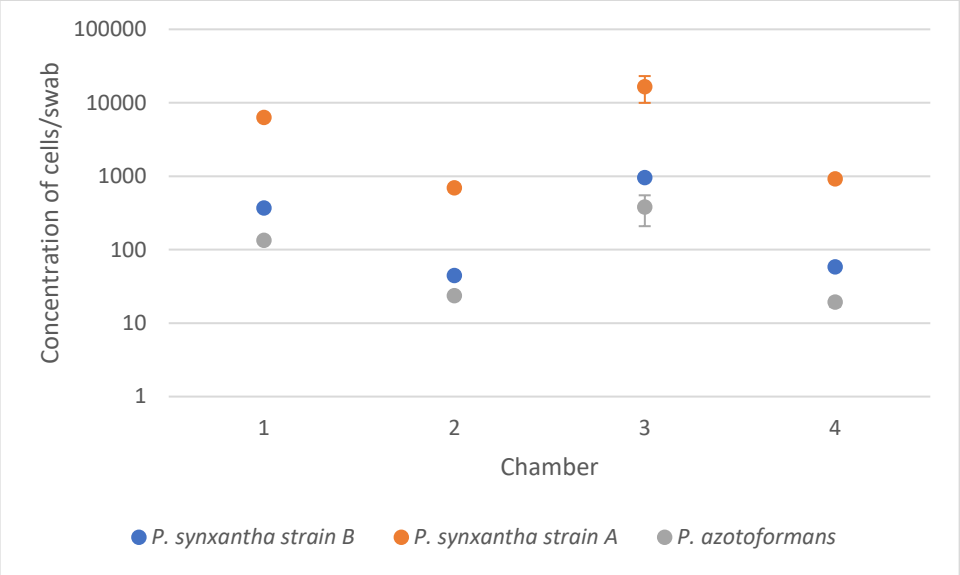
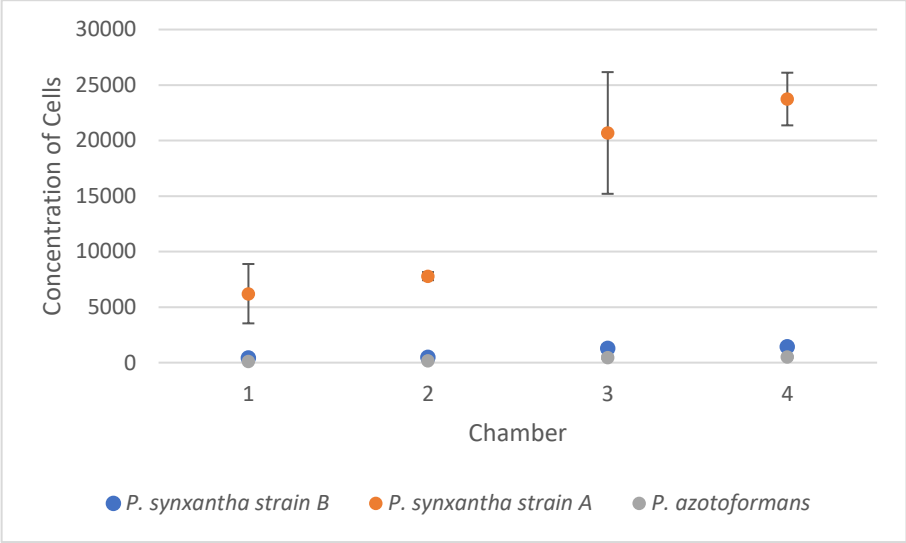


Figure 3.2. qPCR probiotic data comparison of *P. azotoformans,* and strains A and B of *P. synxantha* from 4 swabbed chambers of Bat Box 2 from Stave Lake on August 22nd. Note the logarithmic scale.

A.



B.

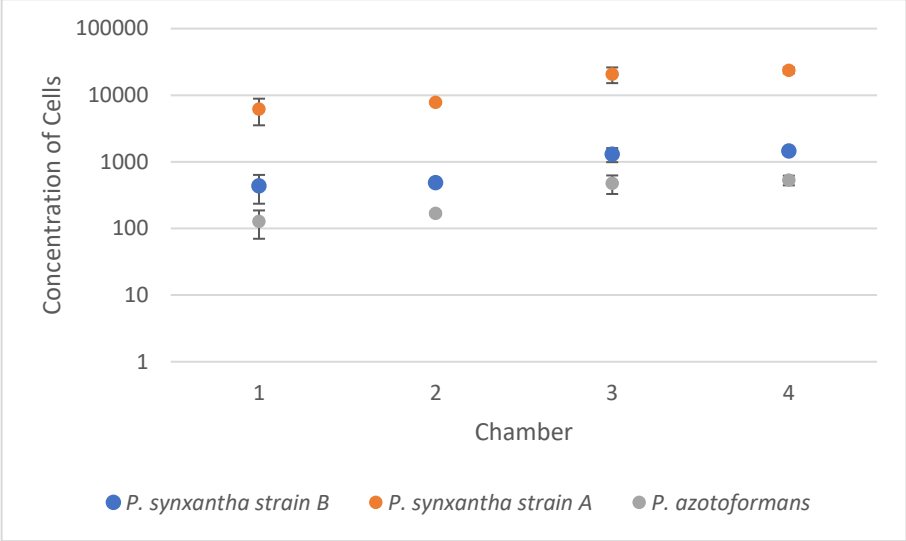


Figure 3.3. qPCR probiotic data comparison of *P. azotoformans,* and *P.* synxantha A and B from each of four swab-sampled chambers of Hayward Bat Box from Stave Lake on August 22nd. A. shows linear scale and the notable magnitude of difference in cell concentrations, with >10x greater *P.* synxantha A strain. B. Same data plotted on logarithmic scale to visualize concentrations of the other two probiotic isolates.

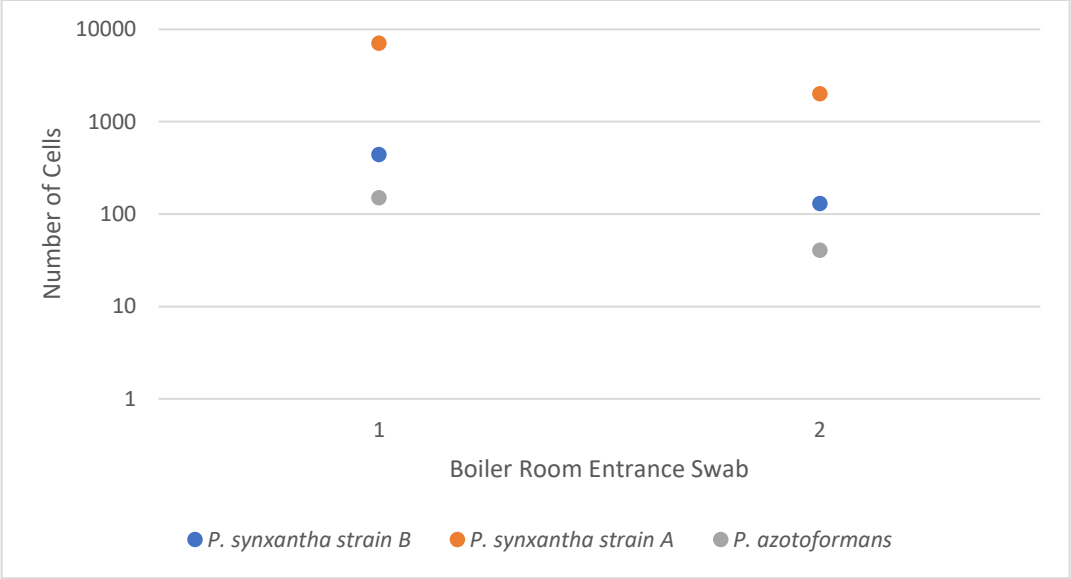


Figure 3.4. qPCR probiotic data of *P. azotoformans,* and strains A and B of *P. synxantha* from 2 swabs of the boiler room entrance at Stave Lake on August 22nd, 2019. Note the logarithmic scale.

PIT tag readers at probiotic-treated Stave Lake roost sites confirmed that bats used all 3 bat boxes as a night roost for at least 5 days after inoculation with probiotic. The boiler room roost was consistently occupied. At Hayward Bat Box, 33 bats were captured with harp traps and swab-sampled on August 27th, almost three weeks since the roosting chambers were inoculated on August 8th. The majority of swabbed bats were adult female *M. lucifugus*, except for one juvenile female and one adult *M. yumanensis* (Table 3.1). Ten of the 33 samples (30%) had detectable amounts of probiotic on their wings.

Of the 10 bats with a quantifiable number of probiotic bacteria on their wings, bacterial strains and concentrations varied widely. *P. synxantha* strain B was the most widespread (8 of 10 swabs) and its starting quantities were higher than *P. azotoformans* in samples where both bacteria were found; however, the most abundant probiotic bacteria was clearly *P. synxantha* strain A (5 of 10 swabs; Figure 3.5). *P. azotoformans* was detected on 3 of the 10 swabs (Swab 1, 4, and 9 in figure 3.5).

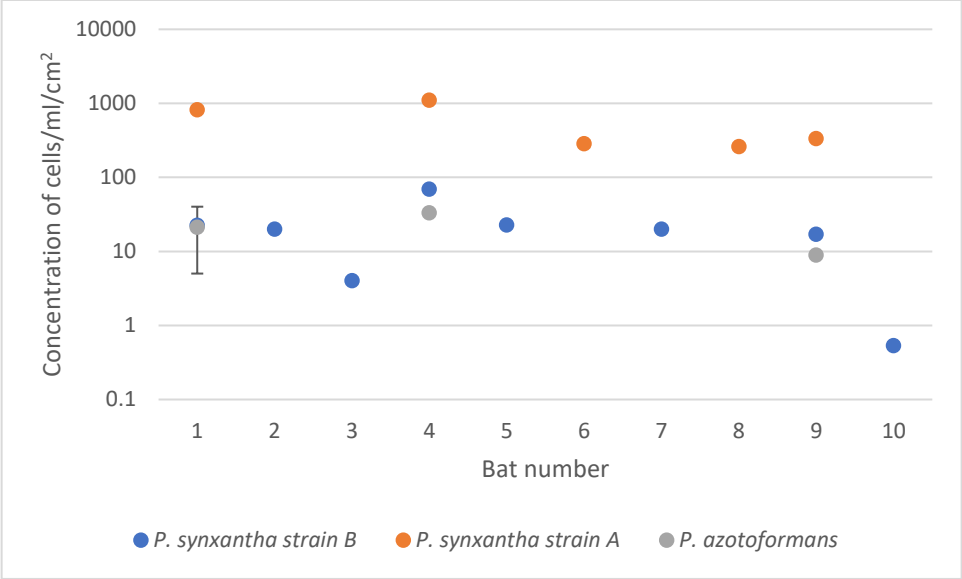


Figure 3.5. Quantitative PCR analysis of wings of 10 captured bats (for which probiotic was detected) on August 27th, 2019 at Stave Lake study site. Presence of three probiotic bacteria were tested: *P. azotoformans,* and strains A and B of *P. synxantha*. Note the logarithmic scale.

Table 3.1. Characteristics of bats caught at Stave Lake on August 27th, 2019.

Bat Swab #	Probiotic Species	# of qPCR replicates	Age	Sex	Species
1	*P. synxantha* B	2	A	F	MYLU
1	*P. synxantha* A	1	A	F	MYLU
1	*P. azotoformans*	1	A	F	MYLU
2	*P. synxantha* B	1	J	F	MYLU
2	*P. synxantha* A		J	F	MYLU
2	*P. azotoformans*		J	F	MYLU
3	*P. synxantha* B	1	A	F	MYLU
3	*P. synxantha* A		A	F	MYLU
3	*P. azotoformans*		A	F	MYLU
4	*P. synxantha* B	1	A	F	MYLU
4	*P. synxantha* A	1	A	F	MYLU
4	*P. azotoformans*	1	A	F	MYLU
5	*P. synxantha* B	1	A	F	YULU
5	*P. synxantha* A		A	F	YULU
5	*P. azotoformans*		A	F	YULU
6	*P. synxantha* B		A	F	MYLU
6	*P. synxantha* A	3	A	F	MYLU
6	*P. azotoformans*		A	F	MYLU
7	*P. synxantha* B	1	A	F	MYLU
7	*P. synxantha* A		A	F	MYLU
7	*P. azotoformans*		A	F	MYLU
8	*P. synxantha* B		A	F	MYLU
8	*P. synxantha* A	1	A	F	MYLU
8	*P. azotoformans*		A	F	MYLU
9	*P. synxantha* B	1	A	F	MYLU
9	*P. synxantha* A	1	A	F	MYLU
9	*P. azotoformans*	1	A	F	MYLU
10	*P. synxantha* B	1	A	F	MYLU
10	*P. synxantha* A		A	F	MYLU
10	*P. azotoformans*		A	F	MYLU
11	*P. synxantha* B		J	M	MYLU
11	*P. synxantha* A	1	J	M	MYLU
11	*P. azotoformans*		J	M	MYLU

** Concentration deemed to be an outlier and thus this sample was removed from analysis.*

Spring Swab Samples of Roosts

P. azotoformans, *P. synxantha* A, and *P. synxantha* B were not present in any of the swabs of roost substrates in March 2020. Broth was inoculated with swab suspension as a way to try to grow any trace probiotic cells, however and only one swab from a bat box at Colony Farm (Bat Box 2) grew one of the strains -- *P. synxantha* A, however it was not present in the water sample.

DISCUSSION

We determined that probiotic could be transferred to wild free-flying bats by applying it to their roost substrates. We found evidence at both of our treatment sites, that bats had probiotic bacteria on their wings several weeks after roost inoculation. At Colony Farm, only 2 bats were captured, and they had both detectable amounts of probiotic on their wings one week after bat box inoculation. Inoculated bat boxes from Colony Farm were empty after inoculation throughout the rest of the trial, which is consistent with their annual pattern of leaving the bat box array by mid-August each year (John Saremba, Burke Mountain Naturalists, unpublished data). These bats may leave the area for mating and hibernation, and this colony may leave earlier than others if they have a long ways to travel to hibernacula (Norquay et al. 2013), or they have more suitable roosts for late summer/early fall). Bats were still present at Stave Lake at least three weeks after inoculation of the three bat boxes and the boiler room roost entrance. Three of the 4 probiotic species were detected at two of the three bat box roosts and at the Lodge roost entrance at Stave Lake, several weeks following roost inoculation. It was not possible to test for the presence of the fourth bacteria species, *P. antarctica* because the PCR probe did not anneal properly during qPCR DNA amplification. The other 3 probiotic species were present in abundant numbers with *P. synxantha* A being greater than the others by 17.1-43.9 fold in Bat Box 2, 15.9-44.7 fold in Hayward Bat Box, and 15.9-47.8 fold at the boiler room roost entrance. Although the protocol used to create the probiotic-clay and the application of the clay into each roosting chamber was designed to apply each probiotic bacteria in approximately equal concentrations, one of the bacteria (*P. synxantha* A) amplified over time on roost and

wing substrates. The reason is unknown, but may due to its ability to proliferate in the warm bat roost conditions. This same uneven proliferation was not seen in hibernation conditions (see Chapter 2). What has not been confirmed is whether the bacterial cells of all probiotic cells swabbed from bats and roosts are viable or whether the DNA of nonviable cells remains and is being amplified. For example, if *P. synxantha* B and *P. azotoformans* are not viable and only DNA remains to be amplified, but *P. azotoformans* is viable, then it would appear that this latter strain has drastically outnumbered the other strains when in fact its cell division is just indicative of viability. This strain, however, might truly be more prolific in the warm summer roosts conditions and on warm-bodied bats (adult females raising young maintain warm bodies, e.g., Lausen and Barclay 2003). Further experimentation and sampling will need to be done to shed light on viability in relation to relative concentrations of the four probiotic bacteria. We had planned for our broth inoculations of each sample to test for viability, but this proved to be unreliable; this may stem from an underestimate of the time needed for cell division, or from competition of other microbes in a rich nutrient environment.

We know from our captive bat trials that probiotic remains on bat box substrates for several months after application in summer months (see Chapter 2). However, this was the first test of whether the probiotic would still be detectable on bat box roosting substrates the following spring. We detected none of the probiotic species in the overwinter subsampling of bat boxes at Stave Lake in March. Only one sample of one chamber in one box at Colony Farm showed a trace quantity of *P. synxantha* A from a broth sample. This failure to find probiotic cells on the roosting surfaces suggests significant die off of the probiotic in the bat boxes overwinter. However the presence of *P. synxantha* A in one broth sample may suggest a small quantity of cells were able to grow when exposed to broth, but were too small to be quantified in water. Application protocol, frequency, and/or cell concentrations should be adjusted in future field activities to verify conclusions and determine a potential cause for the overwintering die-off; for example, freezing temperatures might be responsible for no detection of cells in the broth, but the fact that the water samples also proved to have no bacterial DNA to amplify suggests that the DNA of the bacteria was also gone and perhaps there was

enzymatic breakdown of the probiotic cells by other microbes on the roosting surface. Application and sampling of a roost substrate in a heated building will help to determine if temperature is a factor, or whether there other potential causes like microbial competition. In this study we did not swab at any other time points between winter and spring, therefore we do not know the exact time frame in which the probiotic species were present. Multiple sampling periods and environmental condition analysis, such as temperature and humidity, throughout winter could provide better insight to each bacteria species survivability.

Bat boxes were not swabbed at Colony Farm due to no bats being observed within the bat boxes after the first and second week after inoculation. Unlike at Stave and Alice Lake sites, bats at this site were not PIT-tagged, and so presence of bats could not be known unless visually observed. This together with the fact that this colony is known to leave these bat boxes annually in early to mid August, resulted in few bats being sampled.

Bat hibernacula in western United States is largely unknown, with 95% of counts containing less than or equal to 10 myotis species in each roost (Weller et al., 2018). At this time it is impossible to apply probiotic to hibernating sites, and even then, they would likely be heavily disturbed. Future methods should consider a quicker, and more efficient method of application that would only be conducted with 1 or 2 people.

Our results found that 30% of swabbed bats from Stave Lake had a detectable amount of applied probiotic on their wings. All bats found with our probiotic on their wings were female MYLU species. The period between bat box inoculation and bat swabbing was 19 days from August 8th until August 27th. This would presume that swabbed bats likely came into direct contact with the inoculated probiotic within the bat boxes. To further re-iterate our findings, we saw none of our probiotic species in the baseline wild bat swabs from the Stave Lake bat swab, the Colony farms bat swab, and the 2 bat swabs from Alice Lake from July and early August. Like in the bat box chambers, *P. synxantha* A was much more abundant on each bat swab compared to the other probiotic species. These results give us an idea of how much probiotic was

present on their wings with the dosage we used. Although an estimated 250 million cells of each anti-*Pd* bacteria species were inoculated onto each bat box, our highest result was 2.37 ± .237 $x10^4$ (.190 - 2.64 $x10^4$) cells of *synxantha* A as seen in chamber 4 of the Hayward Bat Box. The highest result seen on bats was $1.11x10^3$ ± 0 cells/cm^2 of *P. synxantha* A that used the Hayward Bat Box. Interestingly, the 2 most highly used chambers of the Hayward box (chambers 1 and 2 near the landing platform entrance) were noticeably lower in quantity of all microbes compared to the least used chambers (3 and 4;Figure 3.3). This may reflect the overall use of the bat box by bats and the adhering of cells to bat skin and fur that is expected to occur. The bats caught at Colony Farms had a much higher result of 3.87 ± 3.18 $x10^4$ (8.38 - 102 $x10^3$) *P. synxantha* cells/cm^2, however, we do not have bat box swabs to compare to. A larger abundance of bacteria on bats and within bat boxes may stimulate biofilm growth. Ideally, we would like to see biofilm formation within the bat box and eventually hibernacula from bats transferring it. Biofilm could form and survive in bat hibernacula because of its above freezing, humid and stable refrigerator-like temperatures (Fenton and Robert 1980; Gennari and Dragotto 1992). Therefore spore loads within the environment and on individual bats would likely be lower from the anti-*Pd* metabolites released from the biofilm.

For further studies we would recommend using repeated, and possibly higher, dosages of anti-*Pd* bacteria from the *P. fluorescens* species complex and further monitoring of the bat box chambers throughout winter and into spring. Consideration of repeated dosing of bat boxes is warranted because of the high concentrations of probiotic bacteria seen in the captive trial hibernation experiment (chapter 2). The captive bats from the hibernation trial in chapter 2 used a bat box that was doused twice with probiotic on September 30th and October 7th before moving into the hibernation trial on November 7th. Probiotic concentrations greatly increased throughout the hibernation trial into the hundreds of thousands per cm^2. Wild bats may have a greater concentration of detectable probiotic on their wings if the bat boxes were inoculated multiple times, similar to our captive trial. Clay has previously been documented as a great mineral source for *Pseudomonas* bacteria (Alimova, et al. 2009). Furthermore,

repeated dosages of anti-*Pd* bacteria to maintain bacteria proliferation and growth has been recommended by other researchers when using anti-*Pd* bacteria to reduce WNS impacts (Hoyt et al. 2019). Utilizing *P. synxantha* A, *P. azotoformans* and *P. synxantha* B colonization could be a useful management practice when hibernacula are not known. These results give an insight into new types of management practices that rely on natural anti-*Pd* probiotics found on a smaller percentage of wild bats and spreading it throughout bat species populations.

In conclusion, we inoculated bat boxes and a building roost with our anti-*Pd* probiotic naturally found on wild bats, and later detect the same probiotic species on bats that were using the bat boxes. Inoculating the roost substrate directly would have likely been a better choice than the roost entrance of the building, as not all bats likely encountered the probiotic. Probiotic species were not detectable in the same bat boxes following exposure to winter conditions and seem to have died off by spring. Further research is needed to examine different dosage concentrations and treatment frequency needed to sustain probiotic cell numbers both on roost surfaces, and bat wings throughout the entire hibernation period into spring. Monitoring cell concentrations on bat species while they hibernate in the wild would be ideal, however, this may be impossible due to bat hibernacula being mostly unknown. Instead, sampling efforts should be bolstered to increase sampling size and frequency throughout the fall season to create a timeline of probiotic abundance on bats and bat boxes. Methods should also be implemented to determine viability and metabolite abundance of probiotic bacteria on the bat box and bats throughout fall and into winter if possible. This study is one step towards alleviating the selective pressure that western WNS impacted species are facing during hibernation. In the future, affected bats may survive hibernation and *Pd* exposure if they were to maintain anti-*Pd* bacteria numbers and indirectly defend themselves from mass mortality.

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Chapter 4: Inoculation and Quantification of *Pseudomonas fluorescens* Species Complex Bacteria and *Pseudogymnoascus destructans* on Myotis Patagia

INTRODUCTION

White-nose syndrome (WNS) is a deadly infectious disease that results in bat mortality during hibernation (Frick et al. 2010). This disease is caused by the fungal pathogen *Pseudogymnoascus destructans* (*Pd*) which has caused millions of bat deaths across eastern North America (Frick et al. 2010; USFWS 2012). Surviving bats will groom off the fungus and persist, though survival rates for some populations have been less than 10%; , *Pd* fungus can persist in caves and infect bats in each subsequent hibernation period (Frick et al. 2015; Lorch et al. 2011; Hoyt et al. 2015). *Pd* is a psychrophilic fungus and grows optimally between 12.5-15.8^{o}C in the presence of high relative humidity (80.5%; Verant et al. 2012; Marroquin et al. 2017). These temperature and humidity conditions are found in caves in which many bats choose to hibernate (Davis 1970; Fenton 1970; Anderson and Robert 1971; Raesly and Gates 1987; Cryan et al. 2010; Vanderwolf et al. 2012; Kurta 2014). White-nose syndrome was introduced into North America in 2006 and bat mortalities were first noted in 2007 near Albany, New York (Blehert et al. 2009; Frick et al. 2010). Today it now resides in 39 states and seven Canadian provinces (USFWS 2020). Bats are thought to fill crucial roles in ecosystems, agriculture, and forests (Boyles et al. 2011; Kunz et al. 2011). They are voracious predators of insect pests and provide a natural method of insect control without the use of pesticides. Vector-borne diseases that utilize insects as a method of transmission may in part managed be by insectivorous bats. Natural pest control services of bats there is estimated to be worth billions of dollars to the US agricultural industry (Boyles et al. 2011; Kunz et al. 2011).Therefore it is especially important to understand how to control WNS infection in bats to prevent or reduce further mortalities. This is especially important as the disease spreads in western North America where the species diversity of bats far exceeds that of the eastern part of the continent. In British Columbia (B.C.) in particular, bats represent nearly 20% of small mammal diversity (CDC 2021). White-nose syndrome affects hibernating bats. Fourteen of the 17 bat

species in B.C. are known to hibernate (Naughton 2012), suggesting that WNS could substantially decrease mammal diversity in the province (and in Canada). With a growing understanding of the importance of biodiversity to the function and health of ecosystems (e.g., Battista et al. 2016), control over this invasive bat fungus takes on even greater urgency as it infects new western bat species for which susceptibility is as of yet unknown.

White-nose syndrome fungal infection is subtle to detect in bat wings and deadly during hibernation (Meteyer et al. 2009). Signs of infection are inconsistent but typically include rough patches on the face, ears, forearms, wing membranes, and feet. Skin tissues may show irregular pigmentation or small tears in wing membranes from *Pd* infection. The wing membrane epidermis is composed of 2 single cell layers that are separated by a thin layer of blood and lymphatic vessels, nerves, muscles and specialized connective tissues (Makanya et al. 2007). *Pd* has shown to highly express 94 genes during the cutaneous infection of M. lucifugus, and 34 genes that directly contribute to its virulence (Reeder at al. 2017). These genes include expression of enzymes lipase 1 and squalene monooxygenase which degrade surface lipids and sebum found on the bat wing epidermis. Other genes significantly improved *Pd*'s heat shock response, cell wall remodelling and micronutrient acquisition to likely evade host pattern recognition receptors and antibody responses. *Pseudogymnoascus destructans* will commonly produce cup like erosions that are filled with fungal hyphae on bat wing membranes and ulcerate the underlying connective tissue as infection continues. *Pseudogymnoascus destructans* hyphae are septate and will branch in either uniform, parallel walls, or irregular bulging walls that are larger in diameter (Makanya et al 2007; Meteyer et al. 2009). Hyphae will destroy apocrine glands, hair follicles and sebaceous glands, and replace host connective tissue, blood and lymphatic vessels, glandular structures, elastin and muscle fibers with *Pd* digests.

Ulceration and invasion of *Pd* into host tissue can cause many physiological complications for its host. Damage to the epidermis has been shown to cause eventual electrolyte depletion, hypotonic dehydration and respiratory acidosis during torpor and

hibernation (Verant et al. 2014; Warneke et al. 2013). *Pseudogymnoascus destructans* hyphae will damage underlying connective tissue and increase vascular permeability, which further accelerates fluid loss and can cause hypovolaemia which would trigger bats to awake from torpor and drink water (Warnecke et al. 2013). Furthermore, destruction of epidermis can prevent normal blood flow and proper CO_2 expiration, increasing ventilation rates and possibly hyperventilation, which further contributes to arousal frequency and evaporative water loss (EWL; Verant et al. 2014; Warnecke et al. 2013). Sodium and chloride levels will drastically decrease due to hibernacula not containing sufficient electrolytes (eg. insects) to replenish losses caused by the disease (Cryan et al. 2013). Imbalance of electrolyte levels can interfere with homeostatic function and lead to possible impaired neural and/or heart function as seen in other mammals. It has been proven that bats exhibit heightened energy expenditure and EWL after infection during torpor and this directly contributes to WNS pathophysiology (McGuire et al. 2017). Bats will initially express a heightened torpor metabolic rate (TMR) and this is possibly due to a small immune response when bats initially try to fight off the infection (Field et al. 2015, Lilley et al. 2017). However, torpor will typically suppress a bat's immune system making them more vulnerable to infection as hibernation continues (Bouma et al., 2010; Geiser 2004; Moore et al., 2011). Put simply, bat mortality is the result of premature arousal from torpor because of physiological imbalances and results in depletion of energy reserves for winter survival (Storm and Boyles 2010; Cryan et al. 2013; Warneke et al. 2013; Verant et al. 2014).

Methods of controlling *Pd* infection in wild bats are being researched, but more than a decade after the disease's discovery in North America, field-ready preventative or treatment tools do not yet exist. Anti-fungal agents such as fungicidal drugs that are commonly used in agriculture, vaccines, and UV light are being studied (Palmer et al. 2018; Rocke et al. 2019.). Fungicidal drugs were among the first treatment methods tested and proven to be unsuccessful. Not only can anti-fungal drugs be dangerous to a bat's natural microbiome and expensive, but they can be toxic and have effects on non-target taxa when deployed in bat roosts. Vaccines are expensive, require extensive testing, and are difficult to implement into wild animals. Finally, UV light is non-specific

and does not reach under bats arms when they hibernate, leaving reservoirs for the fungus to persist. Utilizing anti-*Pd* bacteria has been a promising method of controlling *Pd* infection and an increasingly interesting form of controlling WNS infection. *In vitro* tests utilizing antifungal metabolites from bacteria can limit growth of *Pd* mycelia and spores (Cheng et al. 2016; Chaturvedi et al. 2011; Cornelison et al. 2014a; Cornelison et al. 2014b; Lemieux-Labonté et al., 2017; Micalizzi and Smith 2020; Hoyt et al. 2015). Researchers from University of California have developed an *in vivo* screening method using explant chambers to examine *Pd* infected bat tissue in the presence of *R. rhodochrous* (strain DAP 96253 induced with urea; Cornelison et al. 2014b). *In vivo* tests prevent other fast growing fungal microbes and mold from interfering with *Pd* and anti-*Pd* bacterial inoculation.

Here, I assess whether severed *Myotis yumanensis* bat wings treated with a cocktail of anti-*Pd Pseudomonas* bacteria can prevent or slow *Pd* spore germination and/or hyphae growth. The anti-*Pd* cocktail contains four synergistic bacterial strains from Western Canada that were originally found on wild bat wings and inhibit the growth of *Pd* (see Chapter 2). Here I investigate whether the probiotic reduces *Pd* growth or germination. I used two different approaches to testing this: one using wings of bats who had been indirectly inoculated via application of a probiotic-clay powder (see Chapter 2) on their roosting surfaces; the other using direct inoculation of live wing skin excised from a freshly euthanized bat. Here I present results of *Pd*-challenge experiments. where I exposed captive bats to the probiotic at their bat box roosts, humanely euthanized the bats, severed their wings, and challenged them with *Pd* to quantify and describe subsequent *Pd* spore germination or *Pd* hyphae growth time. I also performed an experiment using wings of wild bats that were not exposed to probiotic, whereby the skin tissue is kept partially alive using tissue media in custom explant chambers. Probiotic cells and Pd spores are applied to the explants and the subsequent germination is examined and compared between explants with and without probiotic cells.

METHODS

Probiotic Cocktail

The first *Pd* challenge experiment used two synergistic bacterial strains of *Pseudomonas*. Both were isolated from an adult female *Eptesicus fuscus* caught in a mine outside of Salmo in the Kootenay area of British Columbia. Only one of these were carried through to the next probiotic cocktail and it is now identified as *P. synxantha* B.

The present anti-*Pd* cocktail contains four synergistic bacterial strains from western Canada: *Pseudomonas synxantha* strains *A* and *B, P. azotoformans,* and *P. antarctica*. Each isolate was previously sourced from bats in BC. *Pseudomonas synxantha* strain A was isolated from a *M. yumanensis* at a mine hibernaculum near Salmo, BC; strain B was isolated from an adult female *E. fuscus* captured free-flying at a mine near Salmo, BC (as told above); *P. azotoformans* was isolated from a *Corynorhinus townsendii* adult bat caught at a maternity roost in Deroche, BC; and *P. antarctica* was sourced from a female juvenile *M. evotis* at from a mine hibernaculum near Nelway, BC.

Pd Challenge - Ex vivo wing culture

After application of the probiotic cocktail to captive bats indirectly via treatment of their roosting surfaces (see Chapter 2), wings of euthanized bats were challenged with *Pd* to investigate growth of *Pd* on these previously inoculated wings versus wings of bats that had not roosted on an inoculated roosting surface.

In all experiments, bats were humanely euthanized and separated into control (no probiotic) and treatment (probiotic) groups for fungal *Pd* inoculation. Each wing was inoculated with fungal *Pd* spores as described below. Reduced *Pd* growth on treatment group wings compared to control group wings would suggest previously applied probiotic was inhibiting *Pd* growth.

In Experiment A, after removal from the carcasses, the wings were immediately spread out onto a sterile cork board and pinned using sterile toothpicks within a sealed

bioassay plate (n=12 wings). Two petri dishes were filled with water to generate high humidity and the bioassay plates were placed into a 15^{o}C incubator to simulate wild *Pd* growth conditions. Six wings were used from our trial for testing *Pd* inoculation. Three 'types' of wings were used: wings of control bats, and wings from two types of treatment groups -- one that stopped receiving probiotic several weeks before the end of the trial, and one that received the higher dose of probiotic near the end of our trial. Two sets of these three types of wings were used.

One set of 3 wings were inoculated with 5x10^{5} conidia/mL water suspension on two equal portions of the wing. One portion was scratched with an inoculation loop to promote germination and this was compared to unscratched areas of the wings. *Pd* hyphae are known to penetrate bat wing tissue (Meteyer et al. 2009) and scratching could assist *Pd* growth. The other set of three wings were grown in the same conditions; however, they were placed directly onto SDA agar instead of cork board. Wings were left for one month and examined daily for fungal growth. Observed fungal growth was swabbed with sterile polyester tips and transferred onto SDA plates. It should be noted that the probiotic cocktail present on these captive bats were a pilot blend of two synergistic bacterial strains of *Pseudomonas*.

In Experiment B, I *Pd*-challenged wings of another set of captive bats (euthanized in July 2019; control vs treatment; refer to Chapter 2 for details) that had been inoculated with the final probiotic cocktail blend of four bacteria (see Probiotic Cocktail section above). There were 6 treatment bat wings (n = 6 bats) that were previously exposed to the probiotic that inhibits WNS, and 5 control wings (n = 5 bats) that had not been exposed to the probiotic bacteria. The bat wings were used immediately after necropsy to reduce wing decay as a confounding factor. The other wing that was not used, was for the histological examination (see chapter 2).

Two different methods of *Pd* application were used. The first method used a polyester swab that was rubbed onto a mature *Pd* plate that was established onto Sabouraud Dextrose Agar (SDA) and had uniform mycelia growth. A swab was pushed into the fungus and rubbed uniformly over the plate to collect spores with the swab. The

swab was then rubbed onto a wing. An individual swab was rubbed onto two treatment group bat wings and two control bat wings, for a total of four swabs. With this method, there was an unknown amount of *Pd* added, but hyphae would be added not just spores to ensure sufficient inoculation of *Pd* to the wings.

The second method used a different approach to applying *Pd*, which required application of spores only, not mycelia. Seven different doses of 150,000 spores were added together in Phosphate Buffer Solution (PBS) and vortexed in two intervals at 5000rpm for 10 minutes. Each pellet was diluted with 1ml of yeast extract broth for 5 hours on a 200rpm shaker at 14^{o}C to assist with germination. Spores were vortexed at 7500 rpm for ten minutes after the 5 hours and resuspended with sterile water, twice. Before the spores were applied to the wings, they were vortexed at 7500 rpm for ten minutes and the sterile water was decanted. A sterile swab was pushed into the centrifuge tube and rubbed against the pellet in the tube before being rubbed onto the wings. There were four doses for treatment bat wings and 3 doses were for control bat wings.

Wings were placed into petri dishes and stretched into place with water agar anchor points. The water agar prevented the wing from closing and raised humidity within the plate. Petri dishes containing the wings were sealed profusely with a generous amount of parafilm and placed into a 14^{o}C cooler for one month. After one month the wings were removed and placed into 50ml centrifuge tubes containing PBS solution. The wings were gently vortexed at medium-high for 30 seconds before being sent to McMaster University for qPCR analysis.

In Experiment C, three bats (n=6 wings) from the captive hibernation trial were euthanized and immediately the skin tissue was exposed to *Pd*. These bats had shown substantial growth of probiotic bacteria on their wings during a 35 day hibernation period (see Chapter 2 Results). A new in-house method was used to grow *Pd* without interference from competing mold or fungi. Methodology was changed because of other distinguishable fungi growing on the bat wings previously, presumably due to the slow

growth rate of *Pd.* We developed a new method that attempted to prevent fast growing fungi from outcompeting *Pd.*

Five SDA plates growing a uniform *Pd* mycelium were scraped and blended in saline solution for 30 seconds. The suspension was then centrifuged into concentrated micro doses of 0.2 mL PBS solution containing *Pd* mycelia – defined as a 'dose' for this experiment. A single 'dose' was inoculated onto each treatment wing (n = 6) and a previously frozen severed wing (n = 1) from an untreated bat of the same species. For comparison, as a 'standard positive' for *Pd*, one 'dose' was immediately frozen and sent as a comparison to treated wings. Treated wings were placed into a 14^{o}C incubator for 1 week before being placed into PBS solutions and frozen to stop *Pd* growth. Wings were analyzed by qPCR for growth over the time period.

To analyze *Pd* growth/quantities in control versus treatment wings post incubation, in all experiments, we visually inspected the wings and fungus for similarities to *Pd* using a dissecting microscope before sending for qPCR analysis. Fungi was re-isolated onto SDA and examined for growth rate and morphology. Examination of isolates was compared to *Pd* cultures previously grown on SDA plates.

Pd Challenge - Ex vivo explants

The purpose of the explant experiment was to test the *Pd* inhibition threshold of the anti-*Pd* bacteria -- *Pseudomonas synxantha*, strains A and B; *P. azotoformans*; and *P. antarctica* -- on live bat tissue. Severed tissue explants can be kept partially alive within specialized chambers in an *ex vivo* experiment, thus preventing the associated interference of decomposition (Figure 4.1). Wild captured *M. yumanensis* bats (n=3) were euthanized by overdosing via isoflurane at Thompson Rivers University in Kamloops, BC. Each bat's patagium was separated into 1 cm diameter samples using a biopsy punch to collect full-thickness samples of skin (n=24). Biopsy punches were placed into the individual explant chambers quick enough to ensure constant source of nutrients to maintain cellular activity in skin cells. One side of the explant was exposed to Eagle's modified minimal essential medium supplemented with or without antibiotics (gentamycin, 10 µg/ml).

Explants were separated into two groups, 3 days and 7 days, and two subgroups, with and without gentamycin (Table 4.1). Two lengths of time were used because, due to this being a novel experiment, we were unsure how long the *Pd* would take to grow and/or colonize the skin tissue within the explant chambers. Ultimately, the time periods ended up being 3.5 and 7.5 days to test different timeframes. Seven and a half days was chosen to give the *Pd* enough time to grow but hopefully not be outcompeted by other fungi. Three and a half days was chosen to see if *Pd* numbers grew in quantity and to avoid any competing fungi possibly growing on the explant if left longer.

Samples that were exposed to antibiotic media (n = 13) were compared to samples that did not have any exposure to antibiotic media using qPCR (n=11). Post-experiment, a subsample of the explants (n=5) were examined using Scanning Electron Microscope (SEM) analysis to visualize *Pd* colonization and microscopic interactions between the probiotic bacteria, *Pd* and gentamycin, at the end of the 3 and 7 day trials. SEM samples were also swabbed for qPCR and were used in both the qPCR and SEM groups.

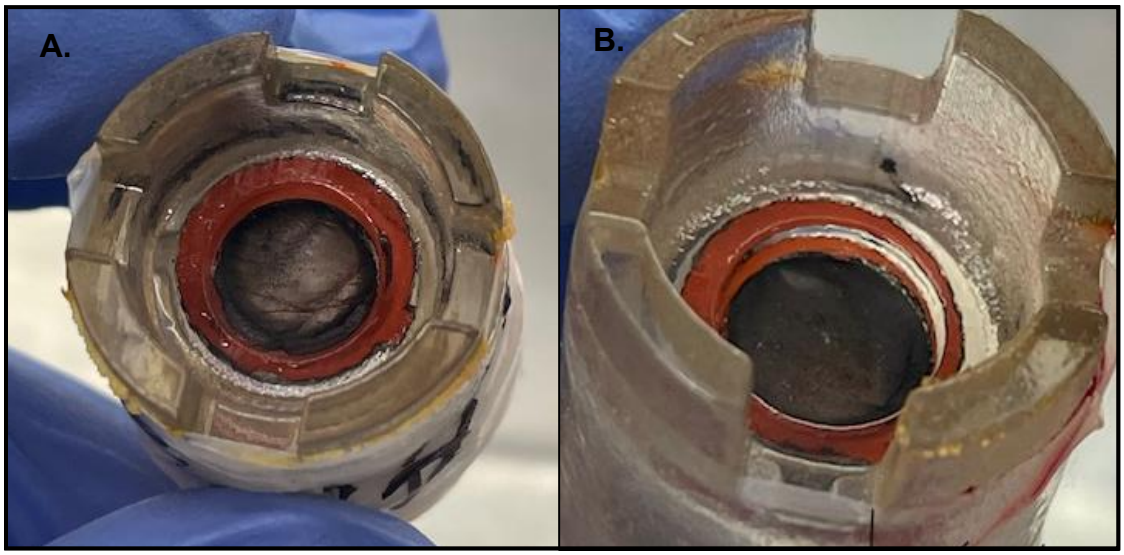

Figure 4.1. Explant chambers that contain separated myotis patagium A) Myotis patagium with antibiotic, *Pd* and probiotic; and B) Myotis patagium with only *Pd* and no probiotic or antibiotics, with white fungal growth evident.

Table 4.1. Number of samples in each category that were exposed to media with antibiotics, media without antibiotics, and sent for SEM analysis. Group 1 consists of samples that had endpoints of 3.5 days, and group are samples with an endpoint of 7.5 days.

	Media with antibiotic (qPCR)	Media without antibiotic (qPCR)	Sent for SEM-Media without antibiotic	Sent for SEM-Media with antibiotic
Add Probiotic (3.5 days)	3	2	0	0
No Probiotic (3.5 days)	2	2	0	0
Probiotic + *Pd* (3.5 days)	2	2	0	0
Pd only (3.5 days)	0	0	1	0
Add Probiotic (7.5 days)	2	1	0	1
No Probiotic (7.5 days)	2	1	0	1
Probiotic + *Pd* (7.5 days)	2	2	0	1
Pd only (7.5 days)	0	1	1	0

Probiotic bacteria were grown in individual flasks of LB broth in a 25^{o}C incubator 4 hours before bat euthanasia. Cell concentrations in each probiotic dosage were as follows: 500 cells of *P. synxantha* strain B, 8500 cells of *P. synxantha* strain A, 200 cells of *P. azotoformans*, and 200 cells of *P. antarctica*. Cell concentrations were estimated using Colony Forming Units (CFU) vs Optical Density (600nm) curves (Figure 4.2). Six hundred nanometer wavelength was used due to the yellow/golden tint of LB broth that cells were grown in. Each inoculated broth was portioned based on CFU curve estimations and centrifuged at 4000 rpm for 5 minutes in a tabletop centrifuge. This process was repeated three times and rinsed with Phosphate Buffer Solution (PBS) to remove any broth from the final dosage. The final probiotic dosage contained all four bacteria species in 20µl of PBS which was pipetted onto the opposite surface of explants either exposed or not exposed to Eagle's modified minimal essential medium.

Pd spores were separated into individual dosages from a larger stock concentration and centrifuged at 10,000 rpm for 5 minutes. The *Pd* spores stock concentration was constituted of PBS with 0.02% Tween 20 to achieve even distribution of spores within inoculum once pipetted onto the explant. Eighteen hundred *Pd* spores were inoculated onto explants to monitor interactions with probiotic bacteria and antibiotics.

Explant chambers were sealed with parafilm and placed into a 14^{o}C incubator. After 0.5 days, half of the surface area of each explant (except the *Pd* control) were swabbed with a sterile polyester-tipped swab before being placed into the incubator. This swab was placed into sterile water and frozen until qPCR analysis. The other side of the explants were swabbed at the end of the experimental periods (3.5 and 7.5 days, for Groups 1 and 2, respectively) to quantify probiotic and *Pd* spore quantity. Swabs were placed into sterile water and frozen until qPCR analysis.

Five explants were sent for SEM analysis: two with *Pd* spores inoculum only with no exposure to antibiotic media over 3.5 and 7.5 days, respectively; one with probiotic inoculum only and exposed to antibiotic media over 7.5 days; one with no inoculum and was exposed to antibiotic media for 7.5 days; and one with probiotic and *Pd* inoculum and was exposed to antibiotic media for 7.5 days. The quantitative PCR was conducted using the previous methodology in this thesis (See chapter 2 and 3 methods).

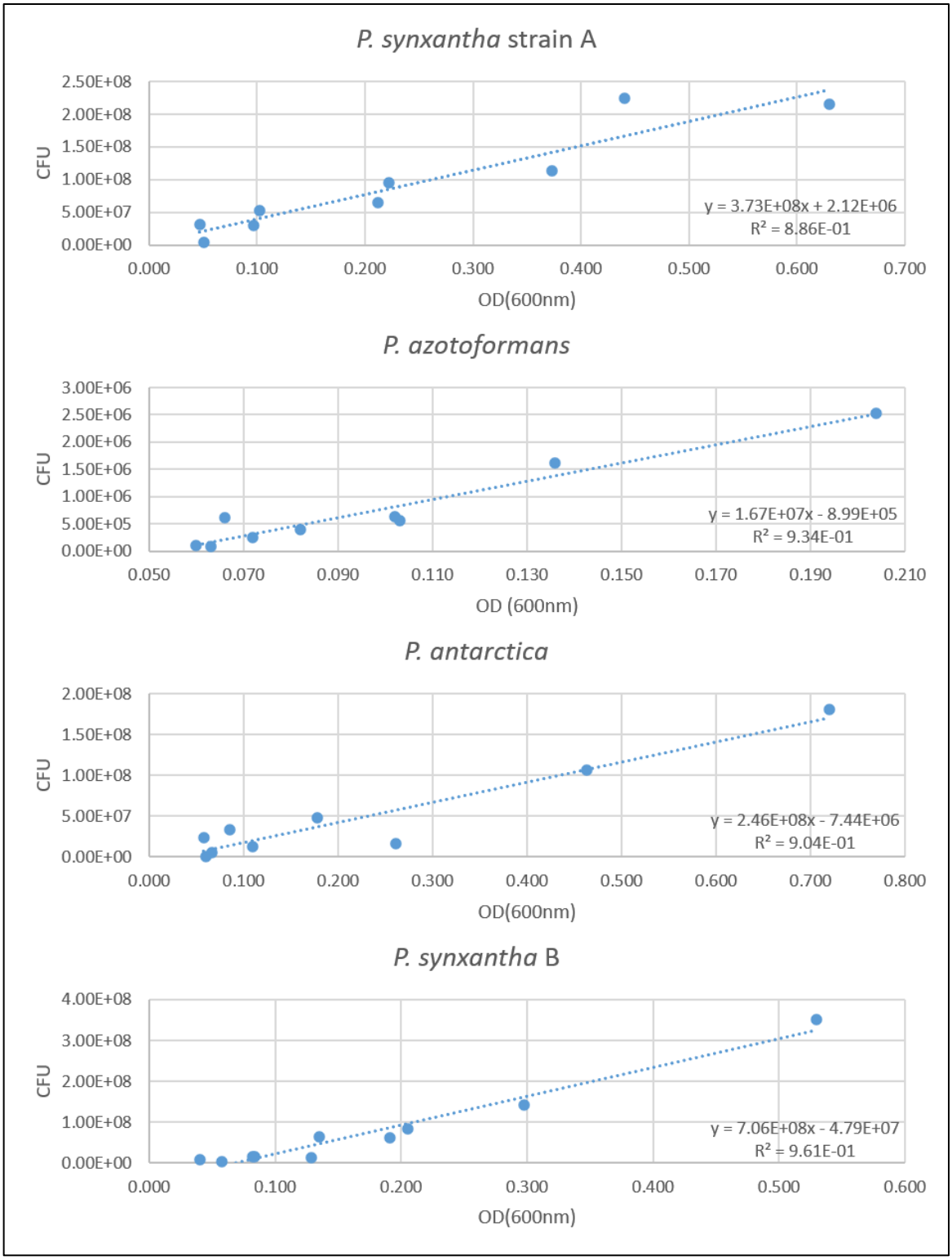

Figure 4.2. Plotted CFU versus OD at 600nm of *Pseudomonas synxantha*, Strains A and B; *P. azotoformans*; and *P. antarctica* when grown in LB broth at 25ºC.

RESULTS

Ex vivo Wing Cultures

Neither treatment nor control group wings exhibited any significant *Pd* growth in experiments A, B and C. This was determined through all methods of evaluation: visual examination, examination under a microscope, and through qPCR analysis of mycelia DNA quantity. Other species of fungi grew on the wings that were different visually and morphologically different from *Pd*. Fungi isolates exhibited rapid, irregular growth once they were plated onto SDA agar and visualized for growth rate and morphology. I concluded that bat wings plated onto SDA agar exhibited an increased number of bacteria and fungi growth that were not associated with our experiment. As this was first noted in Experiment A, a revised protocol was used in Experiment B, as described in Methods, to minimize the likelihood of other microbes dominating the bat tissue, however, this too was unsuccessful – *Pd* growth on both groups of wings was minimal and other forms of unidentified fungi and mold were present (Figure 4.3).

Figure 4.3. Separated bat wing from a control bat one week after *Pd* inoculation using the first application method, in which *Pd* was swabbed onto the bat tissue. Obvious fungal growth is evident that does not represent *Pd*.

The germination of *Pd* spores was minimal despite having grown *Pd* spores in yeast broth before inoculation. One bat in Experiment B exhibited increased number of *Pd* mycelia (L Metal R Grey individual; Figure 4.4), however, this was the only sample. In Experiment C (Figure 4.5), qPCR results suggests that *Pd* either degraded on the wing tissue or, more likely, sampling and qPCR procedures interfered with the analysis. The frozen 'dose' was approximately the same number of *Pd* that was placed onto each wing in the frozen, control and treatment groups. Therefore, measuring the number of *Pd* in the mycelial sample in PBS may have worked much better in comparison to sending the entire wing submerged in PBS in for analysis. This major difference in quantity between the mycelial sample and wing samples is likely attributed to the sampling procedure.

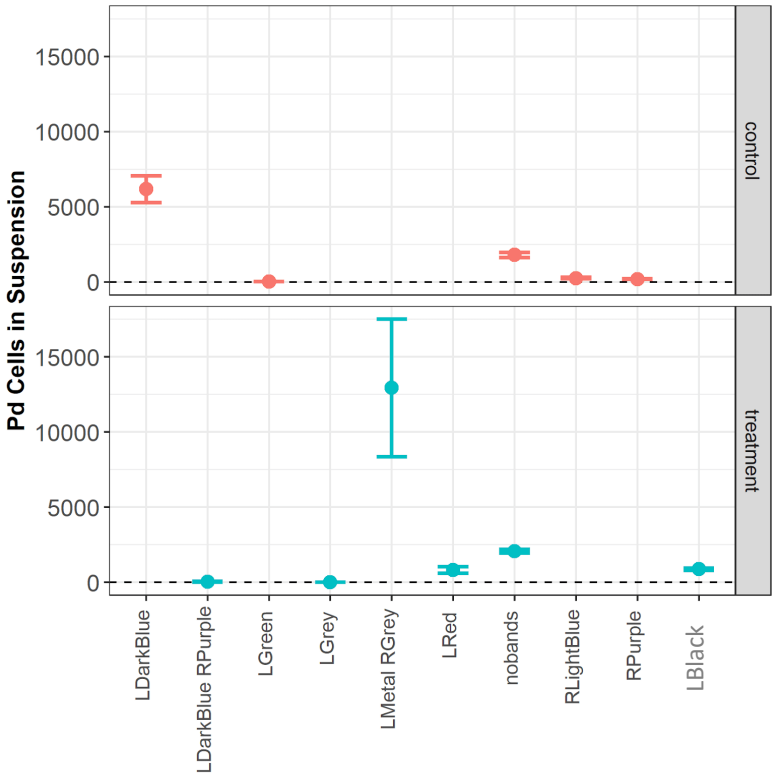

Figure 4.4. Number of *Pd* mycelia detected on control and treatment wings from the summer trial. The x axis is each individually identified captive bat. There were two bats designated as "nobands", one in the control group and one in the treatment group. Figure prepared by Adrian Forsythe.

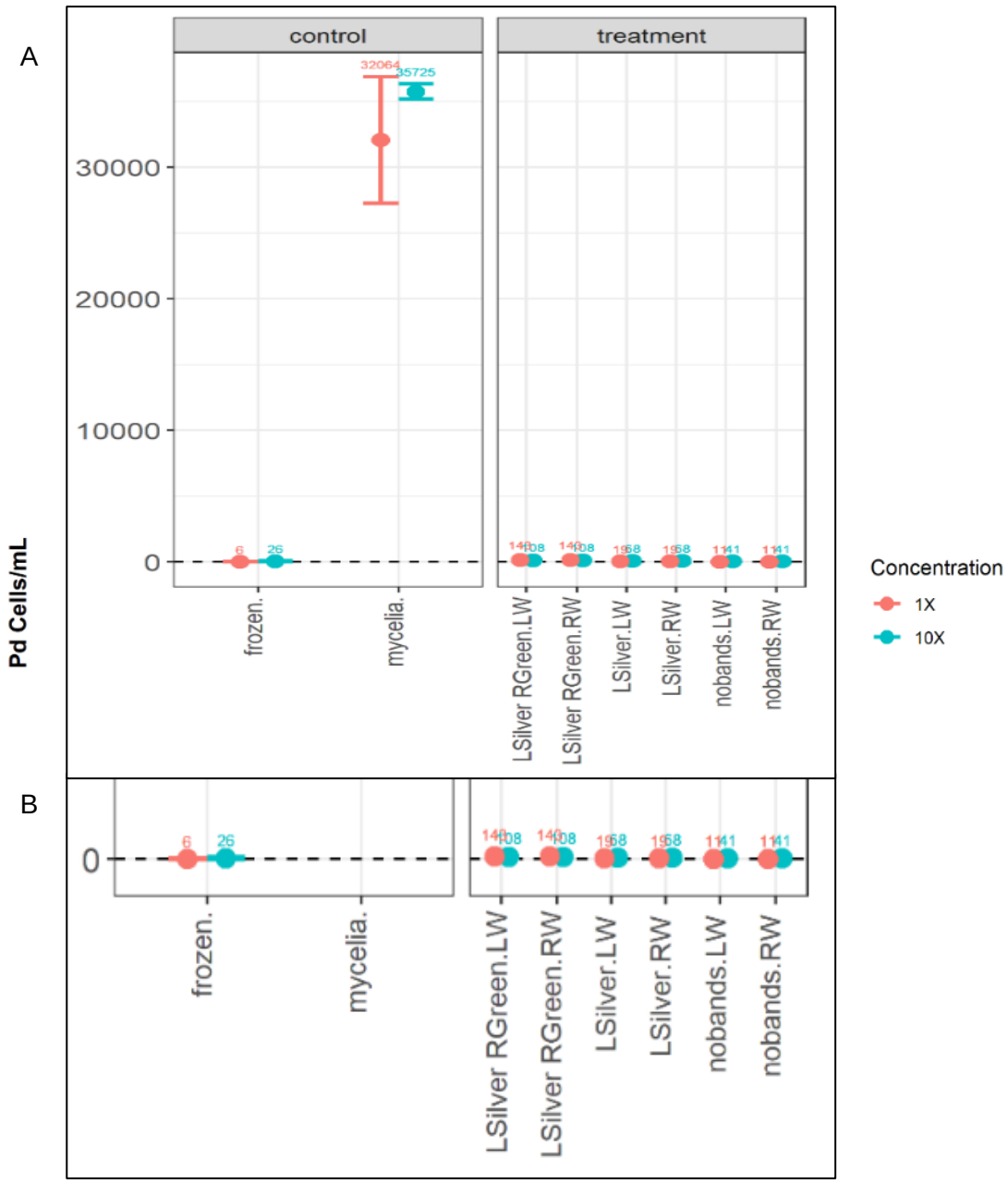

Figure 4.5. Average *Pd* mycelia concentrations on the treated bat wings and controls. A wing from a previously frozen bat of the same species was used as a control and dosed with the same concentration as the treated wings (frozen). The sample 'mycelia' is not a wing sample, but the *Pd* stock solution (standard) used for comparison. Suspensions were concentrated 10x in an attempt to quantify lower values. Picture B is a zoomed in version of A. Prepared by Adrian Forsythe.

Ex vivo Explant Results

Pd Quantities

Quantitative PCR Cq results were compared between the 3.5 and 7.5 day groups and the subgroups that did or did not use antibiotics when inoculated with either *Pd* alone, probiotic alone*, Pd* and probiotic, or nothing (Table 4.2). All samples exposed to antibiotic medium had inconsistent results and/or showed little to no *Pd* or probiotic in the qPCR analysis. This suggests that gentamycin antibiotic destroyed *Pd* spores and probiotic cells on the explants, ultimately resulting in DNA degradation of these cells and inconclusive qPCR results. Inoculated samples in both Groups 1 and 2 provided evidence of *Pd* spores upon first swabbing. However, at the termination of each experiment, 3.5 and 7.5 days later, respectively, qPCR of swab samples revealed no *Pd* was evident (Tables 4.2).

Swabs of wing tissues with probiotic that were not exposed to antibiotic were more consistent. *Pd* concentrations in Group 1 (3.5 days) decreased when exposed to bacteria inoculation on the same surface. The *Pd* Cq numbers changed from 37.75 to 38.44, and from 38.16 to 38.19, signifying decreases in the amount of *Pd* DNA present on these two explants (Table 4.2). One of the explants in Group 2 (7.5 days) produced similar *Pd* reduction (Cq from 36.5 to 37.0; Table 4.2). The other explant changed in Cq values from 36.72 to 39.54, signifying a massive increase in *Pd* concentration. The inconsistencies observed may be largely due to swab sampling error, confounded by small sample sizes. Some contamination of *Pd* was evident among some of the explants and could largely be due to the explants sharing the same incubator.

The *Pd* control was not exposed to antibiotic and was swabbed after 3.5 days and 7.5 days, demonstrating a higher detection count (lower Cq) after 7.5 days than at 3.5 days (Table 4.2). Notably, although only a single sample, the Cq concentration of *Pd* is highest in this control sample compared to all other explant samples.

Table 4.2. Baseline and endpoint swab sample Cq results of *Pd* from the Group 1 and 2 explants. Inconsistent or unexpected results are highlighted in yellow.

With antibiotic			**Without antibiotic**		
Group 1	Cq after 0.5 days	Cq after 3.5 days		Cq after 0.5 days	Cq after 3.5 days
With probiotic (A)	30.44	0	With probiotic (A)	0	0
With probiotic (B)	0	0	With probiotic (B)	0	0
With probiotic (C)	0	0			
With no probiotic (A)	0	35.7	With no probiotic (A)	0	37.74
With no probiotic (B)	0	0	With no probiotic (B)	0	0
Probiotic + *Pd* (A)	38.5		Probiotic + *Pd* (A)	37.75	38.44
Probiotic + *Pd* (B)	39.49		Probiotic + *Pd* (B)	38.16	38.19
			Pd Only		37.36
Group 2	Cq after 0.5 days	Cq after 7.5 days		Cq after 0.5 days	Cq after 7.5 days
With probiotic (A)	0	0	With probiotic (A)	0	39.02
With probiotic (B)	0	0			
With no probiotic (A)	0	0	With no probiotic (A)	0	0
With no probiotic (B)	32.79	0			
Probiotic + *Pd* (A)	0	0	Probiotic + *Pd* (A)	36.5	36.99
Probiotic + *Pd* (B)	40.4	0	Probiotic + *Pd* (B)	39.54	36.72
			Pd Only		37.26

Probiotic Quantities

Each of the three probiotic bacteria, *P. azotoformans, P. synxantha* strain A, and *P. synxantha* strain B were analyzed for each of the two experimental groups: 0.5 day baseline, 3.5 day endpoint, and 7.5 day endpoint, with and without antibiotics. Results for the fourth bacteria, *P. antarctica*, are largely inconsistent due to difficulties with custom gyrB probe for this species not annealing and thus resulting in poor DNA amplification. A change in probiotic Cq values could be calculated for 8 explants only due to failure of some samples to amplify DNA. Six of these 8 samples showed an overall decrease in probiotic cells from the start of the experiment to the end of the

experiment (ie. Cq values increased; Tables 4.3 and 4.4). Two explants, one with (3.5 day group) and one without (7.5 day group) *Pd* inoculation, showed an overall increase in probiotic cells during the course of the experiment.

In conclusion, I saw many inconsistencies in the explants, most notably those exposed to the gentamycin antibiotic, similar to the results of the *Pd* qPCR results above (Tables 4.3 and Table 4.4). However, other inconsistencies point towards additional methodological concerns such as small sample sizes, sources of cross-contamination of *Pd* spores, sampling biases (possibly due to adherence of cells on swabs and small surfaces being swabbed).

Table 4.3. qPCR results of *P. azotoformans, P. synxantha* strain A, *P. synxantha* strain B, and *P. antarctica* from the 3.5 day group 1. Inconsistent or unexpected results are highlighted in yellow.

Group 1 Cq values from qPCR								
	Starting Point (qPCR 0.5 days) with antibiotic				**Endpoint (qPCR 3.5 days) with antibiotic**			
	P. synxantha B	*P. synxantha* A	*P. azotoformans*	P. *antarctica*	*P. synxantha* B	*P. synxantha* A	*P. azotoformans*	P. *antarctica*
With probiotic (A)	0	0	0	0	0	0	0	0
With probiotic (B)	33.61	35.81	33.81	37.37	38.96	38.98	38.34	0
With probiotic (C)	38.09	39.64	38.97	40.58*	40.08	42.34	40.01	0
With no probiotic (A)	44.95	0	38.37	0	0	0	0	0
With no probiotic (B)	0	0	0	0	0	0	0	0
Probiotic + *Pd* (A)	0	0	0	38.62	25.27	27.71	25.53	27.5
Probiotic + *Pd* (B)	37.2	38.79	37.93	39.82	35.29	37.27	35.5	36.55
	Starting Point (qPCR 0.5 days) without antibiotic				**Endpoint (qPCR 3.5 days) without antibiotic**			
With probiotic (A)	38.78	39.88	39.07	0	0	0	0	0
With probiotic (B)	35.21	36.98	35.46	39.56	38.86	39.01	38.99	38.26
With no probiotic (A)	0	0	0	0	0	0	0	0
With no probiotic (B)	0	0	0	0	43.94	0	40.28	0
Probiotic + *Pd* (A)	36.83	38.5	37.15	0	0	0	0	0
Probiotic + *Pd* (B)	36.52	38.08	36.92	38.62	36.87	38.42	37.34	41.11

Table 4.4. qPCR results of *P. azotoformans, P. synxantha* strain A, *P. synxantha* strain B, and *P. antarctica* from the 7.5 day group 2. Inconsistent or unexpected results are highlighted in yellow.

Group 2 Cq values from qPCR								
	Starting Point (qPCR 0.5 days) with antibiotic				**Endpoint (qPCR 7.5 days) with antibiotic**			
	P. synxantha B	*P. synxantha* A	*P. azotoformans*	P. *antarctica*	*P. synxantha* B	*P. synxantha* A	*P. azotoformans*	P. *antarctica*
With probiotic	42.11	43.9	42.64	0	0	0	0	0
	37.95	39.55	38.72	0	41.49	0	42.04	42.12
With no probiotic (A)	0	0	0	0	40.92	42.24	42.02	38.73
With no probiotic (C)	0	0	0	0	41.29	42.79	42.1	0
Probiotic + *Pd* (A)	0	0	0	0	36.24	38.53	36.88	0
Probiotic + *Pd* (B)	37.74	39.36	38.24	37.88	0	0	0	0
	Starting Point (qPCR 0.5 days) without antibiotic				**Endpoint (qPCR 7.5 days) without antibiotic**			
With probiotic (A)	38.97	40.82	38.82	42.05	23.71	25.75	24.11	31.66
With no probiotic (A)	0	0	0	0	0	0	0	0
Probiotic + *Pd* (A)	37.2	38.53	37.77	45.04	37.93	39.32	38.52	0
Probiotic + *Pd* (B)	36.9	37.86	36.88	0	40.68	42.35	41.24	0

DISCUSSION

Here we tested methods of inoculating *Pd* onto both dead and live bat tissue in multiple experiments, as well as testing interactions between the anti-*Pd* probiotic bacteria and *Pd*. We found that there were inherent difficulties with utilizing dead tissue as a replicate for *Pd* infection and other variables may have impeded the results. Using live wing tissue explants allowed us to hurdle some of the dead tissue complications and *Pd* was more easily detectable by qPCR and did not have other competing molds to interact with. However, low cycle numbers were evident and likely due to inadequate sampling procedures. Each experiment provided insight into what *Pd* inoculation studies should consider before an experiment begins.

Wings that were inoculated *ex vivo* produced few spores or mycelial growth when analyzed by qPCR. *Pd* was difficult to visualize based on the fast growth of competing decay molds. Natural microbes on the wing grew faster on the decaying tissue and out competed *Pd* even while growing in optimum *Pd* growth conditions of 14^{o}C and high humidity. A growing fungi was easily distinguishable as not being *Pd* when it was isolated into subculture, based on its morphology and fast growth within 1-2 days compared to *Pd* which naturally takes at least 3-5 days to see any growth on SDA. Spores were grown in yeast extract to promote mycelia growth before inoculation still could not out compete decaying molds on the bat wings. Utilizing blended *Pd* mycelia was successful on frozen bat wings and SDA agar in previous trials (Not shown here), however it was unsuccessful on the final set of bat wings from the hibernation trial. There was some slow growing white fungal growth seen on the bat wings before submerging it into PBS for analysis. *Pd* mycelia were detected in the PBS solution control in the correct concentration however it was not detectable on the severed bat wings when the submerged PBS was tested for *Pd*. This is likely due to interference from skin tissue preventing qPCR probes from detecting *Pd*, or difficulties with sampling and extracting the spores from the large suspension in which the wing was submerged in. Future efforts would need to consider utilizing a swab or smaller wing sample for qPCR, histological cross examination, or SEM methods to better quantify growth and

colonization of wing tissues. Furthermore, although *Pd* mycelia can grow quickly on frozen wings, this dosage does not reflect natural *Pd* infection in wild bats regarding concentration and mechanism (Makanya et al 2007; Meteyer et al. 2009). Finally, dead wing tissue is considerably different than live tissue in terms of growth conditions. Sebum and skin composition, which are crucial factors for skin microbes, are particularly different in dead tissue than live tissue and do not replicate typical growth medium for *Pd* (Wei et al. 2020).

Non-antibiotic results from the explant experiment suggest that there was some interaction between *Pd* spores and anti-*Pd* bacteria. Both “Probiotic+*Pd*” explant samples from group 1 that were not exposed to antibiotic decreased in *Pd* concentration in relation to the *Pd* control, which instead increased. This could signify that probiotic bacteria were antagonistic towards *Pd* growth. *Pd* concentration in one sample increased in group 2 by nearly 3 fold (“Probiotic+*Pd* (B), without antibiotics”), however bacteria concentration was quite low in comparison to other explants after 7 days. We failed to detect any probiotic after 3.5 days on samples “With probiotic (A)” and “Probiotic+*Pd* (A). *P. antarctica* was largely the most inconsistent result of all the probiotic bacteria and was not detected when other bacteria were. This can be seen in nearly all samples of probiotic+*Pd* in group 2. Low Cq numbers are likely stemmed from swab sampling and inadequate pick-up of spores and/or probiotic cells. Due to the design of the explant chamber and low number of cells and spores involved, swabbing was likely an inferior method of extraction compared to utilizing the entire explant submerged in PBS for qPCR.

Antagonism is still highly suggestive from every other sample exposed to probiotic bacteria decreased in *Pd* concentration. It is difficult to draw conclusions from qPCR bacteria results, however nearly all samples decreased in probiotic concentration when compared to their baselines. One sample from group 2 increased nearly 14 fold (“Probiotic (A)”), however this is likely due to sampling or inoculation error. Some samples had probiotic and *Pd* contamination which may have led to further ambiguous results. Future experiments should consider separate incubators to prevent this.

A similar experiment by Cornelison et al. in 2014b saw inhibition of *Pd* growth on explants that shared the same airspace as anti-*Pd* bacteria. In our experiment, each explant chamber was sealed with parafilm and inoculated on the same surface instead of different surfaces. The anti-*Pd* bacteria in our study has been shown to inhibit *Pd* on cultured agar, presumably through naturally released antibiotic metabolites such as 2,4-Diacetylphloroglucinol (Delany et al. 2000; Bangera and Thomashow 1999). Bacteria were inoculated onto the surface of the explant before their growth phase and exponential growth to encourage its anti-*Pd* properties. Furthermore, the only explant in which we saw any fungal growth was on the control 3.5 and 7.5 day explants, in which a slow growing white fungus was forming without any probiotic interaction. This is encouraging to state that the inoculated probiotics may have impeded *Pd* growth however without a larger sample size it is difficult to make any assumptions from our results.

Our results also suggested that utilizing antibiotic's to prevent unwanted bacteria infection and mold growth were not necessary from our observations. No mold or visible morphological change of the explants occurred and explants that were not exposed to antibiotics were indistinguishable from ones that did. Furthermore, many qPCR results were ambiguous from explants that were exposed to antibiotic media for both the 3.5 and 7.5 day groups. Although we were very diligent in attempting to prevent antibiotic exposure to the inoculum surface and did not visibly see leakage, there is a possibility that gentamycin antibiotic perfused through the epithelial matrix and interacted with both the probiotic and/or *Pd* inoculum. Future experiments should consider avoiding antibiotics unless necessary to prevent this interfering variable.

We did not test for skin viability once the experiment concluded for both groups of explants. Although skin had the same texture and no signs of decay were present, we did not conclude this with a cross section and/or histological examination. No decay mold and similar feel to a live bat patagium may hint at live skin tissue. Future experiments should also consider utilizing a larger sample size for more robust results. Here, nearly half our explant samples were unusable due to antibiotic interference. We

would recommend using a larger sample size to monitor *Pd* and probiotic interactions on the live explant tissue.

In conclusion, there are many obstacles and variables to consider when inoculating *Pd* onto bat tissue for *ex vivo* experiments. Refining *ex vivo* experiments are important considerations when live bats are not available, or researchers do not want to replicate the rigorous methods of hibernating wild animals. Here we found that live tissue explants can be an encouraging possibility for future *Pd* inoculation experiments. Researchers need to consider the difficulty of using explant chambers and the forewarning of small sample sizes as seen here. Experiments should seriously consider not using antibiotics to prevent unwanted variables. We did not see any visual difference between explants that were or were not exposed to antibiotics, however qPCR results were mixed and ambiguous. The explant experiment was novel; however it has tremendous potential for future WNS research given enough refinement to its methods.

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Chapter 5. CONCLUSION

This thesis covered three aspects of implementing an anti-*Pd* probiotic to fight WNS: Captive trial testing, field trial testing, and *Pd* challenge lab experiments.

Following two separate captive trials, the probiotic has been shown to have no detrimental effects to bats, and can easily be transferred to bats indirectly using a mixture of clay powder and freeze-dried bacteria, a method of application that I developed. Probiotic bacteria were persistent on captive bats and bat boxes months after application, including when exposed to high summer heat in the 40-50^{o}C range. Although viability of cells following these extreme temperatures has yet to be tested, *P. synxantha* A showed signs of proliferating in the bat boxes. Probiotic cells grew substantially, confirming viability when exposed to hibernation conditions.

The field trial was on-the-ground evidence that probiotic can be successfully transferred from inoculated human-made structures onto wild bat wings. Wings of wild *Myotis lucifugus* and *M. yumanensis* bats roosting in bat box or building roosts at our study sites had detectable amounts of the probiotic species used in our probiotic cocktail, following inoculation of their roosting substrates.

While the probiotic cocktail has performed well *in vitro* against *Pd*, it has yet to be properly tested to quantify its efficacy *in vivo* in the lab and in the field when bats hibernate in *Pd* infected sites. Through multiple experiments, I determined that *Pd* does not grow on dead bat tissue, presumably due to competition from fast growing molds. Explant experiments were an improvement because they utilized live tissue; although challenging and requiring euthanizing of live bats, explant chambers present a promising tool for answering the question of *in vivo* efficacy of probiotic against *Pd* germination. However, low sample sizes of the pilot that I performed made it difficult to make conclusions. Refined methodology including using separate Bio-safety Cabinets (BSC) will help reduce cross-contamination; more euthanized bats will enable larger sample sizes; direct DNA extraction of tissue explants instead of swabs may reduce high variability; histology examination will enable viability testing; and inoculating probiotic bacteria one day before *Pd* could encourage bacteria inhibition of *Pd.*

In the field, realized reduction of WNS-caused mortality will not be possible until the bats in our study area are exposed to *Pd* in winter hibernation. Each spring *Pd* surveillance efforts are ongoing, and automated PIT tag readers track return rates of bats after hibernation allowing for these to be compared between Control and Treatment sites.

There were many challenges associated with this project. It is near impossible to replicate a roost in a captive setting without an extensively large enclosure and minimal disturbance. Stress and capture myopathy are unpredictable variables that may prevent desired results due to unprecedented events such as weight loss, un-natural behaviour, and death (Jung et al. 2002). These factors were evident when conducting the captive trial experiments, in which some bats stopped eating and/or were not able to participate in the trial further. Most experiments had an acceptable sample size however the hibernation experiment, which is arguably one of the most important experiments in this thesis, falls short in sample size because of our bats falling victim to environmental conditions and stress from captivity. Further experiments should heavily consider these variables and start with a larger sample size to offset any unpredicted losses.

In particular, the captive trial in 2019 (Chapter 2) had limitations such as number of assistants, space to construct enclosures, number of bats, having to develop an application method, problems with the *P. antarctica* primer, and using a novel study design. This led to smaller sample sizes over a shorter period than what we would have preferred. These problems resulted in an irregular study design that was not well suited to comparing treatment 1 to treatment 2 groups over different time periods, although comparing these two groups was not an objective. What would have been useful, in hindsight, was to have had a 3rd treatment group which could have received one dose of probiotic with exposure for a few weeks only, and then swapping of the bat boxes to see how long the bats retained probiotic on their wings. While this was done at the end of the captive trial, it was cut short after a few weeks to apply probiotic to the bats that would then enter into the hibernation experiment. If a second hibernation fridge and experiment could have been conducted, it would have been interesting to see how the

probiotic increased from small concentrations present, more closely simulating what might be the case if a bat in the wild receives probiotic application from its late summer roost, and then leaves to transition roosts for a few weeks or months prior to hibernation.

The study design could have been improved by utilizing separate treatment enclosures in different locations with their own control enclosures also in different locations (to separate treatments into their own groups better), to ensure absolutely no cross-contamination. Treatments could have occurred at similar times and tested different concentrations or more frequent dosages. A larger sample size of bats would have been beneficial for more rigorous comparisons, but was limited by permitting. Similarly, testing the probiotic on additional species of bats was not possible due to permitting, but may have been interesting to compare results.

Finally, we did not look at the metagenomics of the bat skin. In the future, researchers should strongly consider looking at the entire microflora of the inoculated bat species, to examine the shifts in their microbiome from introducing the probiotic species bacteria. While we attempted to do this, our samples contained small samples of DNA that were not easily analyzed. Some results from the metagenomic sampling may be forthcoming, but were not included in this thesis.

Researchers have explored many routes to prevent WNS infection in hibernating bats. Chitosan, polyethelene glycol, *P. fluorescens* species complex bacteria, *Rhodococcus rhodocrous*, *Trichoderma* sp, terbinafine, vaccines, valencia orange oil, and propolis have all been considered for preventing *Pd* infection, as summarized in a paper by Hoyt et al. (2019). Similarities are evident between every manuscript because they overlap with objectives of developing an effective method of preventing *Pd* germination. This can otherwise be said in the general hypothesis, “If I apply X to this media (or bat) then *Pd* will not grow”. Two field trials using bacteria from the *P. fluorescens* species complex have already been successful (Cheng et al. 2016; Hoyt et al. 2019). The study by Hoyt et al. (2019) provides strong evidence that bats can be augmented to survive overwinter despite exposure to *Pd*. They found that bats

inoculated with anti-*Pd* bacteria from the *P. fluorescens* species complex emerged from hibernation later with a five fold increase in survivability. The study by Cheng et al. suggested that bats must be inoculated upon exposure to *Pd* but still provided evidence of certain bacteria within the *P. fluorescens* species complex lowering disease severity when *Pd* is present. Current knowledge and proposals of fighting *Pd* infection in wild bats are consistently changing however anti-*Pd* probiotics have been considered the most promising method, particularly in British Columbia where hibernacula are not known and preventative measures are needed instead (Fletcher et al. 2020; Weller et al. 2018). Probiotics are beneficial because anti-*Pd* bacteria can be isolated from bat wings and reintroduced into other local populations with little risk to or the bat's health or natural microbiota (Thomas and Willis 1998). *E. fuscus* and some persisting populations of bats that survive WNS are known to have an abundance of anti-*Pd* microbiota that can prevent *Pd* infection during hibernation (Langwig et al. 2017; Lemieux-Labonté et al. 2020). I am hopeful that the anti-*Pd* bacterial cocktail described in this thesis can be applied as a prophylaxis to bats in BC to reduce overwinter WNS mortality rates bats from this province come in contact with *Pd*.

Bats are an important contributor of downwards pressure on arthropods, specifically nocturnal flying insects such as mosquitos, moths, and beetles, and are considered one of the most important predators of nocturnal flying insects (Nagorsen 1995). Adult little brown females eat thousand of insects a night (Anthony and Kunz 1977; Kurta et al. 1989; Nagorsen 1995). Studies have shown that exclusion of bats dramatically increase the abundance of insects prevalent on plants (Kalka et al. 2008; Williams-Guillen et al. 2008), sometimes 2 fold. Boyles et al. (2011) estimated the value of insectivorous bats to be 3.7-53 billion USD a year in North America. Cost savings are due to decreased use of pesticides and applications needed to fight resistance in agricultural pests. This is worrying, because White-nose syndrome, among other conservation threats such as wind turbines, roads, habitat fragmentation and loss, and deforestation have incredibly devastated bat populations (Berthinussen et al. 2014). White-nose syndrome is responsible for over 6 million bat deaths and recently emerged in Washington state (Frick et al. 2010; Frick et al. 2015; WNS 2019). Extinction risk for

many species is real and often the result of emerging infectious diseases (Daszak 2000; De Castro and Bolker 2004).

The overarching conservation goal of this project was to prevent, or lessen, the number of deaths caused by WNS once it arrives in British Columbia. This project is unique in all of North America as there are no other studies of using probiotic as a prophylaxis applied at maternity roosts. Much of the methodology employed in this project, and use of bio-augmentation via probiotics is novel (Thomas and Willis 1998). This work has established a foundation for other researchers to replicate and build upon.

Future research directions are limited in preventing and/or stopping WNS from causing mass mortalities in western hibernating bat species. Studies should consider two goals, 1. Build on the preventative measures that we suggest in this thesis, and 2. Urgently increase bat population monitoring and assess hibernacula for signs of WNS. Bats mostly hibernate in small clusters in the western North America (Weller et al. 2018) making it difficult to locate bat hibernacula. Here we propose a solution using bat boxes and man made structures to transfer anti-*Pd* probiotic onto roosting bats. However, this may limit the application potential of rural areas where bat boxes may be impossible to maintain or dose properly. Ideally, we would want to apply our probiotic to known areas of hibernacula and monitor for *Pd* and probiotic levels. Because of the short time frame, incredible difficulty and sparse groupings, it is likely impossible to directly apply the probiotic to bat hibernacula. Refining a preventative measure is the only plausible option currently available. Bat monitoring should continue with the thought of direct hibernacula inoculation, but not relied on. Future field trials would benefit from a repeated, and higher dosage of anti-*Pd* bacteria on bat boxes in October with minimal disturbance to the roosting colony. It would be beneficial to investigate treatment frequency and dosages needed to maintain a viable concentration of bacteria that release the anti-*Pd* metabolites needed to prevent WNS infection. Further, if a nutrient supplement that stimulated *pseudomonas* metabolite production was proven safe in another captive trial, perhaps it could be added to the final prophylactic cocktail (Duffy and Défago 1999).

There are many different research directions to take in preventing WNS in western bat species and time is running out. Researchers will likely have to compromise on the best possible approach with whichever prophylaxis they finalize on and where to apply it. WNS will inevitably be found in British Columbia and it is up to researchers to decide on what is the best possible approach to prevent mass bat mortality.

Studies performed in this thesis were in collaboration with the BC Ministry of Agriculture, McMaster University, UBC Okanagan, and Wildlife Conservation Society Canada. Capture information came from Leah Rensel of UBCO, and the qPCR results which I present in many tables and figures were generated by Adrian Forsythe, McMaster University.

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APPENDIX A. Synergistic Testing of Anti-*Pd* Isolates

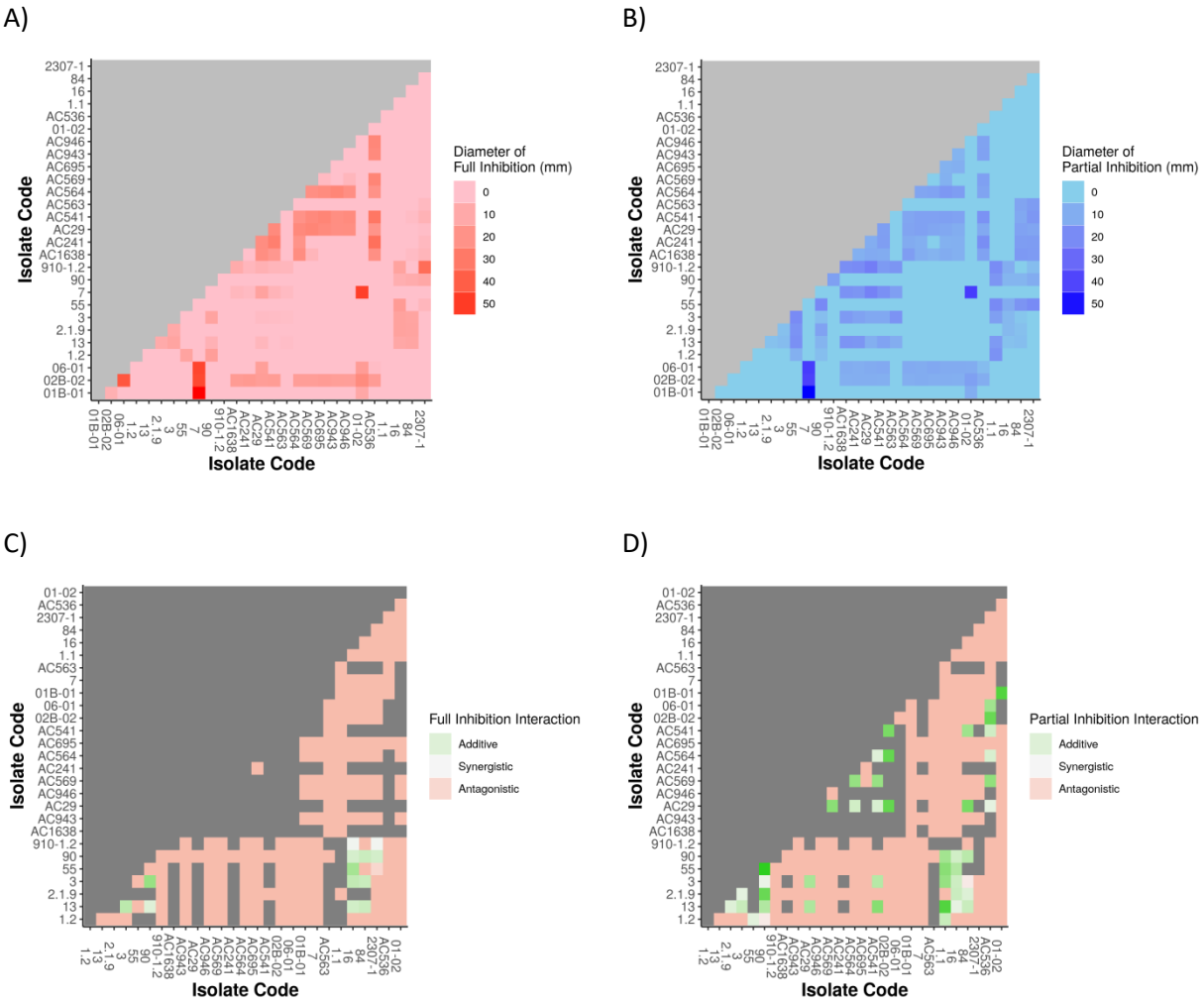

Figure A.1. Inhibition of *Pd* in pairwise combinations A) measuring full zone of clearing; B) partial zone of clearing; C) Fractional Inhibitory Concentration Index for full inhibition; and D) partial inhibition. Figures prepared by Adrian Forsythe.

APPENDIX B. Animal care

Summer 2019 Trial

Twenty *M. yumanensis* were used in our captive trial, and were taken from Creston, Kuskanook, Chase, and Lardeau areas in British Columbia. Bats were brought into captivity in two batches. The first batch of 15 bats were captured within two days apart and were progressively placed into small, medium and large cages. Each bat was banded with one or two coloured bands on their forearms to differentiate them from each other. They were taught the principle of self feeding (mealworms) and self-drinking out of dishes throughout the acclimation process. This was performed by having mealworms and a water dish present within the mesh chamber for several days. Similarly to our fall trial, bats were manually fed mealworms and water for the first few days, with close observation on subsequent nights to ensure they are each drinking and eating without human intervention (observing colored bands). Bats were released from the large mesh enclosure and placed into our control enclosure for further acclimation. They were monitored daily for weights until evidence of self feeding and watering were shown. The amount of food and water accepted by each bat was recorded daily. Food stations consist of half aluminum foil cupcake tins filled with mealworms held in place by wooden boards to prevent shifting if the bats climbed into the food dishes. A water station was a dish kept topped up with an upside down bottle of water, and each dish was filled with marbles and had sponges on top which were regularly changed; these measures were taken to ensure sufficient water and prevent accidental entrapment or submersion of bats. Additionally, a mesh overlay on one end allowed bats to easily land above the water dish and climb down to approach its surface or the soaked sponges. Once the bats learned how to feed and water on their own, hand feeding and watering were not required. Bats were separated into Control and Treatment 1 groups when they were comfortable living within captivity, sustained their weight, and independently drank water. Mealworms were fed the same rich Omega 3 fatty acid diet used in the fall captive trial. This diet is a better approximation of one consisting of wild caught insects. The second batch of 5 bats were introduced into the Treatment 2 group at a later date with the same protocol followed as above.

Bats were monitored daily within their enclosure and were visited on a consistent time schedule each day. Little time was spent within the enclosure and noise was kept to a minimum to prevent disturbance and stress to the bats. They were removed from their bat house once a week for a health inspection by a registered Veterinarian Technician at the Kamloops BC Wildlife Park. Bats were placed into cloth bags and individually weighed before looking for details such as behaviour, body condition, injury, dehydration, and the condition of the eyes.

Hibernation Trial

The bats were introduced to the beverage cooler and it was set at 12-13^{o}C, mimicking outside temperatures. Humidity was immediately introduced at >90% with mealworms, water and a roost pouch readily available. Each subsequent day, the temperature was dropped by 1^{o}C until it reached 7-9^{o}C. Bats were taught the principle of self-feeding and self-drinking out of a water dish during this time. Bats were manually fed mealworms and water for the first few nights, and closely observed on each subsequent night to ensure they are eating and drinking without human intervention. Each bat's weight was monitored for weight loss or gain to presume self feeding. Once the optimum temperature was reached and bats had a steady weight of >6.0g to survive hibernation, we removed the mealworms and the roost pouch to entice hibernation. Two weeks later the temperature was dropped by another 1^{o}C to approximately 6-8^{o}C to further entice the bats to hibernate. Bats were meticulously monitored throughout the acclimation process and were checked daily with a Bluetooth infrared camera placed inside the fridge. Past and current bat activity was examined with the camera and identified with a coloured forearm band previously attached onto their wing. A red light was also used to watch the bats through the glass door and the checks were always completed within a dark room to prevent further disturbance. Notes were taken daily such as, roosting position of each bat, individual behaviour of each bat, temperature and humidity levels, water and food levels, and level of water in terrarium humidifier. Particularly, notes such as torpor time and environmental conditions were of greatest importance.

APPENDIX C. Swabbing Area and Sampling Dates

Table C.1. Swabbing area of the left arm, right arm, and both arms combined of bats from the 2019 captive trial. Excluded width of body.

Bat Code	Left arm length	Right arm length	Left+right arm length combined
LMRP	9.91	10.16	20.07
LMRG	10.80	10.29	21.08
LM	9.33	9.21	18.54
LMRB	9.72	10.41	20.13
LMRLB	9.46	9.72	19.18
LG	9.21	9.33	18.54
LMRG	9.72	9.84	19.56
NB	9.97	9.78	19.75
LB	10.73	10.60	21.34
LMRB	9.65	9.59	19.24
LR	9.53	9.65	19.18
LBRP	9.97	10.10	20.07
LB	9.46	9.33	18.80
LBRLB	9.53	9.65	19.18
LG	10.22	10.35	20.57
LLB	9.53	9.59	19.11
RP	10.16	10.48	20.64
LDB	9.72	9.84	19.56
Mean	9.81	9.88	19.70
SE	0.10	0.10	0.19

Table C.2. Swab schedule of treatment 1 and 2 bats.

Treatment group	Date	Swab Location	Length
Baseline Bat Swabs	April 28th	Right wing	9.88
Treatment 1	Entire Sampling period	Both wings	19.70
Treatment 2	June 15th	Right wing	9.88
Treatment 2	June 29th	Left wing	9.81
Treatment 2	July 27th	Left wing	9.81
Treatment 2	Aug 9th	Right wing	9.88
Treatment 2	Aug 14th	Left wing	9.81
Treatment 2	Aug 24th	Left wing	9.81

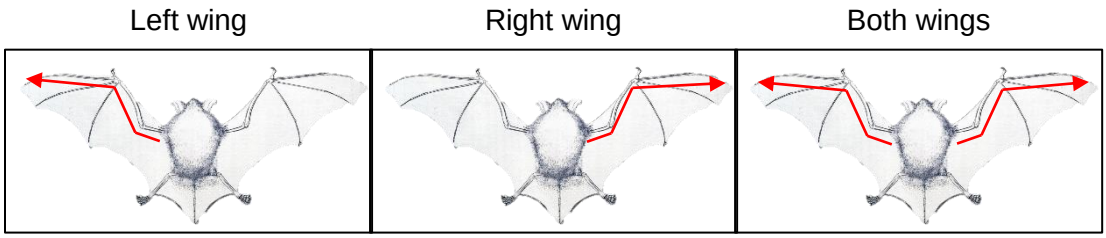

Figure C.1 Representation of bat swab areas for Treatment group 1 and 2. Note: scale of photo is not accurate.

Table C.3. Swab schedule of bats in the hibernation trial.

Sample Dates	oct 30 and dec 21	07-Nov	Nov 18 and 28	dec 8 and 18
Wing section	Shoulder to elbow (1)	First and Second finger bone (2)	Third finger bone (3)	Fourth finger bone (4)
L Silver	4.3815	5.334	5.08	2.921
L Silver R Green	4.445	6.35	4.6355	4.191
No Bands	4.0005	5.334	4.8895	4.445
Mean	4.27482	5.67182	4.86664	3.85064
SE	0.054645321	0.133333333	0.050689688	0.185592145

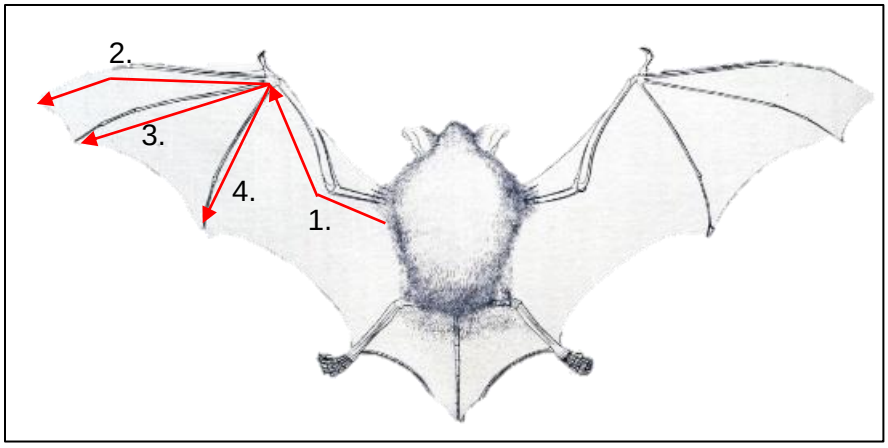

Figure C.2 Representation of bat swab areas for the Hibernation group. Note: scale of photo is not accurate.

APPENDIX D. Hibernation Fridge Temperature and Relative Humidity**.**

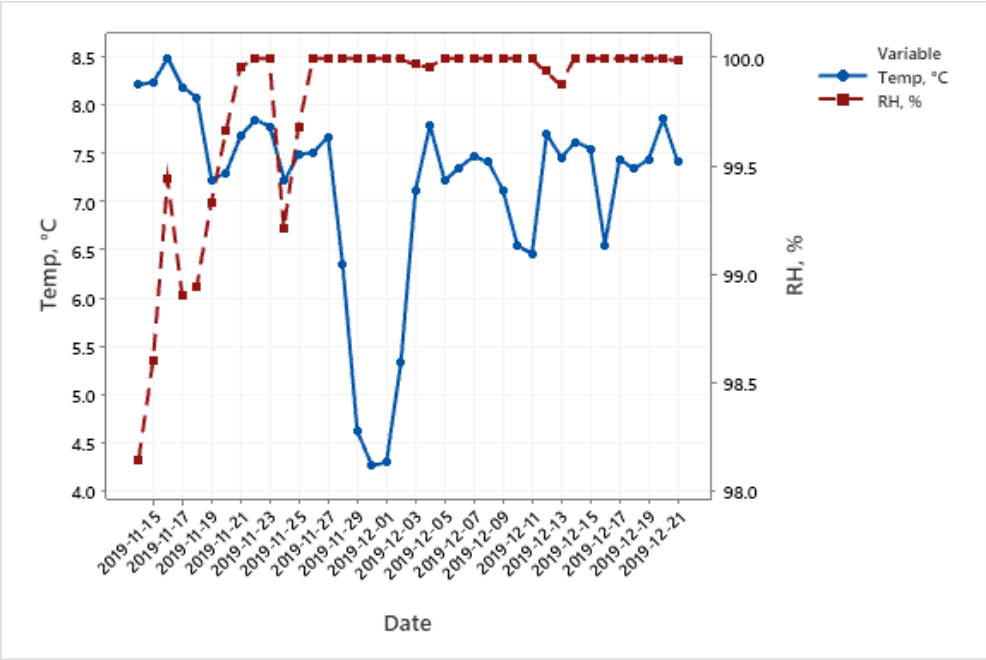

Figure D.1. Temperature and relative humidity percentage within the hibernation chamber throughout the trial period.

APPENDIX E. Swab Broth Suspension Results.

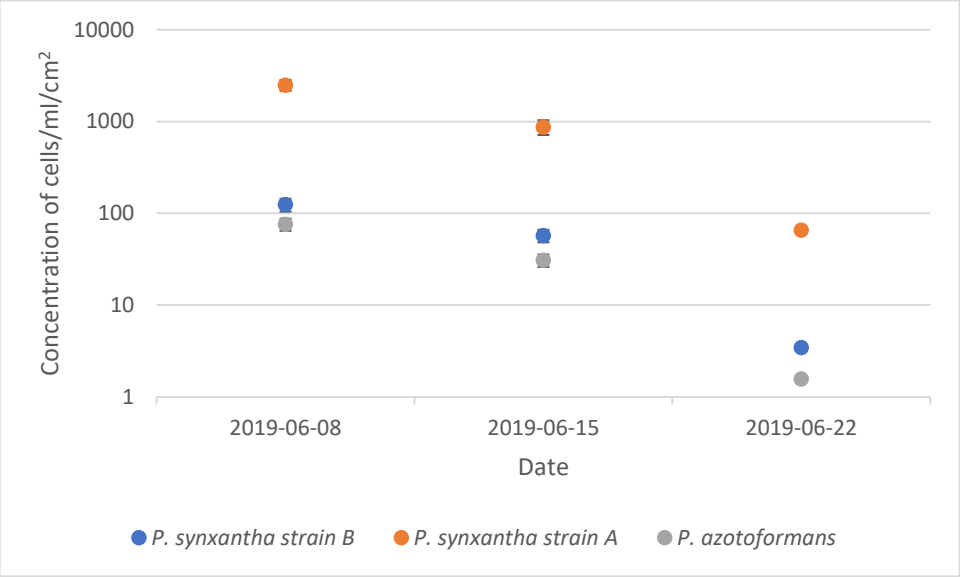

Figure E.1. Concentration of probiotic cells detected on treated bats in the Treatment 1 group using qPCR for each probiotic bacteria species. Swab suspension was grown in broth for 12 hours and incubated at 25ºC. Concentrations are corrected by wing area to represent cm^2. Note the logarithmic scale.

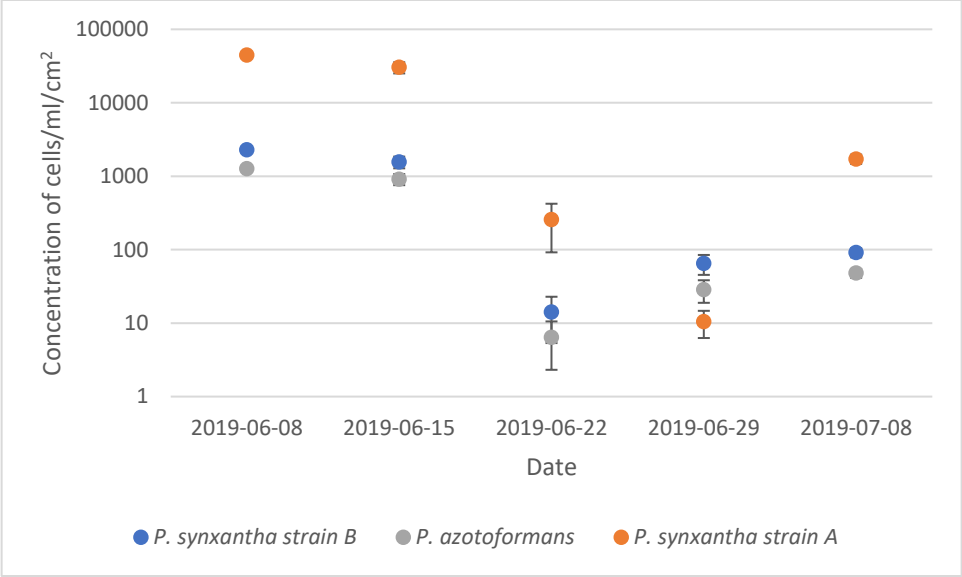

Figure E.2. Concentration of probiotic cells detected on the bat box in the Treatment 1 group using qPCR for each probiotic bacteria species. Swab suspension was grown in broth for 12 hours and incubated at 25ºC. Note the logarithmic scale.

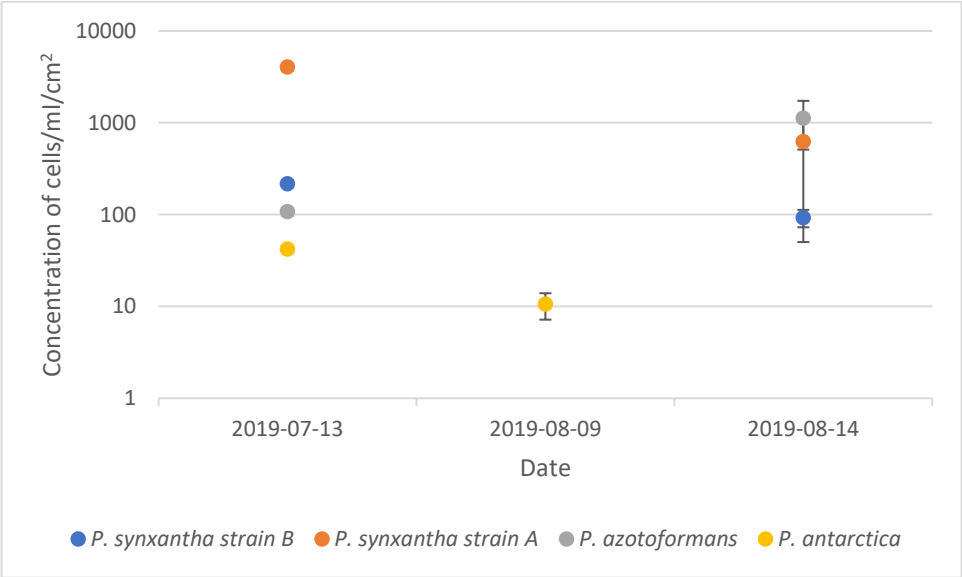

Figure E.3. Concentration of probiotic cells detected on treated bats in the Treatment 2 group using qPCR for each probiotic bacteria species. Swab suspension was grown in broth for 12 hours and incubated at 25ºC. Concentrations are corrected by wing area to represent cm^2. Note the logarithmic scale.

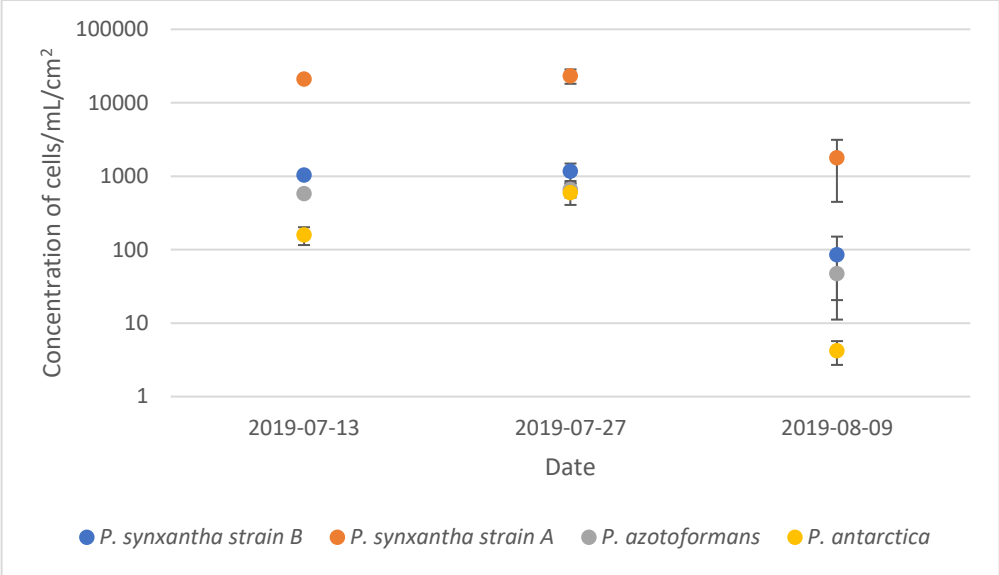

Figure E.4. Concentration of probiotic cells detected on the bat box in the Treatment 2 group using qPCR for each probiotic bacteria species. Swab suspension was grown in broth for 12 hours and incubated at 25ºC. Note the logarithmic scale.

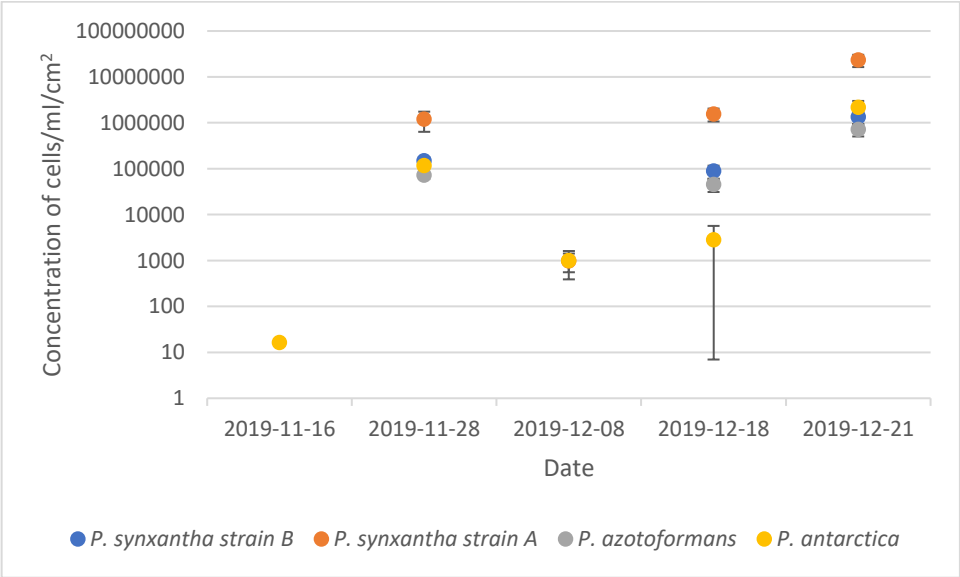

Figure E.5. Concentration of probiotic cells detected from bat swabs in the hibernation trial using qPCR for each probiotic bacteria species. Swab suspension was grown in broth for 12 hours and incubated at 25ºC. Concentrations are corrected by wing area to represent cm^2. Note the logarithmic scale.

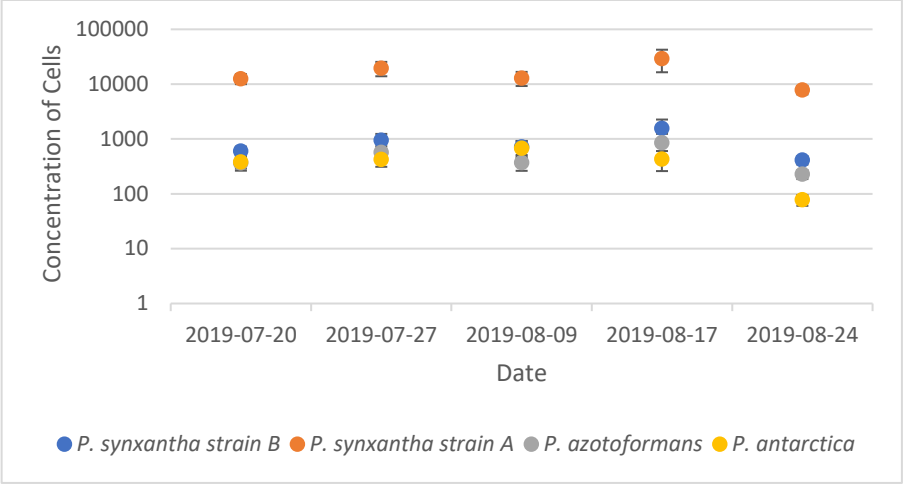

Figure E.6. Concentration of probiotic cells detected within the four-chamber bat box using qPCR. Swab suspension was grown in broth for 12 hours and incubated at 25ºC. Results from all chambers are pooled into one figure. Note the logarithmic scale.

APPENDIX F. HISTOLOGY SCORE RESULTS

Table F.1. Histology score results from the 2018 captive bat trial.

Bat Identification#	Band Colour	Treatment/control	Start mass (g)	End mass (g)	Date deceased	Post mortem preservation	Necropy Comments (G. McGregor)
19-1004-A	Purple C	Control	5.3	7.1	4-Nov-18	Good	
19-1004-C	Blue C	Control	3.6	6.2	4-Nov-18	Good	
18-5528-C	Light blue	Control, deceased before treatment began	4.2	4.2	11-Sep-18	mod, freeze-thaw	
18-5528-A	Dark blue	Treatment, deceased before treatment began	3.9	3.9	11-Sep-18	mod, freeze-thaw	
18-5528-B	Grey	Control*	4.6	4.7	9-Oct-18	mod, freeze-thaw	
19-1004-F	Black	1st Treatment Period only	4	6.3	4-Nov-18	Good	
19-1004-B	Purple P	1st Treatment Period only	4.6	7	4-Nov-18	Good	A LOT of large foamy macrophages in the lungs in septa and alveoli with grey material inside.** Mild renal coccidiosis.
19-1004-D	Blue P	1st & 2nd treatments	4.8	6.8	4-Nov-18	Good	Moderate numbers of large foamy

							macrophages in lung. **
19-1004-E	Green	1st & 2nd treatments	5.2	6.2	4-Nov-18	Good	Mod number of large foamy macrophages in lung. **
**bat died of fall while torpid* * This was attributed to bats preparing for hibernation (Bouma et al. 2010; see Chapter 2 Results).							

B)

	BAT								
	No Probiotic Applied					Probiotic Applied			
BODY PART	Purple C	Blue C	*Light blue**	*Dark blue**	*Grey**	Black	Purple P	*Blue P*	*Green*
WING	30	23	*0*	*2*	*1*	1	24	*2*	*16*
TAIL	24	15	*13*	*0*	*0*	2	20	*19*	*17*
EAR	0	0	*0*	*0*	*0*	0	1	*0*	*0*
NOSE	33	12				0	0	*0*	*0*
LUNG - neutrophil count	3	3				3	4	*2*	*3*
SPLEEN - neutrophil count	4	3				3	5	*4*	*1*
OVERALL COUNT	**94**	**56**	***13***	***2***	***1***	**9**	**54**	***27***	***37***
AVERAGE SCORE	33.2					31.75			

*	these 3 bats died and were sent for necropsy; the others were all euthanized at the end of the trial period in early Nov.
Note:	Segs and Monos - these tests are on a scale of 0 - 100 but are shown here instead as 1/100 (e.g. 25 shown as 2.5) to enable a crude weighting for summing and average scoring.
Dark shade	bats that were Controls or died before treatments began; italics are bats that were not in captivity as long as the other bats (died prematurely)
Light shade	bats that received probiotic treatment; italics are bats that received a large dose of probiotic in last week or trial vs non-italics are Treatment bats that did not receive treatment for the last 2 weeks of trial

Table F.2. Histology scores of wing tissue from bats within the 2019 summer captive trial. These consisted of Control and Treatment 1 groups.

Wing								
PM preservation	Tx group	acanthosis	hyperkeratosis	overall inflammation	epidermal inflammation	dermal inflammation	Segs	Monos
Good	control	1	0	2	0	2	0	100
Good	control	2	1	2	2	1	0	100
Good	control	3	2	3	0	3	0	100
Good	control	1	0	2	1	1	0	100
Good	control	2	1	3	1	2	25	75
Good	control	1	0	3	0	3	0	100
Good	T1	2	1	3	1	3	25	75
Good	T1	3	1	3	0	3	0	100
Good	T1	2	0	1	0	1	0	100
Good	T1	2	0	3	0	3	25	75
Good	T1	1	0	1	0	1	0	100
Good	T1	2	2	2	0	2	25	75
Good	T1	2	1	3	3	3	50	50
Control mean		1.67	0.667	2.5	0.667	2		
Treatment mean		2	0.714	2.29	0.571	2.286		

Hyphae	Bacterial epidermal	Bacterial invasion	Ulceration	Hemorrhage	Mites	Yeast
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0

Table F.3. Histology scores of tail tissue from bats within the 2019 summer captive trial. These consisted of Control and Treatment 1 groups.

Tail								
PM preservation	Tx group	acanthosis	hyperkeratosis	overall inflammation	epidermal inflammation	dermal inflammation	Segs	Monos
Good	control	0	0	2	0	3	25	75
Good	control	1	1	0	0	0	0	0
Good	control	2	0	4	1	3	25	75
Good	control	2	1	3	0	3	25	75
Good	control	1	0	1	0	1	25	75
Good	control	1	0	2	0	2	0	100
Good	T1	1	1	1	0	1	0	100
Good	T1	1	0	2	0	2	0	100
Good	T1	0	0	0	0	0	0	0
Good	T1	1	0	1	0	1	25	75
Good	T1	0	0	0	0	0	0	0
Good	T1	0	0	2	2	0	100	0
Good	T1	1	0	1	0	1	0	100
Control mean		1.17	0.333	2	0.167	2		
Treatment mean		0.571	0.143	1	0.286	0.714		

Hyphae	Bacterial epidermal	Bacterial invasion	Ulceration	Hemorrhage	Mites	Yeast
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0

Table F.4. Histology scores of ear tissue from bats within the 2019 summer captive trial. These consisted of Control and Treatment 1 groups.

Ear						
PM preservation	Tx group	acanthosis	hyperkeratosis	overall inflammation	epidermal inflammation	dermal inflammation
Good	control	0	0	2	0	2
Good	control	0	0	2	0	2
Good	control	0	0	2	0	2
Good	control	0	0	0	0	0
Good	control	0	0	0	0	0
Good	control	0	0	0	0	0
Good	T1	0	0	4	0	4
Good	T1	0	0	1	0	1
Good	T1	0	0	2	0	2
Good	T1	0	0	3	0	3
Good	T1	0	0	0	0	0
Good	T1	0	0	0	0	0
Good	T1	0	0	1	0	1
Control mean		0	0	1	0	1
Treatment mean		0	0	1.571	0	1.571

Hyphae	Bacterial epidermal	Bacterial invasion	Ulceration	Hemorrhage	Mites	Yeast
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0

Table F.5. Histology scores of nose tissue from bats within the 2019 summer captive trial. These consisted of Control and Treatment 1 groups

Nose						
PM preservation	Tx group	overall inflammation	epidermal inflammation	dermal inflammation	Segs	Monos
Good	control	0	0	0	0	0
Good	control	0	0	0	0	0
Good	control	5	5	5	75	25
Good	control	1	0	1	0	100
Good	control	0	0	0	0	0
Good	control	0	0	0	0	0
Good	T1	0	0	0	0	0
Good	T1	0	0	0	0	0
Good	T1	0	0	0	0	0
Good	T1	0	0	0	0	0
Good	T1	0	0	0	0	0
Good	T1	1	0	1	0	100
Good	T1	0	0	0	0	0
Control mean		1	0.833	1		
Treatment mean		0.143	0	0.143		

Hyphae	Bacterial epidermal	Bacterial invasion	Ulceration	Hemorrhage	Mites
0	0	0	0	0	0
0	0	0	0	0	0
0	3	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0.5	0	0	0	0
0	0	0	0	0	0

Table F.6. Histology scores of lungs and spleens from bats within the 2019 summer captive trial. These consisted of Control and Treatment 1 groups.

	Lung		**Spleen**
Tx group	Lung neuts	Lung histocytic cells	spleen neuts
control	2	3	1
control	1	2	1
control	2	1	1
control	2	2	2
control	2	3	2
control	1	3	1
T1	1	1	n/a
T1	2	3	1
T1	1	1	1
T1	1	2	n/a
T1	1	2	1
T1	1	3	0
T1	2	3	n/a
Control mean	1.67	2.33	1.33
Treatment mean	1.28	2.14	0.75

APPENDIX G. Captive Trial Experimental Design and Justification

The experimental design involves 3 groups of bats which belong to the Treatment 1 group, Treatment 2 group, and the Hibernation group. Each group was independent of one another because of how they differed in their experimental design. The Treatment 1 group was inoculated with two probiotic dosages which were on May 12th and May 24th, followed by weekly swabbing (swab areas can be found in Appendix C) of their wings. Treatment 2 group was only inoculated with one dosage of probiotic bacteria on June 15th. The bat box in the Treatment 1 group was changed out upon inoculating another dosage, whereas the Treatment 2 group bats used the same bat box that was initially inoculated. Therefore we are comparing two groups of bats that were exposed to differing concentrations of probiotic, one dosage versus two dosages of probiotic bacteria. The Hibernation group was independent of the other treatment groups because it was placed into entirely different environmental conditions within a hibernacula chamber (Appendix D).

All replicates from each species of bacteria and on each swab date formulate a mean abundance of cells in each category (Table G.1, G.2, G.3). The standard error is calculated among all replicates due to the mean of probiotic bacteria being derived from these individual replicates. Of which, the mean of means of each bacteria from each bat would derive the same result as the mean of all replicates they originated from.

Table G.1. Number of swabbed bats in the Treatment 1 group and qPCR data replicates of each bacteria species found across all bats.

Date of bat swab	Number of bats swabbed	*P. synxantha* strain B replicates	*P. synxantha* strain A replicates	*P. azotoformans* replicates
2019-05-26	7	14	15	15
2019-06-01	7	9	8	7
2019-06-08	7	2	2	2
2019-06-15	7	7	9	7

Table G.2. Number of swabbed bats in the Treatment 2 group and qPCR data replicates of each bacteria species found across all bats.

Date of bat swab	Number of bats swabbed	*P. synxantha* strain B replicates	*P. synxantha* strain A replicates	*P. azotoformans* replicates	*P. antarctica* replicates
2019-06-29	3	4	4	4	0
2019-07-13	3	1	1	1	0
2019-08-09	3	1	1	1	2
2019-08-14	3	2	2	2	0

Table G.3. Number of swabbed bats in the Hibernation group and qPCR data replicates of each bacteria species found across all bats.

	Number of bats swabbed	*P. synxantha* strain B replicates	*P. synxantha* strain A replicates	*P. azotoformans* replicates	*P. antarctica* replicates
2019-11-16	3	9	9	9	6
2019-11-28	3	9	9	9	9
2019-12-08	3	9	9	9	9
2019-12-18	3	3	3	3	0
2019-12-21	3	6	6	6	6

APPENDIX H. Swab Protocol

Step-by-step protocol

INTRODUCTION:

The objective of this sampling is to collect metagenomics data on the bat species and populations for conservation efforts. Metagenomic sampling of bats will provide total genomic data of a bat's wing microbiome.

Materials needed ***for each bat / environmental sample****:*

Included in kit:
1 vial of sterile NaCl (0.15M) buffer solution (for moistening swab and storing swab after sampling)
1 ziploc bag (to place vial in when sampling is completed)
1 sticker **OR** 1 piece of rite on rain paper (label with sample information)
1 sterile swab with a predefined breakpoint on the stick

Included in kit:
One large Ziploc bag is included for each *sampling session*

Also needed per sample:
1 small Ziploc bag for individual samples
1 pair of latex/nitrile gloves
1 permanent marker
Decontamination chemical (for WNS spread prevention) for any surface that happens to touch the bat

Methods: swabbing

i. Put on latex/nitrile gloves.

ii. Fill in information on sticker/rite on rain paper. Include the following 7 information fields:

NAME of Principal Investigator:

DATE (MMM DD/YY)

SITE

BATID#

SPECIES: (4 letter code)

SEX: M/F

AGE: A/Juv

If stickers are provided in your kit, this information should be filled out on the label with permanent marker and adhered to the ***inside*** of the small Ziploc bag. But if there is only rite on the rain paper, please write this information on the paper and place it inside the Ziploc bag.

iii. Open the Whatman Omni swab package so that the swab handle is presented to the handler. Keep the tip of the swab inside the package to keep it sterile. Avoid contact with the tip of the swab.

iv. ***<u>Soak the tip of the swab in 0.15M NaCl buffer tube. Remove excess liquid from the swab by pressing the swab tip against the inside of the tube wall.</u>***

v. Part 1 (to be completed for all bats). Swab the bat:

a) Please read the precautions at the bottom of this protocol.

b) Carefully swab the inside of the right wing (forearm and wing) in linear strokes. Cover the entire wing surface, slowly rolling or lightly brushing the swab over the skin. This should take at least 12 seconds.

c) Immediately place the swab into the vial -- Release the tip of the swab into the collection tube by pressing on the swab handle.

d) Place collection tube into small Ziploc bag. Be sure there is a label on the inside of this bag (either a sticker or piece of paper).

vi. Part 2 (To be completed only once at each capture site (**<u>Environmental Reference Swab -- to be completed only if there are known bat roosts at the sampling area).</u>**

a) Using another Whatman omni swab, vial and fresh gloves, create a label with the following information fields:

NAME of Principal Investigator:

DATE (MMM DD/YY)

SITE:

ENVIRONMENTAL SUBSTRATE SWABBED: (e.g., inside rock crevice, mine wall, rafter of attic)

KNOWN USE BY ROOSTING BATS? y/n

b) Swab the wall/surface closest to where the bats are roosting or would typically roost for 10 seconds in a circle of approximately 5cm.

- For example, if bats are captured outside of a rocky bluff area, and bats are thought to be using the rock crevices in the area, swab the inside of a randomly selected crevice. If an actual crevice roost is known, sample this instead.

c) Release the tip of the swab into the collection tube by pressing on the swab handle.

d) Store swab sample immediately after swabbing by releasing tip into collection tube

APPENDIX I. Explant Protocol

Myotis Explant SOP

Introduction:

The purpose of this SOP is to test the *Pd* inhibition threshold of the anti-*Pd* bacteria *Pseudomonas synxantha*, Strains A and B; *P. azotoformans*; and *P. antarctica* on live bat tissue. Severed tissue explants can be kept partially alive within specialized chambers, thus preventing the associated interference of decomposition**.**

Materials needed:

Autoclave and/or sterilize the following:

→ Isoflurane
→ Cotton balls
→ Small mason jars with lids
→ Large sterile scissors
→ Surgical scalpel
→ Clean cutting board
→ Biohazard waste bag
→ Sterile forceps
→ Biosafety cabinet (BSC)
→ Fume hood
→ Calipers
→ Gloves
→ P20 & P200 pipettes and tips
→ Explant chamber
→ Explant hole-punch
→ Eagles minimal essential medium
→ Gentamycin
→ Probiotic dosage
→ Gas mask
→ Prepared probiotic bacteria
→ Prepared *Pd* spores

→ Scanning Electron Microscope (SEM)
→ Phosphate Buffer Solution (PBS)
→ Centrifuge
→ Tinfoil

Procedure

1. Gather necessary material in both the fume hood and BSC. Make sure to wear a mask that protects against volatile gases (i.e. Isoflurane). Add media with and without gentamycin antibiotics into the explant chambers and seal off with tinfoil. Leave the chambers in the BSC until the explants are extracted from the bats.

2. To prepare for euthanasia, soak a cotton ball with a generous amount of isoflurane and place it into the bottom of a mason jar. Be sure to have positive airflow in the fume hood to avoid leaking of the anesthetic.

3. Take the bat and place it into the jar containing the cotton ball soaked in isoflurane. After 1-2 minutes, the bat should be knocked out and unconscious from the anesthetic. The bat should overdose quite quickly.

4. After the bat is no longer moving and at least 5 minutes have passed, remove the bat from the jar and move it to the BSC. Quickly decapitate the bat using a sharp pair of scissors to confirm euthanasia. Do not stop halfway through.

5. Turn off laminar airflow going into the BSC. The anesthetic is contained within the separate fume hood and a sterile environment is not needed for sampling the explants.

6. Using the explant hole-punch, sample skin tissue from the myotis wing patagium one at a time. Work quickly and diligently because samples can dry up and become unusable.

7. Place the sampled tissue into the explant chamber and screw both pieces of the apparatus together to seal the explant into place. Place tinfoil over top of the explant chambers. Repeat steps 6-7 until sufficient explants have been sampled or the tissue is starting to dry out.

8. Turn on the laminar air flow and prepare probiotic dosages for application.

    a. Bacteria will be grown before application in LB broth. Utilizing growth curves, OD readings, and dilution calculations, a proper dosage can be achieved for all explants. Remove designated amount of each of the 4 probiotics and mix them together. Prepare the same number of dosages as explants.

    b. Centrifuge at 4000rpm for ten minutes. Remove supernatant and flush with 1ml of PBS. Vortex on medium high.

    c. Repeat step b and centrifuge once more to remove any LB broth from the bacteria. Remove supernatant and add 250ul of PBS. Vortex on medium high to displace the bacteria pellet.

9. Inoculate each of the explants with the probiotic.

10. Inoculate the designated number of explants with *Pd* spores from previously prepared spore isolations. Refer to the spore isolation SOP for more info.

11. Fully seal the explant chambers and place the bat corpses into the -80 freezer. Monitor the explants daily.

12. After 3-4 days, half of the explants will be removed and monitored through SEM for spore germination and hyphae growth. Control will be compared to probiotic treatments at different spore loads, with and without antibiotics.

13. After 1 week, monitor the other half of the explants for spore growth and germination. Remove explants and place them into PBS solutions. Freeze the samples. *Pd* and probiotic numbers can then be monitored through qPCR analysis.